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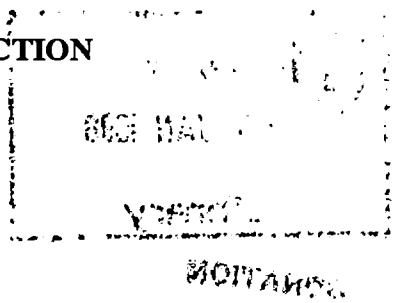
# MONOCLONAL ANTIBODIES BINDING TO MALARIAL MEROZOITE SURFACE PROTEIN 1 PROTECT *IN VIVO* AGAINST *PLASMODIUM YOELII* INFECTION



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**A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Ph.D**

**August 1997**

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T o my parents, Mavis and Gilberto; my grandmother, Amanda; and my aunt Adita, for their support, encouragement and respect for my work.

## ABSTRACT

The malarial merozoite invades the erythrocyte and develops within the host cell. One of the most important and well studied parasite surface proteins at this stage of the life cycle is merozoite surface protein-1 (MSP-1). *Plasmodium falciparum* MSP-1 undergoes proteolytic processing into several fragments, of which only the carboxyl-terminal 19 kDa fragment (MSP-$1_{19}$) remains on the merozoite surface during invasion of a new erythrocyte. To study the importance of antibodies to the 19 kDa fragment of *Plasmodium yoelii* MSP-1 in providing protective immunity against infection, MSP-1-specific monoclonal antibodies were produced and characterised. Six antibodies were studied in detail. When naïve mice were passively immunised with these antibodies and then challenged with *P. yoelii* YM; which is a virulent and lethal parasite line, four of the antibodies showed a protective effect, reducing the level of parasitaemia and increasing host survival. There was some correlation between antibody subclass and protection. The epitopes for the antibodies were located on MSP-1, two antibodies bound to a recombinant protein comprised of the whole of MSP-$1_{19}$, and two others bound to a smaller sub-domain of it. These antibodies also recognised a similar recombinant protein from *P. yoelii* 265 BY; this cross-reaction occurred despite quite extensive sequence differences between the two polypeptides. Two other MSP-1 specific antibodies did not recognise recombinant MSP-$1_{19}$, one bound to an epitope in the protein sequence adjacent to MSP-$1_{19}$ and the second recognised a larger structure that included MSP-$1_{19}$. This panel of biologically active antibodies enables the mechanisms of antibody inhibition of erythrocyte invasion to be studied in detail.

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Thanks to my family and friends for encouragement during the development of this thesis.

**ABBREVIATION**

| | |
|---|---|
| BSA | bovine serum albumin |
| DMSO | dimethyl sulphoxide |
| DTT | dithiothreitol |
| EDTA | ethylene diamine tetra acetic acid |
| EGF | epidermal growth factor |
| ELISA | enzyme-linked immunosorbent assay |
| FCA | Freund's complete adjuvant |
| FCS | foetal calf serum |
| FIA | Freund's incomplete adjuvant |
| FITC | fluorescein-isothiocyanate conjugate |
| *g* | acceleration due to gravity |

| | |
|---|---|
| g | gram |
| GST | glutathione *S*-transferase |
| h | hour |
| HAT | hypoxanthine aminopterine thymidine |
| HGF | hybridoma growth factor |
| HT | hypoxanthine thymidine |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| IFA | indirect immunofluorescence assay |
| Ig | immunoglobulin |
| IPP | immunoprecipitation |
| IPTG | isopropyl-β-D-thiogalactoside |

| | |
|---|---|
| kDa | kiloDalton |
| KGS | Kreb's glucose solution |
| M | molar |
| mA | milliAmpere |
| Mab | monoclonal antibody |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| nm | nanometre |
| NMS | normal mouse serum |
| OPD | o-phenylene diamine dihydrochloride |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| PMSF | phenylmethylsulphonylfluoride |
| PRBC | parasitised red blood cell |
| RPMI | Roswell Park Memorial Institute |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| U | units |
| UV | ultraviolet |
| v | volt |
| v/v | volume/volume |

| | |
|---|---|
| w/v | weight/volume |
| °C | degrees Celsius |
| µg | microgram |
| µl | microlitre |

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# CHAPTER 1: INTRODUCTION

## 1.1 Malaria and the health problem

Malaria remains one of the most important priorities for improving public health in the tropical and subtropical areas of the world. The World Health Organisation estimates that there are 300 to 500 million clinical cases and approximately 2 to 3 million deaths due to malaria per year. The development of a vaccine against malaria is urgently needed in countries where the disease is endemic. In these regions there are serious problems in the control and eradication of malaria due to the increasing prevalence of insecticide-resistant mosquitoes, drugs-resistant parasites, and logistical problems in anti-malaria campaigns (Cohen, 1994; Desowitz, 1996; Maurice & O'Brien, 1997). The need for a vaccine was emphasised by the committee set up by the Institute of Medicine, USA in 1984. A malaria vaccine topped the list of desperately needed vaccines (Cohen, 1994).

Malaria is caused by protozoan parasites of the genus *Plasmodium* that belongs to the phylum Apicomplexa; protozoa that are characterised by the presence of an apical organelle complex at some stages in the life cycle. This apical complex consists of rhoptries and micronemes, secretory organelles that allow the invasion of host cells and tissues (Holder, 1994; Doury et al. 1993). There are four species of *Plasmodium* that infect humans and three of them, *P. vivax*, *P. ovale* and *P. malariae*, may cause severe illness. However, the species which causes the most serious illness and sometimes death is *P. falciparum* (Pf). The malaria parasite is a complex organism that is carried by Anopheline mosquitoes and enters the bloodstream of the vertebrate host when the

mosquito takes a blood meal. In man (a vertebrate host) the parasite grows through many stages, eventually bursting open red blood cells and causing a disease typified by fever and other symptoms. One of the major causes of pathology and mortality is the adherence of parasitised erythrocytes to the endothelium of cerebral and other capillaries, which can lead to vascular obstruction, frequently followed by coma and death. The principal objective of vaccines against blood stage parasites is the prevention or reduction of malaria and death in developing tropical countries, especially in Africa.

Chemotherapy is the main means of control of malaria in infected individuals, but there is a growing global problem of parasite multi-drug resistance. For example, mefloquine resistance was reported even before the drug had been routinely used, and halofantrine resistance has emerged concomitantly. Quinine and chloroquine have been used for longer periods, but in many places of world, chloroquine is no longer an effective treatment for *falciparum* malaria, and *P. vivax* has developed resistance in some parts of Oceania. Quinine has been used for more than 350 years, and a logical question is: "What is so special about quinine?". There are several possible explanations. First, quinine's intraparasitic target might be so specific that mutations which confer resistance can occur only at an exceptionally slow rate. It is also possible that modern strains of malaria parasites might be more resistant to quinine when compared to the strains of parasite that existed 300 years ago, so that quinine resistance has developed slowly over centuries of use and an increase in the required dose has gone unnoticed. On the other hand, perhaps quinine had not been used frequently enough to exert a selective pressure until recently (Meshnick, 1997).

Combination chemotherapy is a rational approach to tackle drug resistance in malaria. If drug combinations are used, these should have compatible pharmacokinetics and pharmacodynamics, no adverse pharmacological interaction and no additional toxicity. Pyrimethamine plus long-acting sulphonamide (S/P) has been used very successfully, but it is no longer effective in areas of South-east Asia and its efficacy is fading in West Africa. Quinine is most often combined with tetracycline, doxycycline or S/P for the treatment of uncomplicated malaria, but the long treatment course required makes it impractical in most settings. A three-day combination of quinine and clindamycin has proved effective in semi-immune subjects in Brazil and Cameroon, but its efficacy in non-immune subjects has not been evaluated. (White & Olliaro, 1996).

### 1.2 Life cycle of the malaria parasite *Plasmodium* spp.

The life-cycle of the malaria parasite within the human host is complex (Figure 1.1). This cycle is initiated by the inoculation of sporozoites by the *Anopheles* mosquito. When the mosquito bites, it injects sporozoites and they move quickly through the bloodstream to the liver. The sporozoite invades an hepatocyte and then the parasite undergoes asexual multiplication, eventually forming merozoites (the hepatic schizogony phase). Finally the liver cells burst, releasing merozoites into the blood stream. In a few seconds, these merozoites invade red blood cells and within these cells the parasite undergoes further nuclear division. After intracellular multiplication within the red blood cell (the erythrocytic schizogony phase), the host cell is burst open, releasing more infectious merozoites, which rapidly reinvade fresh red blood cells to repeat the asexual erythrocytic cycle. Some parasites within infected red blood cells develop into gametocytes to initiate the parasite's sexual stage. When the mosquito bites an infected

human, it may suck up gametocytes, which produce male and female gametes in the mosquito's stomach. Each gamete is haploid and after fertilisation, the diploid zygote matures over the next few hours into a mobile ookinete. Eventually the ookinete reaches the stomach wall, passes between the epithelial cells to reach the basement membrane and forms an oocyst. Within the oocyst multiplication occurs and eventually it releases thousands of sporozoites (sporogony phase). These sporozoites migrate to the mosquito's salivary glands to initiate the cycle of infection again (Cherfas, 1990; Knell, 1991).

### 1.3 Rodent models of malaria infection

The unlimited supply of mice, and the availability of some species of rodent malaria parasites, have been invaluable in research towards development of malaria vaccines. These models have been very useful to identify and characterise different antigenic proteins that may be common to all malaria parasites. For example, it was first shown that mice vaccinated with merozoite surface protein-1 (MSP-1, see below) of *P. yoelii* were protected against a parasite challenge with lethal *P. yoelii* YM (Holder & Freeman, 1981). This was extended to show that vaccination with a recombinant protein consisting of the C-terminal part of *P. yoelii* MSP-1 induced protective immunity in mice (Daly & Long, 1993; Ling et al., 1994). The study of murine models can give valuable information relevant to the study of human parasites such as *P. falciparum*.

The pathology of rodent malaria is perhaps more difficult to define with the same clarity as is the pathology of the human disease. In *P. berghei* infected mice (without cerebral malaria), uncontrolled parasitaemia may simply exhaust the ability of the host to generate

new blood cells, and the mice die as a result of anaemia. Other features of pathology in rodent malaria infections that may parallel those in humans, such as anaemia and hypoglycaemia, can be measured. In these situations, high tumour necrosis factor (TNF) concentrations in plasma are often observed, but not all the clinical signs observed can be attributed to an increase in TNF concentration alone. Other factors such as the strain of mouse, the species or strain of parasite and the effective parasite dose have great influence (Butcher, 1996).

In order to have an insight into protective immunity *in vivo,* various murine models have been studied, using parasites isolated from wild rodents. These offer some advantages over simian malaria models because they can be easily handled, and are relatively cheap to maintain. In addition, the mouse immune system is well characterised and large-scale infection and vaccination studies may be performed and readily repeated if necessary. Some rodent parasite species such as *P. berghei* and *P. vinkei*, and some strains of *P. yoelii* and *P. chabaudi* cause lethal infection in mice. Others, including *P. yoelii*, *P. chabaudi adami*, *P. chabaudi chabaudi* and *P. vinckei petteri*, can cause limited infections in most laboratory strains of mice. There is an initial parasitaemia followed by either complete elimination of parasites (e.g. *P. yoelii*), or smaller patent recrudescent populations that can persist for several months (e.g. *P. c. chabaudi* and *P. vinckei petteri*) (Taylor-Robinson, 1995). The lethal infection models are often used to screen compounds for chemotherapeutic activity, and the non-lethal infections serve as models to investigate the mechanisms of acquired immunity.

It must be considered that although rodent malaria models are quite good systems for studying the immune response, laboratory mice are not the natural hosts of malaria

parasites. Therefore the information derived from analysis of murine infection models must be extrapolated with care to understand human malaria. For example in reference to the interpretation of parasitaemia, for most species of murine malaria acute parasitaemias peak at >30%, a level that far exceeds that of *P. falciparum* in humans, which only exceptionally rises above 10% (Taylor-Robinson, 1995). Despite this, the murine models offer numerous advantages for the study of malaria and should continue to provide information which currently can not be obtained from other models for either ethical or technical reasons.

### 1.3.1 *Plasmodium yoelii*

Rodent malaria parasite are classified into three groups, *berghei, vinckei* and *chabaudi*. The blood stages of the *berghei* group are morphologically indistinguishable and are notable for their marked predilection for immature erythrocytes in the blood of experimentally infected laboratory rodents. Parasites of this group (*P. berghei* and *P. yoelii* ssp.) are clearly distinguished by this characteristic from *P. vinckei* and *P. chabaudi*, neither of which has a preference for immature erythrocytes. The subspecies of *P. yoelii* are distinguishable by morphological characteristics of other stages, their geographical distribution and differences in isoenzyme patterns (Killick-Kendrick, 1974).

*Plasmodium yoelii* was isolated from a thicket rat (*Thamnomys rutilans*), trapped in the Central African Republic (Landau and Chabaud, 1965). The original isolate from a wild rat was adapted for growth in laboratory mice, producing a mild and chronic infection. When mice were inoculated with $10^5$ parasites, most were capable of spontaneously resolving the infection. By the fourth day the parasitaemia had risen to a level of 2-5%

and all available reticulocytes were parasitised; only 5% of the mice developed increasing parasitaemia, and died within two weeks, (Wery, 1966; Yoeli et al., 1975). When the 17X strain of *P. berghei yoelii* was inoculated into sixteen mice, only one of them developed high parasitaemia and died. However, following 110 days storage in a deep-freeze, a line of this strain immediately exhibited enhanced virulence causing high parasitaemia and the death of all infected mice. This virulence did not alter after eight blood passages or following cyclic transmission through *Anopheles stephensi.* This was the origin of the line that was renamed YM (or 17XL [L = lethal]) (Yoeli et al., 1975). It is well known that prolonged and continuous syringe passage of rodent malaria parasites under laboratory conditions can increase their virulence, perhaps by increasing their adaptation to a particular host, but the genetic relationship of the virulent YM parasite to other parasites in the 17X isolate remains obscure. The virulent (YM) and avirulent (17X) parasites have each been cloned; the growth characteristics of each clone conformed to that described for the parental type (Owen, 1994).

### 1.4 The host immune response to malaria parasites

The malaria parasite like other parasites has developed sophisticated strategies to adapt to its host and evade the attack of the immune system. Immunity to malaria has been studied in humans and the most appropriate murine models. Although the mechanisms that operate in the control of infections are very complex, and have not been clearly established, it is known that malaria parasites stimulate humoral and cell-mediated immune responses, both of which play a role in the protection against malaria parasite infection (Taylor-Robinson, 1995).

Protection induced by vaccination can be correlated with the production of specific antibody. However, T-cell mediated immunity may also be involved, with activation of macrophages as possible effector mechanisms with or without specific antibodies being involved. The production of cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) (Taylor-Robinson, 1995) may also be important in stimulating B-cells to secrete antibody and in activating macrophages. Macrophages can act as effector cells in malaria by releasing cytotoxic substances such as reactive oxygen radicals and nitric oxide which destroy parasites within erythrocytes. This cytotoxic mechanism may be mediated through the release of TNF (lymphokine production) by macrophages, because it has been suggested that TNF can kill several species included *P. falciparum in vitro*. However the parasite sensitivity to TNF may differ *in vivo* (Taverne et al., 1986).

$CD4^+$ T helper (Th) cells can be separated into two major subsets which mediate qualitatively distinct immune responses; for example, cell-mediated responses (Th1) and humoral responses (Th2). A characteristic of murine malaria parasites is that primary infections with asexual erythrocytic forms generate a host protective immune response with a spectrum of Th1 and Th2-type $CD4^+$ T-cells. The way in which $CD4^+$ T cells effect their protective role appears to be different in various murine models (Taylor-Robinson, 1995).

Butcher (1996) discusses models for malaria, and points out the fact that acquired resistance to malaria involves an initial phase of non-specific macrophage/neutrophil activation with concomitant increase in the concentration of TNF in serum. This may result in the characteristic symptoms and pathology such as high temperature and limited

parasite replication. However it is difficult to distinguish these effects from those caused by oxygen free radicals or other cytotoxic factors that may also be present. This process is associated with the host controlling parasite replication. It is followed by a rise in antibody levels and the gradual replacement of a Th1-cell type response by a Th2-cell dominated response, with further reduction in the parasite numbers. There is also evidence for macrophage activation in both humans and murine models during the early acute phase of infection, and the role of antibodies in subsequently controlling and eliminating parasites. In terms of humoral response, the antibody-mediated immunity takes time to become effective. This may be because parasite antigens show considerable diversity. This antigenic diversity induces a switch in the host immune response ( Butcher, 1996). Both polyclonal and monoclonal antibodies have been useful to identify protective protein antigens and to establish their physicochemical characteristics. The genes that encode these proteins have been cloned, sequenced and expressed in a variety of expression vectors.

Weidanz and Long (1988), found that splenic T cells from mice immune to *P. yoelii*, as well as from non-immune mice, proliferated when exposed to parasitised erythrocytes or to lysates of these cells. The proliferative response of the T cells derived from immune mice occurred earlier than that of the T cells from non-immune mice. This result may reflect the presence of mitogenic factors in the crude preparations of malaria antigens used to stimulate lymphocyte culture in vitro. Others studies have shown that both murine and humans T cell lines and clones proliferate in responses to plasmodial antigens from different stages of the malaria parasite. These responses are antigen-specific and major histocompatibility complex (MHC)-restricted (Riley et al., 1991). Another

indication of specific T cells activation by malaria antigens is the expression of delayed-type hypersensitivity (DTH) in immunised mice when challenged with malaria antigens.

### 1.5 Malaria vaccine candidates

There are various proteins expressed at different stages of the life cycle that have been defined as targets of the immune response and potential vaccine candidates. For example, the circumsporozoite protein (CSP) on the surface of sporozoites, merozoite surface protein-1 and -2 (MSP-1 and MSP-2) and sexual stage antigens, such as a 48/45 kDa gamete surface protein (Pfs48/45) and a 25 kDa zygote/ookinete surface protein (Pfs 25). Although many antigens can be shared between the different stages, it appears that immunity is species and stage specific; for example, immunisation with sporozoites does not protect against blood stage challenge and *vice versa*. Each vaccine strategy has certain limitations. For example, in the case of a vaccine targeted at sporozoites, it may prevent the initial infection but it would be ineffective against subsequent blood stages. Since a single sporozoite is capable of initiating a full-blown asexual blood stage parasitaemia, an anti-sporozoite vaccine may need to be 100% effective to provide protection in individuals not previously exposed to malaria. Although a vaccine directed against the asexual blood stage may reduce or abolish clinical symptoms, it may have no effect on the development of the sexual stages and the transmission of the disease. A vaccine against the sexual stage of the parasite's life cycle would not protect the individual but may reduce transmission and the subsequent infection of others (Holder, 1993).

Within the context of a pre-erythrocytic stage vaccine, CSP has been studied most intensively with the aim of developing a vaccine to prevent the initial infection. The identification of this antigen and the demonstration that a Mab specific to a central repetitive element ([Asn-Ala-Asn-Pro]$_n$ in *P. falciparum,* but different in different parasite species) within it could passively protect mice against sporozoites, formed the conceptual basis of the vaccine trials that have been carried out. Despite early problems with immunogenicity and limited evidence of protection, recent studies using RTS,S a hybrid CSP-Hepatitis B surface antigen particle expressed in yeast cells and mixed with a powerful adjuvant, have shown impressive levels of CSP-specific antibody and very good protection against sporozoite challenge in volunteers (Stoute et al., 1997).

The proteins that have been identified as possible targets of immune responses which inhibit erythrocyte invasion have been placed in three groups (Holder, 1996), a classification that is based on the location of the proteins. These proteins are (1) merozoite surface proteins (MSPs), (2) soluble proteins that may be loosely associated with the merozoite surface, and (3) proteins in the apical organelles. These groups include (1) merozoite surface proteins-1 and -2 (MSP-1and MSP-2); (2) serine repeat antigen (SERA), secreted polymorphic antigen associated with merozoites (SPAM/MSP-3), glutamate acid-rich protein (GLURP), acidic basic repeat antigen (ABRA), and S-antigen; and (3) proteins in the apical organelles, comprised of microneme and rhoptry proteins. Within the microneme is the *P. falciparum* 175 kDa erythrocyte binding antigen (EBA-175) that appears to bind to the host molecule glycophorin A, and is related to the Duffy-blood group binding protein of *P. vivax.* The rhoptry protein group includes apical membrane antigen 1 (AMA-1) and rhoptry-associated proteins -1 and -2 (RAP-1 and RAP-2).

The characteristics of these proteins are outlined briefly below, except for those of MSP-1, which is described in detail later in this chapter:

MSP-2: This is a 43- to 56-kDa protein on the merozoite surface, and it is recognised by antibodies that inhibit *P. falciparum* growth *in vitro* (Fenton et al., 1991; Ramasamy et al., 1990). The protein is anchored on the surface by a glycosyl phosphatidyl inositol but, unlike the 19 kDa fragment of MSP-1 it does not appear to be carried into the erythrocyte at invasion. For this protein there are two families that are largely defined by the highly variable repetitive and non-repetitive variable regions; one is typified by the sequence in the FC27 strain and the other by the sequence in the ICI and 3D7 strains. MSP-2 is a vaccine candidate because of its location and because Mabs reactive with MSP-2 inhibit parasite growth in vitro. The sequence and antigenic diversity also suggest that MSP-2 is under immunological pressure. A vaccine based on this protein may include either a structure conserved between the families or two structures representative of the two forms.

SERA: The serine repeat antigen, also known as serine-rich protein, p113, p126, or p140; is a soluble protein synthesised by the late-erythrocytic-stage parasite and secreted into the parasitophorous vacuole. At the end of schizogony, the protein is cleaved into 50 and 73 kDa fragments. N-terminal sequencing showed that the 73-kDa fragment is composed of a 47-kDa polypeptide from the N-terminus disulphide linked to an 18-kDa fragment from C terminus of SERA. The evidence that this antigen is a potential vaccine candidate has been obtained largely from direct immunisation data, although SERA-specific growth inhibitory Mabs have been described, and the protein has been shown in

antibody-merozoite aggregates. Two recombinant antigens, SERA 1 and SERA N have been expressed in yeast and tested in Aotus immunisation studies. Both proteins are recognised by a Mab that blocks parasite invasion (Barr et al., 1991; Iselburg et al., 1991).

SPAM/MSP-3: This antigen was described recently and identified by purified human IgG antibodies active in an in vitro antibody dependent cell inhibition (ADCI) assay. Concurrently, SPAM, a protein with the same partial sequence was also reported (Oeuvray et al., 1993 & 1994). This is a soluble protein only loosely associated with the merozoite surface.

GLURP: Glutamate-rich protein has been examined extensively in field studies, but there are no in vivo or in vitro studies to implicate it as a target of protective immunity. The protein has an apparent molecular mass of 220 kDa, and it is expressed in liver and asexual blood stages. This antigen is considered to be a vaccine candidate based on its location, and because sero-epidemiological studies have shown a correlation between antibody response and low parasitaemia (Dziegiel et al.,1993).

ABRA: Acidic basic repeat antigen is a soluble protein of the parasitophorous vacuole and it is also found in agglutinated clusters of merozoites. This protein has been poorly studied, and the evidence to support its use a vaccine candidate is weak.

S-antigen: S-antigens are highly diverse, heat-stable, soluble proteins of the parasitophorous vacuole. Four families of S-antigens have been defined based on the N- and C-terminal sequences. The protein can be cross-liked to MSP-1 and therefore may

be loosely associated with the merozoite surface. An specific Mab against this protein inhibits parasite growth in vitro (Saul et at., 1995), but there is no other evidence that S-antigens are involved in protective immunity against the merozoite stage.

Erythrocyte binding proteins located in the micronemes: The parasite's ability to invade the erythrocytes depends on the host, the expression of particular molecules on the erythrocyte surface, and the age of the erythrocyte. Parasite protein binding to specific molecules of the erythrocytes surface is an integral part of the invasion process (Mitchell et al., 1986). A 175-kDa *P. falciparum* erythrocyte-binding protein (EBA 175) is a sialic acid-binding protein and may be targeted by a vaccine strategy designed to interfere with specific receptor-ligand interactions.

Rhoptry proteins: The rhoptry organelles contain a number of proteins, all with unknown function, but some implicated as targets of protective immunity. There is compartmentalisation within the rhoptries, with different proteins not being uniformly distributed throughout. A 225-kDa protein derived from a 240-kDa precursor is located in the necks of rhoptries, and a similar localisation has been described for apical membrane antigen 1 (AMA-1). Two macromolecular complexes have been identified in the body of the rhoptries (Doury et al. 1994): a high-molecular-mass complex of 150, 130 and 110 kDa proteins and a low-molecular-mass complex of rhoptry-associated protein 1 (RAP-1) and RAP-2. These proteins are associated with membranous structures released from merozoites and after invasion they are associated with the parasitophorous vacuole membrane-parasite plasma membrane (Crewther et al., 1990). Immunofluorescence studies and radiolabeling indicate that AMA-1 is also located on

the merozoite surface at the apical end of the parasite (Thomas et al. 1990 & Waters et al., 1990).

An effective vaccine may require a mixture of several elements from different antigens of different stages, and it will be a costly and formidable task to identify and test all the candidates for inclusion in such a cocktail (Holder, 1993).

### 1.6 Merozoite Surface Protein-1: structure, processing and function

MSP-1 is a high-molecular-mass protein synthesised as a precursor during schizogony and found on the surface of the merozoite as a complex of fragments derived by proteolytic processing of the precursor (Holder 1988). In *P. falciparum*, MSP-1 is a195-200 kDa protein. The protein has also been given a number of different names, including the precursor to major merozoite surface antigen (PMMSA), polymorphic surface antigen (PSA), glycoprotein 195 (gp 195) and merozoite surface antigen-1 (MSA-1). MSP-1 has been found in all malaria species, including *P. vivax* (Pv 200) and the rodent malaria parasites, such as *P. yoelii* (a 230 kDa protein) and *P. chabaudi* (a 199 kDa protein) (Diggs et al., 1993).

MSP-1 is a precursor protein for a number of smaller polypeptides found on the blood-stage merozoite (Holder & Freeman, 1984; McBride & Heidrich, 1987). The proteolytic processing of *P. falciparum* MSP-1 has been studied in most detail, but similar proteolytic cleavage appears to occur in other species (David et al., 1984; O'Dea et al., 1995). The processing occurs in two steps. Primary processing involves cleavage of the precursor to give four fragments of approximately 83, 30, 38 and 42kDa (called MSP-

$1_{83}$, MSP-$1_{30}$, MSP-$1_{38}$ and MSP-$1_{42}$). These fragments are present in a complex on the merozoite surface, attached by the glycosyl phosphatidyl inositol-anchored C-terminal 42 kDa fragment.. At the time of invasion, secondary processing of the membrane-bound 42 kDa fragment occurs between a leucine and an asparagine residue to produces a N-terminal cleavage product of 33kDa and a C-terminal 19 kDa fragment. The 33kDa fragment (MSP-$1_{33}$) is shed from the merozoite surface as part of a soluble complex with other fragments of MSP-1 and the19 kDa fragment (MSP-$1_{19}$) remains membrane bound and is carried into the invaded erythrocyte, where it can be detected in early ring stage parasites (Blackman et al., 1990). The enzyme involved in this secondary cleavage is a calcium-dependent serine protease (Blackman & Holder, 1992). The importance of the primary processing still needs to be elucidated. However, in successfully invaded parasites the enzymatic processing goes to completion.

The amino acid sequence of MSP-1 corresponding to MSP-$1_{19}$ resembles two EGF-like motifs (Blackman et al., 1991). The sequence contains a series of cysteine residues which are highly conserved. Comparison of MSP-1 sequences from different malaria parasite species, reveals the conservation of 10 cysteine residues and the preservation of their relative positions within the C-terminal region of molecule (Daly et al., 1992). The arrangement of the cysteines suggests that they form disulphide bonds within each EGF-like motif and thereby stabilise the 3-dimensional structure of the protein. Single-site substitutions may have profound effects on this conformational structure which may consequently affect the accessibility of antibodies to key regions of molecule (Holder & Blackman, 1994).

Several authors have discussed the function of MSP-1. The precursor protein has been reported to bind to sialic acid on erythrocytes (Perkins & Rocco, 1988). Cooper (1993) suggests that MSP-1 is involved in the invasion of erythrocytes, because antibodies specific for MSP-1 can block invasion *in vitro*. Holder and Blackman (1994) suggested that MSP-1 could be involved in the initial recognition of red blood cells, but that this interaction is weak. They also suggested that the fragment retained on the parasite surface may act like other membrane-bound growth factors and interact with a specific receptor protein, perhaps on the red blood cell surface. Alternatively the fragment could also have other biochemical functions, such as a signal-transduction system leading to the activation of specific protein kinases, to communicate to the cell that successful invasion has occurred and differentiation should continue.

**1.7 Expression, antigenicity and immunogenicity of recombinant MSP-1.**

MSP-1 purified from the parasite has been shown to induce partial or complete protection against challenge infection of malaria parasites in animal models (Diggs et al., 1993). Therefore, this protein is considered as one of the best candidates for an anti-malarial vaccine, and extensive research is being carried out in order to produce a MSP-1 recombinant protein, which maintains the conformation of the native antigen. Several studies have been carried out to produce parts of MSP-1 in heterologous expression systems so that their antigenicity and immunogenicity can be studied. The C-terminal region of MSP-1 protein has been produced using the eukaryotic baculovirus insect cell expression system. A recombinant protein is produced which has a similar conformation to that of native MSP-1 (Murphy et al., 1990; Chang et al., 1992). Recently the 19kDa C-terminal fragment has been expressed in recombinant bacteria and yeast (Chappel &

Holder, 1993; Kaslow et al., 1994; Burghaus & Holder, 1994). The integrity of disulphide-constrained epitopes, defined by antibodies reactive with the native protein, is intact in the recombinant proteins. Reduction and alkylation of *P. falciparum* MSP-$1_{19}$ abolishes its recognition by Mabs and polyclonal antibodies; this treatment of native MSP-1 also abolishes its ability to bind these antibodies. Epitopes of inhibitory Mabs are either present on individual EGF-like motifs or require both motifs in order to be present. Most of the antibody-binding sites in MSP-$1_{19}$ appear to be conformational; the two EGF-like domains are required for the formation of dominant epitopes (Holder, 1996).

### 1.8 The immune response to MSP-1

MSP-1 is considered to be a vaccine candidate. This is based on an understanding of the biology of the parasite, such as the production of MSP-$1_{19}$ by a proteolytic process and the retention of this fragment on the intracellular ring stage, and experimental studies in which the MSP-1 molecule purified from the parasite, was able to induce protection in animals models. Antibodies to the 42 kDa fragment of MSP-1 and, in particular that part which comprises MSP-$1_{19}$ , inhibit parasite development *in vitro*, possibly by inhibiting protease cleavage (Blackman et al., 1994). Immunisation with MSP-1 purified from extracts of *P. falciparum* either partially (Etlinger et al., 1991) or completely (Siddiqui et al., 1987) protected against blood stage parasite challenge in primate models. Partial protection has also been obtained with peptides or recombinant proteins. The locations of epitopes recognised by Mabs with a biological function have also been identified; some antibodies against MSP-1 are known to inhibit merozoite invasion of erythrocytes *in vitro* (Pirson & Perkins,1985; Blackman et al., 1990; Cooper et al., 1992). Cooper

and colleagues (1992) determined the antibody binding sites for a panel of Mabs against the *P. falciparum* MSP-1. The epitopes were mapped by using the naturally occurring processing fragments, by chemical cleavage of the protein, and by comparison of the isolate-specificity of binding with known sequence variation. The Mabs also gave an indication of the relative antigenicity of different parts of the protein, this analysis suggested that C-terminal region contains an immunodominant domain. The 19 kDa polypeptide is the target of inhibitory antibodies (Blackman et al., 1990; Chappel & Holder, 1993). There are multiple mechanisms by which antibodies to C-terminal region of MSP-1 may inhibit parasites, including inhibition of proteolytic processing of MSP-1 (Blackman et al., 1994).

Immunisation studies using the *P. yoelii* in mice model have suggested that MSP-1, and in particular the C-terminal region of MSP-1, is able to induce protective immune responses. Immunisation with native *P. yoelii* MSP-1 protects mice against challenge infection (Holder & Freeman, 1981). Immunity is passively transferred with a Mab against an epitope in the EGF-like motifs of *P. yoelii* MSP-1 (Majarian et al., 1984; Burns et al., 1989). In recent years, immunisation with recombinant proteins based on the 19 kDa fragment at the C-terminus of *P. yoelii* MSP-1 gave very good protection against parasite challenge; this protection was mediated by antibodies and required both EGF-domains and the conformational structure of protein to be preserved (Daly & Long, 1993, Ling et al., 1994; Ling et al., 1995). Direct analysis of the functional response in the *P. yoelii* model have confirmed that the protective response induced by immunisation with recombinant MSP-$1_{19}$ is mediated by antibodies. This is supported by the kinetics of the effector response, and passive immunisation studies with purified immunoglobulin or immune serum, indicating a significant role for humoral immunity in

protection (Daly & Long, 1995). Also, it was shown that immunisation with reduced and alkylated protein did not induce an antibody response to the native protein (Ling et al., 1994).

Hui and colleagues demonstrated that the immunogenicity of the C-terminal 19-kDa fragment of Pf MSP-1 expressed in yeast ($YMSP1_{19}$) was dependent on the mouse strain and the adjuvant formulation. They suggested that adjuvants may modulate the immunogenicity of $MSP\text{-}1_{19}$ in genetically diverse populations. This observation and other experiments suggest that T helper epitope(s) on MSP1 were effective in helping to producing anti-MSP1 antibodies. They hypothesised that the nature and/or specificity of T helper epitopes may be crucial in inducing a parasite inhibitory, anti-MSP-1 antibody response. Hence, the presence of these epitopes may determine the efficacy of an MSP-1 vaccine (Hui et al., 1994). The antibody response induced in humans will need to be studied in great detail.

Recently, Holder and Riley (1996) reviewed what is known about the human immune response to MSP-1. In epidemiological studies both cross-sectional and longitudinal surveys have shown that antibodies and T-cell and cytokine responses to certain parts of MSP-1, in particular the C-terminus, are associated with a reduced susceptibility to clinical malaria. In the work of Udhayakumar et al.(1995), it was found that T-cell proliferative responses were stimulated much more by peptides from the N-terminus of $MSP\text{-}1_{42}$, suggesting better T-cell recognition of linear peptides, rather than those in highly disulphide bonded regions. This is supported by the observation that reduction and alkylation of antigens increases proteolytic processing and presentation of immunogenic peptides bound to MHC class II. Experiments with synthetic peptides may

not be the most appropriate way to assess the T-cell response, although the use of peptides in T-cell assays obviates the need for processing and presentation of the native molecule if the peptides bind directly to HLA molecules.

