Erythrocyte invasion by the rodent malaria *Plasmodium berghei*

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I hereby declare that this material, which I now submit for assessment on the program of study leading to the award of Ph.D. is entirely my own work, and has not taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed : JOHN McNALLY

John McNally

Date : 8/8/94

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ABSTRACT

Erythrocyte invasion is a process essential to the survival of malaria parasites. An understanding of the mechanism of erythrocyte invasion by malarial merozoites is important, since interruption of any of these events would halt the parasites asexual cycle and prevent disease. Much work has focused on the human malaria *Plasmodium falciparum*, however it is expensive to maintain in the laboratory. The research described here concentrates on the rodent malaria *Plasmodium berghei*, which in many ways is similar to *P. falciparum*, but is more convenient to obtain, cheaper to maintain and remains relatively uninvestigated.

A percoll density gradient centrifugation step was developed to purify mature parasite-infected erythrocytes from other infected and uninfected erythrocytes. This purification step allowed the development of *in vitro* invasion assays and erythrocyte receptor binding assays for *P. berghei.* The invasion assays confirmed *in vitro,* the invasion specificities observed *in vivo* for *P. berghei,* in particular the preference of *P. berghei* merozoites for invasion of immature erythrocytes, the reticulocytes. The binding assays identified a 130 kDa *P. berghei* molecule that bound exclusively to murine erythrocytes, and which undoubtedly has a role in merozoite invasion of erythrocytes.

Polyclonal antisera was raised to various protein preparations of *P. berghei,* and these were used to screen a cDNA expression library of *P. berghei* constructed in gt11. This cDNA library was also screened with radiolabelled oligonucleotide probes corresponding to fragments of a gene encoding an erythrocyte binding protein of the human malaria *Plasmodium falciparum.*

A cDNA library constructed in gt11 from the sexual stages of *P. falciparum* was screened with an antisera specific for sexual stage antigens. Isolated clones were characterised and ß-galactosidase fusion proteins prepared. Antisera was raised to these fusion proteins.

ABBREVIATIONS

BCIP	5'-bromo-4-chloro-3-indoyl phosphate
bp	base pairs
BSA	Bovine serum albumin
CS	Circumsporozoite
DAP	Duffy adhesion protein
DEPC	Diethyl pyrocarbonate
EBA	Erythrocyte binding antigen
EBP	Erythrocyte binding protein
EDTA	Ethylenediaminetetraacetic acid disodium salt
EE	Exoerythrocytic stage
E-64	trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane
FCS	Foetal calf serum
HEPES	N-[2-hydroxyethyl] piperazine-N'[2-ethanesulfonic acid]
i.p.	intra-peritoneal
IPTG	Isopropyl-ß-D-Thiogalactopyranoside
kDa	kilodalton
NBT	Nitro blue tetrazolium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
RBP	Reticulocyte binding protein
SDS	Sodium dodecyl sulphate
STI	Soybean Trypsin inhibitor
TPCK	N-Tosyl-L-Phenylalanine chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

MALARIA

Throughout the tropics and subtropics, over 2000 million people are at risk from infection with malaria. Of these, between 200 and 400 million are infected and between one and two million, mainly children, die each year. The disease is caused by a protozoan parasite of the genus *Plasmodium,* which is transmitted to man by the anopheline mosquito. Four species of the parasite affect man: *Plasmodium falciparum* and *Plasmodium vivax* are the most common, with the former being the most dangerous and responsible for nearly all malaria related deaths. *Plasmodium malariae* and *Plasmodium ovale* are much less prevalent.

Traditional methods of malaria control involve the use of insecticides against the mosquito vector and drugs for the prevention and cure of infection. However these methods have merely contained the disease, and have had little impact on its eradication. Even these control measures are now becoming less effective with the worldwide spread of insecticide-resistant mosquitoes and the emergence of malaria parasites that are resistant to the available antimalarial drugs. In addition, factors such as the growth in world population, the destabilisation of developing regions of the world and the problems of administering control programs all increase the threat of malaria.

Most malariologists agree that a vaccine will have to be an essential component of a future control and eradication scheme and consequently great resources have been put into developing one. However the task has been much more difficult than had been envisaged, due both to the parasites ability to avoid or adapt to immune pressure and to our incomplete understanding of man's immune system.

Although the *Plasmodium* species attacking man have been the most extensively studied, considerable research use has been made of malarial species which infect laboratory animals, especially *Plasmodium knowlesi* in monkeys, *Plasmodium gallinaceum* in birds and *Plasmodium berghei* in rodents. The problem with these model host-parasite systems are that they can provide only indirect information about what might happen in a human infected with malaria, though several important leads have come from studies on owl monkeys (*Aotus trivirgatus*) and Squirrel monkeys (*Saimiri sciureus*), both of which can be experimentally infected with *P. falciparum*. However the number of these monkeys available for

experimental purposes is limited and they are now only used to a significant extent in their native South America. A murine model has now also been developed for *P. falciparum,* which is hoped will aid research into certain stages of the malarial life cycle (Sacci *et al.*, 1992). It remains likely that the rodent and avian malarias like will continue to be provide useful information on the parasite and the disease it causes for some time as they remain easier and cheaper to maintain in the laboratory, and may themselves be evolutionarily closer to *Plasmodium falciparum* than the monkey malarias are (McCutchan *et al.*, 1984). However in the light of past mistakes, and failed vaccine candidates, greater care is being taken in the inferences drawn from immunological data provided by animal malarias.

All malarial *plasmodia* that infect animals, spend a part of their life in a vertebrate host and part in a mosquito host. Their life cycle is therefore complex, comprising several stages, each of which is structurally, biochemically and antigenically distinct. Despite the large number of species and the range of hosts affected by the malaria parasite, they share a common life-cycle and a similarity in the morphology of the various developmental stages.

THE LIFE CYCLE OF THE MALARIA PARASITE

The life cycle of malaria in the vertebrate and invertebrate hosts is shown in Fig. 1.1. Infection is initiated by the injection of the sporozoite form of the parasite into the victim by the female Anopheline mosquito during feeding. Since the mosquito usually feels about with its proboscis until it strikes a capillary, the sporozoites are introduced directly into the bloodstream. Injected sporozoites released from the mosquito salivary gland circulate in the blood and are carried to the liver where they invade the hepatocytes and begin to replicate. This stage of the life cycle is termed the exoerythrocytic (EE) stage. In the case of *P. falciparum* , the sporozoite develops into merozoites over the course of 5-7 days and up to 40,000 merozoites are released from each infected cell.

The merozoite initiates the asexual erythrocytic cycle of the parasite, rapidly invading erythrocytes where it develops from ring-form to schizont, the stage which undergoes nuclear division. In *P. falciparum* nuclear division gives rise to 10-20 new merozoites each capable of invading a