### 1.9 Aim of the project

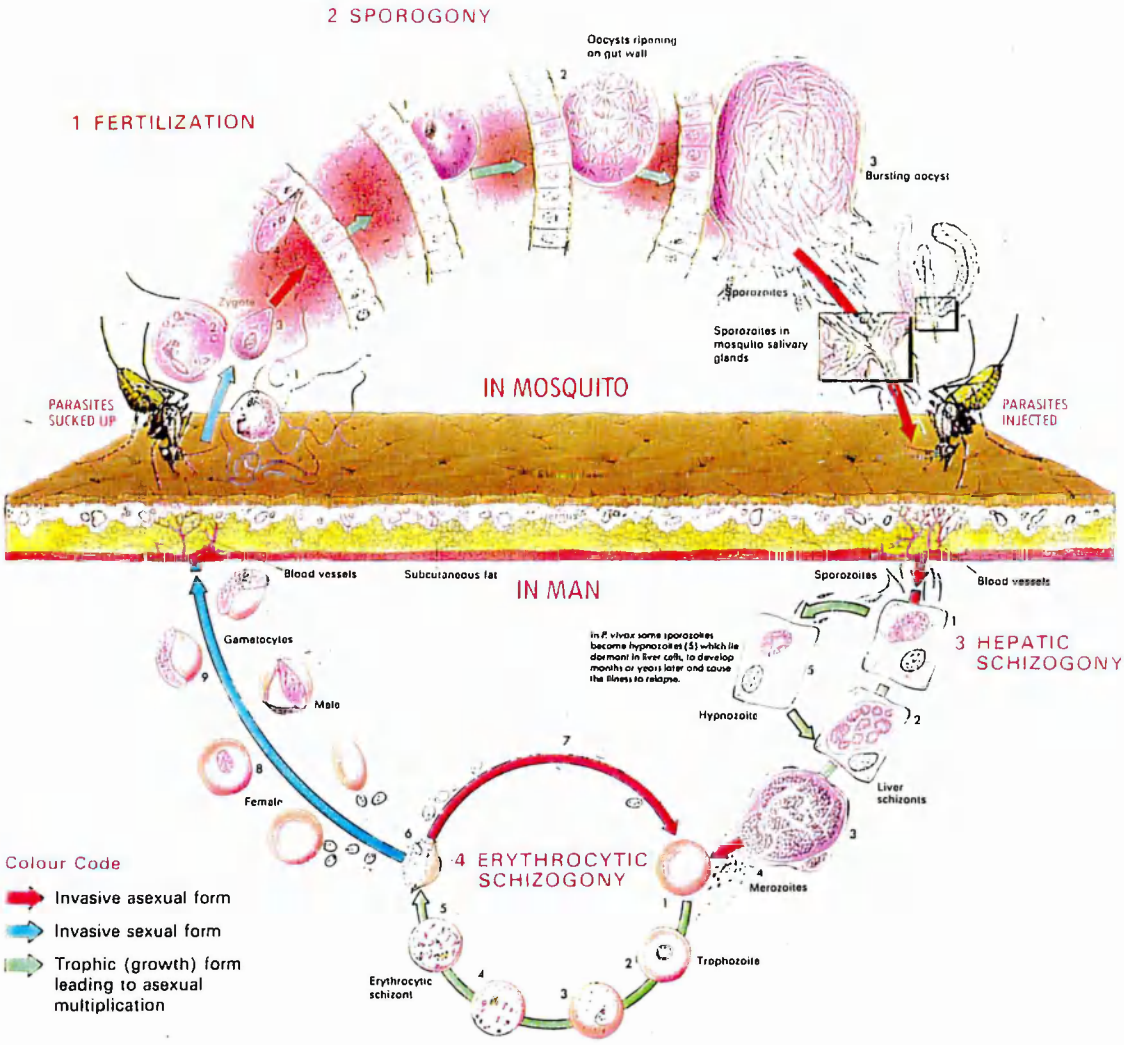
The results of the protection studies in mice after immunisation with recombinant MSP-$1_{19}$ have increased interest in the potential of this antigen as a vaccine candidate. Monoclonal antibodies (Mabs) are good tools with which to study MSP-1 because they may recognise important epitopes and inhibit parasite invasion *in vivo* and *in vitro*, However, there are few Mabs that can be used in the *P. yoelii* murine model and it was therefore necessary to produce more Mabs against *P. yoelii* MSP-1 for further studies.

My project was the production and detailed characterisation of Mabs to the C-terminus of MSP-1, with the particular goal of defining the epitopes recognised by antibodies capable of inhibiting invasion *in vivo*.

**Figure 1.1.**

The life cycle of *Plasmodium*. In this diagram is shown the sequence of the four phases of the life cycle. The first asexual phase takes place in an *Anopheles* mosquito, leading to the formation of sporozoites. The second and the third asexual phases (liver and erythrocytic schizogony, respectively) occur in a vertebrate host, in this case a human. The sexual phase is initiated in the mosquito gut from gametocytes ingested from an infected individual.

Source: The Trustees of Wellcome Trust.


2 SPOROGONY
Oocysts ripening on gut wall
1 FERTILIZATION
3 Bursting oocyst
Sporozoites
Sporozoites in mosquito salivary glands
IN MOSQUITO
PARASITES SUCKED UP
PARASITES INJECTED
Blood vessels
Subcutaneous fat
IN MAN
Sporozoites
Blood vessels
3 HEPATIC SCHIZOGONY
In P. vivax some sporozoites become hypnozoites (5) which lie dormant in liver cells, to develop months or years later and cause the illness to relapse.
Hypnozoite
Liver schizonts
Gametocytes
Male
Female
4 ERYTHROCYTIC SCHIZOGONY
Merozoites
Trophozoite
Erythrocytic schizont
Colour Code
Invasive asexual form
Invasive sexual form
Trophic (growth) form leading to asexual multiplication

# CHAPTER 2: MATERIALS AND METHODS

## 2. 1 The malaria parasite

A clone of *Plasmodium yoelii yoelii* YM was used in all experiments (Yoeli et al., 1975). The parasites were maintained as a frozen stock in liquid nitrogen and passaged by intraperitoneal inoculation into adult BALB/c mice. Four to 5 days after inoculation, blood was harvested from the brachial plexus of infected mice, into a solution containing 50 U of preservative-free sodium heparin (Sayles & Wassom, 1988). Parasitaemia was measured by microscopic examination of thin blood smears stained with Giemsa's reagent. At low levels of infection, the number of parasites in 50 fields each containing 250 erythrocytes was counted. At high levels of infection (more than 10 parasites per field) the number of parasites in 200 red blood cells was counted. In each case the percentage parasitaemia ([the number of parasite-infected cells counted /total number of erythrocytes counted] x 100%) was determined. Animals were considered to be clear of infection if no parasites could be detected on thin smears.

## 2. 2 Expression and preparation of recombinant protein containing the C-terminal amino acid sequence of *Plasmodium yoelii* MSP-1

*P. yoelii* MSP-1 sequences were expressed in *Escherichia coli* using the plasmid pGEX3x. The pGEX plasmids are designed to induce high level intracellular expression of a gene fragment as a fusion protein at the C-terminus of *Schistosoma japonicum* glutathione S-transferase (GST) (Smith & Johnson 1988). The GST-MSP1 fusion

proteins were used to immunise mice and as antigens to analyse the specificity of monoclonal antibodies (Mabs).

The DNA sequence of the C-terminal 19 kDa fragment of *P. yoelii* YM MSP-1 (MSP-$1_{19}$) which contains the two EGF-like motifs (residues 1649 to 1754 in the amino acid sequence numbered according to Lewis, 1989) was inserted into the *E. coli* expression vector pGex3x (Ling et al., 1994). Expression of the GST-MSP$1_{19}$ fusion protein was induced and then the bacterial cells were harvested. A soluble lysate of the cells was prepared and passed through a column of glutathione-agarose. After extensive washing of the column the GST fusion protein was eluted with 8 M urea (Ling et al.,1994). Fractions containing the recombinant protein were pooled and then dialysed extensively against phosphate buffered saline (PBS) in order to remove the urea. A similar protocol was used for the expression of each individual EGF-like motif of MSP-1 (MSP1EGF1 is the first motif and MSP1EGF2 is the second motif) as fusion proteins with GST (Ling et al., 1995). The DNA sequence coding for MSP-$1_{19}$ was also amplified from genomic DNA of *P. yoelii* 265BY and expressed as a GST fusion protein. The plasmids were kindly provided by Miss Irene Ling.

Colonies of *E. coli* containing plasmid were initially grown overnight at 37°C on an agar plate containing 50 µg/ml ampicillin. One colony was picked and transferred to 10 ml of L-broth supplemented with 0.1mg/ml ampicillin. The culture was then incubated for 12 or 24 hr at 37°C with continuous shaking. After this time, the cells were transferred to 100 ml of medium, and 60µg/ml of isopropyl β-D-thiogalactoside (IPTG) were added to induce protein expression. After incubation at 37°C for 1-2 h with continuous shaking the cells were harvested by centrifugation (7,000g for 10 min at

4°C) to sediment the cells. The supernatant was discarded and the bacterial cell pellet was resuspended in 10 ml of suspension buffer (25 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.2 % Nonidet P40 (v/v); and 100 mM phenyl methyl sulphonyl fluoride [PMSF]) and then the cells were lysed by sonication. DNA was then digested by adding 20 µl DNAse (10 mg/ml) and 20 µl 1M $MgSO_4$ to 10 ml of the bacterial cell lysate for 2 hrs. Cellular debris were removed from the lysate by centrifugation (15,000g) for 1 hrs. The cleared lysate was then applied slowly to a glutathione-Sepharose 4B column (Sigma). After the bacterial lysate had passed through the column, the column was washed with 100ml of suspension buffer. This was followed with a further wash with 100ml of PBS. The GST-fusion protein was eluted with 8M urea in 50mM Tris-HCl, pH 7.5, collecting fractions of 1ml. The fractions were monitored for the presence of GST-fusion protein by electrophoresing a small sample on SDS-PAGE (see Section 2. 12). The peak fractions containing most of the protein of interest were pooled and then dialysed against PBS.

The yield of fusion protein was estimated by measuring the Absorbance of the protein solution at 280 nm and calculating the concentration of the protein by use of the formula: an Absorbance of 1.0 at 280nm (1 cm path length) corresponds to a protein concentration of 0.5 mg/ml.

### 2. 3 Preparation of recombinant MSP-$1_{19}$

Recombinant MSP-$1_{19}$ was obtained by treatment of the GST-MSP$1_{19}$ fusion protein with Factor Xa protease (Abath & Simpson, 1990). The fusion protein was bound to glutathione-agarose and then treated with Factor Xa protease (from Boehringer Mannheim), which cleaves at a unique site between the GST-carrier and the recombinant

MSP-$1_{19}$. After cleavage the GST remains bound to the column and the released soluble MSP-$1_{19}$ can be eluted and purified. The recombinant MSP-$1_{19}$ was used to develop an enzyme-linked immunosorbent assay (ELISA) for the initial screening of hybridoma supernatants and for the characterisation of Mabs.

To produce recombinant MSP-$1_{19}$ a batch of GST-MSP$1_{19}$ was bound to glutathione-agarose in a column. Factor Xa was dissolved in 30µl of water and added to 4ml of cleavage buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM $CaCl_2$) to give a final concentration of 50µg/ml. The enzyme solution was then allowed to permeate the column, which was then sealed and incubated overnight at 4°C, in order to allow digestion of the GST fusion protein. The released MSP-$1_{19}$ was then eluted from the column, leaving GST remaining bound to the matrix. GST was then eluted from the column with 8M urea in 50mM Tris-HCl, pH 7.5, and following this elution the column was washed with PBS. Both proteins were dialysed against PBS and the concentration of proteins was measured as described above. The purified proteins were analysed by SDS-PAGE on 15% polyacrylamide gels according to the method of Laemmli (1970), as described in Section 2. 12.

## 2. 4 Reduction and Alkylation of GST-MSP$1_{19}$

In order to determine whether or not the epitopes recognised by Mabs were dependent upon disulphide bonds, GST-MSP$1_{19}$ was reduced and alkylated with sodium iodoacetate prior to analysis by western blotting. Briefly, 500µl of the GST-MSP$1_{19}$ solution was treated by addition of dithiothreitol (DTT) to 50mM, and incubation for 30 minutes at room temperature, followed by addition of sodium iodoacetate to 200mM and

a further incubation for 15 minutes at room temperature, in a solution adjusted to pH 8.5 with NaOH. The protein was then dialysed extensively against PBS at 4°C (Ling et al., 1994) prior to use for immunoblotting as described in Section 2. 13.

**2. 5 Mouse immunisation protocol**

A group of five 6-week old BALB/c female mice were each immunised intraperitoneally with 0.13 mg GST-MSP1$_{19}$ recombinant protein in Freund's complete adjuvant (FCA). The response was boosted by three further injections of the protein in Freund's incomplete adjuvant (FIA) 7, 21 and 35 days later. On days 28 and 80 after the first immunisation, blood samples were taken from the tail to determine the level of serum antibodies against MSP-1$_{19}$ by ELISA. If the ELISA titre was at least 1:3200, the spleen of the mouse was removed and used to prepare hybridomas.

In a second series of experiments, mice that had been passively immunised with monoclonal antibodies, challenged with parasites and then had cleared the infection, were inoculated on three occasions with 5 x$10^8$ parasitised erythrocytes prior to harvesting their spleens to prepare hybridomas.

**2. 6 Preparation of monoclonal antibodies**

Spleen cells from mice immunised with GST-MSP1$_{19}$, or by parasite infection, were fused with SP2/O-Ag14 mouse myeloma cells in the presence of 50 % polyethylene glycol (PEG). The cell ratio was 4:1 (8x$10^7$ spleen cells to 2x$10^7$ myeloma cells). In general, the fusion protocol was as described by Harlow and Lane (1988). Before fusion

with spleen cells, the myeloma cells were cultured in the presence of 8-azaguanine to ensure their sensitivity to the hypoxanthine-aminopterine thymidine (HAT) selective medium, and then five days prior to cell fusion, they were transferred to a medium without 8-azaguanine. At the time of fusion a high cell viability is required and this was verified by the trypan blue exclusion method, in which non-viable cells acquire a blue cytoplasm and viable cells retain an unstained cytoplasm. After the fusion, the suspension of hybridoma cells was dispensed into 96-well culture plates (Nunclon TC microwell plates) using 100µl/well in a diluent of HAT-supplemented RPMI 1640 medium. After 10 days of culture, the supernatant medium from each well was screened for antibody reactivity by ELISA with the recombinant MSP-$1_{19}$ (as described in Section 2. 7.1). Supernatants from the positive wells were then tested further for the presence of antibodies reacting with the parasite by using the indirect immunofluorescence assay (IFA; Voller & O'Neill, 1971), as described in Section 2. 7. 2).

The hybridoma cells from wells containing reactive antibody were cloned at least three times by using the limiting dilution method in hypoxanthine-thymidine medium (HT), following the protocol described by Harlow and Lane (1988). A soluble hybridoma growth factor (HGF) supplement (Immune System) was used during the hybridoma cloning steps and afterwards during the expansion of cell lines from the microwells. The hybridomas were grown in RPMI 1640 complete medium supplemented with 20% (v/v) foetal calf serum (FCS) and 1% glutamine. The number of hybridomas that lost their ability to produce antibodies decreased with successive cloning and all the cultures that remained antibody-positive cultures after the recloning procedure were considered stable. To maintain samples of the hybridoma cells at each cloning step they were frozen

at -70°C in RPMI complete medium containing 10% dimethylsulphoxide (DMSO) and 50% FCS.

## 2. 7 Screens to detect MSP-1 specific monoclonal antibodies

### 2. 7. 1 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA test was used for the initial screen to detect hybridomas secreting MSP-$1_{19}$ specific antibodies. Ninety six well plates (Immulon 4 from Dynatech) were coated with 100µl of antigen solution (recombinant MSP-$1_{19}$) per well, using1 µl/ml of the antigen solution diluted in coating buffer (0.1 M sodium carbonate/bicarbonate pH 9.6, containing 0.02% sodium azide). Plates were then incubated overnight at 4°C and washed three times (3X) with PBS containing 0.05% Tween 20 (PBS/T). Plates were then blocked by addition of PBS containing 1% bovine serum albumin (BSA) for 30 minutes at room temperature, followed by further washing with PBS (3X). One hundred microlitres of the supernatant from wells containing hybridomas were added to each well and incubated at 37°C for 1 hour. The plates were then washed 3X with PBS and 100µl/well of horseradish peroxidase-conjugated (HRP-) rabbit anti-mouse polyvalent immunoglobulins (IgG, IgA, and IgM-specific, Sigma) were added at a 1:1000 dilution. After incubation for 1 hour at 37°C the plates were washed again with PBS. Binding of the conjugate to bound antibody was detected by the addition of 100µl of o-phenylenediamine dihydrochloride (OPD, Sigma) as a 0.4mg/ml solution in 0.05M phosphate-citrate buffer, pH 5.0 containing 0.03% sodium perborate. The reaction was stopped by addition of 50µl 2M $H_2SO_4$ per well. The plates were read in a Titertek

Multiscan MCC/340, using Titer-soft Software (Flow) to measure the Absorbance at 492 nm.

A mouse polyclonal serum against recombinant MSP-$1_{19}$ of *P. yoelii* was used as a positive control and normal mouse serum (NMS) was used as a negative control.

### 2. 7. 2 Indirect Immunofluorescence Assay (IFA)

After the initial screening by ELISA, supernatants from the positive wells were tested by IFA to detect antibody reaction with the parasite. The antigen used in the preparation of the IFA slides was a blood film of erythrocytes containing *P. yoelii* YM, and obtained from infected mice with a parasitaemia of about 30%. The mice were bled into Kreb's glucose solution (KGS) containing 5% heparin and then the blood was passed through a column of Fibrous Cellulose Power (CF-11) to remove the leukocytes (Richards & Williams 1973). The erythrocytes were pelleted by centrifugation at 19,000 rpm (IFC Centra-4B centrifuge), and then resuspended at a dilution of 1:20. A volume of 10µl of this antigen was added to each well of an IFA slide (Dynatech), the slides were air dried and then fixed by immersion in acetone. The IFA procedure was performed as described by Voller and O'Neil (1971). Hybridoma supernatants, or solutions of purified Mab were incubated with the acetone-fixed *P. yoelii* infected erythrocytes in a humid chamber for 1 hour, then the slides were washed with PBS and incubated with a secondary antibody for 1 hour. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (FITC-conjugate from Sigma) diluted 1:1000 in PBS. Slides were washed again, dried, covered with 80% (v/v) glycerol in PBS and examined using a fluorescence microscope (Leitz, Germany) to detect the binding of antibody. The titre of

samples was determined; the values represent the last dilution of the test Mab solution which yielded visually detectable fluorescence.

The titre of antibodies in the sera of mice after passive immunisation was also determined by IFA. Serum samples were diluted in two-fold steps, starting at a dilution 1:50 in PBS.

## 2. 8 Purification of monoclonal antibodies by Protein G chromatography

Monoclonal antibodies were purified by Protein G Sepharose 4 Fast Flow (Pharmacia ; Akerstrom et al., 1985) according to the manufacturer's recommendations. The matrix binds all mouse IgGs (IgG1, IgG2a, IgG2b and IgG3) at 5mg antibody per ml of swollen gel. Protein G Sepharose is supplied pre-swollen in 20% ethanol, therefore the gel was washed with binding buffer (20mM sodium phosphate, pH 7.0) to remove the ethanol and then packed into a column and equilibrated with binding buffer at room temperature, using 200 ml of binding buffer at flow rate of 60ml/hour. After the sample was loaded, the column was washed extensively and then bound IgG was eluted with 15 ml of 0.1M glycine-HCl buffer, pH 2.5-3.0. In order to neutralise the eluted IgG solution a few drops of 1M Tris-HCl, pH 9.0 were added and then it was dialysed against PBS.

The purity of the eluted Mabs was analysed by SDS-PAGE (Section 2. 12) after reduction to detect the presence of light (25 kDa) and heavy (50 kDa) chains.

## 2. 9 Passive Immunisation with Mabs and parasite challenge

Mice were passively immunised with purified Mabs to investigate their effect on the course of a parasite infection. Female BALB/c mice from 8 weeks of age bred under specific pathogen-free conditions were obtained from the National Institute for Medical Research SPF Unit. Groups of 3-10 mice were inoculated on three occasions by intraperitoneal injection with purified Mabs (0.5mg/injection; a total of 1.5mg IgG/mouse). The Mabs were administered one day before, one day after and on the day of a challenge infection. The parasite used for challenge was *P. yoelii* YM which causes a lethal fulminating infection in these mice. The parasite stock, stored at -70°C, was thawed and then passaged once in a BALB/c mouse before use. The parasite challenge was administrated by intravenous (i.v.) injection into the lateral vein of the tail, using $5x10^3$ parasitised red blood cells (PRBC) per mouse. Parasitaemia was then assessed daily on blood films stained with Giemsa's reagent.

## 2. 10 Determination of antibody isotype and subclass by ELISA and Immunodiffusion (ID)

The antibody isotype or subclass was determined by double diffusion in agar (Ouchterlony, 1958). A solution of 1% agarose in PBS was prepared and using a pipette, 5ml was applied onto a glass slide. The agar was allowed to solidify, wells were cut out, and then the slide was stored in a humidified chamber at 4°C. Ten microlitres of the Mab (0.5mg/ml) to be tested were placed in a centre well and 5µl of isotype or subclass specific antibodies (Goat anti-Mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) were

placed in surrounding peripheral wells. After 48 hours incubation in the humidified chamber at 4°C, a reaction was detected by the presence of a precipitation band.

The subclasses of the Mabs were confirmed by using a capture ELISA and an indirect ELISA with an isotyping kit from Sigma. The capture ELISA test was carried out according to the manufacturer's recommendations. The subclass specific antibodies were diluted 1:1000 in PBS and 100µl of each were dispensed into 2 wells of a microtitre plate (Immulon 4 from Dynatech) and incubated for 1 hour at 37°C. The plates were washed 3X with PBS/T, then 100µl of each Mab (5µg/ml) to be tested were dispensed into each well and incubated for 1 hour at 37°C. The Mab solution was then removed and the plates were washed. Peroxidase-conjugated Goat anti-Mouse IgG (Fab Specific) antibody was diluted 1:600 in PBS/T and 100µl added to each test sample and the plates were incubated for 30 minutes at 37°C. The conjugate was removed and the plates were washed again. The plates were developed with 100µl of OPD (0.4mg/ml in 0.05M phosphate-citrate buffer, pH 5.0 containing 0.03% sodium perborate), then the reaction was stopped and the Absorbance measured as described in Section 2.7.1.

In the indirect ELISA, Mabs to be tested were diluted to1µg/ml in PBS, dispensed into wells of a microtitre plate, and incubated for 1 hour at 37°C. The coated plates were washed 3X, then subclass specific antibodies diluted 1:1000 in PBS were dispensed to each well (100µl) in duplicate and incubated for 30 minutes at room temperature. The plates were washed again and 100 µl detection conjugate (diluted 1:5000) was added. After a further incubation for 30 minutes at room temperature the plates were washed again. Finally, the substrate was added and after colour development the Absorbance was measured.

## 2. 11 Biotinylation of Mabs and their use in a Competitive ELISA

Mabs were biotinylated using Sulfo-NHS-Biotin (Pierce) as recommended by the manufacturer, for use in a competition ELISA together with an unlabelled second Mab.

To carry out the competition experiments the wells of 96-well microplates (Dynatech Immulon-4) were coated with 1μg/ml of antigen (recombinant MSP-$1_{19}$) as described in Section 2. 7. 1. The excess protein binding sites in the wells were then blocked by the addition of 1% of bovine serum albumin in PBS. One hundred microlitres of a solution of unlabelled Mab were added to each well and incubated for 1 hr at 37°C, the wells were washed 3 times with washing buffer, and then 100 μl of a solution of the biotinylated Mab were added and incubated for 1 h at 37°C. The wells were washed again 3 times and then incubated with 100 μl of 10 μg/ml Streptavidin-Horse Radish Peroxidase conjugate for 1 h at 37 °C. Finally the plates were washed 3 times, followed by addition of 100 μl OPD substrate per well. The colour reaction was allowed to develop and then stopped by the addition of 50 μl of 1M sulphuric acid per well, and the Absorbance was read at 492 nm.

Statistical calculations were performed using GraphPad Prism® software. Comparisons of the mean Absorbance values between the different Mabs evaluated by competition ELISA were made using the Kruskal-Wallis analysis of variance. The Kruskal-Wallis test is a non-parametric test that can be used to compare three or more independent groups. The P value addresses this question: If the populations do have the same median, what is the chance that random sampling would result in medians as far apart as those observed

in the experiment? If the P value is 0.05, it means that there is a 5% chance of observing a difference as large as that observed even if the two population means are identical. Therefore, there is a 95% probability that the difference observed reflects a real difference between populations and a 5% probability that the difference is due to chance. The P value is compared with a threshold value, (usually $P< 0.05$). If the P value is less than the threshold, the difference is statistically significant, but if the P value is greater than the threshold, the difference is not statistically significant (Glanztz, 1997).

**2. 12 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Analysis of the purified recombinant proteins and Mabs was done by SDS-PAGE on 15% or 12.5% polyacrylamide gels (Laemmli, 1970). The protein samples were solubilised in SDS sample buffer (0.15M Tris-Cl, pH 6.8, 4.6% SDS, 23% glycerol, and 0.2M DTT in 0.1% w/v bromophenol blue) and heated at $100^0$C for 5 min. Proteins were then loaded onto an SDS-PAGE gel using 4µg of protein per track. The SDS-PAGE gel consisted of a separating gel (15% w/v acrylamide/bis-acrylamide, 0.37M Tris-Cl, pH8.8, 0.1% SDS, set by addition of 0.3% TEMED, 0.03% ammonium persulphate) with a 3% stacking gel (3% w/v acrylamide/bis-acrylamide, 0.1M Tris-Cl pH 6.8, 0.1% SDS , plus 0.1%TEMED and 0.05% ammonium persulphate). Electrophoresis was carried out at 20 mA for 1 hour using a Mighty Small II vertical slab gel unit (Hoefer Scientific Instruments). Low molecular mass markers obtained from Pharmacia (14,400-97,000 daltons) or prestained high molecular mass markers (14,400-200,000 daltons) from Gibco BRL were used. After electrophoresis the gel was stained with 0.1 % w/v Coomassie Brilliant Blue R-250 (Sigma) in methanol:water:ethanoic acid (5:5:1) and then destained with methanol:water:ethanoic acid (1:17:2).

## 2. 13 Immunoblotting

After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose paper (NCP, Schleicher & Schuell, 0.45 μm pore size) to allow immunodetection of the proteins which could be recognised by the Mabs. The proteins were transferred in a Hoefer TE Series Transphor apparatus at 120 mA, for 1 h at $4^0$C, using 25 mM Tris-HCl, 150 mM glycine, 20% v/v methanol, by the method of Towbin et al. (1979).

After transfer the blots were blocked by incubation with a solution of 3% non-fat milk powder in PBS for 30`

min at room temperature, and were then washed three times (3X) in PBS, containing 0.05% v/v Tween-20 (Sigma) (PBS/T). Blots were then incubated with a solution of Mab (5μg/ml) in PBS/T for 1 hour at room temperature. Blots were then washed 3X, and incubated in a solution of affinity purified goat anti-mouse polyvalent immunoglobulin conjugated to peroxidase, at a 1:1000 dilution (Sigma), for 1 hour at room temperature. Blots were washed again in PBS (3X) and then antibody binding was detected by incubation in a solution consisting of 10 ml of 3 mg /ml 4-chloro-1-napthol (Sigma) in methanol, mixed with 50 ml of 50 mM Tris-HCl pH 7.5, and 30 μl of 30% $H_2O_2$ . The reaction was stopped by washing three times in $H_2O$ (Blackman et al., 1991).

## 2. 14 Immunoprecipitation (IPP)

This technique allows the identification of small amounts of a single protein in a complex mixture, by interaction with antibodies. The biggest advantage of using Mabs

for this method is the specificity of their interaction to allow the precipitation of a single antigen from a radiolabelled mixture of proteins, which can be analysed by polyacrylamide gel electrophoresis and fluorography.

Trophozoites, schizonts and merozoites were obtained from *P. yoelii* infected mouse blood, after passage through a CF-11 column to remove leukocytes (Richards & Williams 1973). The trophozoites and schizonts were isolated by centrifugation through a percoll gradient (the trophozoite and schizont layer was at between 60-70 % percoll). The parasites were washed twice in cysteine and methionine free RPMI 1640 medium, and then incubated for 1 hour at a 10% haematocrit in cysteine and methionine free RPMI medium, containing 2 MBq $ml^{-1}$ TRAN$^{35}$SLABEL (70% [$^{35}$S] L-methionine, 15% [$^{35}$S] L-cysteine, ICN). The supernatant was first harvested by centrifugation at 500 g for 5 min, then the pellet containing the parasitised erythrocytes was washed and solubilised in lysis buffer (50 mM Tris-HCl pH 8.0 containing 5 mM EDTA, 5 mM EGTA and 1 % NP40) (Holder and Freeman,1981).

Five microgrammes of each Mab were added to aliquots of the parasite lysate and incubated for 1h in order to form an immune complex. Then 10µg of rabbit anti-mouse IgG (Serotec) were added, and finally protein A sepharose beads were added and incubated for 1 hour at 4°C. The beads were washed well with 50mM Tris-HCl pH 8.0, 5mM EDTA and 0.5% NP40 and the purified immune-complexes were analysed by SDS-polyacrylamide gel electrophoresis on 10 and 12% polyacrylamide gels. Gels were stained with Coomassie blue, destained, soaked for 15 minutes in Amplify (Amersham) and then vacuum dried on to paper at 80°C and exposed to X-ray film (X-OMAT-AR from Kodak) at -70°C to identify radiolabelled proteins by fluorography.

Immunoprecipitation was used to evaluate the Mabs. A mouse polyclonal serum against recombinant MSP-$1_{19}$ (Ling et al., 1994) and Mab 25.1 (Holder & Freeman, 1981) were used as positive control antibodies and normal mouse serum was used as a negative control.

**2. 15 Preparation of parasite extracts for western blotting**

A group of 10 female BALB/c mice at least 8 weeks-old, were infected by intraperitoneal injection of $10^4$ *P. yoelii* YM parasitised red blood cells. When the parasitaemia was about 30% the mice were sacrificed and parasites were isolated by centrifugation through a 60-80% percoll gradient ( Richards & Williams, 1973). The parasites were solubilised in lysis buffer at 4°C for 1 h and then the solution was centrifuged at 500 g for 30 minutes. The soluble parasite extract was used as antigen in Western blotting (Section 2. 13).

# CHAPTER 3: MONOCLONAL ANTIBODY PRODUCTION AND CHARACTERISATION

## 3.1 Introduction

The asexual development of the malaria parasite in the blood of the vertebrate host is characterised by cyclic growth and multiplication within the erythrocyte. MSP-1 is synthesised as a large protein precursor during the later stage of parasite development in the erythrocyte known as schizogony, and is proteolytically cleaved into a complex of smaller polypeptide fragments found on the merozoite surface. The C-terminal 19kDa fragment of MSP-1 is the only fragment retained on the surface and carried into the erythrocyte at invasion. MSP-1 is one the most prominent candidates for a vaccine against the asexual blood stage; based on experiments conducted using the human malaria parasite *P. falciparum* and other species such as the simian parasite *P. knowlesi* (David et al., 1984) and the rodent parasite *P. chabaudi* (Lew et al., 1989; O'Dea et al., 1995). The C-terminal fragment is in particular the focus of vaccine design (Holder, 1988; Cooper, 1993; Daly & Long, 1995) since it is the most logical target for blocking erythrocyte invasion (Locher et al., 1996). Epitopes within the C-terminal region that are recognised by Mabs may be important in the induction of a protective humoral response during malaria infection.

Polyclonal and monoclonal antibodies specific for the 42 and 19 kDa fragments of MSP-1 inhibit parasite growth *in vitro* (Blackman, et al., 1991; Pirson & Perkins, 1985; Chang, et al., 1992; Chappel & Holder, 1993), but only a limited number of studies have investigated whether or not Mabs can provide passive immunity *in vivo* (Majarian et al.

1984). Recently, the C-terminal portion of *P. yoelii* MSP-1 (the 19kDa fragment) was produced in a bacteria expression system as a fusion protein with glutathione S-transferase. Immunisation with this fragment (containing both EGF-like modules) protected mice against lethal parasite infection (Daly & Long, 1993; Ling et al., 1994). There are 10 highly conserved cysteine residues within the two EGF-like motifs of MSP-$1_{19}$ (Daly *et al.*, 1992) which are predicted to form 5 disulphide bonds that stabilise the formation of a complex three-dimensional structure. It has been shown that protein that is unfolded by reduction and alkylation is unable to induce protective immunity (Ling et al., 1994). Consequently a recombinant MSP-$1_{19}$, which is suitable for vaccination of mice, has to be correctly folded.

Study of the immunological response to immunisation with the C-terminal region of MSP-1 and a detailed characterisation of the epitopes within this region will provide a greater understanding of the protective immune response to this antigen and of its function. In this study Mabs were produced and characterised to define potential protective epitopes.

### 3.2 Purification of various recombinant MSP-1 proteins for analysis of the Mabs

In order to characterise the monoclonal antibodies for reactivity with the 19kDa fragment of MSP-1, three recombinant proteins were produced and used in western blotting experiments. The first, second and combined EGF-like motifs of MSP-1 were expressed in *E. coli* as fusion proteins with glutathione S-transferase (GST-MSP1EGF1, GST-MSP1EGF2 and GST-MSP$1_{19}$, respectively). These fusion proteins were purified from bacterial lysates by glutathione-affinity chromatography. The eluted fractions were

monitored by SDS-PAGE on15% polyacrylamide gels and those containing the highest concentration of each protein were collected, pooled and dialysed against PBS . In this way the three different proteins were purified (see Figures 3.2, 3.3 & 3.4), together with the GST carrier (Figure 3.5). Figure 3.6 shows an analysis on a 12.5% SDS-PAGE gel of the purified recombinant proteins after glutathione-affinity chromatography.

Recombinant MSP-$1_{19}$, cleaved from the GST carrier with Factor Xa, was also produced. As shown in Figure 3.7, the MSP-1 sequence was released in soluble form after cleavage by digestion with blood coagulation Factor Xa.

### 3.3 Selection of hybridomas secreting MSP-1-specific monoclonal antibodies

In an initial study, five BALB/c mice were immunised with MSP-$1_{19}$ recombinant protein at two weekly intervals. A total of five i.p. injections (0.13mg/ml of antigen for each immunisation) were given. For primary immunisation, Freund's complete adjuvant (FCA) was used to emulsify the antigen and subsequent immunisation was conducted using Freund's incomplete adjuvant (FIA). Prior to removal of the spleens for hybridoma production the antibody titres of sera obtained from tail vein bleeds of mice were determined by IFA. Mice were boosted until the antibody titres of the sera were at least 1:3200 (Harlow & Lane, 1988) (see Chapter 2). The spleens of these BALB/c mice that were immunised with the recombinant protein MSP-$1_{19}$ were removed and the cells fused with SP2/O-Ag14 myeloma cells. MSP-1-specific Mabs were initially identified by screening hybridoma supernatants using an ELISA with 1µg/ml affinity-purified MSP-$1_{19}$. Two hybridoma cell lines that produced antibodies reactive with the recombinant MSP-$1_{19}$ were obtained and named B10 and G3.

A second set of hybridomas was made using the spleens of mice which had survived infection with *P. yoelii* YM. Mice from a passive immunisation experiment in which the animals received either Mab B10 or G3 and then were challenged with parasitised erythrocytes, were subsequently inoculated with 5 x $10^8$ parasitised red blood cells on three occasions and the spleens harvested 7 days later. Figure 3.1 shows the immunisation protocol. This additional fusion experiment resulted in the production of several hybridomas, including P2-F5, P6-B6, P3-D3, and P5-D9. Positive antibody reactions obtained by using ELISA were checked for all the monoclonal antibodies by using IFA. One hundred and four of three thousand hybrid cell wells produced antibodies reactive with recombinant MSP-$1_{19}$ in ELISA and of these a small number of hybridoma supernatants gave a positive result by IFA. Each one of the Mabs showed a characteristic "bunch of grapes" staining pattern with the *P. yoelii* infected erythrocytes, corresponding to merozoites in schizont infected red blood cells.

I have produced a panel of twelve hybridoma cell lines that secrete antibody specific for *P. yoelii* MSP-1. Hybridomas B10 and G3 were produced after immunisation with the recombinant GST-MSP$1_{19}$. The other hybridomas were produced from the spleens of mice which survived the parasite infection after passive immunisation with either Mab B10 or G3. The antibodies from only six hybridoma cell lines (B10, G3, P2-F5, P6-B6, P3-D3, P5-D9) have been characterised in detail by a number of different techniques, and were chosen based on initial passive immunisation studies which indicated that these antibodies were fully or partially protective against a blood stage parasite challenge (Chapter 4).

### 3.4 Purification of Mabs by Protein G chromatography

The individual Mabs were purified before being characterised in detail, and for this purpose affinity chromatography on Protein G-sepharose was used. Protein G is an immunoglobulin G (IgG)-binding bacterial cell wall protein isolated from group G streptococci. The avidity of protein G for various monoclonal and polyclonal antibodies of the IgG class is greater than that of protein A. Protein G binds all monoclonal IgG from mouse: IgG1, IgG2a, IgG2b and IgG3, and rat: IgG2a, IgG2b, and IgG2c. In addition, it has been shown to bind polyclonal IgG from a variety of mammals, including human, cow, rabbit, goat, rat, and mouse antibodies, whereas chicken IgG does not bind. (Akerstrom et al. 1985).

To produce large amounts of the Mabs, the hybridomas were grown in vitro and the secreted antibody purified from the culture medium. The medium from the cultured cells was harvested and concentrated by a factor of approximately 10, before being passed through a Protein G-sepharose 4 Fast Flow column to purify the individual antibodies. The bound antibody was eluted from the column, dialysed, and analysed by SDS-PAGE for purity. Figure 3.8 shows an analysis of the different purified Mabs, using SDS-PAGE under reducing conditions (15% polyacrylamide) and detecting the proteins with coomassie blue. For each sample the component heavy (~50kDa) and light (~25kDa) chains of the IgG molecules are visible.

SDS-PAGE analysis was also used to discriminate between two Mabs of similar properties. In mouse the light chain exists in two distinct forms called kappa and

lambda. The different light chain types may combine with any of the heavy chain types, but in any one molecule both light chains are of the same type (Roitt et al. 1993). Two antibodies, P6-B6 and P2-F5 had similar biological activity, providing protection against challenge infection (see Chapter 4) and were of the same subclass (IgG3, see below), however they could be distinguished from one another by the mobility of the respective light chain in SDS-PAGE electrophoresis (see Figure 3.9). This result indicates that these Mabs are the products of two distinct different hybridoma clones.

### 3.5 Determination of Mab subclass

The IgG subclasses of the purified antibodies were determined by immuno-diffusion in agar using rabbit anti-mouse Igs, and by capture ELISA, using the methods described in Chapter 2. The results are given in Table 3.1 which shows the determination of the subclass of the 12 Mabs which were analysed. The cloned hybridoma cells produced IgG from each of the subclasses. The subclass of each antibody will be discussed in the next chapter in the context of the results from the passive immunisation studies.

### 3. 6 Indirect Immunofluorescence assay identifies two antibody specificities

The ability of the purified Mabs to react with the parasite was confirmed using the IFA test. Hybridoma cell lines had been selected by two assays to analyse the specificity of their secreted Ig: ELISA with the recombinant MSP-$1_{19}$, and immunofluorescence on methanol-fixed smears of parasitised erythrocytes. The Mabs produced by the cloned cell lines were again tested by IFA (Pirson & Perkins, 1985).

In the re-analysis of six Mabs, two different patterns of reactivity were noted. Mabs B10, G3, P2-F5, P6-B6 stained schizont, merozoite and ring forms (see Figures 3.10, 3.11, 3.12, and 3.13). However, the Mabs P3-D3 and P6-B4 produced a slightly different pattern, they stained around the schizont and merozoite surface, but did not react with ring forms (see Figures 3.14 and 3.15 respectively). In *P. falciparum* the C-terminal 19kDa fragment of MSP-1 is the only part of the molecule that remains on the merozoite during invasion and can be detected in ring stage parasites (Blackman et al., 1990). By analogy, it is likely that the first group of four antibodies is directed against the equivalent part of *P. yoelii* MSP-1, whereas the second group of two antibodies is against another part of the molecule (see below for a more detailed analysis of the epitopes for these antibodies). In view of these findings, it is difficult to explain why these two hybridomas were selected, since the initial selection was for those that secreted antibody that reacted with MSP-$1_{19}$ in ELISA.

### 3.7 Immunoprecipitation (IPP) of MSP-1 from extracts of parasites

Detergent extracts of merozoites and schizonts, radiolabelled with $^{35}$S-methionine/cysteine, were mixed with individual Mabs, as well as normal mouse serum and an anti-serum to recombinant *P. yoelii* MSP-$1_{19}$ (Ling et al., 1994) as negative and positive controls, respectively. The immune complexes formed were precipitated and then analysed by SDS-PAGE (12.5% polyacrylamide gel) under reducing conditions. In this immunoprecipitation all Mabs recognised a major band of 230kDa in lysates from schizonts (Figs. 3.16 and 3.17), which was also recognised by Mab 25.1 (Holder & Freeman, 1981) and a polyclonal serum specific for MSP-$1_{19}$ which were used as positive controls. MSP-1 has been shown to be cleaved to a series of lower molecular

mass polypeptides and therefore the other polypeptides in these tracks may represent some of these fragments.

### 3.8 Use of immunoblotting with recombinant proteins and parasite extracts to define the antibody binding sites

To confirm the reactivity of the Mabs with MSP-1, and identify more precisely the epitopes they recognise, the ability of the antibodies to bind to recombinant proteins expressed from parts of the *MSP-1* gene was investigated by western blotting. Recombinant proteins (GST-MSP1$_{19}$, GST-MSP1EGF1, GST-MSP1EGF2 and GST-MSP-1$_{33}$ ), or parasitised erythrocytes extracted with 50mM Tris-HCl pH 8.2, 5mM EDTA, 0.5% w/v NP-40, were denatured in SDS-PAGE sample buffer, resolved by PAGE on 12.5% polyacrylamide gels and then electrophoretically transferred to nitrocellulose. The purification of the recombinant proteins based on MSP-1$_{19}$ was described in Section 3.2 (see Figure 3.6).The results of these experiments are shown in Figures 3.18, 3.19 and 3.20.

Mabs B10, G3, P2-F5 and P6-B6 all bound to GST-MSP1$_{19}$ , indicating that they recognise epitopes in the C-terminal cysteine-rich region of MSP-1. None of the Mabs bound to GST-MSP1EGF2, but both P2-F5 and P6-B6 bound to GST-MSP1EGF1. This reactivity was abolished if the recombinant proteins were reduced and alkylated with iodoacetic acid before SDS-PAGE, a treatment that prevents refolding of the proteins after electrophoresis and destroys epitopes that require disulphide bonds for their maintenance (Burghaus & Holder, 1994). These results suggest that B10 and G3 recognise epitopes that require both EGF-like motifs to be present in the recombinant

protein. The epitopes recognised are formed when both motifs are expressed together and these epitopes could contain amino acids from both motifs or the precise sequence configuration required for antibody binding in one motif is constrained by the presence of the other one. This observation also has been made with Mabs to *P. falciparum* MSP-1, such as Mabs 12.10 and 111.2 (Chappel & Holder, 1993; Burghaus & Holder, 1994).

Mabs P6-B4 and P3-D3 did not bind to GST-MSP1$_{19}$, GST-MSP1EGF1 or GST-MSP1EGF2, although both Mabs recognised MSP-1 by immunoprecipitation (Fig. 3.16). These are the antibodies that did not react with the ring stage parasites by immunofluorescence (Section 3.4). The epitope of Mab P6-B4 was mapped to MSP-1 sequence immediately N-terminal to MSP-1$_{19}$, corresponding to the 33kDa fragment of *P. falciparum* MSP-1 that is produced as a result of secondary processing (Blackman et al., 1993). A new recombinant protein corresponding to a large part of the predicted *P. yoelii* MSP-1$_{33}$ fused to GST was used to map the epitope of Mab P6-B4 (Fig 3.21). The recombinant protein contains the amino acid sequence corresponding to residues 1394 to 1633 in the *P. yoelii* MSP1 sequence (Lewis, 1989). The other Mabs did not bind to the recombinant GST-MSP-1$_{33}$ protein in Western blotting (data not shown).

Western blotting was also performed with extracts of parasites to try to identify fragments of MSP-1 that reacted with Mab P3-D3. This Mab appeared to recognise a 42kDa fragment of MSP-1 in merozoite extracts (Fig 3.22). The same polypeptide was recognised by the other Mabs. Since Mab P3-D3 seems to recognise the C-terminal 42kDa fragment of MSP-1 by western blotting, but not GST-MSP1$_{19}$ or GST-MSP1$_{33}$

that comprise this fragment, it is possible that the epitope recognised by this antibody requires the larger conformational structure for formation.

### 3. 9 Evaluation of the fine specificity of Mab binding to MSP-$1_{19}$ by competition ELISA

Four monoclonal antibodies appeared to bind to the C-terminal MSP-$1_{19}$ sequence by western blotting (Section 3.6) , therefore the fine specificity of this binding was analysed in more detail by competition ELISA (Table 3.2). In this assay the antigen (recombinant MSP-$1_{19}$) was bound to a microtitre plate and the ability of an unlabelled antibody to block the binding of a labelled antibody was determined by titration.

The purified Mabs were biotinylated and the titre of each was measured by serial dilution to establish the proper working dilution at which to use the antibody in the competition ELISA. Table 3.2 shows the titres of biotinylated Mabs and the working dilution used for each Mab in the competition ELISA. As expected from the western blotting studies, B10, G3, P2-F5 and P6-B6 reacted with the recombinant MSP-$1_{19}$, whereas P3-D3 did not. Nevertheless this antibody was included in the experiments as a control preparation. To analyse the competitive ELISA results, the minimum significant Absorbance value for each antibody was established from the Mean + 2 Standard Deviations of the Absorbance of the Mab inhibited by itself. Data from the competition ELISA between B10 and G3, and P6-B6 and F2-F5 were analysed using the Kruskal-

Wallis statistical test (Figures 3.23, 3.24, 3.25 and 3.26) with a values of P = 0.04, 0.03, 0.04 and 0.01 for Mabs P2-F5, P6-B6, B10 and G3 respectively.

Figures 3.23 and 3.24 show the mean Absorbance values obtained in the competition ELISA experiments, using biotinylated P2-F5 and P6-B6. The results shown in Figure 3.23 indicate that the mean Absorbance value with the biotinylated Mab P2-F5 was lower when the Mab P6-B6 was used as the primary antibody when compared with the control (non-biotinylated P2-F5 vs. biotinylated P2-F5). This result suggests that P6-B6 interferes with the binding of P2-F5. This effect was not observed when Mabs P3-D3, B10, G3 and P6-B4 were used as primary antibodies as shown in Figure 3.23, i.e. in these cases the mean Absorbance values for the biotinylated Mab P2-F5 were greater than in the control. These differences were statistically significant (P=0.04) by the Kruskal-Wallis test. Figure 3.24 shows a similar experiment using biotinylated P6-B6. In this case the mean Absorbance value for the biotinylated antibody was greater than the control (non-biotinylated P6-B6 vs. biotinylated P6-B6) when P2-F5 was used as the primary antibody, suggesting that the binding of P2-F5 did not affect the binding of P6-B6. Similar results were observed with all the other non-labelled Mabs employed (P=0.03, Kruskal-Wallis test). These data indicate that the epitopes recognised by these Mabs are different from the one recognised by P6-B6. Similar analyses were carried out for each of the Mabs included in this study. Mab G3 competed with B10, although B10 was not able to compete effectively with G3 (Figures 3.25 and 3.26).

The results of the competition ELISA can be summarised as follows and are presented in Table 3.3. Mab P6-B6 competed with P2-F5, but not with any of the other antibodies, although P2-F5 was not able to compete effectively with P6-B6 or with any of the other

antibodies. Mab G3 competed for binding with Mab B10 but not with any of the other antibodies. Mab B10 did not compete with G3 or with any of the other antibodies. Apparently, therefore, each pair of Mabs, G3 and B10 and P6-B6 and P2-F5 do not recognise identical epitopes, because there is competition in only one direction, i.e. when the Mabs G3 or P6-B6 are initially bound, but not in the opposite case when B10 or P2-F5 were used as the primary antibody. These results suggest that the epitopes recognised by the Mabs G3 and P6-B6 are adjacent to the ones recognised by Mabs B10 and P2-F5, respectively. It is feasible that the initial binding of G3 and P6-B6 cause a steric effect or the induction of a conformational change in the antigen, affecting the binding of Mabs B10 and P2-F5. In other words, the binding of the second Mab (B10 or P2-F5) may depend on the arrangement of the first Mab (G3 or P6-B6) or the precise configuration of the MSP-$1_{19}$ molecule. As expected from the western blot data, P3-D3 and P6-B4 did not bind to MSP-$1_{19}$ . These competition ELISA results are consistent with the results of the western blotting studies, suggesting that the epitopes for B10 and G3 are very close to one another and different from the epitopes for P6-B6 and P2-F5, which also appear to be close to each other.

### 3.10 Discussion.

In the present investigation, I have produced a number of hybridomas and characterised their antibody products. In initial studies the mice were first immunised with a recombinant MSP-$1_{19}$ protein, based on the observation that mice immunised with the recombinant *P. yoelii* MSP-$1_{19}$ could elicit a protective antibody response (Daly & Long, 1993; Ling, et al., 1994). However, a low frequency of hybridomas that produced MSP-1-specific Mabs which were biologically active (see Chapter 4), was obtained.

Therefore, to try to increase the chance of isolating hybridomas that secreted biologically active antibody, a different immunisation regime was employed. Mice that had been passively immunised with Mab B10 and G3 and had survived the challenge infection, were inoculated with live parasitised erythrocytes to boost the immune response and then the spleens were removed and used for hybridoma production.

In the initial assay for hybridomas of interest a two stage screen consisting of an ELISA with MSP-$1_{19}$ followed by IFA with parasitised erythrocytes was developed. The ELISA was used as the first stage of the screen because of the ease of this format for screening large numbers of cell supernatants for the presence of specific antibody. However, it was found that a large number of false positive results were obtained, and these supernatants did not contain antibody that reacted with the parasite (as assessed by IFA). This was possibly due to contaminants (for example of bacterial cell origin) in the MSP-$1_{19}$ material that was used for the immunisation and the ELISA. However the proteins appeared to be substantially pure as assessed by SDS-PAGE. Another explanation could be that the recombinant protein (or a fraction of it) folds incorrectly and therefore presents epitopes that are not present in the native protein. This second explanation is probably the most likely, but no attempt was made to fractionate either GST-MSP$1_{19}$ or the recombinant MSP-$1_{19}$, for example by HPLC, to test whether or not there were different conformational forms of the protein present in the preparations. IFA was used for the second stage of the screen. This technique has the advantage that it detects native parasite antigens, but it is not convenient for screening large numbers of hybridoma culture supernatants, and although the pattern of fluorescence gives some clue to the location of the antigen and therefore its possible identity, it does not allow the identification of specific parasite proteins. A combination of the two assays was

expected to provide a good screen to identify monoclonal antibodies of the desired specificity. Despite the use of this screen, two hybridomas were identified that produced MSP-1-specific antibodies that did not bind directly to MSP-$1_{19}$.

The results of the IFA and immunoprecipitation studies with the purified Mabs indicate that they recognise epitopes on the 230kDa *P. yoelii* MSP-1 precursor protein (Holder & Freeman 1984). Previous studies using immunoprecipitation of metabolically labelled polypeptides and western blotting showed that the precursor protein can be processed into a series of smaller fragments (Holder & Freeman, 1981; 1984). Two distinct patterns of reactivity were seen in the IFA, despite the fact that the antibodies were all against MSP-$1_{19}$: some antibodies reacted with schizonts and merozoites, but others reacted with early ring stages, in addition to the late stage parasites. This difference can be explained by the processing of MSP-1 that occurs at or just before invasion. Mabs which recognise MSP-$1_{19}$, the C-terminus of MSP-1, will react with ring stages because this part of MSP-1 is retained on the parasite surface during erythrocyte invasion, whereas Mabs that bind to epitopes elsewhere in the molecule would not react with ring stages because the rest of the molecule is shed from the parasite surface at the time of invasion.

Data obtained from the Western blotting experiments indicated that the epitopes recognised by some of the Mabs were expressed by the recombinant proteins. The epitopes for four of the antibodies were located in the C-terminal cysteine rich EGF-like motifs of MSP-1. After electrophoresis of the proteins that were untreated or had been reduced with dithiothreitol, they reacted with the antibodies. However, if the reduction was followed by alkylation to modify any free -SH groups and prevent the reformation

of disulphide bonds occurring after the transfer of the proteins to nitro-cellulose, then the binding of the antibodies was abolished. It is also possible that the presence of the alkyl groups (-$CH_2COOH$) on the cysteine side chains could interfere sterically with antibody binding. However, MSP-$1_{19}$ appears to contain the epitopes that are recognised by Mabs B10, G3, P2-F5 and P6-B6. Furthermore, the epitopes recognised by P2-F5 and P6-B6 were found to be located in the first EGF-like motif, whereas G3 and B10 required the presence of both motifs for reactivity. The epitopes appear to require the presence of disulphide bonds for their maintenance; in the same way, Mab 302 obtained by other investigators (Majarian *et al.*, 1984) is also dependent on the presence of disulphide bridges at the C-terminus of *P. yoelii* MSP-1. The structure of extracellular proteins is often stabilised by the presence of disulphide bonds to maintain their conformational form, which is frequently necessary for efficient protein-protein interactions and required for maintenance of biological activity. In this respect the recombinant proteins show the same properties as the natural molecules (Burns et al., 1989; Burghaus & Holder, 1994). Probably there is a strong selective pressure to maintain the structure and implied function in this region of the MSP-1 molecule because the cysteine-rich region of EGF-like motifs and the proteolytic cleavage sites are conserved in MSP-1 in different Plasmodium species and strains (Burns et al., 1989; Daly et al., 1992; Kang & Long, 1995; Tolle et al., 1995). The available Mabs may also be used to define the epitopes most important in the immune response . This question is addressed in the next chapter, describing passive immunisation trials with these Mabs.

Two Mabs, P6-B4 and P3-D3, did not react with the recombinant proteins corresponding to MSP-$1_{19}$ or the individual EGF-like motifs. Another recombinant protein corresponding to MSP-$1_{33}$ was used to locate the epitope recognised by Mab P6-

B4 to this fragment. Mab P3-D3 (which was protective in passive immunisation studies, see next chapter) did not recognise any recombinant proteins by Western blotting. However it did recognise a band of 42kDa in a western blot using a parasite extract, and this band was also recognised by the other MSP-1-specific antibodies that reacted with the C-terminus. Therefore, it is possible to deduce that the epitope recognised by P3-D3 is on the 42 kDa C-terminal fragment of MSP-1. The fact that this antibody did not react with the recombinant polypeptides suggests that the full length 42kDa polypeptide is required to form the epitope. However, because the recombinant GST-MSP-$1_{33}$ is not quite full length it is possible that the epitope for P3-D3 is at one end of the linear polypeptide sequence. If the full length polypeptide is required, this may be because amino acids from both the 33kDa and 19kDa regions contribute to the antibody recognition site, or the conformation of the polypeptide is changed as a result of the secondary processing. Peptide bond cleavage, for example that which occurs in primary and secondary processing of MSP-1, can both create and destroy epitopes. There is the intriguing possibility that the P3-D3 epitope is formed by the secondary processing site. Since there is good evidence in *P. falciparum* that inhibition of secondary processing can be mediated by antibodies binding to MSP-$1_{19}$ and that this correlates with inhibition of invasion (Blackman et al., 1994), the fine specificity of P3-D3 is of great interest.

In this study the fine specificity of the antibodies that bind to epitopes on MSP-$1_{19}$ was investigated by using a competition ELISA. This test is based on the inhibition of binding of one Mab by another that binds to an epitope that is either identical, overlapping or adjacent. My results with the competition ELISA confirmed the results of the western blot study showing that there are two groups: G3/B10 and P6-B6/P2-F5, and suggest that there may be a steric blocking between Mabs G3/B10 and P6-B6/P2-F5 by the

recognition of adjacent epitopes. It is also possible that if the affinity of the antibodies within the two groups are widely different, and then this type of one-way competition could occur, in which one high affinity antibody displaces a bound lower affinity antibody.

The summary of the proposed interaction between Mabs and MSP-1 is shown in Figure 3.27. This displays MSP-1 and the fragments obtained after proteolytic cleavage (based on the model developed for *P. falciparum* [Blackman et al., 1990; David et al., 1984; O'Dea et al., 1995; Chappel & Holder, 1993; Farley & Long, 1995]) and the suggested location of the epitopes for the antibodies. In total, 6 Mabs were assessed in detail and they bound to different sites at the C-terminus of MSP-1. Mabs B10 and G3 recognise the double EGF-like motifs of MSP-$1_{19}$. Mabs P2-F5 and P6-B6 recognise an epitope within the first EGF-like motif. Mab P6-B4 recognises an epitope in MSP-$1_{33}$ at the N-terminus of MSP-$1_{42}$ and P3-D3 appears to require the complete 42kDa polypeptide to bind. In the next chapter I describe the results of passive immunisation with these antibodies.

**Table 3.1.**

Subclass analysis of the monoclonal antibodies.

| Mab | Subclass |
|---|---|
| P4-D10 | IgG1 |
| P1-C5 | IgG1 |
| P8-D1 | IgG1 |
| P1-A9 | IgG2a |
| P8-D4 | IgG2b |
| P5-D9 | IgG2b |
| P6-B4 | IgG1 |
| P3-D3 | IgG2a |
| P2-F5 | IgG3 |
| P2-F5/P | IgG3 |
| P2-F2/S | IgG3 |
| P6-B6 | IgG3 |
| B10 | IgG2b |
| G3 | IgG1 |

**Table 3.2.**

Titres for biotinylated Mab binding to MSP-$1_{19}$.

| Biotinylated Mab | Titre | Working dilution |
|---|---|---|
| B10 | 1:25,600 | 1:10,000 |
| G3 | 1:25,600 | 1:10,000 |
| P2-F5 | 1:25,600 | 1:10,000 |
| P6-B6 | 1:25,600 | 1:10,000 |
| P3-D3 | 1:200 | 1:100 |

**Table 3.3.**

Comparison of the different Mabs by competition ELISA.

| | Labelled Mab | | | | |
|---|---|---|---|---|---|
| non-Labelled Mab | B10 | G3 | P2-F5 | P6-B6 | P3-D3 |
| B10 | 0 | - | - | - | - |
| G3 | + | 0 | - | - | - |
| P2-F5 | - | - | 0 | - | - |
| P6-B6 | - | - | + | 0 | - |
| P3-D3 | - | - | - | - | 0 |
| P6-B4 | - | - | - | - | - |
| PBS | - | - | - | - | - |

0: The Absorbance value in the competition ELISA corresponds to the value of the non-biotinylated Mab competing with itself.

+: Inhibition of binding of the biotinylated Mab to MSP-$1_{19}$ by the non-labelled Mab.

-: No inhibition of binding of the biotinylated Mab to MSP-$1_{19}$ by the non-labelled Mab.

These results were completely reproducible in three separate experiments.

**Figure 3.1.**

The immunisation protocol used to generate the second set of hybridomas. Mice that had been protected against challenge infection with the lethal parasite *P. yoelii* YM, by passive immunisation with either Mab B10 or G3, were inoculated with parasites on three occasions prior to harvesting the spleens. On each occasion the mice received 5 x $10^8$ parasitised red blood cells containing *P.* yoelii YM.

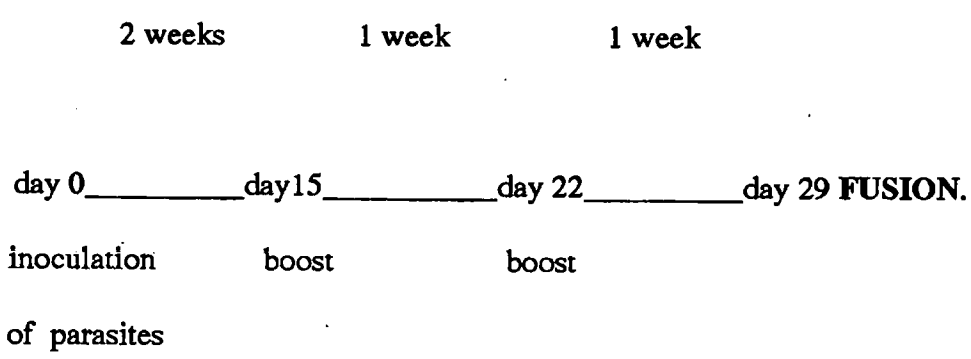


**Figure 3.2.**

SDS-PAGE analysis of purified GST-MSP1EGF1 recombinant fusion protein eluted with 8M urea from a glutathione-agarose column. Fractions containing the purified 35 kDa protein (fractions 6 to 9) were pooled and dialysed against PBS. This protein contains the first EGF-like motif from MSP-1.

On this 15 % polyacrylamide gel, the standard molecular mass marker proteins indicated are: phosphorylase b (97 kDa), bovine serum albumin (66 kDa) , ovalbumin (47 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa).

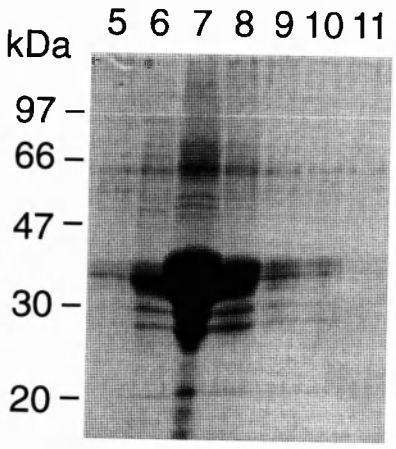



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**Figure 3.3.**

SDS-PAGE analysis of purified GST-MSP1EGF2 recombinant fusion protein eluted with 8M urea from a glutathione-agarose column. Fractions containing the purified 32 kDa protein (fractions 6 to 8) were pooled and dialysed against PBS. This protein contains the second EGF-like motif from MSP-1. On this 15 % polyacrylamide gel, the marker proteins are as indicated in Figure 3.2.

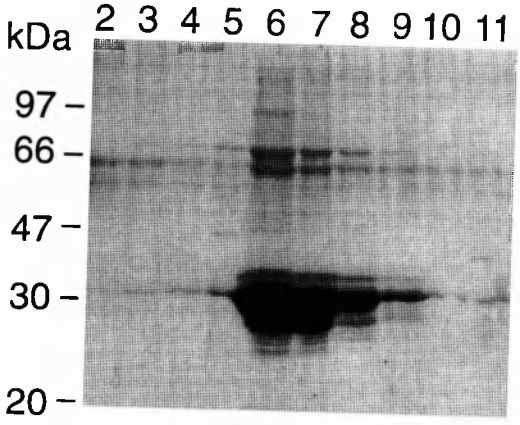


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**Figure 3.4.**

SDS-PAGE analysis of purified GST-$MSP1_{19}$ recombinant fusion protein eluted with 8M urea from a glutathione-agarose column. Fractions containing the purified ~40 kDa protein (fractions 3 to 6) were pooled and dialysed against PBS. This protein contains both the EGF-like motifs from MSP-1. On this 15 % polyacrylamide gel, the marker proteins are as indicated in Figure 3.2.

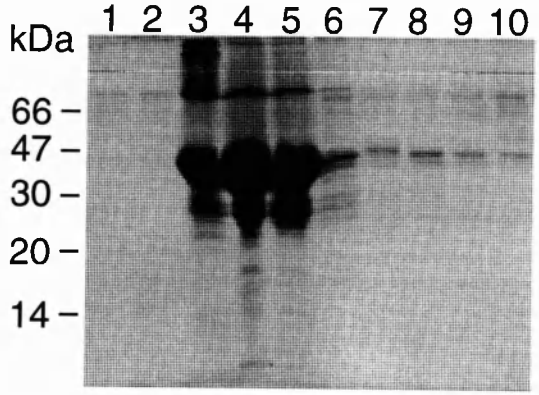


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**Figure 3.5.**

SDS-PAGE analysis of purified GST recombinant protein eluted with 8M urea from a glutathione-agarose column. Fractions containing the purified 26 kDa protein (fractions 5 to 8) were pooled and dialysed against PBS. On this 15 % polyacrylamide gel, the marker proteins are as indicated in Figure 3.2.

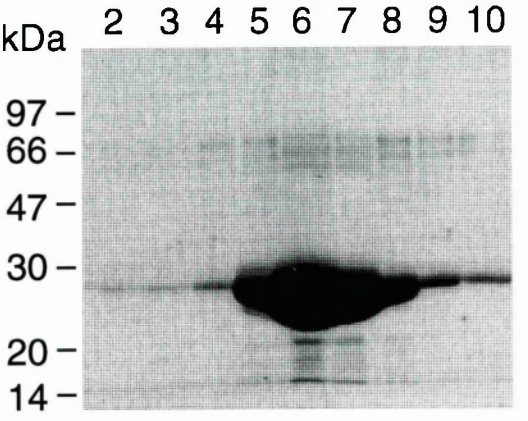


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**Figure 3.6.**

Purified recombinant GST fusion proteins eluted from glutathione-agarose columns and resolved by SDS-PAGE on a 12.5 % polyacrylamide gel. In track 1 is the 26 kDa GST; track 2, the 32 kDa GST-MSP1EGF1 fusion protein containing the first EGF-like motif of MSP-1; track 3, the 35kDa GST-MSP1EGF2 fusion protein containing the second EGF-like motif of MSP-1; and tracks 4 and 5, the 40 kDa GST-$MSP1_{19}$ fusion protein containing both EGF-like motifs of MSP-1. On the left half of the figure the proteins were reduced with dithiothreitol prior to electrophoresis, and on the right half they were not reduced.

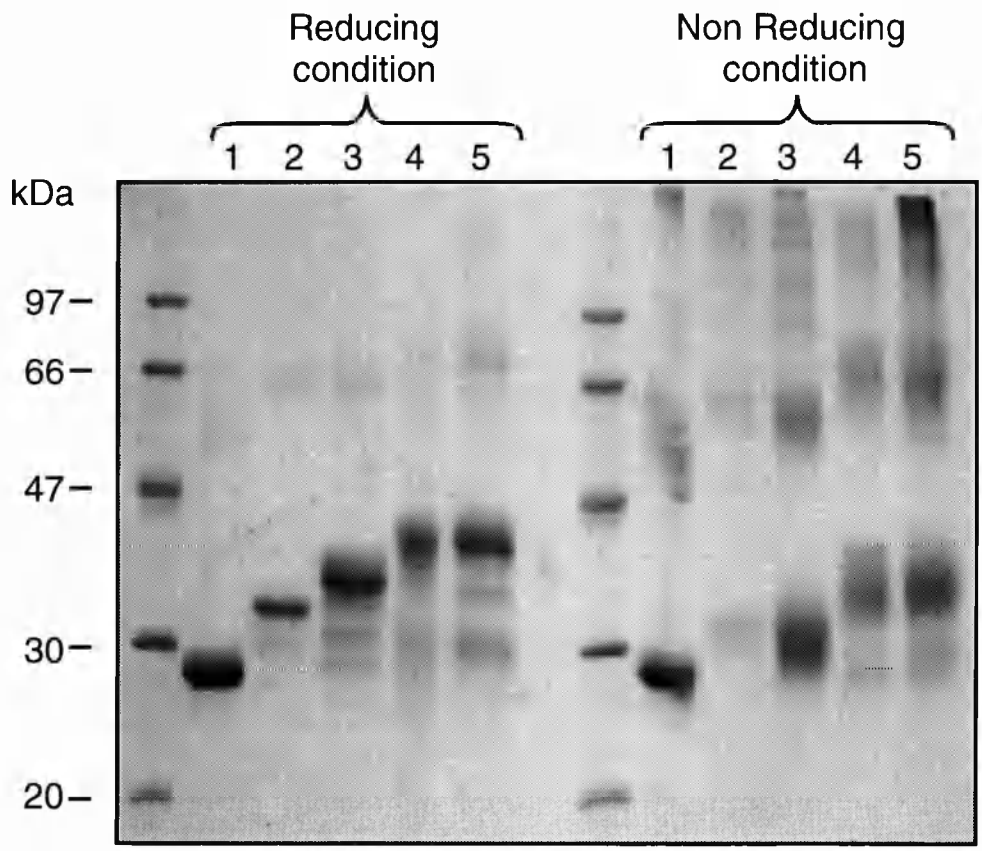


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**Figure 3.7.**

SDS-PAGE analysis on a 15% polyacrylamide gel of recombinant MSP-$1_{19}$ protein after release from the GST-MSP$1_{19}$ fusion protein by digestion with Factor Xa. In Track A can be seen recombinant MSP-$1_{19}$ together with GST, and in track B can be seen the purified ~20kDa MSP-$1_{19}$ protein. The molecular mass markers are as in figure 3.2.

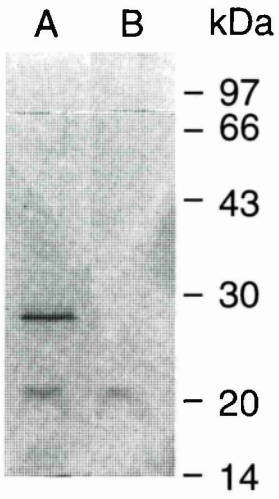


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**Figure 3.8.**

SDS-PAGE analysis (under reducing conditions) of Mabs purified by protein G affinity chromatography: Mab B10 (track 1), Mab P3-D3 (Track 2), Mab P2-F5 (track 3), Mab P6-B6 (track 4), Mab G3 (track 5), Mab P6-B4 (track 6), Mab P5-D9 (track 7) and Mab P1-C5 (track 8). The positions of standard molecular mass markers is indicated on the left. The IgG heavy and light chains at ~50kDa and ~25kDa, respectively, are indicated on the right.

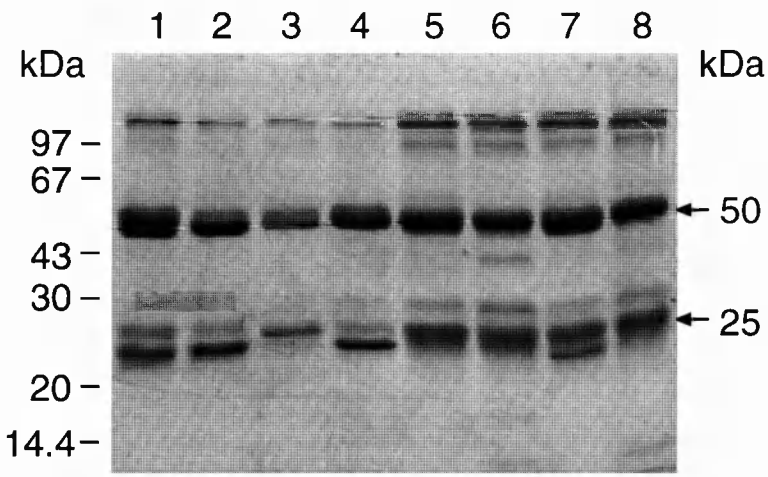


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**Figure 3.9.**

The monoclonal antibodies P2-F5 and P6-B6 were compared by SDS-PAGE on 15 % polyacrylamide gels. The light chain (~25 kDa) and heavy chain (~50 kDa) can be seen for both Mab P2-F5 (track 1) and Mab P6-B6 (track 2), and are indicated. Note difference in size of the light chain from the two Mabs. The positions of standard molecular mass markers is indicated on the left.

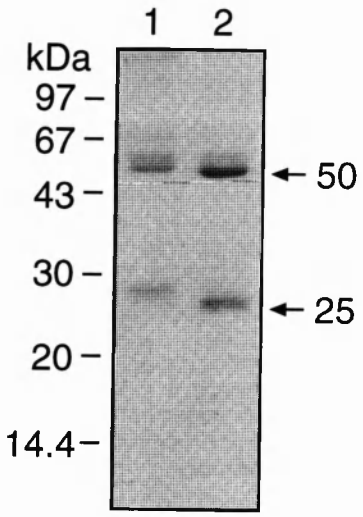


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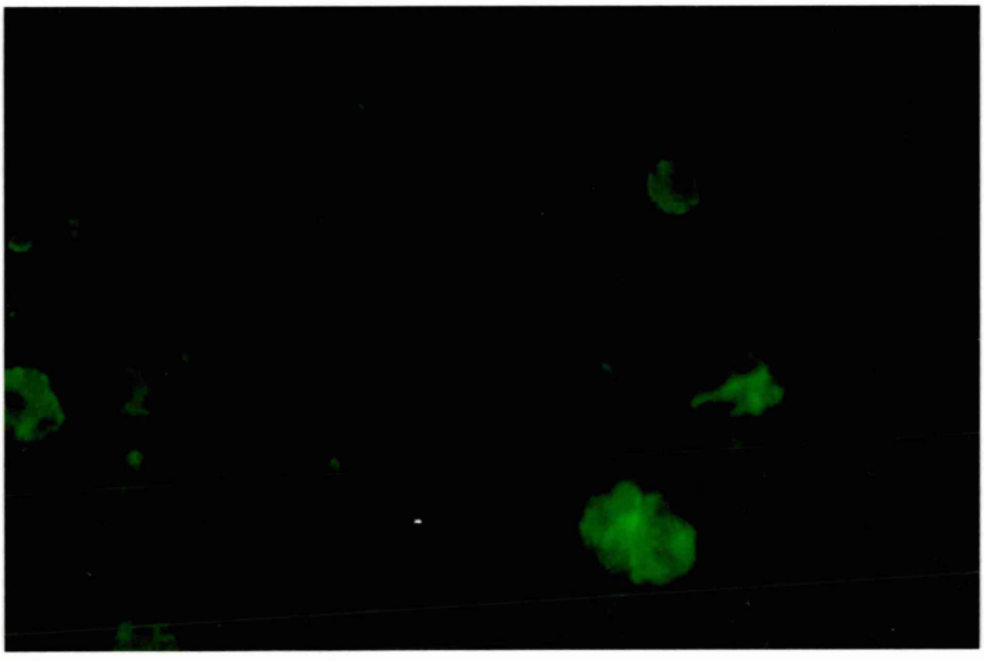
**Figure 3.10.**

Immunofluorescence pattern of Mab B10 reacting with acetone-fixed *P. yoelii* parasites in erythrocytes (x1250 magnification).

**Figure 3.11.**

Immunofluorescence pattern of Mab G3 reacting with acetone-fixed *P. yoelii* parasites in erythrocytes (x1250 magnification).



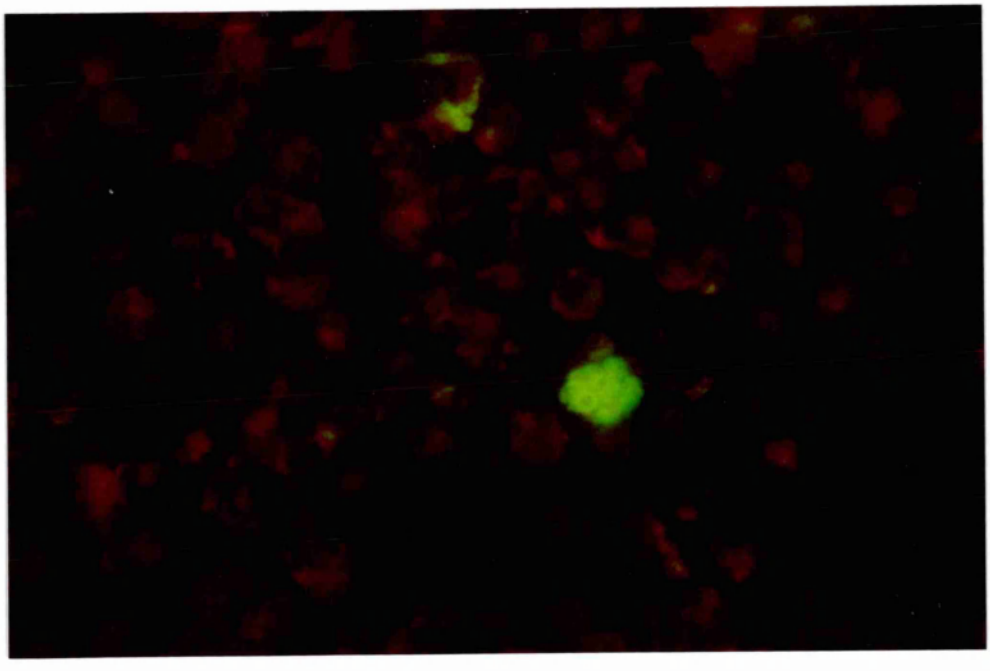
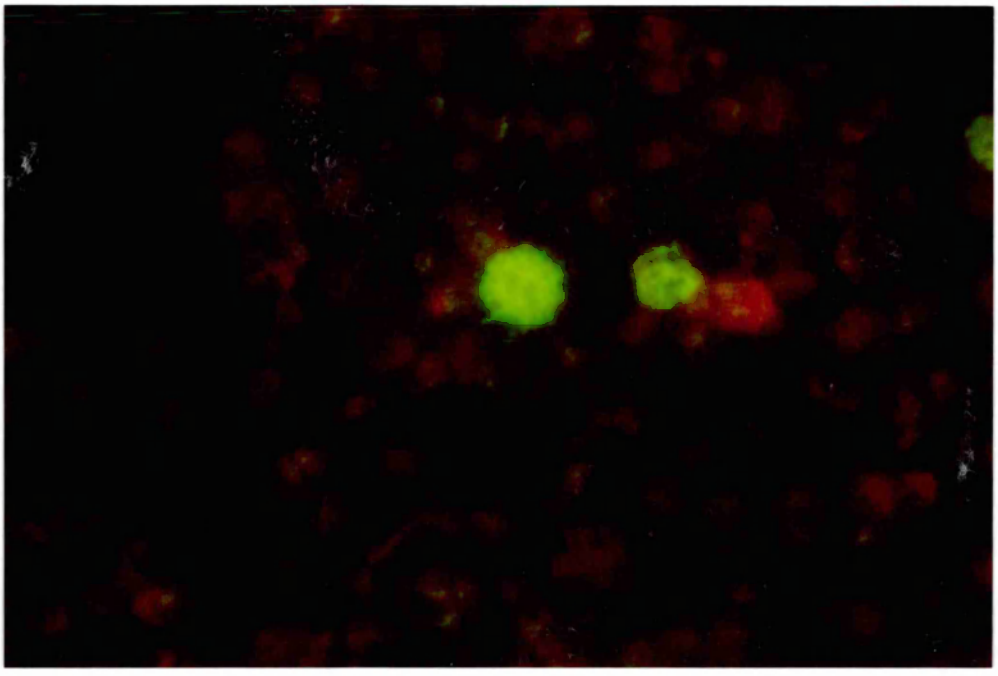


**Figure 3.12.**

Immunofluorescence pattern of Mab P2-F5 reacting with acetone-fixed *P. yoelii* parasites in erythrocytes (x1250 magnification).

**Figure 3.13.**

Immunofluorescence pattern of Mab P6-B6 reacting with acetone-fixed *P. yoelii* parasites in erythrocytes (x1250 magnification).



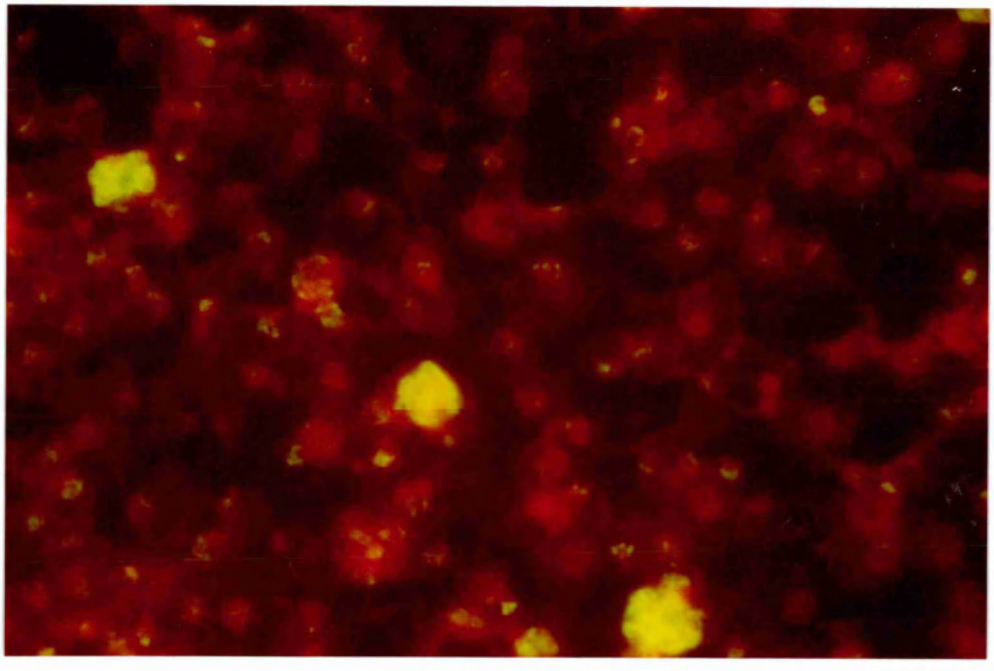
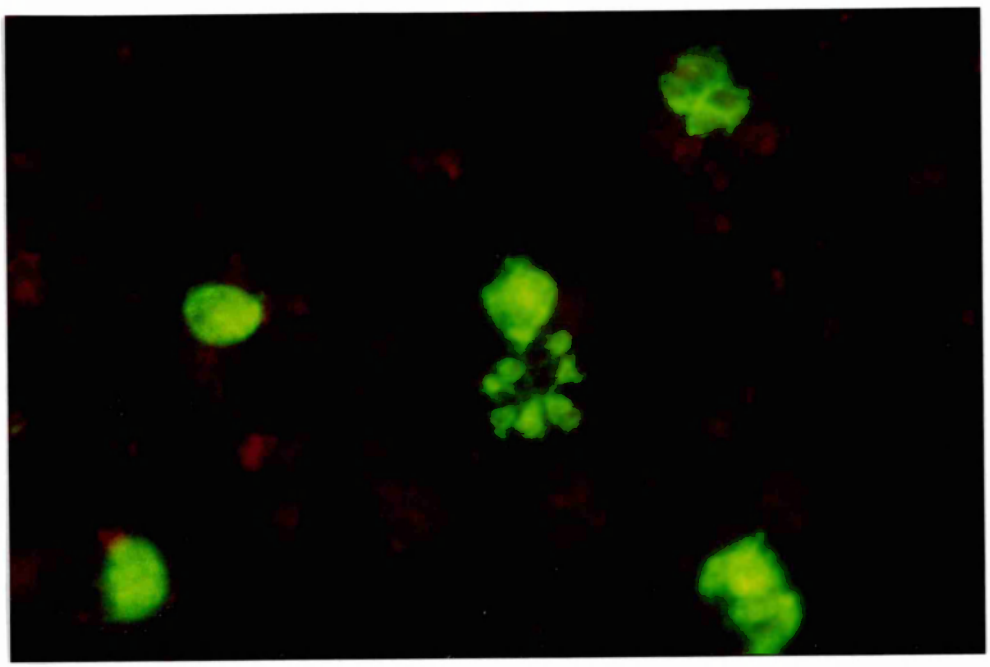


**Figure 3.14.**

Immunofluorescence pattern of Mab P3-D3 reacting with acetone-fixed *P. yoelii* parasites in erythrocytes (x1250 magnification).

**Figure 3.15.**

Immunofluorescence pattern of Mab P6-B4 reacting with acetone-fixed *P. yoelii* parasites in erythrocytes (x1250 magnification).



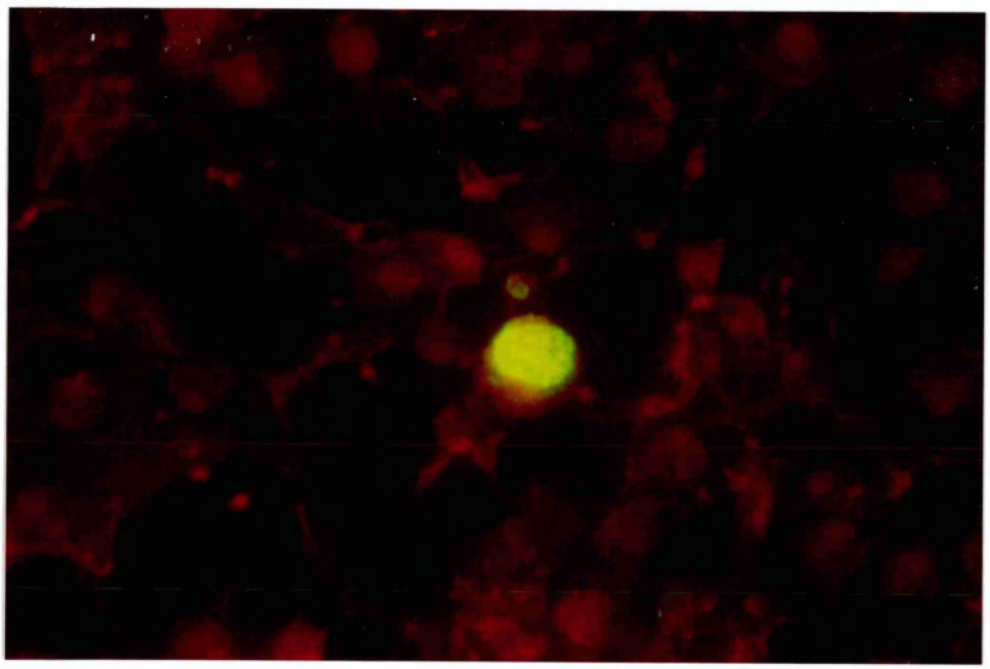


**Figure 3.16.**

[$^{35}$S] methionine/cysteine-containing polypeptides immunoprecipitated from extracts of parasitised erythrocytes by normal mouse serum (track 1), Mab 25.1 (track 2), Mab P5-D9 (track 3), Mab P6-B4 (track 4), Mab P3-D3 (track 5), Mab G3 (track 6), Mab B10 (track 7), Mab P6-B6 (track 8), and Mab P2-F5 (track 9). *P. yoelii* YM parasites were metabolically labelled with [$^{35}$S] methionine/cysteine and solubilised in a buffer containing the detergent NP-40 in the presence of proteinase inhibitors. The immunoprecipitates were analysed by SDS-PAGE on a 12.5% polyacrylamide gel and labelled proteins were detected by fluorography. The mobility of standard molecular mass markers is indicated, together with immunoprecipitated polypeptides marked by arrows.

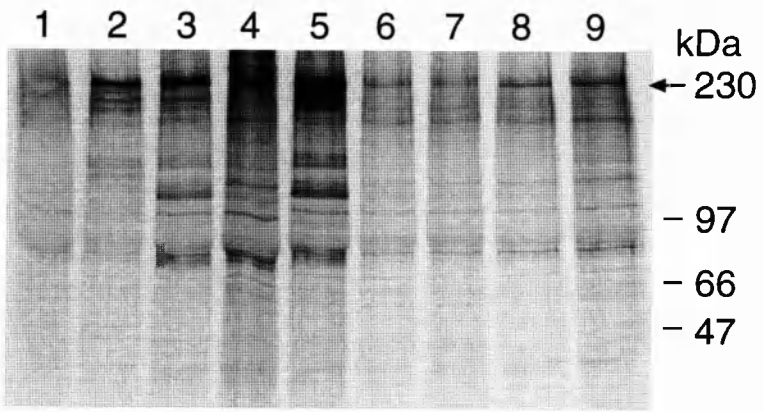



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**Figure 3.17.**

Immunoprecipitation of [$^{35}$S] methionine/cysteine-labelled polypeptides from extracts of *P. yoelii* parasites: Mab P2-F5 (track 1), P6-B6 (track 2), B10 (track 3), G3 (track 4), P3-D3 (track 5), P5-D9 (track 6), 25.1 (track 7), NMS (track 8) and polyclonal anti-serum raised against recombinant MSP-$1_{19}$ (track 9). Other conditions were as described in the legend to Figure 3.16. The immunoprecipitates were analysed by SDS-PAGE (12.5% gel) and fluorography. The mobility of molecular mass markers is indicated, together with immunoprecipitated polypeptides marked by arrows.

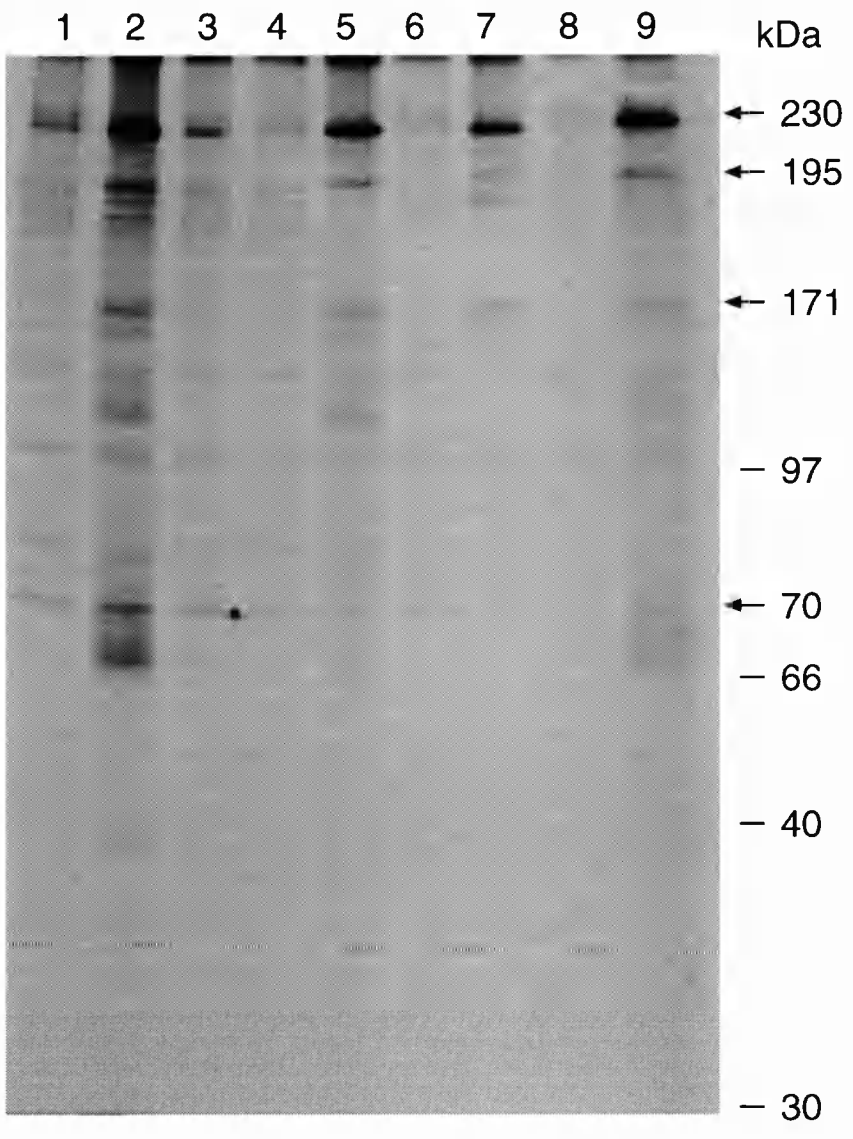


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**Figure 3.18.**

Mabs B10 and G3 recognise recombinant GST-MSP1$_{19}$ under non-reducing conditions (track 1), but after the protein was reduced and alkylated (track 2), the Mabs no longer bound. The recombinant protein was separated by SDS-PAGE on a 12.5% polyacrylamide gel and transferred to nitrocellulose paper, then the blots were probed with Mabs at a dilution 1: 200. Left hand panel: B10; right hand panel:G3.

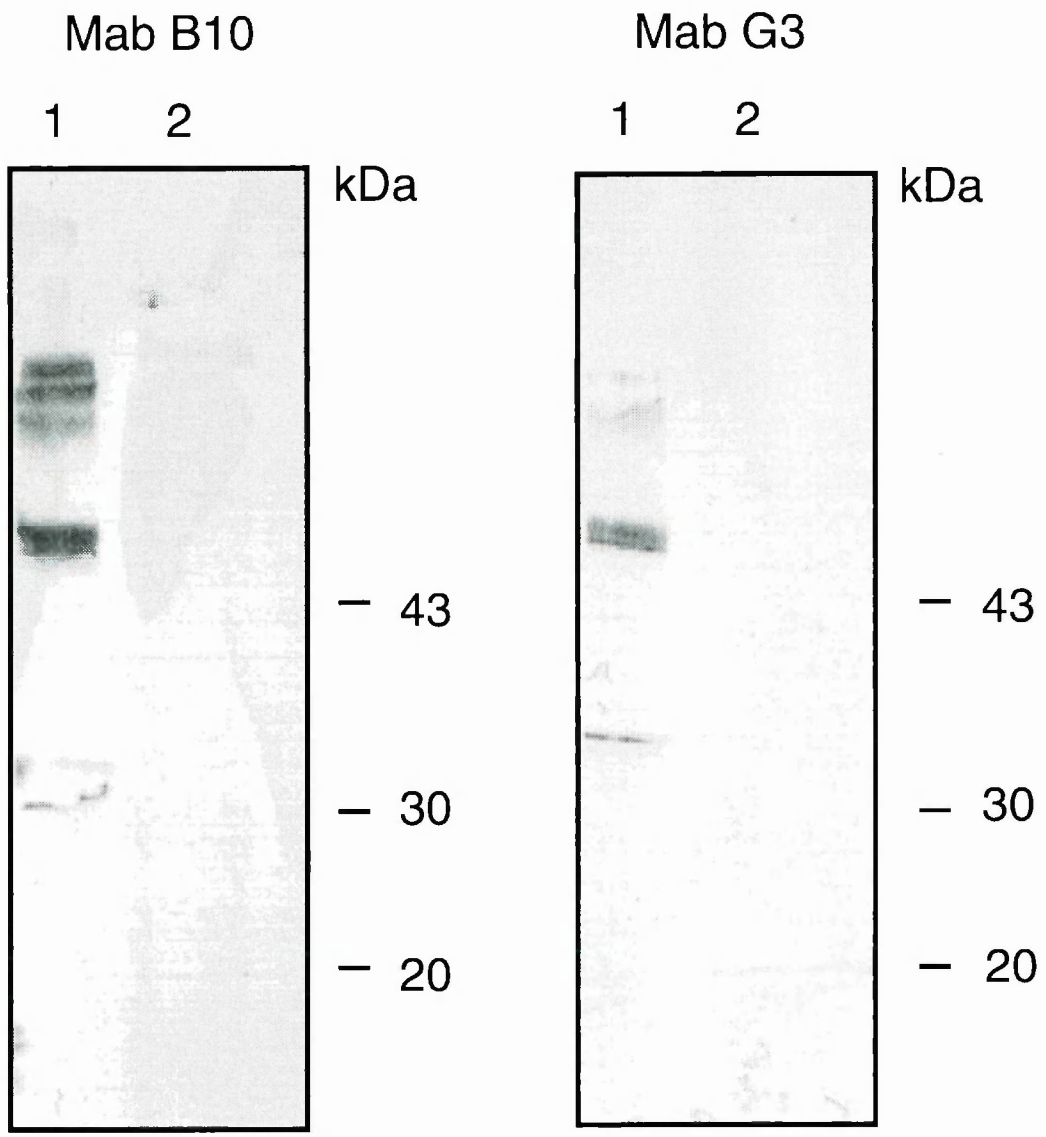


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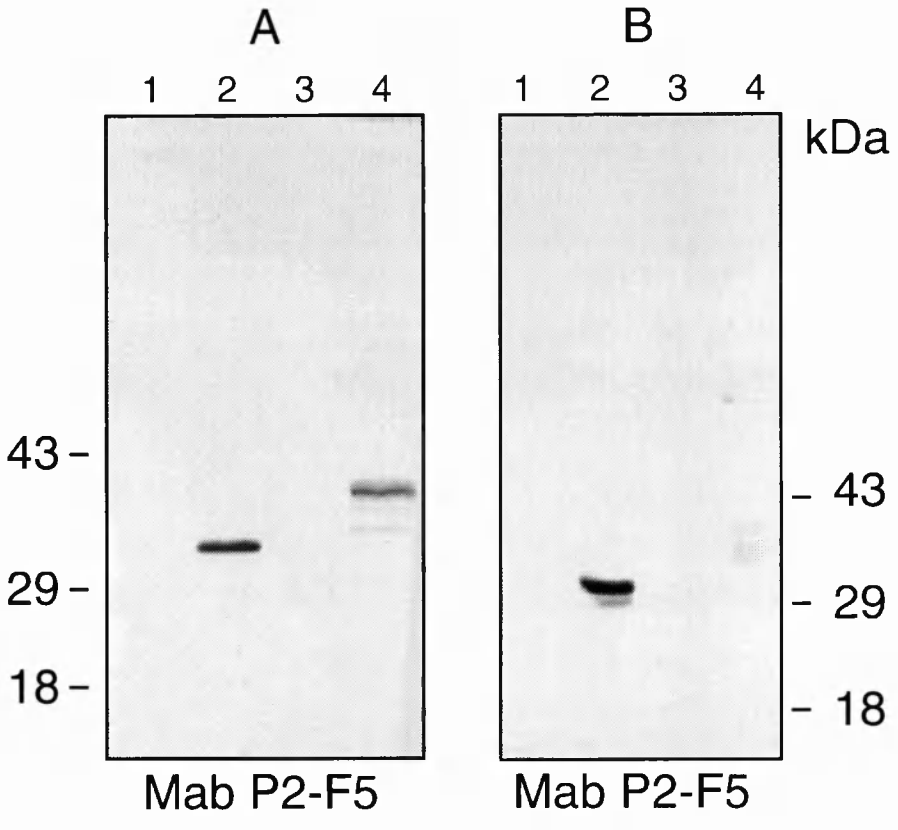
**Figure 3.19.**

The Mabs P2-F5 and P6-B6 recognise recombinant proteins containing the first and both the EGF-like motifs from MSP-$1_{19}$. The proteins were separated by SDS-PAGE on a 12.5 % polyacrylamide gel and the blots were probed with 1:100 dilution of Mabs. Panel A shows analysis of the proteins that have been treated under reducing conditions and panel B an analysis of the untreated proteins (non-reducing conditions). The proteins used were GST (track 1), GST-MSP1EGF1 (first motif fusion protein )(track 2), GST-MSP1EGF2 (second motif fusion protein) (track 3) and GST-MSP$1_{19}$ (track 4). The mobility of molecular mass markers is indicated. Upper panels were probed with P2-F5 and lower panels were probed with P6-B6.



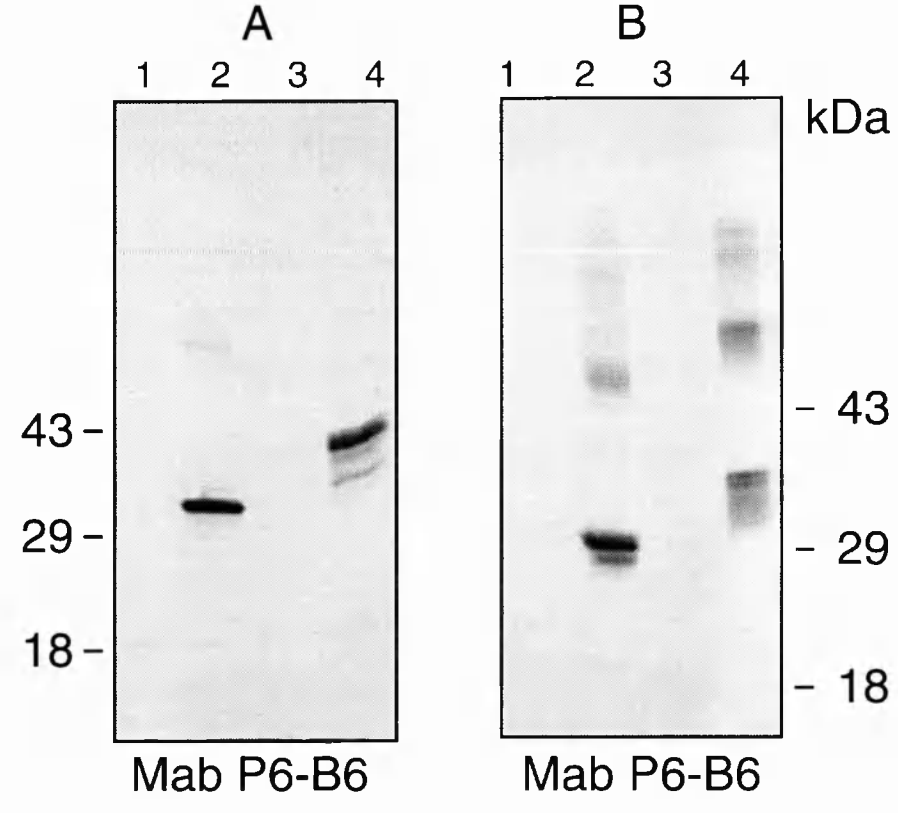




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**Figure 3.20.**

Western blot of using GST-MSP1EGF1 (panel A) and GST-MSP1$_{19}$ (panel B), probed with the Mabs P2-F5 and P6-B6. The antibodies recognised the recombinant proteins containing both the first and the double EGF-like motifs in non-reducing (track 3) and reducing conditions (track 2), but not when the proteins were reduced and alkylated with iodoacetic acid (track 1). The mobility of molecular mass markers is indicated.

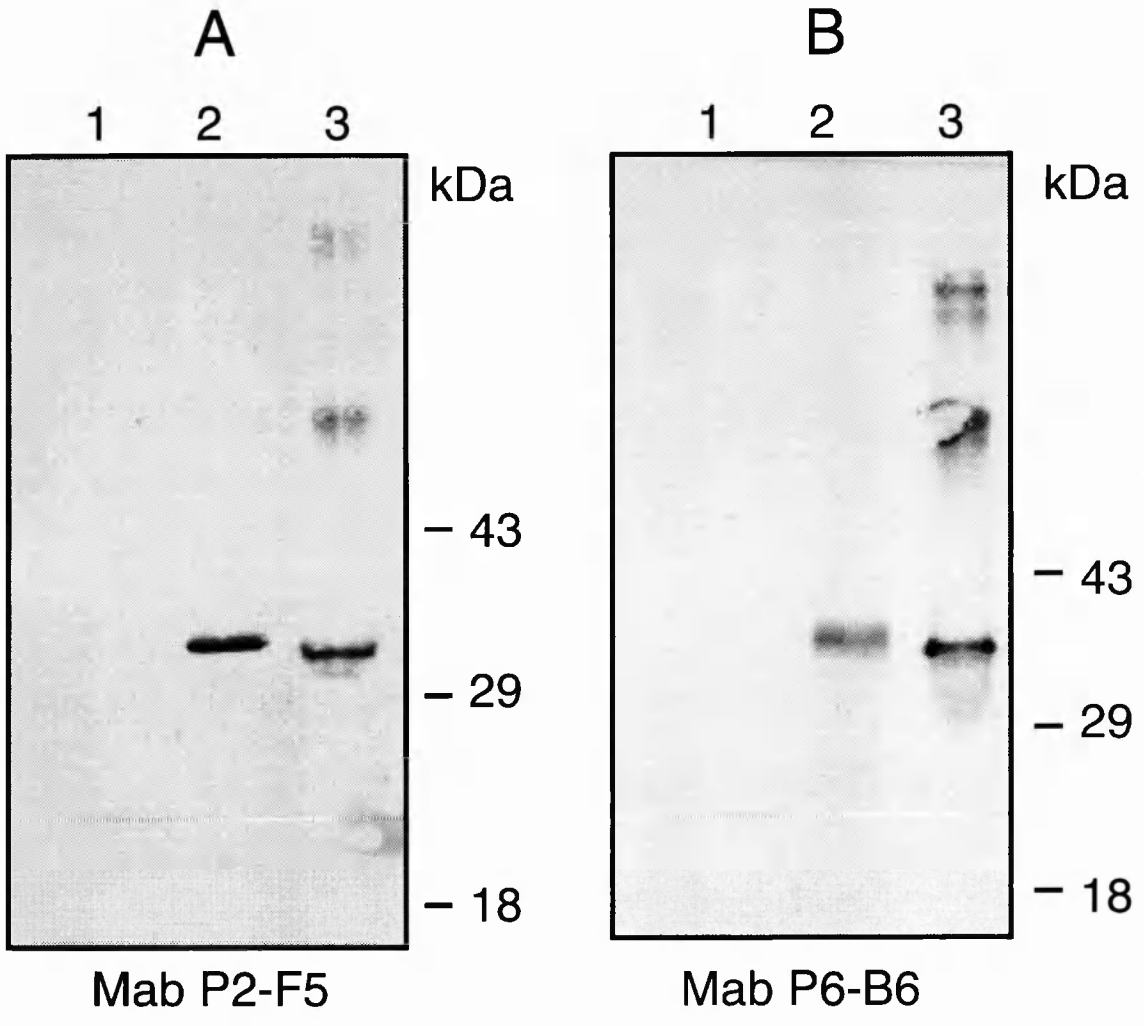


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**Figure 3.21.**

Western blot of GST-MSP1$_{33}$ and GST-MSP1$_{19}$ recombinant proteins probed with Mab P6-B4. The antibody recognised the ~50kDa MSP-1$_{33}$ fusion protein (track 1), but it did not recognise GST-MSP1$_{19}$ (track 2) . The samples were reduced prior to electrophoresis. The mobility of molecular mass markers is indicated.

Mab P6-B4

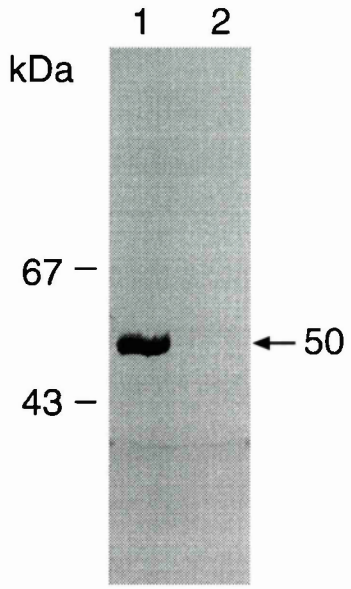
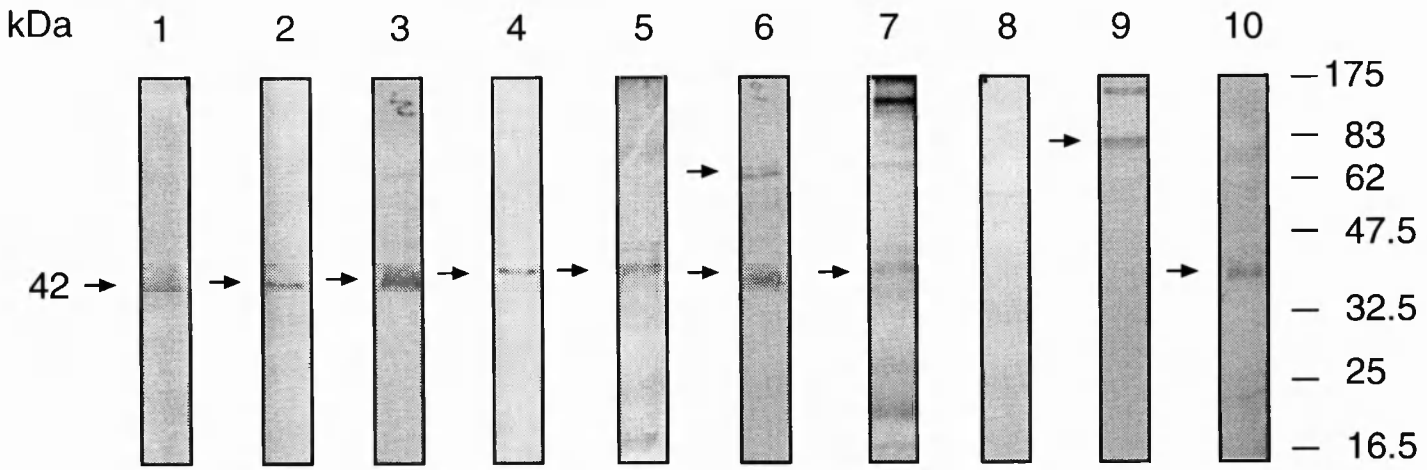


**Figure 3.22.**

Monoclonal antibodies react with polypeptides in an extract of *P. yoelii* parasites on western blots. The samples were reduced, subjected to electrophoresis and then blotted onto nitrocellulose. The blots were probed with: Mab B10 (lane 1); Mab G3 (lane 2); Mab P6-B4 (lane 3); Mab P6-B6(lane 4); Mab P2-F5 (lane 5); Mab P3-D3 (lane 6); Mab P5-D9 (lane 7); normal mouse serum (lane 8); Mab 25.1 (lane 9) and a polyclonal anti-serum against MSP-$1_{19}$ (lane 10). The position of the 42kDa polypeptide, and the mobility of molecular mass markers are indicated.

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**Figures 3.23. and 3.24.**

The mean Absorbance values obtained in the competition ELISA experiments, using biotinylated P2-F5 (Fig. 3.23) and P6-B6 (Fig. 3.24) together with a series of unlabelled Mabs, as indicated on the x-axis.

Figure 3.23: Competition ELISA with biotinylated Mab P2-F5

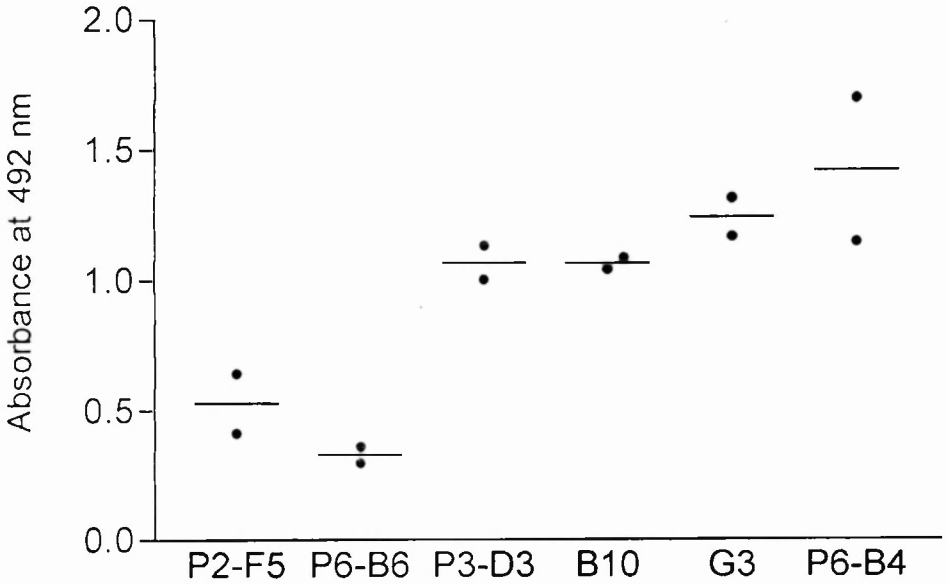
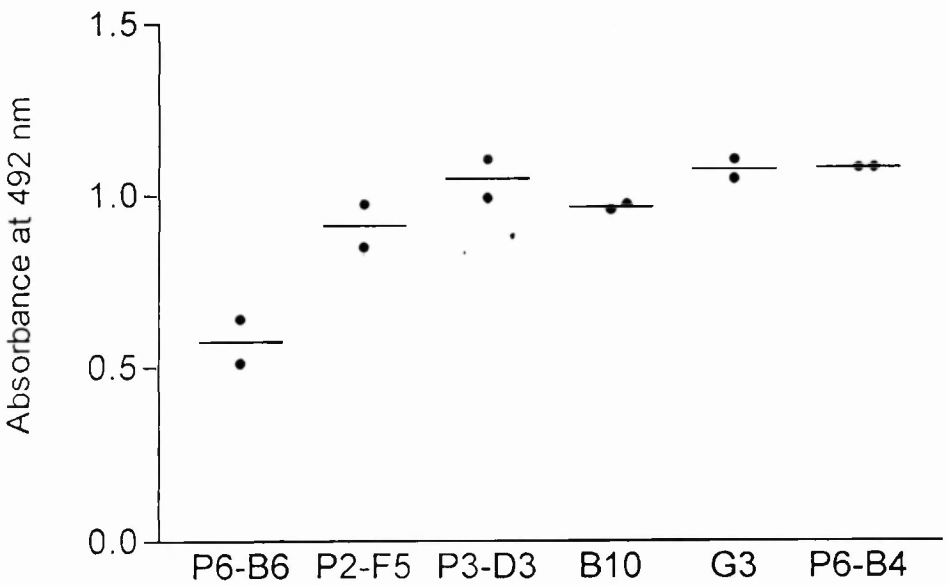



Figure 3.24: Competition ELISA with biotinylated Mab P6-B6



**Figures 3.25. and 3.26.**

The mean Absorbance values obtained in the competition ELISA experiments, using biotinylated B10 (Fig. 3.25) and G3 (Fig. 3.26) together with a series of unlabelled Mabs, as indicated on the x-axis.

Figure 3.25: Competition ELISA with biotinylated Mab B10

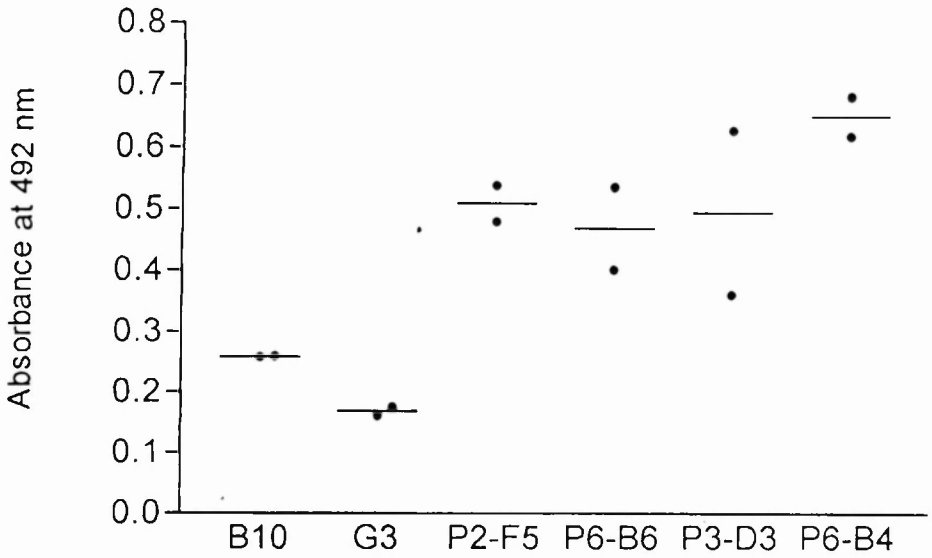



Figure 3.26: Competition ELISA with biotinylated Mab G3

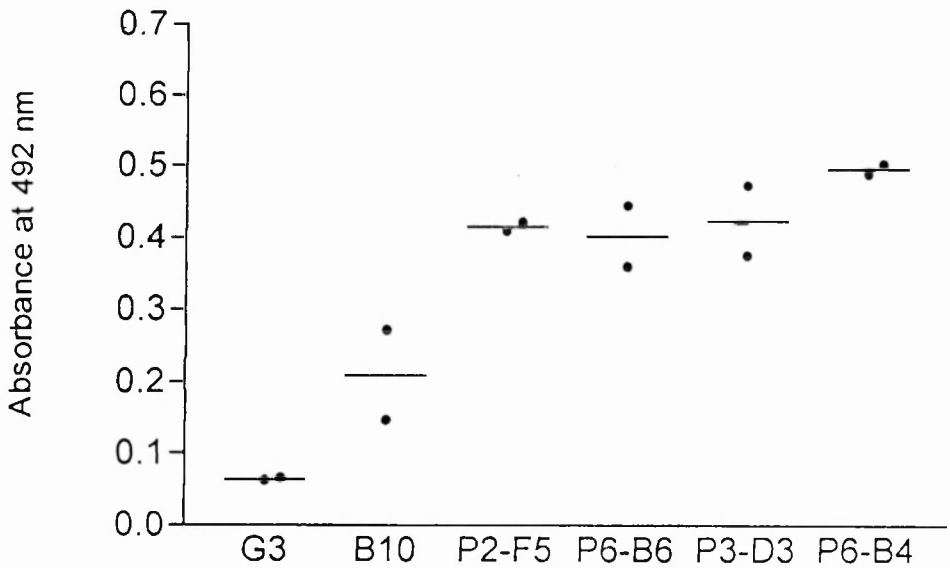
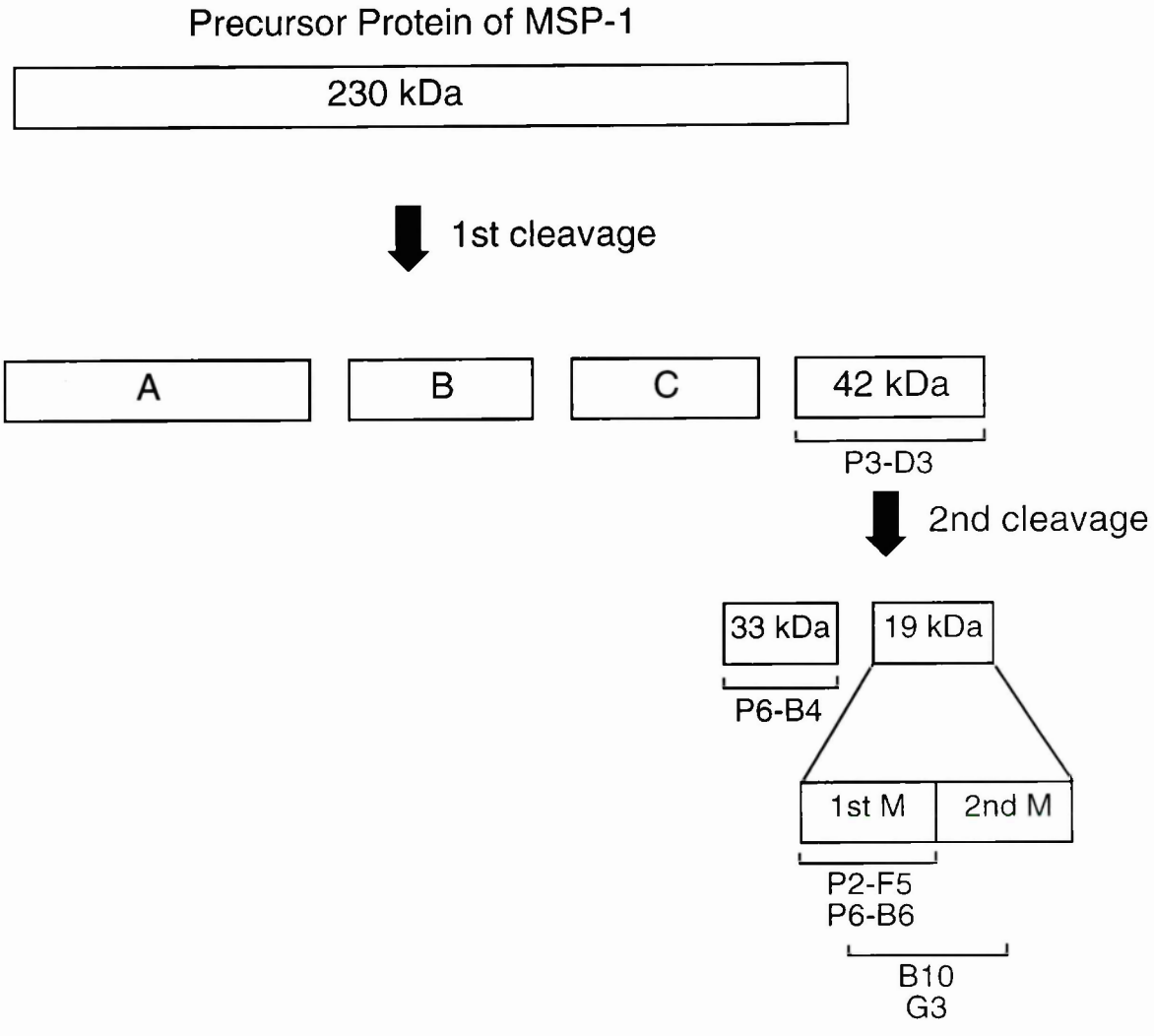


**Figure 3.27.**

A schematic showing the proposed structure of *P. yoelii* MSP-1 and the location of the antibody binding sites. Based on the model developed for *P. falciparum* MSP-1 the protein is present on the newly released merozoite surface as a complex of four polypeptides. A, B, and C correspond to the 83, 30 and 38kDa fragments of *P. falciparum* MSP-1, respectively . The C-terminal 42 kDa fragment undergoes another proteolytic cleavage to give 2 fragments of 33kDa ($MSP\text{-}1_{33}$) and 19 kDa ($MSP\text{-}1_{19}$). $MSP\text{-}1_{19}$ is represented as two EGF-like motifs ($1^{st}$ M. & $2^{nd}$ M.). All Mabs produced in this study bind epitopes on $MSP\text{-}1_{19}$, except Mabs P6-B4 and P3-D3, which recognised the 33kDa and 42 kDa polypeptides, respectively.


Precursor Protein of MSP-1
230 kDa
1st cleavage
A
B
C
42 kDa
P3-D3
2nd cleavage
33 kDa
19 kDa
P6-B4
1st M
2nd M
P2-F5
P6-B6
B10
G3

# CHAPTER 4: PASSIVE IMMUNIZATION OF MICE AND CHALLENGE WITH *PLASMODIUM YOELII*

## 4.1 Introduction

There are a number of ways in which anti-plasmodial antibodies may affect and neutralise the parasite after they interact with the parasite or its specific proteins incorporated into the host erythrocyte cell membrane. Antibody could act by physically coating the parasite's surface and therefore interfering with membrane function. Alternatively the antibody coating could change the adhesive characteristics of the parasite or the parasitised erythrocytes, blocking receptor sites. Antibodies may also interfere with parasite release from the infected cell at the end of schizogony (Kreier & Green, 1980), or prevent the normal growth and development of the parasite by blocking invasion of host cells.

Immunity to the malaria parasite is mediated at least in part by the antibody response to the erythrocytic stages, since passive immunisation with immune serum or immunoglobulin provides some protection against rodent, simian, and human malaria (Boyle et al., 1982). Mabs that inhibit parasite invasion of host cells have been useful tools in the study of malaria antigens, defining important epitopes. Monoclonal antibodies against the surface of *P. knowlesi* merozoite recognise MSP-1, and inhibit reinvasion of erythrocytes *in vitro* (Epstein et al., 1981). A Mab called 5B1 directed against *P. falciparum* MSP-1 was used to characterise the protein and its role in erythrocyte invasion. This Mab recognised two polypeptides of 200 and 50 kDa by IPP

and it reduced merozoite invasion by approximately 45% (Pirson & Perkins 1985). Blackman *et al.* (1990) used a panel of *P. falciparum* MSP-1-specific Mabs and demonstrated that a complex of polypeptides derived from the precursor is present on the parasite surface, and they demonstrated that antibodies to conserved epitopes on the 19 kDa fragment of MSP-1 can inhibit red blood cell invasion. Mabs together with recombinant proteins derived from MSP-1 have allowed the study of the role of antibody-mediated mechanisms in MSP-1-specific immunity to *P. yoelii* infection (Majarian et al, 1984; Long et al. 1993; Ling et al., 1994).

Importantly, the role of circulating antibody in malaria immunity has been studied by passive immunisation of humans. In one study, with children living in The Gambia with severe clinical malaria, the therapeutic effect of γ-globulin prepared from the serum of apparently immune adults was demonstrated (Cohen et al., 1961). Sabchareon and colleagues (1991) assessed the protective effect of African IgG antibodies against *P. falciparum* malaria by passive immunisation of eight Thai patients. These patients were treated by i.v. inoculation of IgG: six with a 100mg/kg dose given over three days, one with a single 20 mg/kg dose, and one with a single 200 mg/kg dose. The IgG caused a decrease in asexual blood stage parasitaemia, while gametocytes were unaffected. After the disappearance of the transferred antibodies, recrudescent parasites from three patients were found to be susceptible to the same IgG preparation, indicating that selection of parasites able to escape the effect of antibodies had not occurred. The authors concluded that the African adult IgG pool exerted a consistent, but non-sterilising effect on each strain tested ; this effect was stage-specific and probably species-specific.

Another study was carried out by Aribot et al. (1996), in which they determined the pattern of whole *P. falciparum* antigen-specific immunoglobulin isotype distribution in the plasma from 145 Senegalese children and adults exposed to continuous and intense parasite transmission, high levels of parasitaemia and the occurrence of malaria attacks. The pattern of anti-malarial immunoglobulin class (IgM and IgG) and IgG subclass (IgG1 to IgG4) distribution was measured by ELISA using a crude blood-stage antigen of *P. falciparum*-infected red blood cells. Adults had higher levels of specific antibodies than children, and IgM, IgG2, and IgG3 accounted for the highest difference. These differences in antibody levels were significant for IgG1 to IgG4 between the lowest and highest transmission season. Only increased levels of IgG3 were found to be associated with a significantly reduced risk of malaria attack. These seroepidemiological data suggest that whereas the total parasite specific IgG is not indicative of any given level of protection against malaria, the level of IgG3 is associated with the relative susceptibility to clinical *P. falciparum* malaria attack.

Much of what has been learned about the role of antibody in the control of malaria parasite infections has come from studies of infected mice. However, these experimental models do not fully represent host-parasite associations that occur in nature and often fail to mimic certain aspects of human disease. Nevertheless, they have been useful in exploring the range of immune responses that may occur following infection (Sayles & Wassom, 1992). Immunity to infection has been passively transferred to naïve mice using hyperimmune serum or Mabs. The ability of hyperimmune serum to alter the course of parasite infection after passive immunisation depends largely on the host-parasite system. In the rodent malaria model *P. yoelii* 17XL in BALB/c mice, the continuous administration of immune serum from mice that had had a single infection, or

a single dose of hyperimmune serum from mice that had been multiply infected and challenged, prevented the development of a patent blood stage infection (Freeman & Parish, 1981). Jayawardena and colleagues (1978) demonstrated that the passive transfer of hyperimmune serum to CBA mice completely protected them against challenge with *P. yoelii* 17XNL. White and colleagues (1991) also studied passive immunisation with hyperimmune serum generated against *P. yoelii* and with purified IgG fractions. Transfer of these antibodies to BALB/c mice or to out-bred ICR mice prior to challenge with virulent *P. yoelii* 17XL, and to CBA/CaJ mice prior to challenge with the avirulent 17XNL, indicated that only the mice that had received IgG2a antibodies had an altered course of infection. On the basis of these studies, White et al. (1991) proposed that antimalarial antibodies of the IgG2a subclass play a dominant role in modulating *P. yoelii* parasitaemia. In addition, previous studies showed that passive immunisation with Mab 302 (an IgG3) directed against *P. yoelii* MSP-1 conferred protection against challenge infection (Majarian *et al.* 1984). *P. yoelii* in laboratory mice is a murine malarial parasite model in which resolution of the infection depends on antibodies.

I have prepared twelve Mabs to MSP-1, an antigen of the intraerythrocytic stage and merozoite surface of *P. yoelii*. Five of the antibodies were found to partially or totally block the invasion of *P. yoelii* YM merozoites into erythrocytes, as measured by *in vivo* assays.

### 4.2 Inoculation of Mabs and challenge infection with *P. yoelii* YM

In the current study, I sought to determine whether or not passive immunisation of naïve mice with a panel of Mabs would protect against a challenge infection with the YM line

of *P. yoelii*. Female BALB/c mice, eight weeks of age, and bred under specific pathogen-free conditions were used. The parasite stock was stored at -70°C and passaged once before use in each experiment. The challenge was administered by intravenous (i.v.) injection into the lateral vein of the tail of $5x10^3$ parasitised red blood cells (PRBC) per mouse. Initially 2 Mabs, B10 and G3 were assessed and Mab 25.37 (an IgG1 isotype, Freeman et al., 1980) was used as a positive control; (data not shown). PBS was used as a negative control because the Mabs were dissolved in this buffer. Mabs (1ml of solution at a concentration of 0.5 mg/ml) were administered by intraperitoneal (i.p.) injection. They were administered on three occasions; one day before, one day after and on the day of the challenge infection, therefore the total amount of antibody was 1.5 mg/mouse. The course of parasitaemia was monitored daily on blood films stained with Giemsa's reagent. Three mice per group were used in the preliminary experiments to assess the protective capacity of the antibodies. Finally ten mice per group were used in experiments to confirm the initial results.

The Mab P2-F5 precipitated after purification by protein G. To determine whether or not this precipitation affected the capacity of the antibody to protect mice against a challenge infection, groups of mice were immunised with three different preparations. The first group of mice was immunised with a solution of the Mab at a concentration of 0.5 mg/ml. The second group was immunised with the precipitated and aggregated Mab suspended in PBS at an estimated concentration of 0.5 mg/ml. The third group of mice was inoculated with aggregated Mab suspended in a 0.5 mg/ml solution of the Mab. This last group of animals therefore received twice the amount of antibody compared to the other two groups.

### 4.3 Titre of Mabs *in vivo*, measured by IFA

The antibody titres in each group of mice in the passive immunisation experiments are shown in Table 4.1. The antibody titres were determined by IFA for those mice which had received either an intraperitoneal injection of Mabs or PBS alone as a control. Serum samples were taken from the tail vein at different times during the experiment: at the time of the initial injection, two days after the challenge with parasites, and then one and two weeks after challenge. The titre of the antibody in the serum samples was determined by fluorescence against *P. yoelii* YM, using multi-well slides with a methanol-fixed infected erythrocyte antigen preparation, as described in Materials and Methods (Chapter 2). Serial two-fold dilutions of serum were added to the wells in duplicate, starting with a 1:50 dilution.

The level of antibody in the peripheral circulation was measured. Serum from mice immunised with P2-F5, P6-B6 and P3-D3 (antibodies that subsequently were found to be highly protective) had a significantly higher IFA titre than serum from mice immunised P4-D10, P1-C5, P8-D1, P1-A9, and P5-D9 (Mabs that subsequently were found to be not protective). The IFA titres of the animals inoculated with Mabs P8-D4, P6-B4, B10 and G3 were also high. However, the titres in all groups decreased after mice were challenged with parasite-infected red blood cells, with the exception of the group that had received Mab P3-D3; in these animals the antibody titre was higher than the initial measurement, two days after the challenge.

Three groups of mice received different preparations of Mab P2-F5, either in solution, as a suspension or in a mix of solution and suspension. In Table 4.1 are shown the antibody

titres of serum samples in groups 9 (which received the soluble P2-F5),10 (which received the suspension of P2-F5) and 11(which received the mix of soluble and suspended P2-F5). The highest antibody titre was observed in group 11. The initial titre of Mab P2-F5 in this group was 1: 25600 and thereafter it decreased to 1:12800 and remained constant at this level during the following two weeks, compared with the titres in groups 9 and 10, which decreased to1:600 and 1:3200, respectively. This difference may be due to the initial higher concentration of Mab used in this immunisation or the fact that the aggregated antibody was solubilised *in vivo* and released gradually into the bloodstream . The titre of the antibody correlated well with the results of the protection studies (See next section).

**4.4 Protection against challenge infection by passive immunisation**

Passive protection studies were used to assess the panel of Mabs that reacted with merozoites and schizonts by immunofluorescence. Passive protection was evaluated by the ability of the Mabs to protect BALB/c mice against a challenge infection with $5x10^3$ PRBC of *P. yoelii* YM. Figures 4.1 to 4.12 show the course of blood stage infections in groups of mice passively immunised with Mabs P4-D10, P1-C5, P8-D1, P1-A9, P8-D4, P5-D9, P6-B4, P3-D3, P2-F5, P6-B6, B10 and G3 respectively. Figure 4.13 shows the course of infection in the group of mice which received PBS alone, which was used as a negative control. In all cases where the Mabs were not protective (P4-D10, P1-C5, P8-D1, P1-A9, P8-D4, P5-D9, and P6-B4), mice developed a high parasitaemia from day 3, peaking at 70-90% on day 5, and died. In contrast, mice immunised with Mabs P2-F5, P6-B6 and P3-D3 developed a lower parasitaemia with peaks of 16% on day 14 (Mab P2-F5), 0.1% on day 9 (Mab P6-B6) and 28% on day 10 (Mab P3-D3). In the mice

immunised with P3-D3 it was noted that at the onset of patent parasitaemia, the parasites were only observed in reticulocytes, an observation not made in any other group. None of the mice in any of these groups died. Of particular note was the finding that Mabs P2-F5 and P6-B6 delayed the onset of patent parasitaemia by up to 5 days (Figs. 4.9 & 4.10). The other two Mabs, B10 and G3, were partially protective because mice immunised with these antibodies developed parasitaemias of 50% and 70% respectively. In the group of mice immunised with G3 only 40% of them survived (3/5 died). In both groups of mice the parasites were observed in tail-blood films until day 22, after this day the parasites were not seen in blood films from any animals.

In order to confirm the results of the preliminary passive immunisation experiments, they were repeated with 10 mice per group. Figure 4.14 shows the mean parasitaemia after challenge in groups of mice passively immunised with Mabs P2-F5, P6-B4, P6-B6, P3-D3, P5-D9, B10 and G3. PBS was again used as a negative control. In spite of the fact that Mab P6-B4 was not protective, this was re-evaluated because it recognised a different fragment of MSP-1. Three of the Mabs (P2-F5, P6-B6 and P3-D3) were confirmed to be protective (100% survival). These experiments gave similar results to those reported by Majarian and co-workers with ascitic fluid containing Mab 302 (Majarian *et al.* 1984). Two others, Mabs B10 and G3 (which are IgG2b and IgG1 respectively), were partially protective in this passive immunisation experiment. These mice developed parasitaemias that were significantly higher than those of mice immunised with the other protective Mabs, the peak parasitaemias were more than 50% on days 13-14 but by day 22 the infection had cleared. The Mabs P6-B4 (an IgG1) and P5-D9 (an IgG2b) were not protective because all the mice developed fulminating

infection and died at around day 8, a pattern identical to the infection in mice inoculated with PBS alone.

Figure 4.15 shows the effects of the different preparations of the protective Mab P2-F5. The protection conferred by this Mab varied between the different preparations. The results suggest that the concentration of Mab in the peripheral circulation (as measured by the IFA titre) affects the course of infection. The mice immunised with the suspension of precipitated Mab produced a more marked reduction in parasitaemia. The group of mice immunised with the soluble antibody had a mean peak parasitaemia of 16% on day 14, in contrast with the groups of mice immunised with the suspended antibody precipitate in PBS (Mab P2-F5/P) and the suspended antibody precipitate in the antibody solution (P2-F5/S) with a mean parasitaemia which did not exceed 0.17% on day 7, or no detectable parasites, respectively.

## 4.5 Attempts to develop an in vitro assay to study the effect of the Mabs on invasion

I tried to demonstrate whether or not the panel of MSP-1 specific Mabs that were protective in passive immunisation experiments, were able to inhibit merozoite invasion of red blood cells in vitro. Analysis in vitro enables the effect of the Mabs to studied in isolation from cellular effector mechanisms that may operate in conjunction with the antibodies in vivo. *P. yoelii* YM-infected cells were isolated from mice, fractionated by centrifugation through a Percoll-glucose gradient (Deharo et al., 1994), and the fraction containing late trophozoites and schizonts was diluted in RPMI 1640 medium containing 10% of NMS. After two washes in this medium by centrifugation for 5 minutes at 250 x

g the cells were resuspended in medium at 1 % parasitaemia and 10% haematocrit (Wahlin et al. 1984). The cultures were incubated for 3 hours in the presence or absence of Mabs, and every 30 min samples were removed and stained with Giemsa's reagent to determine the distribution of different developmental stages (Ahlborg et al. 1996).

In the presence or absence of Mabs, no merozoite invasion in vitro was observed, as assessed by the formation of new ring stages (data not shown). Despite several attempts to optimise the conditions to allow in vitro development, no invasion in vitro was detectable, and after about 3 hours the parasites began to degenerate in culture.

**4.6 Discussion.**

Previous studies have demonstrated that immunisation of mice with hyperimmune serum or Mabs can modulate the course of *P. yoelii* infection (Freeman *et al.* 1980, White *et al.* 1991, Majarian *et al.* 1984). In the present studies a panel of Mabs was assessed for their protective capacity by passive immunisation experiments in mice. The properties of the antibodies are summarised in Table 4.2. Some of these Mabs demonstrated a high level of protection against a lethal parasite challenge infection with *P. yoelii* YM. Specifically five out of the 12 Mabs tested exhibited a protective effect. Mab G3 was partially protective (40% of mice survived the parasite infection). Mab B10 was completely protective (100% survival) although with this Mab the parasitaemia reached a high level (70% of red blood cells infected) on day 14 after infection. Mabs P2-F5, P6-B6 and P3-D3 were all highly protective (100% survival) and the levels of parasitaemia were low throughout the monitored period This study demonstrated that the elimination of parasites is, at least in part, mediated by antibody.

Studies on the protection of mice by active or passive immunisation have indicated some of the IgG subclasses that may be involved. Recent experiments by Akanmori and colleagues (1994) demonstrated that in CBA mice which had been immunised by four successive inoculations of *P. berghei* NK65, each followed by chemotherapy, animals survived a subsequent challenge infection with the parasite. The sera of the mice which survived the challenge had significantly higher anti-plasmodial IgGs, including IgG1 and IgG2a. In particular, the elevated level of IgG2a suggested that this subclass could be involved in a protective humoral response against this rodent parasite. In another study, White *et al.* (1991), using hyperimmune serum generated against *P. yoelii* and IgG fractionated into its constituent subclasses by protein A-sepharose chromatography, showed that only mice receiving IgG2a had an altered course of infection. These experiments were conducted by passive immunisation with the antibody of BALB/c and out-bred ICR mice before challenge with *P. yoelii* 17XL . These results suggest that IgG2a plays a dominant role in the modulation of *P. yoelii* infection. Other protective Mabs have also been described. Two antibodies against a 235kDa rhoptry protein (IgG1 and IgG2a) protected mice against challenge with the virulent *P. yoelii* YM line, converting the parasitaemia to a reticulocyte-restricted self-limiting infection (Freeman et al., 1980; Holder & Freeman, 1981); Mab 302 (IgG3) is a protective MSP-1-specific antibody (Majarian et al., 1984); and Mab 5C10/66 is an MSP-1-specific IgG2a which is protective in mice against *P. chabaudi* infection (Lew A. et al., 1989).

My results presented here are consistent with these other studies. Mabs P2-F5 and P6-B6 are both of the IgG3 subclass, the same as Mab 302 described by Majarian et al. (1984), whilst P3-D3 is an IgG2a antibody, the subclass identified by the Akanmori and

White studies. Mabs previously described and those described in this study are directed against proteins on the surface of merozoites or externalised at the time of invasion, and can probably prevent the interaction between the merozoite and the target erythrocyte. In addition, it is possible, based on other studies, that these antibodies play a role in the phagocytosis of merozoites by macrophages by opsonisation. Antibodies are involved in the macrophage-mediated phagocytosis of parasite-infected erythrocytes after opsonisation of the infected cell (Shear *et al.* 1979 and Tosta *et al.* 1970).

The immunoglobulin titre of sera from mice before and after challenge infection are shown in Table 4.1. These results show that the titre of protective Mabs is significantly higher than that of most of the other Mabs. In addition, a difference in the serum antibody half life was observed between Mab P3-D3 (group 8) and the other two protective Mabs P2-F5 and P6-B6 (groups 9 and 11 respectively), Mab P3-D3 had the highest titre 2 days after the challenge infection, suggesting that the level of IgG2a antibody is perhaps enhanced by activated macrophages (White *et al.* 1991). Taken together with the observations by Taylor and colleagues (1988) who demonstrated that IgG3 antibodies were the first antimalarial antibody of the IgG class detected in serum of several strains of mice infected with 17XL *P. yoelii,* it is therefore tempting to conclude that the IgG2a and IgG3 subclasses are the most important in the humoral response against *P. yoelii* infection. The role of the other IgG subclasses should however be studied further.

Using the *P. yoelii* murine model, I have used Mabs to confer protection against challenge infection and therefore identified regions of MSP-1 that are the target of protective responses. Furthermore, I have identified two Mab subclasses, IgG3 and

IgG2a, that may correlate with protection. These observations, and studies done with the human malaria parasite *P. falciparum* (Rzepczyk *et al.* 1997), suggest that a strategy for the production of a vaccine against malaria must ensure that the most appropriate regions of the antigen are used together with a suitable adjuvant (ten Hagen *et al.* 1993) to generate the appropriate subclass of antibody with protective specificity.

**Table 4.1** IFA titres of monoclonal antibodies in serum samples

| *Group* | *Mab* | *Subclass* | *Initial titre* | *Titre at 2 days* | *Titre at 1 week* | *Titre at 2 weeks* |
|---|---|---|---|---|---|---|
| 1 | P4-D10 | IgG1 | 1:200 | NR | - | - |
| 2 | P1-C5 | IgG1 | 1:100 | NR | - | - |
| 3 | P8-D1 | IgG1 | 1:800 | NR | - | - |
| 4 | P1-A9 | IgG2a | 1:50 | NR | - | - |
| 5 | P8-D4 | IgG2b | 1:51600 | 1:3200 | - | - |
| 6 | P5-D9 | IgG2b | 1:50 | NR | - | - |
| 7 | P6-B4 | IgG1 | 1:51600 | 1:12800 | - | - |
| 8 | P3-D3 | IgG2a | 1:51200 | 1:102400 | 1:51200 | 1:12800 |
| 9 | P2-F5 | IgG3 | 1:51200 | 1:6400 | 1:6400 | 1:600 |
| 10 | P2-F5/P | IgG3 | 1:1600 | 1:6400 | 1:25800 | 1:3200 |
| 11 | P2-F2/S | IgG3 | 1:25600 | 1:12800 | 1:12800 | 1:12800 |
| 12 | P6-B6 | IgG3 | 1:6400 | 1:12800 | 1:12800 | 1:6400 |
| 13 | B10 | IgG2b | 1:12800 | 1:6400 | 1:3200 | 1:1600 |
| 14 | G3 | IgG1 | 1:6400 | 1:3200 | 1:3200 | 1:800 |
| 15 | PBS | - | NR | NR | - | - |

NR: non-reactivity; -: not tested

P2-F5/P: precipitated Mab P2-F5 suspended in PBS solution.

P2-F5/S: precipitated Mab P2-F5 suspended in a solution of soluble P2-F5.

Mabs were inoculated intraperitoneally into groups of mice and then their titre in serum samples was measured by IFA using fixed blood stage parasites. The persistence of the antibody during the course of the experiment was assessed. The antibody titre was

measured using serial serum dilutions, each point represents the average of the results for 5 to 10 mice.

**Table 4.2.** Summary of the characteristics of the Mabs.

| Mab | Subclass | Binding to recombinant proteins | | | | | Passive immunisation |
|---|---|---|---|---|---|---|---|
| | | Reaction with MSP-1 | MSP-1EGF1. | MSP-1EGF2 | MSP-$1_{19}$ | MSP-$1_{33}$ | |
| B10 | IgG2b | + | - | - | + | - | partially protective |
| G3 | IgG1 | + | - | - | + | - | partially protective |
| P2-F5 | IgG3 | + | + | - | + | - | protective |
| P6-B4 | IgG1 | + | - | - | - | + | non-protective |
| P6-B6 | IgG3 | + | + | - | + | - | protective |
| P3-D3 | IgG2a | + | - | - | - | - | protective |

The reaction with MSP-1 was determined by immunofluorescence, Western blotting and immunoprecipitation. The binding to the different recombinant proteins representing fragments of MSP-1 was determined by Western blotting and ELISA.

MSP-1EGF1. = First EGF-like module of MSP-$1_{19}$.

MSP-1EGF2 = Second EGF-like module of MSP-$1_{19}$.

**Figure 4.1.**

Passive immunisation with Mab P4-D10, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

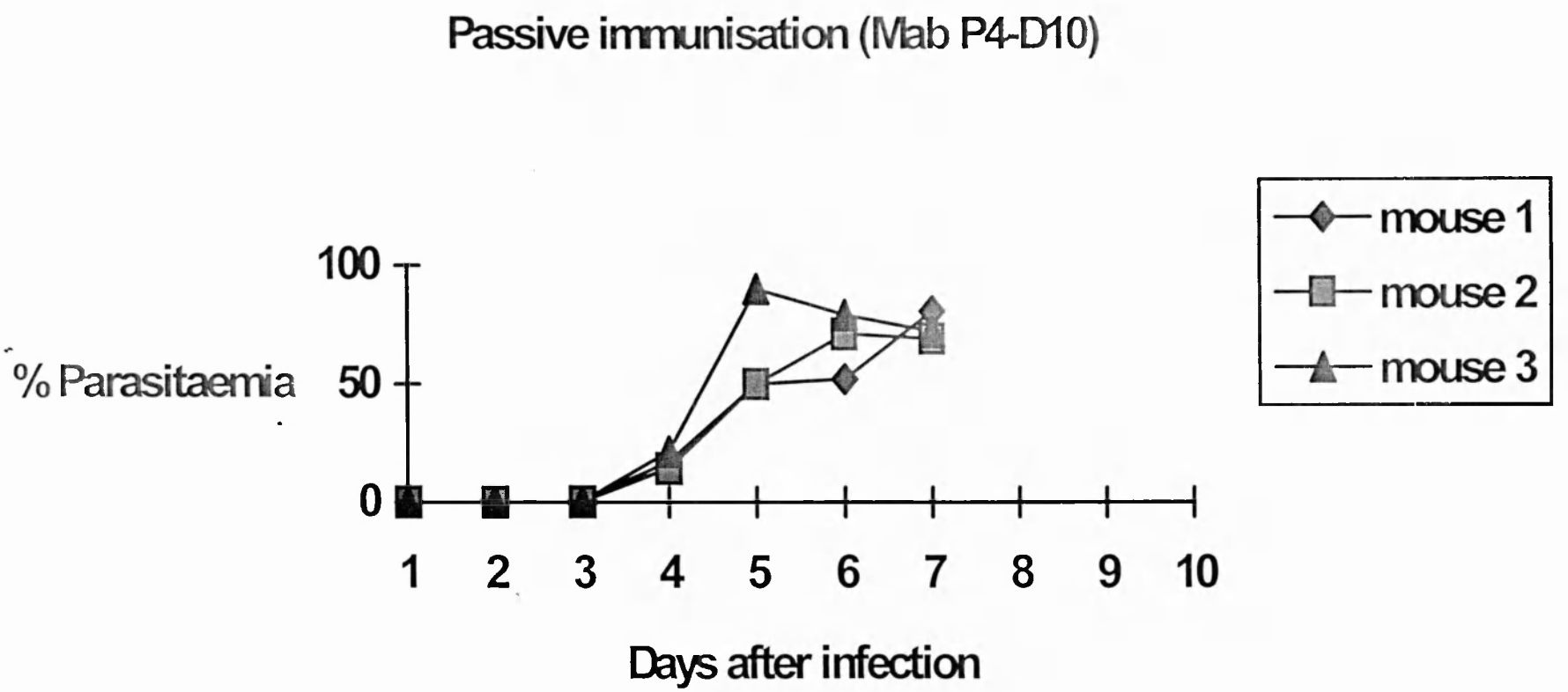

Passive immunisation (Mab P4-D10)
% Parasitaemia
100
50
0
1
2
3
4
5
6
7
8
9
10
Days after infection
mouse 1
mouse 2
mouse 3

**Figure 4.2.**

Passive immunisation with Mab P1-C5, followed by intravenous challenge with $5 \times 10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

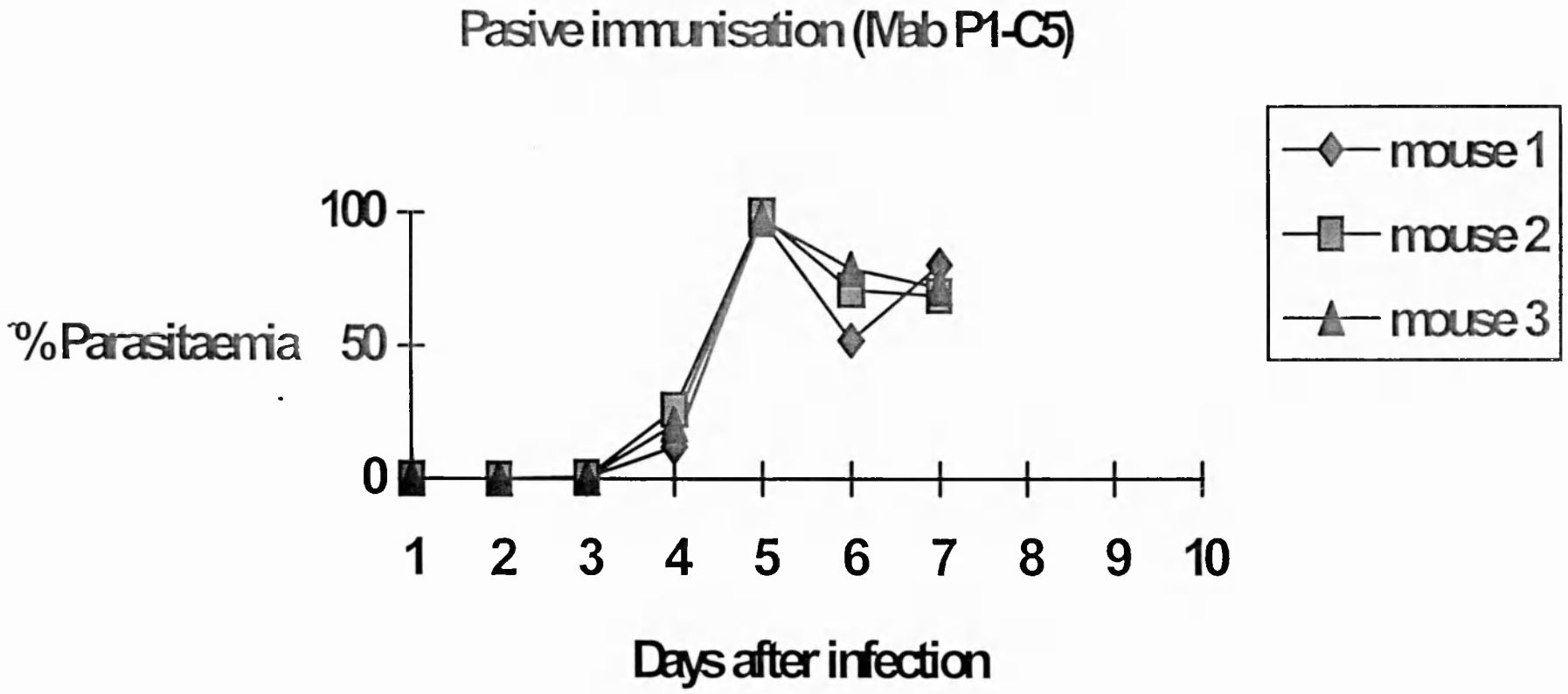

Pasive immunisation (Mab P1-C5)
mouse 1
mouse 2
mouse 3
100
50
0
%Parasitaemia
1
2
3
4
5
6
7
8
9
10
Days after infection

**Figure 4.3.**

Passive immunisation with Mab P8-D1, followed by intravenous challenge with $5 \times 10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

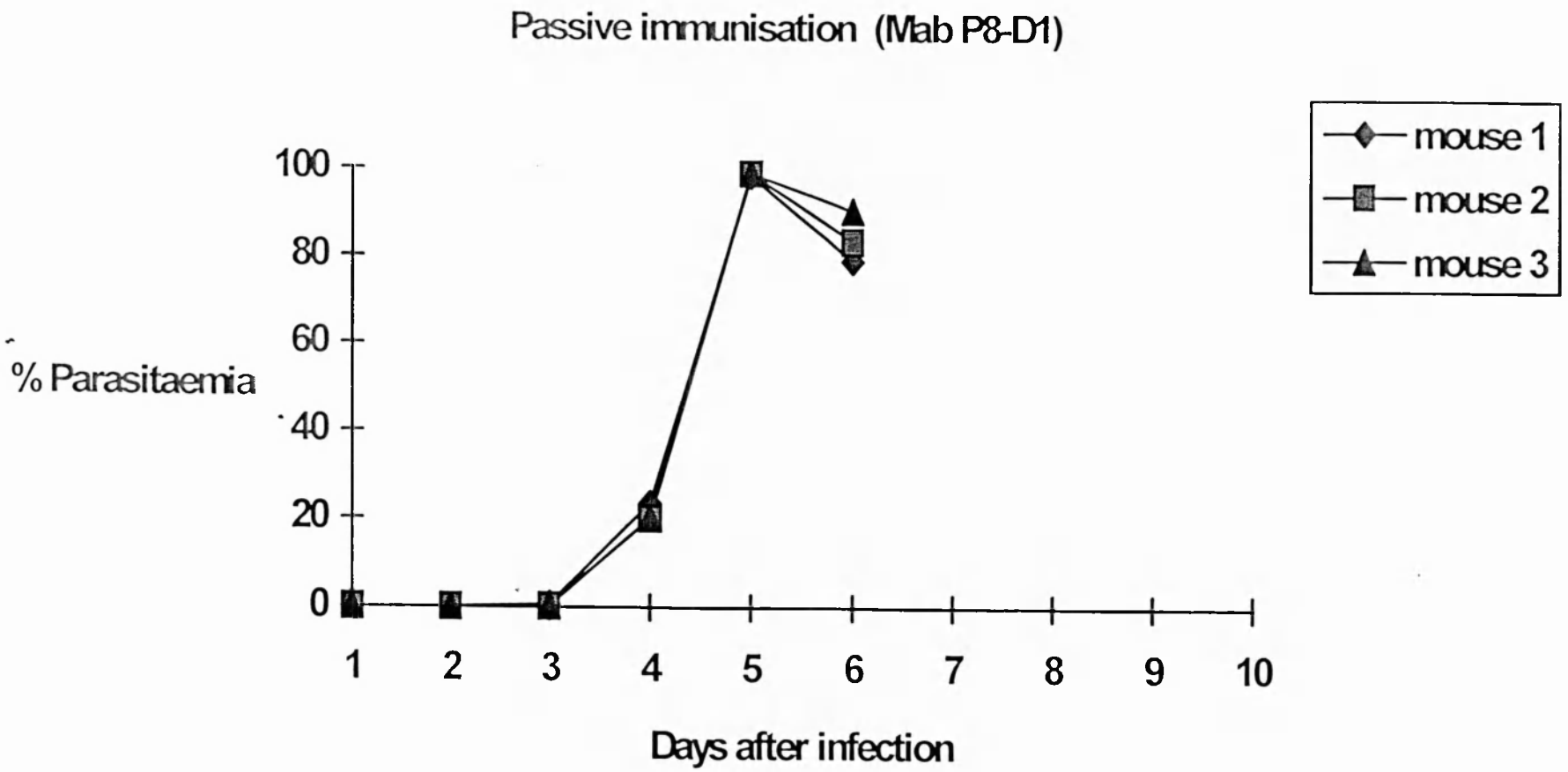

Passive immunisation (Mab P8-D1)
mouse 1
mouse 2
mouse 3
% Parasitaemia
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after infection

**Figure 4.4.**

Passive immunisation with Mab P1-A9, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

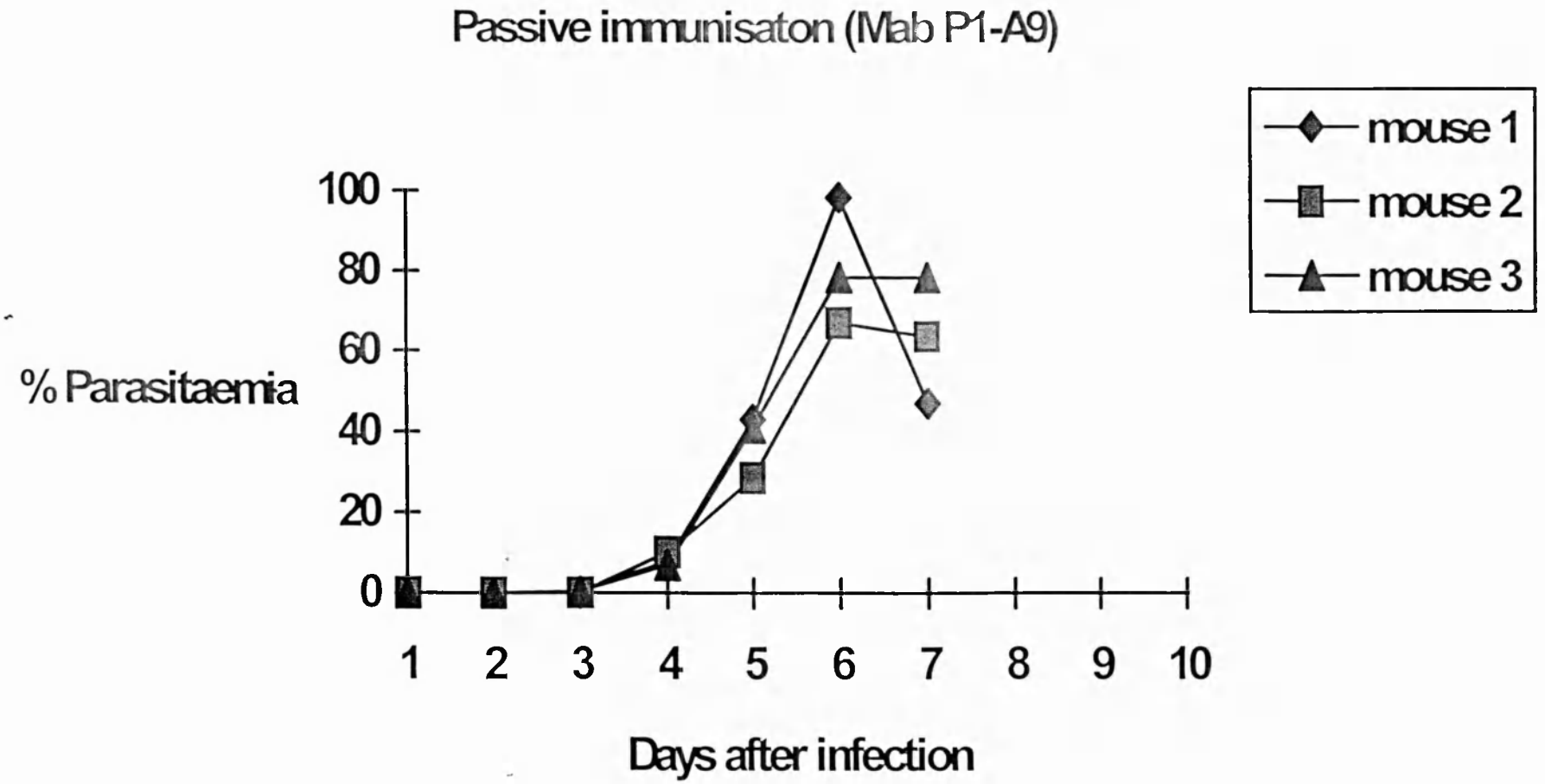

Passive immunisaton (Mab P1-A9)
mouse 1
mouse 2
mouse 3
% Parasitaemia
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after infection

**Figure 4.5.**

Passive immunisation with Mab P8-D4, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

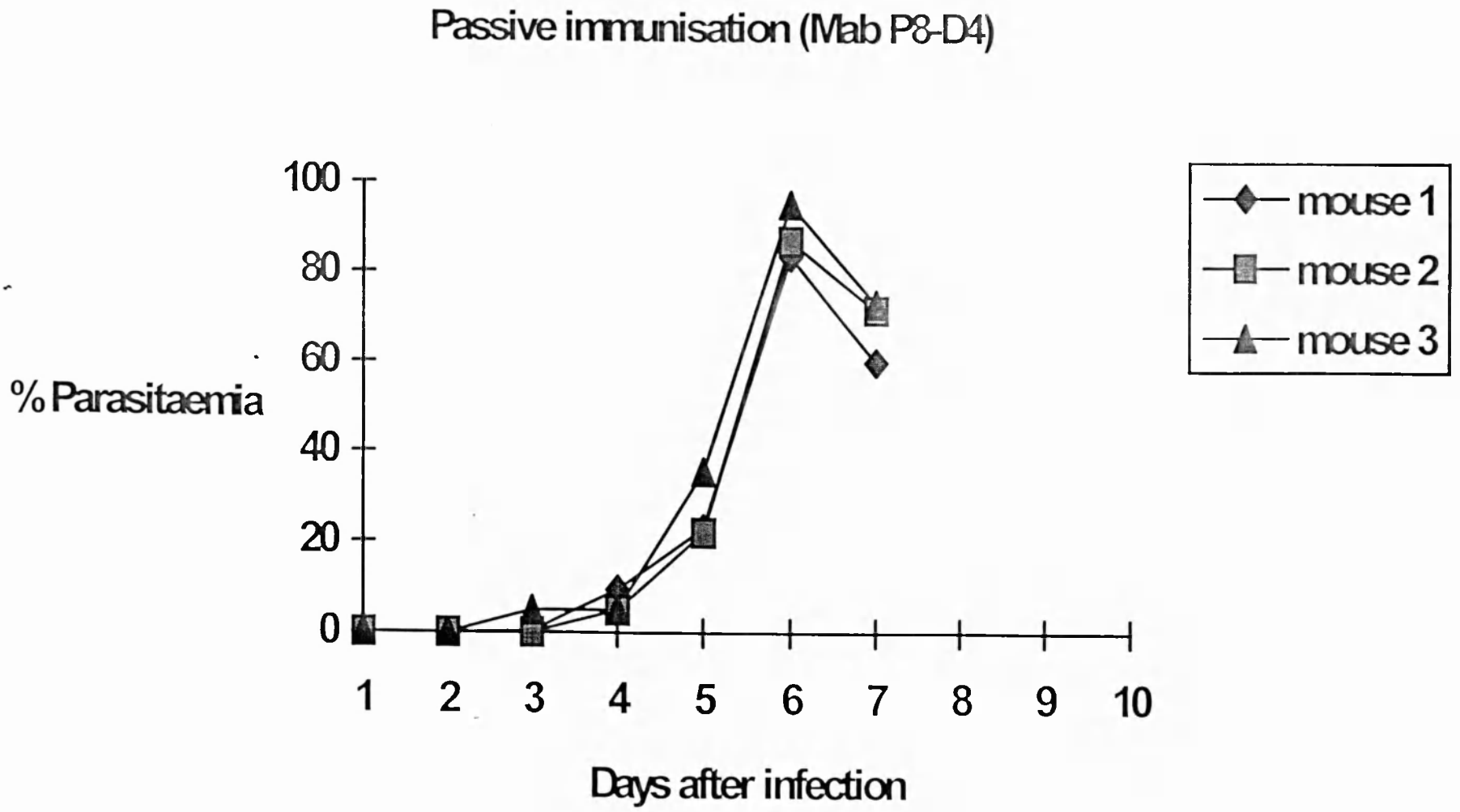

Passive immunisation (Mab P8-D4)
% Parasitaemia
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after infection
mouse 1
mouse 2
mouse 3

**Figure 4.6.**

Passive immunisation with Mab P5-D9, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

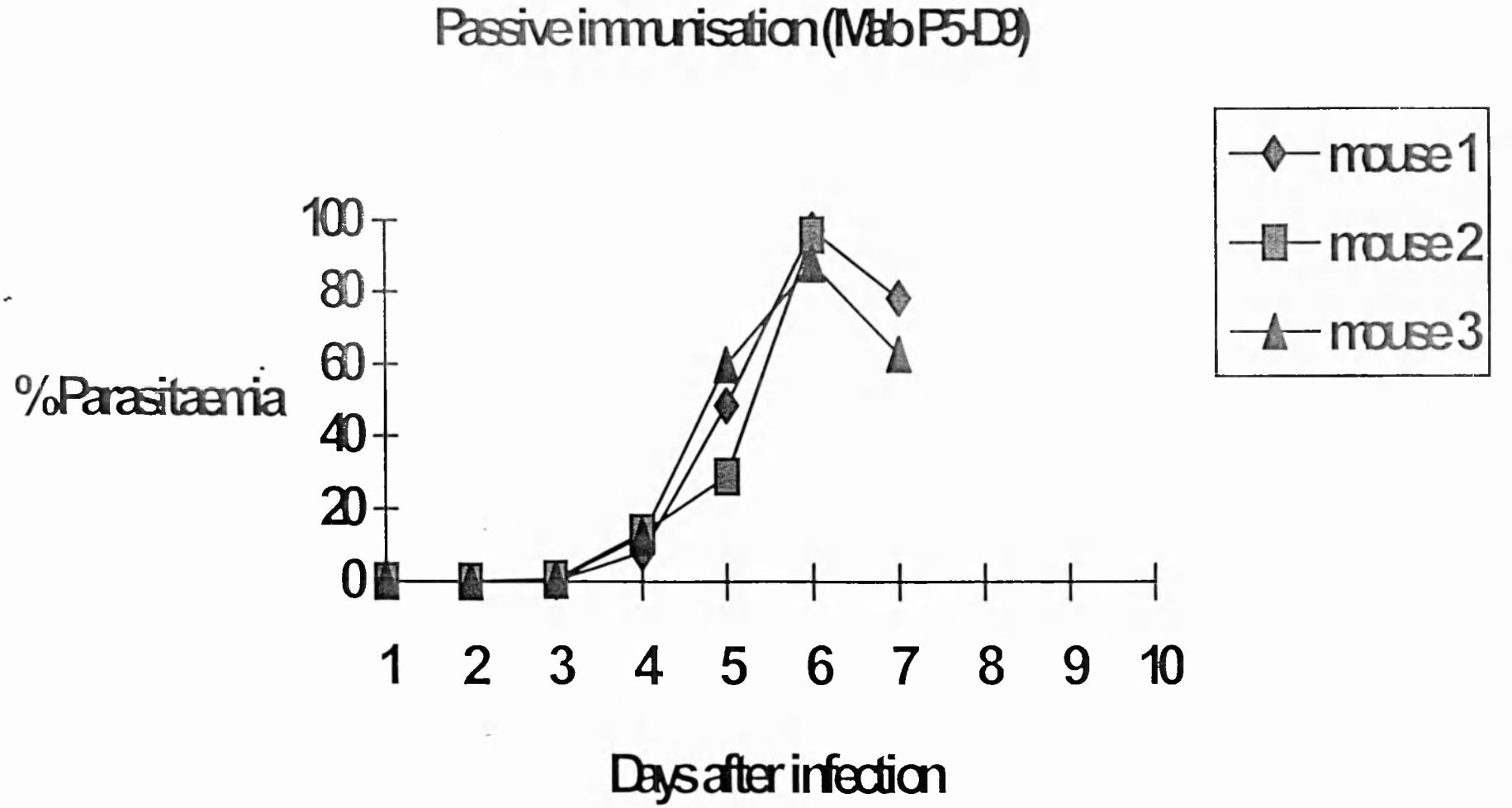

Passive immunisation (Mab P5-D9)
mouse 1
mouse 2
mouse 3
%Parasitaemia
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after infection

**Figure 4.7.**

Passive immunisation with Mab P6-B4, followed by intravenous challenge with $5 \times 10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

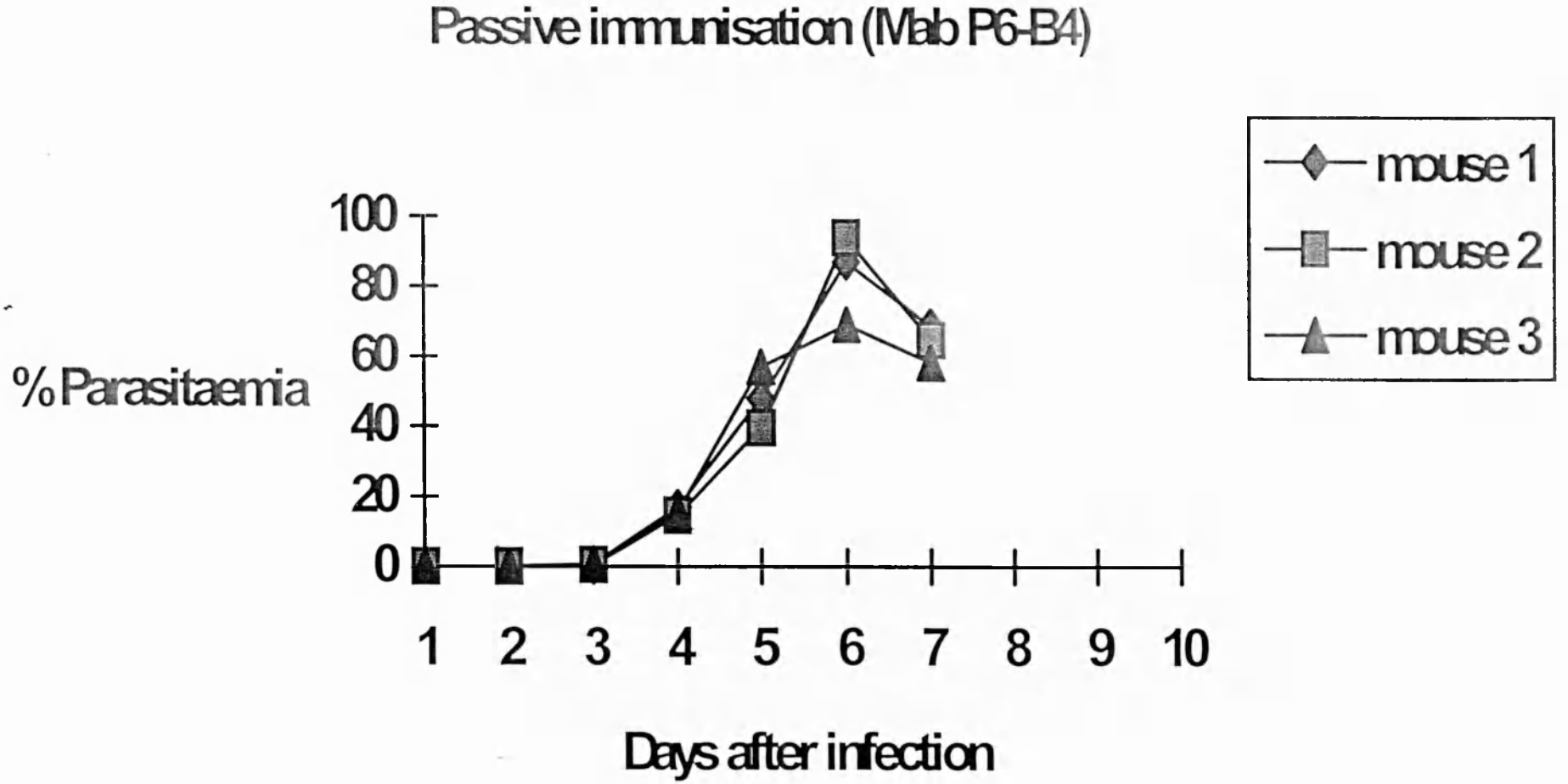

Passive immunisation (Mab P6-B4)
mouse 1
mouse 2
mouse 3
% Parasitaemia
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after infection

**Figure 4.8.**

Passive immunisation with Mab P3-D3, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice survived.

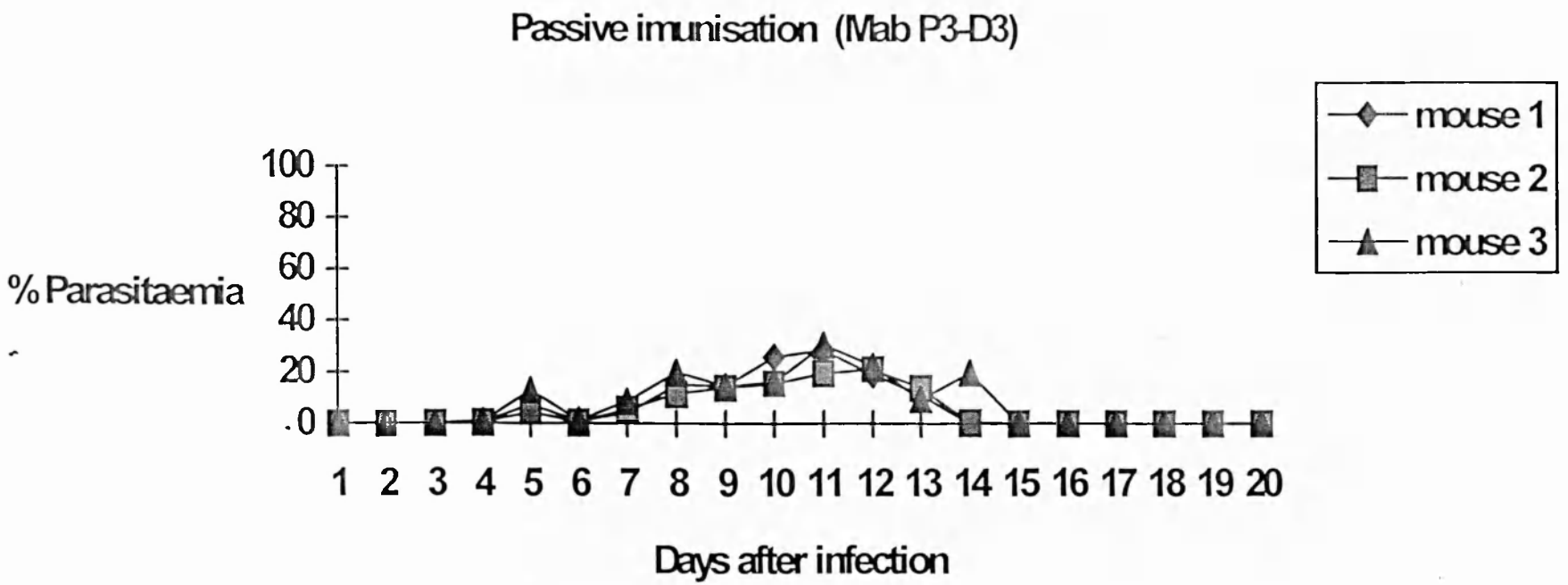

Passive imunisation (Mab P3-D3)
mouse 1
mouse 2
mouse 3
% Parasitaemia
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Days after infection

**Figure 4.9.**

Passive immunisation with Mab P2-F5, followed by intravenous challenge with $5 \times 10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice survived.

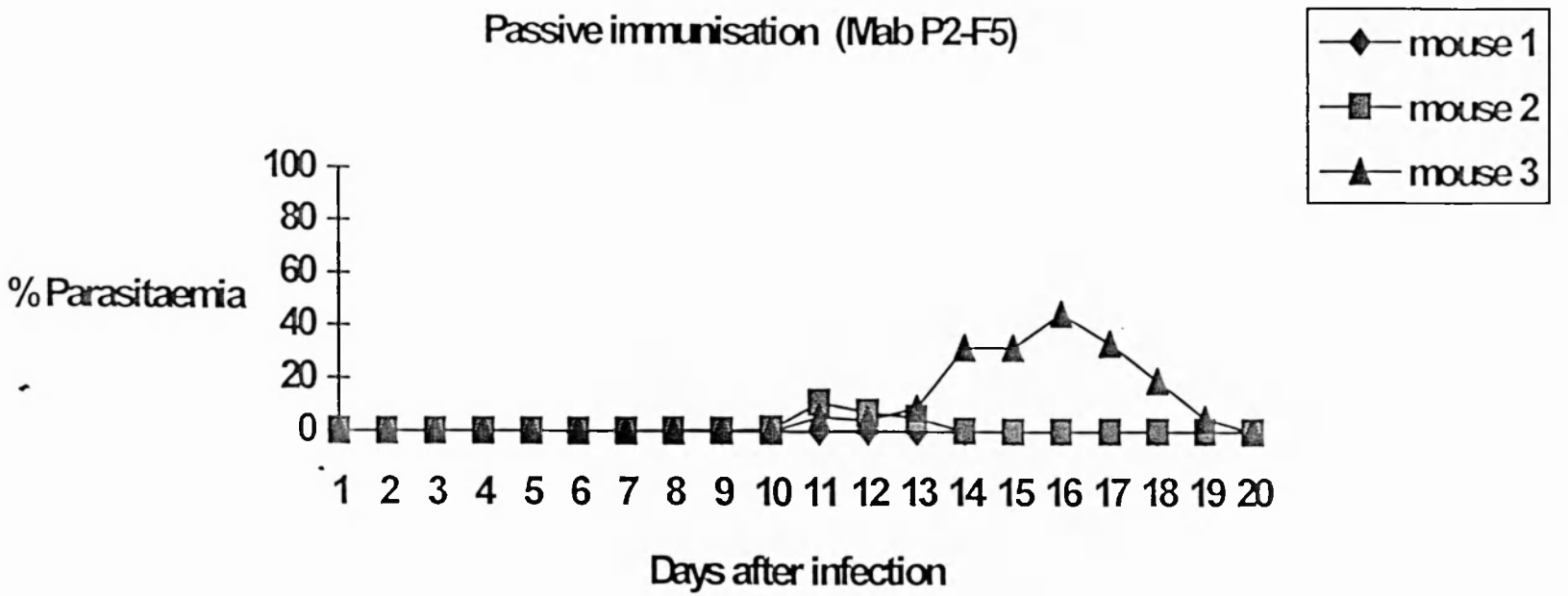

Passive immunisation (Mab P2-F5)
mouse 1
mouse 2
mouse 3
% Parasitaemia
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Days after infection

**Figure 4.10.**

Passive immunisation with Mab P6-B6, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice survived.

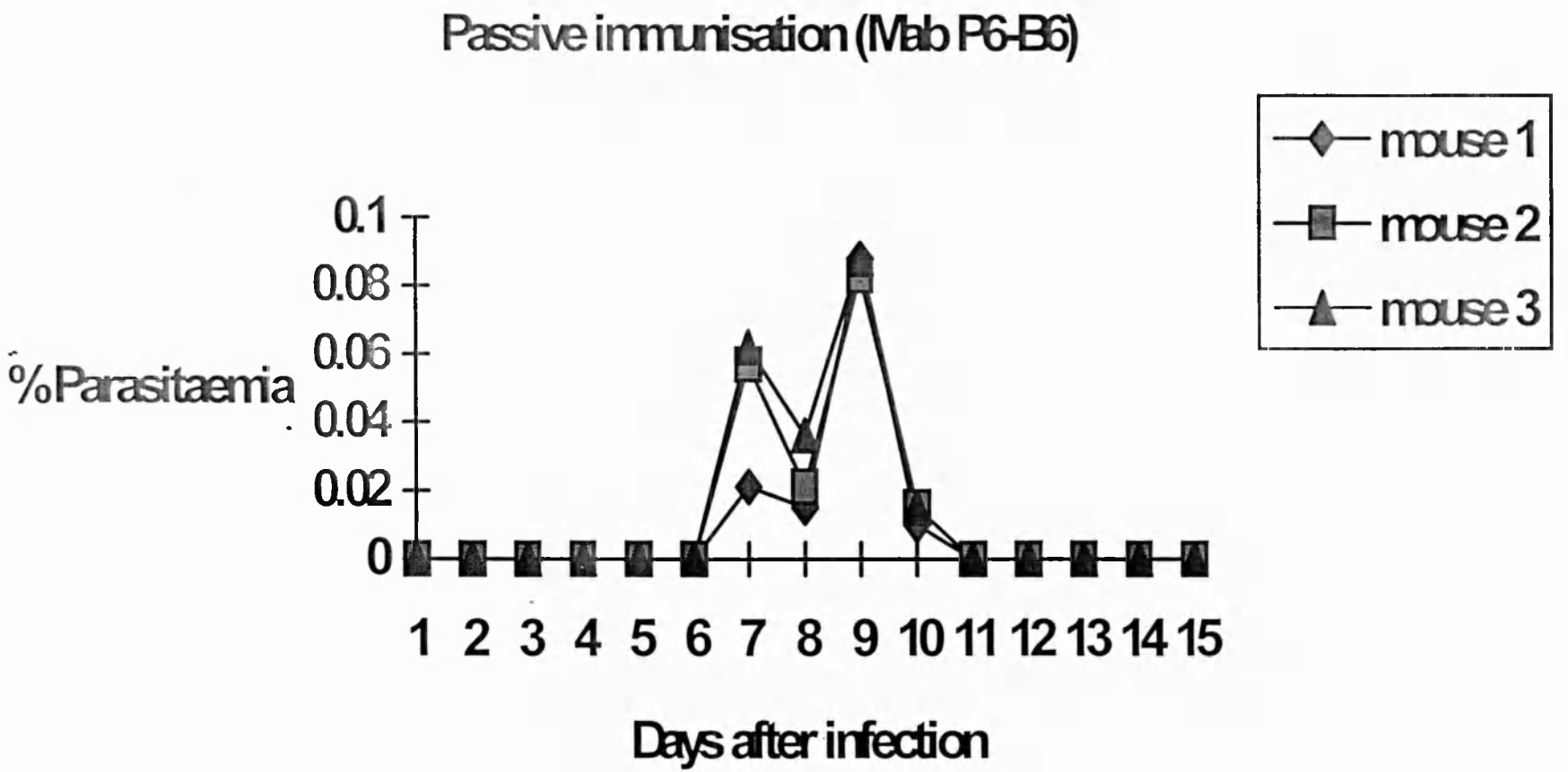

Passive immunisation (Mab P6-B6)
mouse 1
mouse 2
mouse 3
%Parasitaemia
0.1
0.08
0.06
0.04
0.02
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days after infection

**Figure 4.11.**

Passive immunisation with Mab B10, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice survived.

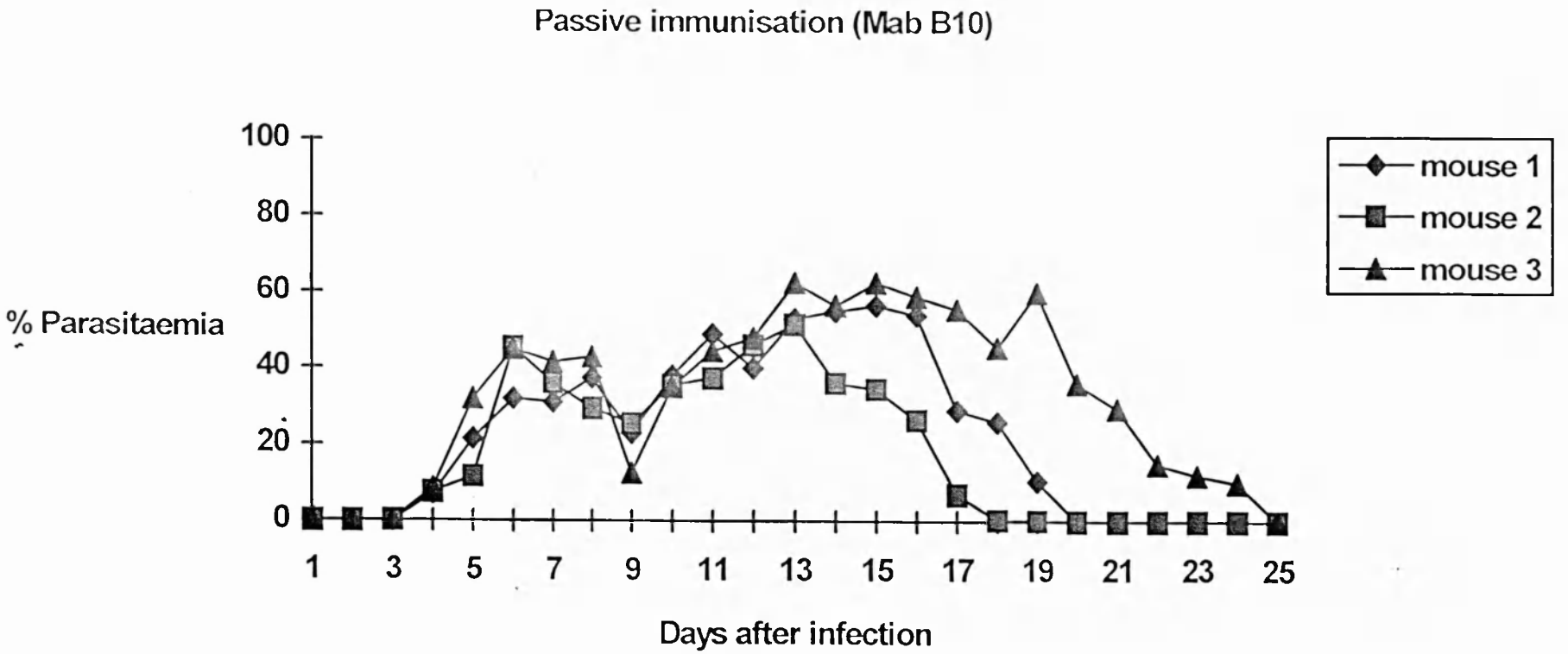

Passive immunisation (Mab B10)
% Parasitaemia
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
23
25
Days after infection
mouse 1
mouse 2
mouse 3

**Figure 4.12.**

Passive immunisation with Mab G3, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of five mice each received 0.5 mg/mouse of Mab intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). Two out of five mice died.

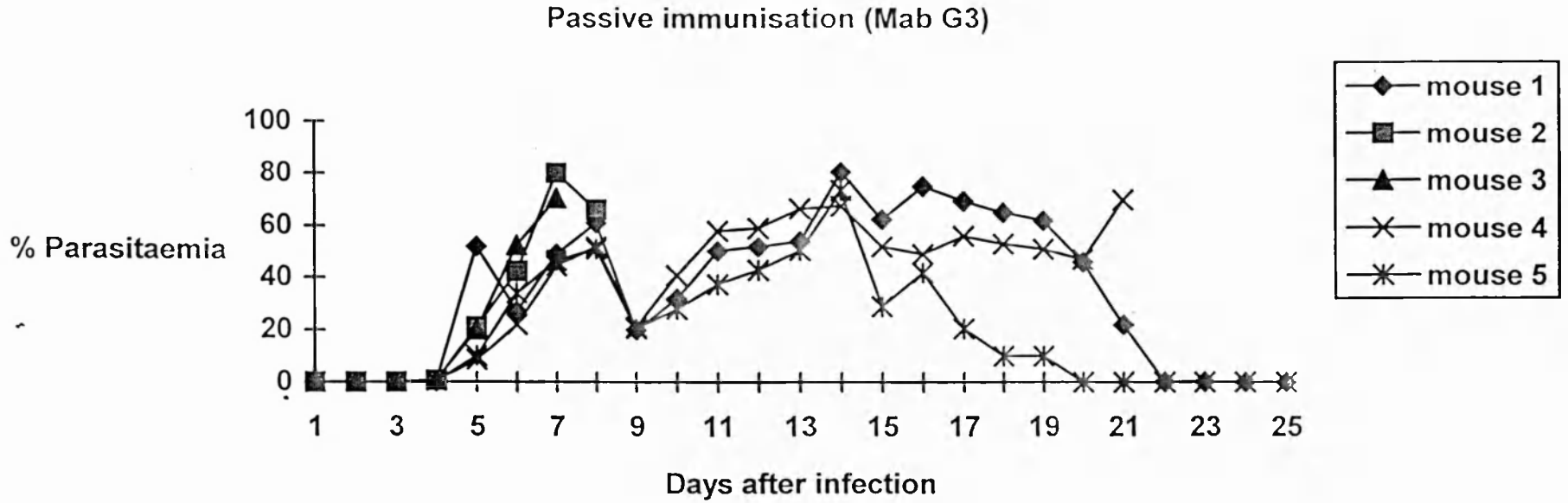

Passive immunisation (Mab G3)
% Parasitaemia
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
23
25
Days after infection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5

**Figure 4.13.**

Inoculation of PBS (as a negative control), followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. This group of three mice each received PBS intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). All mice died.

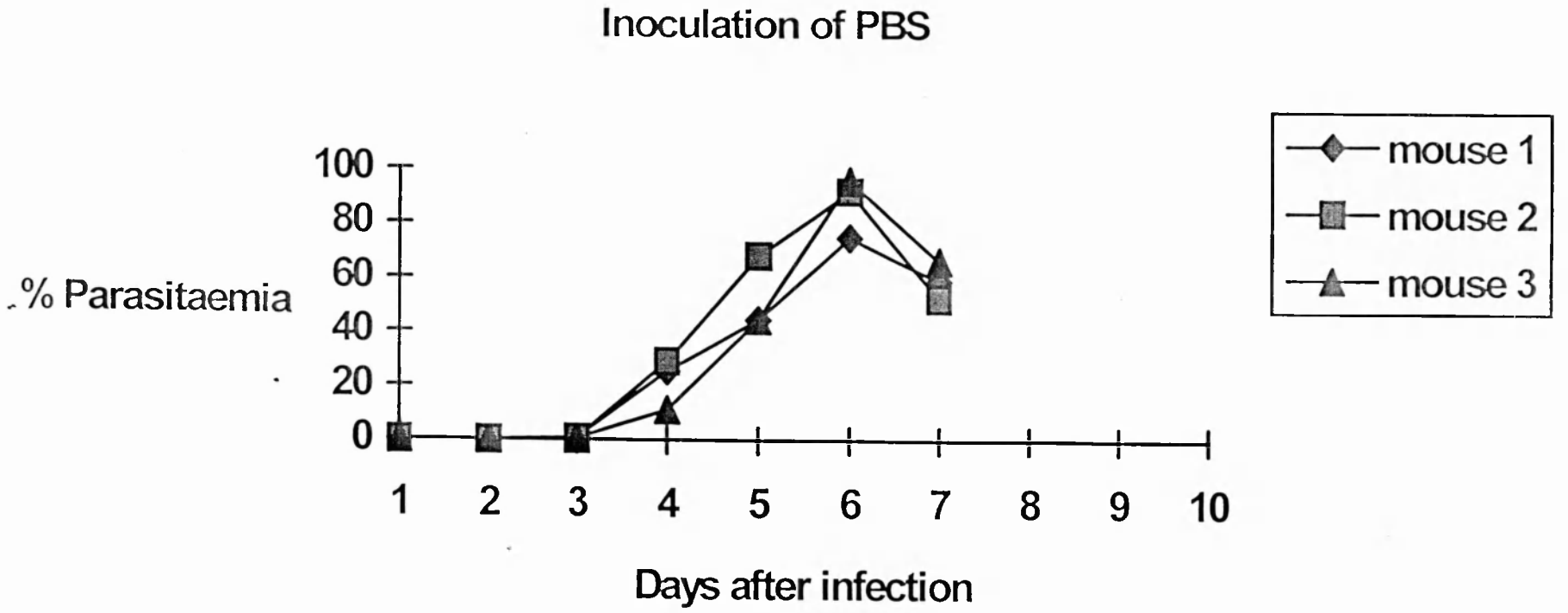

Inoculation of PBS
% Parasitaemia
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Days after infection
mouse 1
mouse 2
mouse 3

**Figure 4.14.**

The course of *P. yoelii* infection in groups of 10 BALB/c mice injected intraperitonially with solutions of Mabs P2-F5, P6-B4, P6-B6, P3-D3, P5-D9, B10 and G3 followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. Mice in each group received 0.2ml of a 1.5mg/ml solution of antibody intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). PBS was used as a negative control. Each point represents the geometric mean parasitaemia of mice in each group and the vertical bars indicate the standard error.

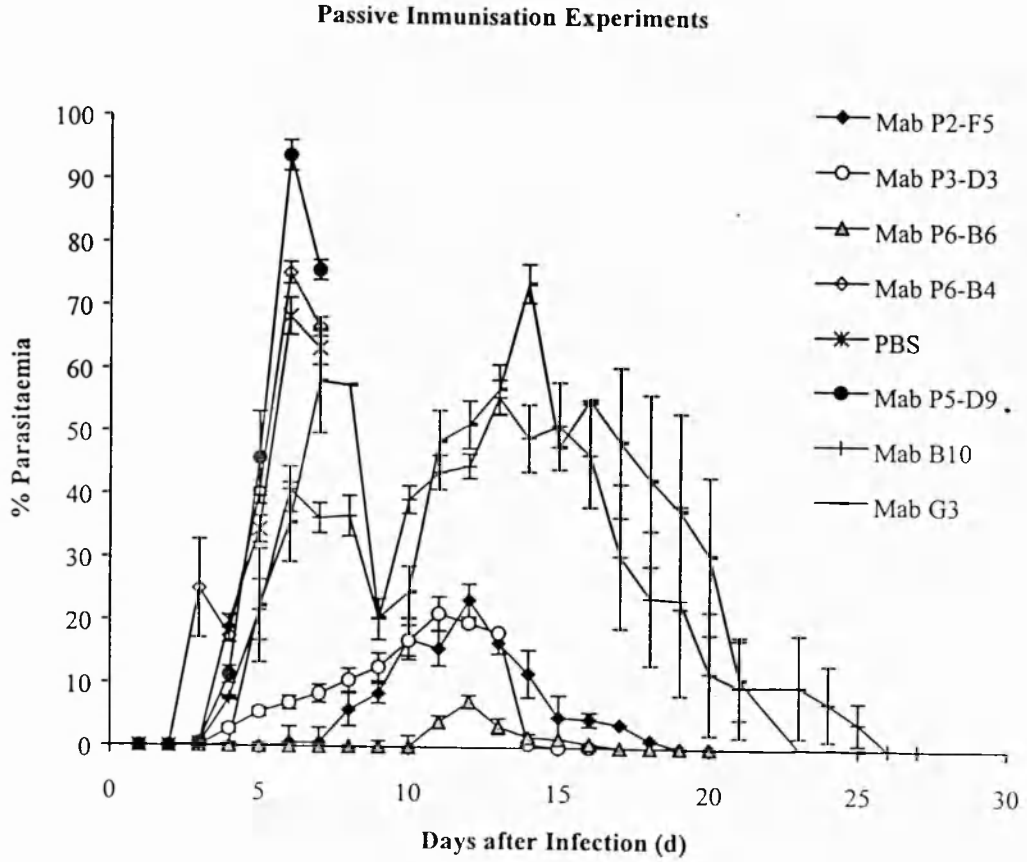
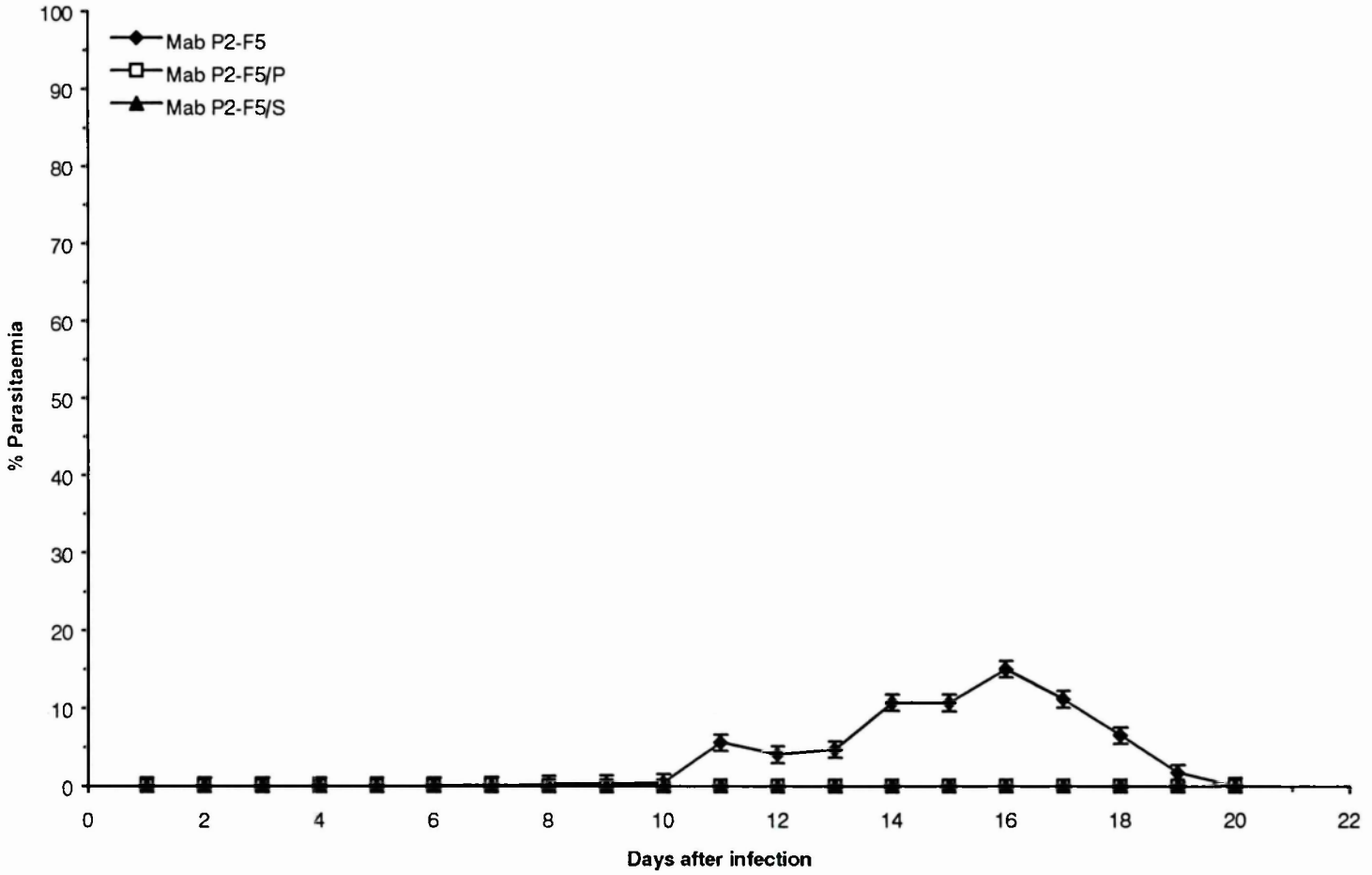

Passive Inmunisation Experiments
% Parasitaemia
Days after Infection (d)
Mab P2-F5
Mab P3-D3
Mab P6-B6
Mab P6-B4
PBS
Mab P5-D9
Mab B10
Mab G3
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30

**Figure 4.15.**

Passive immunisation with different preparations of MabP2-F5, followed by intravenous challenge with 5 x $10^3$ PRBC per mouse. Group of three mice each received either 0.5 mg P2-F5 in solution, 0.5mg P2-F5 as a suspension of precipitated protein in PBS (P2-F5P), or 0.5mg P2-F5 as a suspension of precipitated protein in a solution containing 0.5mg soluble P2-F5 (P2-F5S), intraperitoneally on days -1,0 and 1 relative to the day of parasite challenge (day 0). Each point represents the mean parasitaemia of mice in each group.

**Passive immunisation with different preparations of Mab P2-F5**



## CHAPTER 5: ANTIBODY CROSS-REACTION WITH MSP-1 IN *P. YOELII* 265BY

### 5.1 Introduction

Previous work has demonstrated antibody cross-reactivity between proteins of different malaria parasites. For example, cross-reactivity was detected using an antiserum raised against the 230kDa *P. yoelii* MSP-1. This antiserum contained antibodies which also reacted with high molecular mass proteins of *P. chabaudi* and *P. falciparum,* although individual Mabs specific for each of the proteins did not cross-react with other two species (Holder et al., 1983). Wanidworanun et al.(1989) identified antigens which cross-reacted with antibodies against the Pc96 antigen of *P. chabaudi* in the human malaria parasites, *P. falciparum* (155kDa) and *P. vivax* (220kDa), and in the monkey malaria parasite *P. cynomolgi* (200kDa). To detect cross-reactivity between Pc96 and the other malaria parasite antigens, erythrocytes infected with the others species were incubated with a specific antibody (Mab 7C6) to Pc96 and binding was detected by IFA. Mab 7C6 reacted specifically with *P. falciparum*, *P. vivax* and *P. cynomolgi*. Taylor and colleagues (1981) used eighteen Mabs to study species-specific, stage-specific and serologically cross-reactive antigens of rodent malaria parasites. Twelve distinct plasmodial antigens could be distinguished by using the 18 Mabs. Moreover, 6 of the 18 Mabs identified species-specific antigens and 8 of them detected cross-reactive antigens common to rodent, primate and avian malarias.

In the past it has been difficult to identify and isolate individual plasmodial antigens and evaluate their roles in the immune response. Now, the use of recombinant DNA

technology makes it possible to produce large amounts of antigenic proteins from different malaria parasites and study them in detail. The availability of these proteins together with specific Mabs, which can also be used to identify and purify malaria antigens, have enhanced these studies on the role of various parasite proteins as the targets of, or in the induction of an immune response. One major blood-stage vaccine candidate identified in a number of plasmodial species is MSP-1 (Holder, 1988). The C-terminal fragment of *Plasmodium yoelii* MSP-1 has been expressed as a recombinant protein in *E. coli* and mice immunised with this recombinant protein-1 are protected against challenge with the lethal *P. yoelii* YM strain (Daly and Long, 1993; 1995; Ling et al.,1994). However, little is known about whether or not the protection is specific for homologous parasites. Different *P. yoelii* lines and clones may have sequence differences, and the importance of these in the protective immune response is unclear. For example it is possible that sequence diversity at the C-terminus of MSP-1 leads to antigenic differences and this results in the evasion of a protective immune response directed against MSP-1. Burns and colleagues (1989) using the *P. yoelii* model system demonstrated that the epitope recognised by the protective Mab 302, which recognises the C-terminal 19kDa fragment of MSP-1, is strain variable. The protective capacity of 302 was variant-specific, passive immunisation with this antibody afforded protection against infection with only three of five *P. yoelii* lines.

In Chapters 3 and 4, I have described Mabs which when transferred to naïve mice were able to protect them against a challenge infection with parasites of the YM strain. To extend this work, the ability of these antibodies to cross react with MSP-1 from the 265 BY line of *P. yoelii* was investigated. The 265 BY line was originally isolated by I. Landau, Natural History Museum, Paris France, and was provided by Dr Dominique

Mazier. A pattern of self-limiting infection in mice is caused by 265BY parasites, similar to that of the non-lethal 17X strain (Lucas et al., 1993). A recombinant protein encompassing the two EGF-like motifs of *P. yoelii* 265 BY MSP-1, has been expressed. This protein was recognised by some of the Mabs despite a number of amino acid sequence differences between the proteins from the YM and 265BY lines. Some of the Mabs also showed cross reactivity between these two lines of *P. yoelii* when studied by IFA.

## 5.2. Amino acid sequence differences in the C-terminal region of MSP-1 derived from the YM and 265BY lines of *P. yoelii*

The deduced amino acid sequence of the C-terminal 19 kDa fragment of MSP-1 from *P. yoelii* YM and the differences present in the protein from the 265BY line, are shown in Figure 5.1. This region of MSP-1 is conserved between the lines, but a number of differences can be observed. A number of substitutions of amino acids are evident and are represented by different colours. In the first domain, the amino acids substituted are Aspartic acid (D) by Tyrosine (Y), Threonine (T) by Asparagine (N), Tyrosine by Phenylalanine (F), Glycine (G) was deleted (indicated in a blue colour on Fig. 5.1), Glycine by Asparagine and Asparagine by Aspartic acid. In the second domain the amino acids substituted are Threonine by Serine (S), Isoleucine (I) by Threonine, Proline (P) by Threonine, Threonine by Alanine (A), Asparagine by Glycine, Alanine by Aspartic acid, Glutamic acid by Arginine (R), Threonine by Glycine and Isoleucine by Valine (V). In addition, there is an insertion of a Threonine between Asparagine and Glutamine at residue 69. Overall there is an 11% sequence difference between the two forms of the 19kDa fragment of MSP-1. Although some of these substitutions are conservative, such

as replacement of Tyr by Phe or Ser by Thr, the majority are non-conservative and result in major changes in the size and/or charge of the individual side chains.

### 5.3. Assessment of antibody cross-reactivity by Western blotting

Given the potent biological activity of Mabs P2-F5, P6-B6, P3-D3, B10 and G3 on passive immunisation against the YM line, I have examined the possible variability of the epitopes recognised by these Mabs between two lines of *P. yoelii*. The cross-reactivity of the panel of Mabs was tested by western blotting and ELISA. Western blot analysis was performed essentially as described in Chapter 2. The combined EGF-like motifs of YM and of 265BY MSP-1 were expressed in *E. coli* as recombinant proteins (GST-$MSP1_{19}$ and GST-$MSP1_{19(265BY)}$, respectively), and purified using the method of glutathione-affinity chromatography. Equal amounts of the proteins were solubilised with sample loading buffer, either containing dithiothreitol (reducing conditions) or not (non-reducing conditions), and then subjected to electrophoresis on 15% polyacrylamide gels in presence of SDS. The resolved polypeptides were then transferred electrophoretically to nitrocellulose paper and probed with the Mabs.

The reaction of Mabs B10, P5-D9, P2-F5, P6-B6 and P3-D3 with the recombinant proteins prepared under reducing and non-reducing conditions is shown in Figures 5.2, 5.3, 5.4, 5.5 and 5.6, respectively. Mab B10 recognised the combined EGF-like motifs of the YM MSP-1 in both reducing (Panel A, track 3) and non-reducing conditions (Panel B, track 3),although the reaction with the non-reduced protein was much stronger, but it did not recognise the 265BY protein (Figure 5.2, tracks 4). Mab P5-D9 recognised faintly both the first and combined motifs of the YM protein (Figure 5.3). It also

recognised faintly the combined motifs of the 265 BY protein. In both cases it only recognised the proteins prepared in non-reducing conditions (Panel B). Mabs P2-F5 and P6-B6 (Figures 5.4 and 5.5) recognised both the first and the combined motif recombinant proteins of YM MSP-1. Both Mabs also recognised the combined EGF-like motifs that form the recombinant protein from 265 BY MSP-1. Mab P6-B6 did not recognise the 265BY recombinant protein if this had been reduced with dithiothreitol (Fig 5.5, Panel A, track 4), and detection of the unreduced protein was less sensitive than for the YM protein. Probably the substitution of one or more amino acids in the sequence of the protein modifies the epitope recognised by this Mab or does not allow the protein to refold properly after it has been subjected to SDS-PAGE under reducing conditions. Figure 5.6 shows that none of the recombinant proteins were recognised by the Mab P3-D3. This was not surprising since this Mab only recognises a larger structure consisting of the 42 kDa fragment from the YM MSP-1(see Chapter 3). Finally, the Mab G3 that recognised the combined motifs of YM $MSP1_{19}$ did not recognise the 265 BY recombinant protein in Western blotting experiments (data not shown).

### 5.4. Assessment of antibody cross reactivity by ELISA

Table 5.1 shows the results of the reaction of the different Mabs with the recombinant proteins when assessed by ELISA. Four of the six Mabs cross-reacted with the 265BY protein.

## 5.5. Discussion

The current studies using recombinant proteins derived from MSP-1 from two lines of *P. yoelii* demonstrated that epitopes recognised by a panel of Mabs are variable. However, some cross-reaction between the YM and 265BY MSP-1 recombinant proteins was observed despite the 11% sequence difference between these two polypeptides. Some of the Mabs recognised the recombinant protein from both *P. yoelii* lines by both Western blotting and ELISA. This analysis of the specificity of immune responses by cross-reactivity constitutes a simple approach toward the identification of important conserved antigens. Table 5.2 shows a summary of the characteristics of the Mabs and their cross-reaction between the two MSP-1 recombinant proteins. The demonstration of disulphide-bridge dependent antibody binding in the Western transfer analysis suggests that the cross-reacting determinants are epitopes formed by amino acids sequences which are constrained in a three dimensional structure dependent on the cysteine residues. The reaction of the antibodies with the proteins that had not been previously reduced was always stronger than with the corresponding reduced samples; and there was often reaction with higher molecular mass material that may correspond to inter-chain disulphide-bonded aggregates of two or more molecules. It is possible that reduction of the samples prior to electrophoresis disrupts these intermolecular bonds, allowing the protein to migrate as the monomer, followed by at least partial refolding on the nitrocellulose. In other experiments the ability of the Mabs to react with parasitised red blood cells of *P. falciparum* (Wellcome line) was investigated (data not shown). The Mabs did not recognise the *P. falciparum* parasites in an IFA test, suggesting that the epitopes are not formed by sequence

conserved between these two species. The antigens of malaria parasites are generally species specific, although antibodies raised against one *Plasmodium* species can react with antigens from other species (Holder et al., 1983 and Taylor et al., 1981), due to the presence of homologous molecules with inter-species conserved epitopes (Ray et al., 1994).

Ray and colleagues identified inter-species conserved antigens of *P. falciparum* that appeared to be the target of protective antibodies. They detected antigens in a *P. falciparum* lysate or in whole parasitised cells by using antibodies produced during repeated *P. yoelii* infection of BALB/c mice. Inhibition of *P. falciparum* growth *in vitro* was observed with this mouse anti-*P. yoelii* serum (Ray et al., 1994), but the exact targets of these protective antibodies are unknown.

Of the six Mabs used in this study, only four of them showed some cross-reactivity with the 265 BY MSP-1. Mab P3-D3 does not recognise the recombinant proteins from either line, because this Mab recognises a 42 kDa fragment of MSP-1 in extracts of parasites from the YM line which extends beyond the sequence contained within the recombinant protein. It will be necessary to test whether or not this Mab recognises a common determinant on MSP-1 expressed in erythrocytes parasitised with *P. yoelii* 265 BY, for example by IFA or immunoprecipitation . Unfortunately, I did not have available parasites from *P. yoelii* 265 BY to assess the cross-reactivity. For the same reason, I could not assess the ability of the Mabs to protect against challenge with the 265BY line by passive immunisation.

The fact that the C-terminal region of MSP-1 of two different strains of *P. yoelii* are substantially different, contrasts with the observation of very high sequence conservation in this region of the *P. falciparum* MSP-1. It has been assumed that this protein serves a function essential to biology of parasite. It is not known whether or not the observed sequence diversity in *P. yoelii* represents a selection of variants by a protective immune response or variation due to some other cause. In a recent study Renia and colleagues (1997) investigated the ability of a recombinant MSP-1 based on the YM sequence to protect against challenge with either YM or 265BY parasites. The protection observed appeared to be largely specific for the homologous challenge, suggesting that the sequence differences do have a profound effect on the protective immunity that is induced. Taking into consideration the molecular parallels between the MSP-1 of *P. yoelii, P. falciparum*, and other malaria parasite species, this information may contribute to the development of an anti-malaria vaccine and an understanding of the way in which protective antibody works. To obtain a full understanding, determination of the three dimensional structure of the C-terminal fragment of MSP-1, together with the structure of the antigen-antibody complex, will be necessary. An analysis of the structure of the antigen-antibody complex, for example by X-ray crystallography, will enable the identification of the binding sites for the protective antibodies. Additional studies using either natural sequence variants or mutants in which individual amino acids are changed, will also help define the critical amino acid sequences necessary for antibody binding. Some of the sequence differences between MSP-1 from the two lines may result in a reduced affinity of interaction between the antibody and the antigen, without necessarily completely abolishing reactivity. The importance of antibody affinity and avidity for protection on passive immunisation needs to be investigated further.

Mabs can also be used for taxonomic studies of malaria parasites. Despite the evolutionary adaptation of parasites to various hosts, some antigens have been conserved. The serological cross-reaction of antibodies to the rodent malaria parasites with other species, such as those that infect primates, and between parasites of humans and primates has allowed the partial identification of antigens which may have significance for taxonomic studies (Taylor et al., 1981). However, monoclonal antibodies for this type of analysis should be used with caution, because of the potential of variation in binding, even to parasites within the same species.

**Table 5.1.**

The cross reaction of Mabs with recombinant proteins based on MSP-1 from the YM and 265 BY lines of *P. yoelii*. The antigens used consisted of GST fusion proteins containing the first EGF-like motif from YM MSP-1(MSP1EGF1-YM), the second EGF-like motif from the YM MSP-1 (MSP1EGF2-YM), the combined motifs from the YM MSP-1 ($MSP1_{19(YM)}$ ) and the combined motifs from the 265BY MSP-1 ($MSP1_{19(265BY)}$).

| *Mab* | *MSP1EGF1-YM* | *MSP1EGF2-YM* | $MSP1_{19(YM)}$ | $MSP1_{19(265BY)}$ |
|---|---|---|---|---|
| P2-F5 | + | - | + | + |
| P6-B6 | + | - | + | + |
| B10 | - | - | + | + |
| P5-D9 | + | - | + | + |
| G3 | - | - | + | - |
| P3-D3 | - | - | - | - |

+: Mab recognises the recombinant protein.

-: Mab does not recognise the recombinant protein.

**Table 5.2.**

A summary of the characteristics of the Mabs and their cross-reaction with recombinant proteins based on MSP-1 from the YM and 265BY lines of *P. yoelii*.

| *Mab* | *Subclass* | *Reaction with YM* | *Reaction with 265BY* | *Passive immunity to YM challenge* |
|---|---|---|---|---|
| P2-F5 | IgG3 | + | + | protective |
| P6-B6 | IgG3 | + | + | protective |
| B10 | IgG2b | + | - | partially protective |
| P5-D9 | IgG2b | + | + | not protective |
| P3-D3 | IgG2a | + | ? | protective |

+: Mab recognises the recombinant protein or the native MSP-1 in parasite extracts.

-: Mab does not recognise the recombinant protein, but reactivity with the native MSP-1 has not been tested.

?: Mab does not recognise the recombinant MSP-$1_{19}$ , but it is not known whether or not the Mab can recognise other fragments of the protein.

**Figure 5. 1.**

The deduced amino acid sequence of the C-terminal 19 kDa fragment of MSP-1 from *P. yoelii* YM , showing in red (substitution) and blue (deletion) the residues that differ in the protein from the 265BY line. The polypeptide consists of two EGF-like motifs. Disulphide bonds maintain the conformation and this three dimensional structure may be an important target for protective immunity. The substitutions in the amino acid sequence found in the corresponding part of *P. yoelii* 265 BY MSP-1 are indicated in yellow (substitution) and green (insertion). There is 11% sequence difference between the two forms.

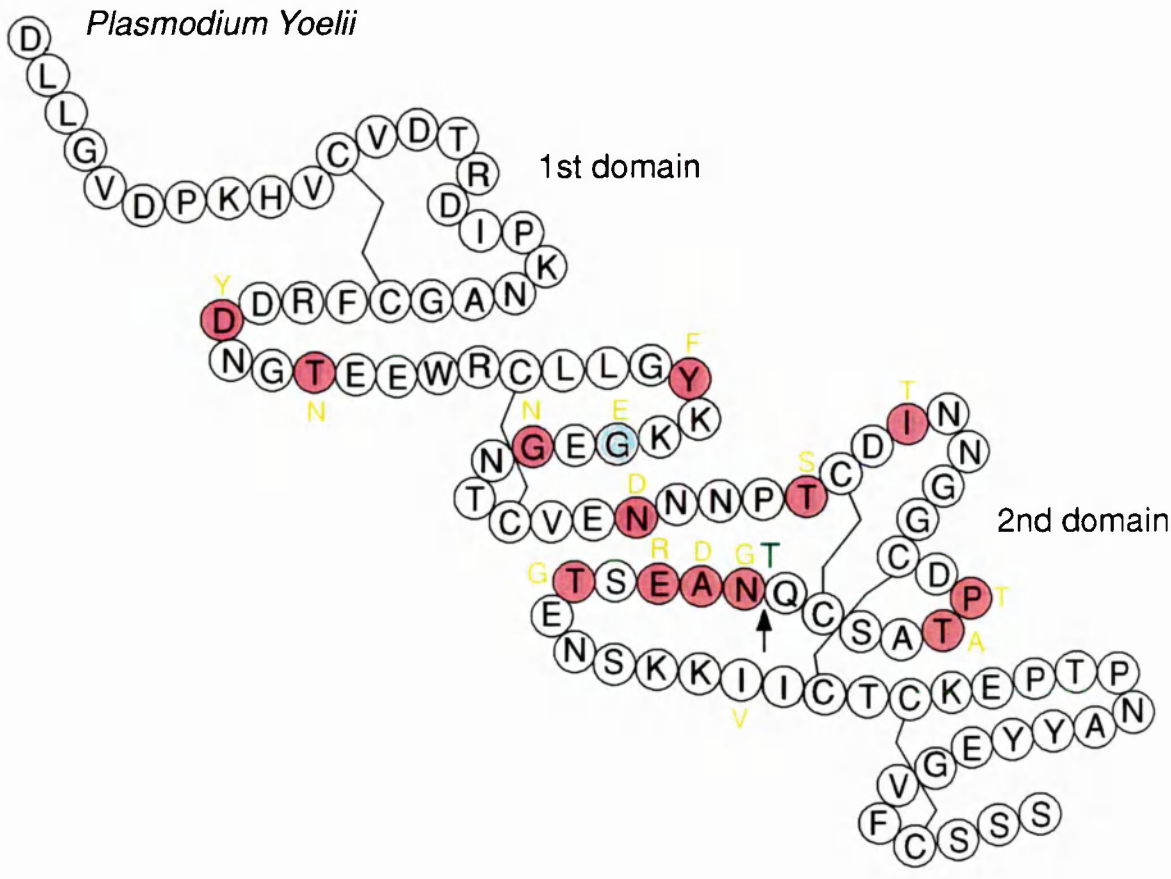
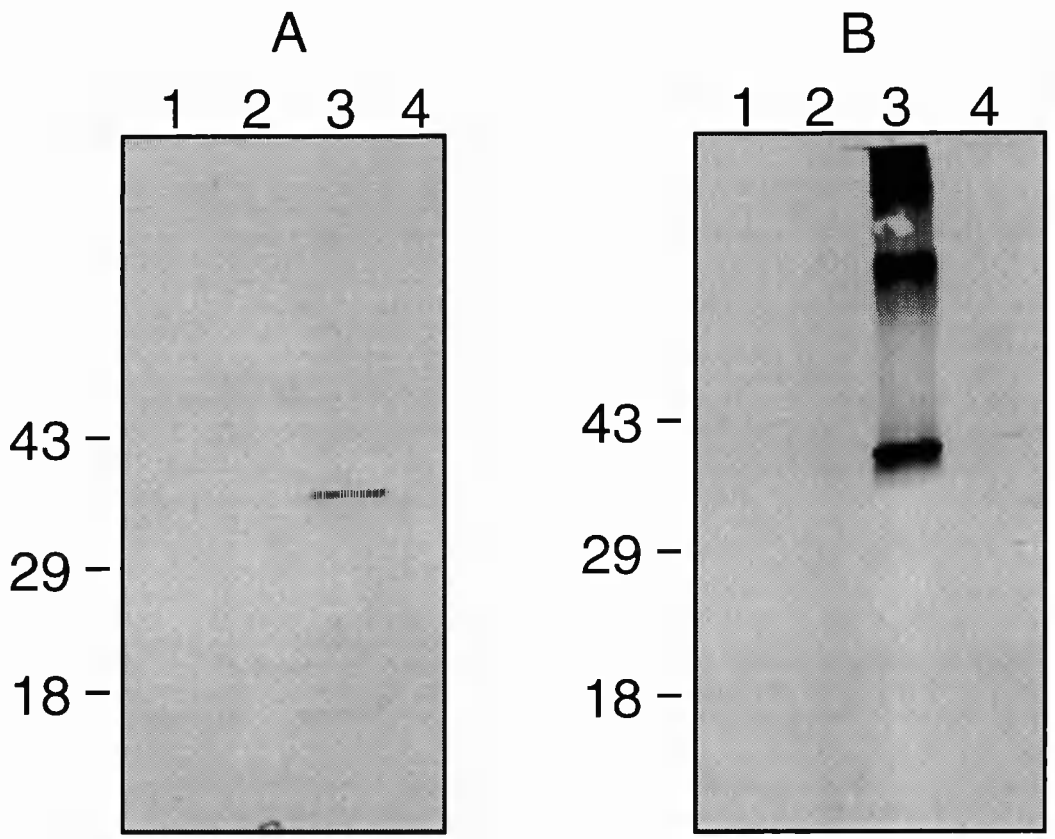

Plasmodium Yoelii
1st domain
2nd domain

**Figure 5.2.**

Mab B10 recognises the recombinant protein containing both EGF-like motifs of MSP-1 from the YM but not the 265BY line. Purified GST-fusion proteins were separated by SDS-PAGE on a 15% polyacrylamide gel, transferred to nitrocellulose and then the blots were probed with a 1:100 dilution of Mab solution.

In Panel A the proteins were reduced with dithiothreitol prior to electrophoresis and in panel B were prepared under non-reducing conditions. The antigens were GST-MSP1EGF1, fusion protein containing the first EGF-like motif of *P. yoelii* YM (track 1); GST-MSP1EGF2, fusion protein containing the second motif (track 2); GST-$MSP1_{19}$, fusion protein containing both EGF-like motifs of MSP-1 from *P. yoelii* YM (track 3); and GST-$MSP1_{19(265BY)}$, fusion protein containing both EGF-like motifs of MSP-1 from *P. yoelii* 265BY (track 4). The mobility of standard molecular mass markers is indicated.



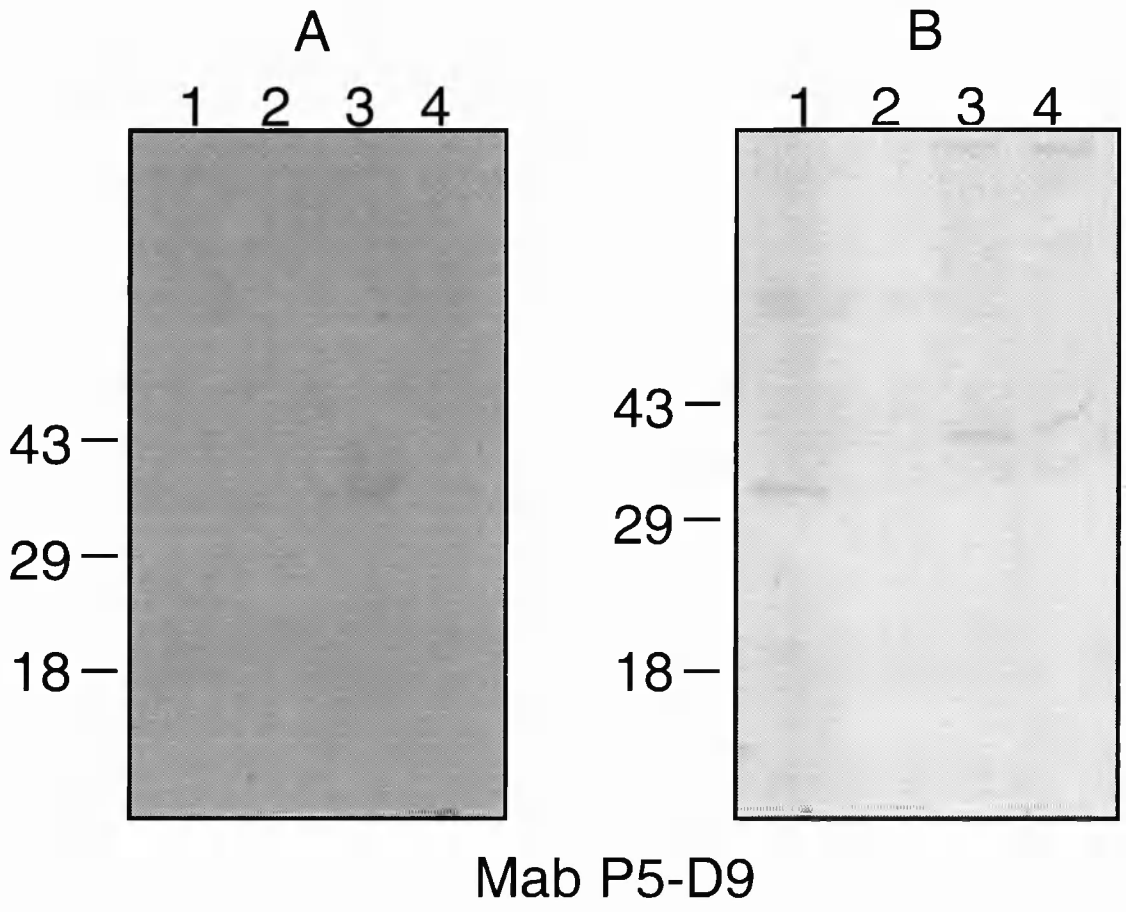

Mab B10

Spencer 1a 5/6/96
N.I.M.R. London, NW7 1AA.

**Figure 5.3.**

Mab P5-D9 recognises recombinant proteins containing the first and the combined EGF-like motifs from YM, as well as the recombinant protein containing the combined EGF-like motifs of MSP-1 from 256BY in non-reducing conditions. After reduction of the recombinant proteins they were no longer recognised by the antibody.

Panel A corresponds to proteins prepared under reducing conditions and Panel B proteins prepared under non-reducing conditions. The samples and other conditions are the same as in Figure 5.2.

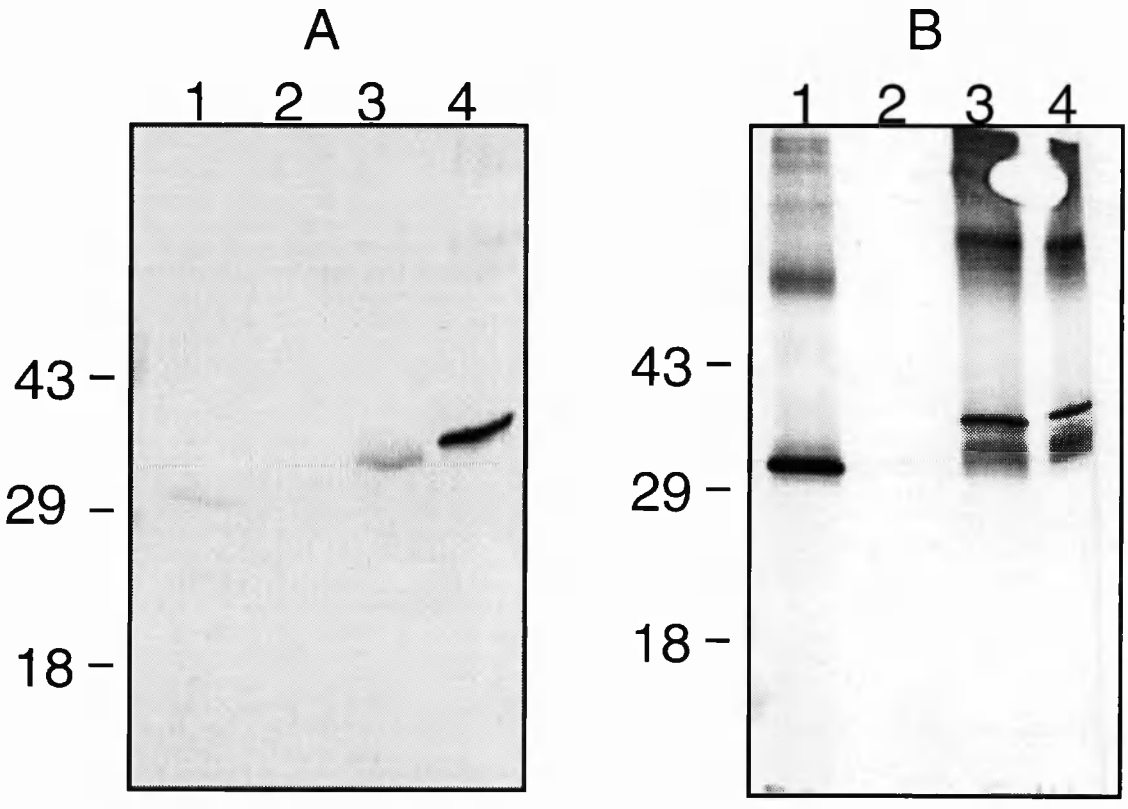



Spencer 1b 5/6/96
N.I.M.R. London, NW7 1AA.

**Figure 5.4.**

Mab P2-F5 recognises recombinant proteins containing the first and the combined EGF-like motifs from YM MSP-1, as well as the recombinant protein containing the combined EGF-like motifs of MSP-1 from 265BY, in samples that had been treated with both reducing and non-reducing conditions.

Panel A corresponds to proteins prepared under reducing conditions and Panel B proteins prepared under non-reducing conditions. The samples and other conditions are the same as in Figure 5.2.



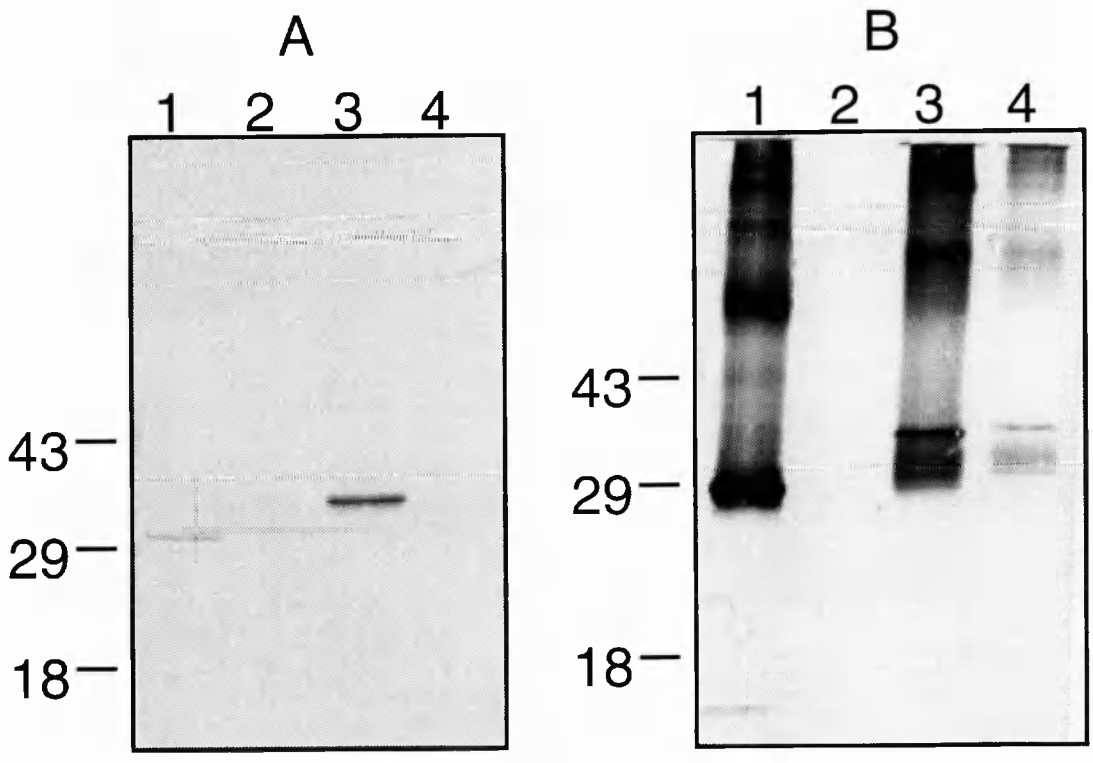

Mab P2-F5

Spencer 2c 5/6/96
N.I.M.R. London, NW7 1AA.

**Figure 5.5.**

Mab P6-B6 recognises the recombinant protein containing the first and the combined EGF-like motifs of MSP-1 from *P. yoelii* YM, and also the recombinant protein containing the combined EGF-like motifs of MSP-1 from 265BY in samples that had not been subjected to reducing conditions. Under reducing conditions the reaction with the 265BY protein was abolished.

Panel A corresponds to proteins prepared under reducing conditions and Panel B proteins prepared under non-reducing conditions. The samples and other conditions are the same as in Figure 5.2.

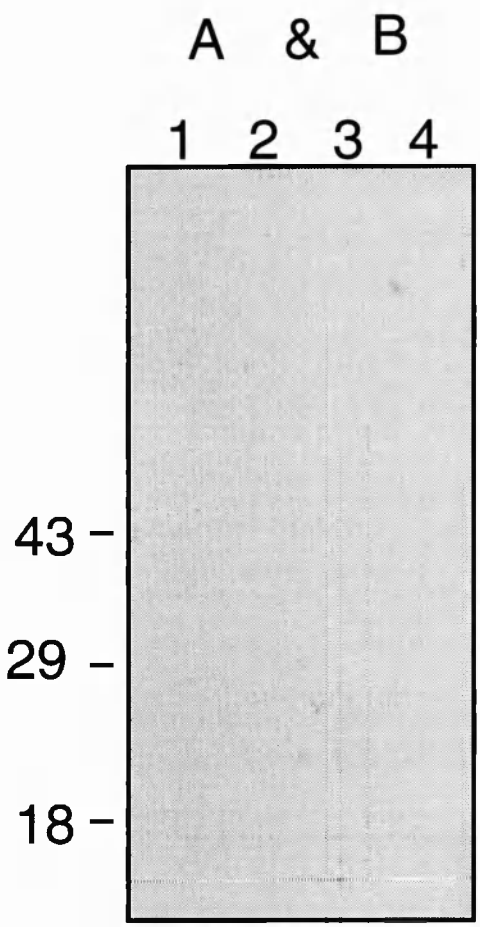



Mab P6-B6

Spencer 2d 5/6/96
N.I.M.R. London, NW7 1AA.

**Figure 5.6.**

Mab P3-D3 does not recognise the recombinant proteins based on MSP-1 from either YM or 265 BY treated with either reducing or non-reducing conditions. The samples and other conditions are the same as in Figure 5.2.




Mab P3-D3


N.I.M.R. London, NW7 1AA.

# CHAPTER 6: GENERAL DISCUSSION AND FUTURE WORK

## 6.1 General Discussion

*Plasmodium yoelii* in laboratory mice represents a model in which antibodies are required for resolution of infection (Weidanz & Long, 1988, Daly & Long, 1993, Daly & Long,1995; Ling et al., 1994). Mab 302 raised against MSP-1, provided protection against challenge infection with this species of *Plasmodium* (Majarian et al., 1984). In this work, I have produce monoclonal antibodies specific for MSP-1. Initially, I used a recombinant protein (GST-MSP1$_{19}$) to immunise the mice, because immunisation with recombinant *P. yoelii GST*-MSP1$_{19}$ could elicit a protective host antibody response (Daly & Long, 1993 & Ling et al., 1994).

The protective and partially protective Mabs (P2-F5, P6-B6, P3-D3, B10 and G3) obtained in this work, which provided passive protection against challenge infection with *P. yoelii,* give good evidence that antibodies play a very important role in the immune response to this parasite. Mabs B10, G3, P2-F5 and P6-B6 recognised epitopes localised to the C-terminal 19 kDa fragment of MSP-1, while P3-D3 recognised a 42 kDa fragment of the MSP-1 molecule, that includes MSP-1$_{19}$. These results, together with the fact that this protein is homologous to the *P. falciparum* MSP-1, show that the C-terminus of MSP-1 is an important candidate for a blood-stage malaria vaccine. This is consistent with an important role for MSP-1$_{19}$, which remains bound to the surface of the merozoite, and the demonstration that the antibodies to this region of the molecule can inhibit *P. falciparum* invasion *in vitro* (Chappel & Holder 1993). Mab 302 is a protective IgG3 antibody, which recognises the first EGF-like motif and the native *P. yoelii* MSP-

$1_{19}$. Calvo and colleagues (1996) hypothesised that the first EGF-like motif could elicit protective responses since Mab 302, which was mapped to this region, could passively protect mice against a lethal infection of *P. yoelii*. However, only the mice immunised with a recombinant protein containing both EGF-like motifs produced antibodies which could recognise the native MSP-1, and were protected against challenge infection with *P. yoelii* (Ling et al., 1995). Two of the protective Mabs (P2-F5 and P6-B6) obtained in this work are of the IgG3 subclass and both recognised the first and combined EGF-like motifs of MSP-$1_{19}$. The other protective Mab (P3-D3) is an IgG2a and it recognised a 42 kDa fragment of MSP-1 when an extract of schizonts and trophozoites was used as antigen in Western blotting. It does not recognise the MSP-$1_{19}$ and MSP-$1_{33}$ recombinant proteins, suggesting that the 42kDa fragment needs to be intact for binding to take place. A striking observation was that all protective Mabs were of the IgG3 and IgG2a subclasses, but not all Mabs are specific for the first EGF-like motif. In fact, only P2-F5 and P6-B6 bind the first motif alone. The partially protective Mabs are of the IgG2b and IgG1 subclasses and they recognised only the combined EGF-like motifs of MSP-1, suggesting that the protection in passive immunisation may depend on the immunoglobulin subclass. The results of this study show that antibody-mediated mechanisms are important for immunity to *P. yoelii*. Antibodies appear to be required in order for most mice to resolve their infections, but it is not clear if the protective antibodies must be of a certain subclass (Sayles & Wassom, 1992).

In the *P. yoelii* model, the *P. yoelii* 17X parasites are reticulocyte restricted through the course of an infection. In the passive immunisation experiments using Mab P3-D3 only a few infected cells were visible and these were reticulocytes. The significance of this

observation is not known, but it will be of interest to confirm whether or not there is a correlation between response to the 42kDa fragment and reticulocyte restriction.

Miller and colleagues (1997) comment that the essential criteria for a successful vaccine are: (1) target epitopes that have limited or no polymorphism; (2) human-compatible adjuvant; and (3) a strategy to successfully identify such an antigen /adjuvant formulation for manufacture and further development. The work described here helps to define the importance of MSP-1 for vaccine development. Mabs can also be used to purify malaria antigens, even if the particular Mab is non-protective or non-inhibitory because the antibodies to other epitopes may block the invasion (Miller et al., 1986). Mab P6-B4, that was not protective against a challenge infection of *P. yoelii* YM bound to a recombinant protein based on the 33 kDa fragment, and this is the first time that such a specificity has been reported.. This monoclonal antibody may be of use to study proteolytic processing of the *P. yoelii* MSP-1.

In the *P. yoelii* model, the secondary structure of MSP-1 is important for both antigenicity and induction of a protective response; reduced and alkylated recombinant proteins (GST-MSP1$_{19}$ , GST-MSP1EGF1 and GST-MSP1EGF2) were not recognised by Mabs on Western blots. Several epitopes common to two different lines of *P. yoelii* have been defined by various Mabs raised against the YM protein. These Mabs have also been used in other collaborative work to study the expression and antigenicity of the C-terminal domain of the *P. yoelii* MSP-1 in *Salmonella typhimurium* (Somner et al., 1997, submitted for publication).

In previous studies with *P. falciparum* it was found that some Mabs directed to the C-terminus of MSP-1 could inhibit the cleavage of the 42 kDa fragment of MSP-1, and that these Mabs were those that inhibited invasion in vitro. Therefore it appears that one of the mechanisms by which antibodies to MSP-1 can inhibit invasion is by preventing secondary processing. It will also be of interest to determine whether or not the protective Mabs defined in this study have similar effects on the processing of the *P. yoelii* MSP-1. The definition of the protease involved in the processing and its specificity may also lead to design of drugs which inhibit this process.

### 6.2 Future work

A future experiment will be to determine the cross-reaction of the Mabs with other species of rodent malaria, to demonstrate specificity for *P. yoelii*. If any of the antibodies react with other malaria parasite species or lines it will be of interest to evaluate the antibodies in passive immunisation experiments. Analysis of the amino acid sequence of the MSP-1 from the other parasites may provide clues to the residues that contribute to the epitopes. It will be of interest to evaluate Mab P3-D3 by passive immunisation against non-lethal *P. yoelii* 17 X, which preferentially infects reticulocytes, in particular because in mice immunised with this Mab any parasites detected in the first days after infection with the YM line were present in reticulocytes. Also, it is necessary to assess the protective Mabs in passive immunisation against the 265BY line, in light of the results obtained by Western blotting and ELISA. It is expected that Mabs P2-F5 and P6-B6 will protect naive mice against challenge infection with *P. yoelii* 265BY, because these Mabs appear to cross-react with this strain.

Passive immunisation with a combination of two Mabs may confer more effective protection than that provided by a single Mab alone. In particular the combination of Mabs which are clearly against different epitopes, for example B10 or G3 with P6-B6 or P2-F5, and the combination of Mabs that interacted in the competitive ELISA, would be of interest. If it is true that the steric inhibition of Mabs P6-B6 with P2-F5, and G3 with B10 happens in the way suggested by the competition ELISA, then, one would expect to see no additive effect when the Mabs are evaluated together by passive immunisation.

It will be important to map the epitopes recognised by the Mabs on the MSP-1 protein, using mutagenesis of selected amino acids and possibly identifying functional domains of the protein (Farley & Long, 1995). These studies will help to define in fine detail regions of the protein that are important in eliciting a protective response and consequently in the development of a vaccine. The three dimensional structure of MSP-$1_{19}$ and of the antigen-antibody complex would provide precise structural information, that could be obtained by X-ray crystallography.

In order to evaluate the importance of the antibody subclass in the protection conferred with the Mabs, passive immunisation of SCID (severe combined immune deficiency) mice and challenge with *P. yoelii* YM, would be very informative. These mice do not generate functional T or B cells because they have a defect in V-(D)-J recombination (Bosma and Carroll, 1991). SCID mice will allow an assessment of the Mabs without any interference from a B or T cell response. A comparison of the percentage parasitaemia and the percentage survival in the SCID mice compared with control normal BALB/c mice immunised in the same way would indicate whether or not additional factors, rather than just antibody blocking of invasion, are important.

Immunoglobulin G (IgG), can undergo partial proteolytic cleavage by papain in the hinge region, to produce two identical fragments that can bind antigen - Fab (Fragment antigen binding) and a fragment which can not bind antigen - Fc (Fragment crystallisable). Using these fragments it would be possible to determine which part of the protective monoclonal antibody is responsible of the protection, and whether or not the intact molecule is required. For example, three groups of mice could be passively immunised with Fab fragment, $F(ab')_2$ fragment and the complete IgG molecule. This experiment would allow the determination of which part of the Ig is responsible for the protection, and whether or not the whole molecule is required.

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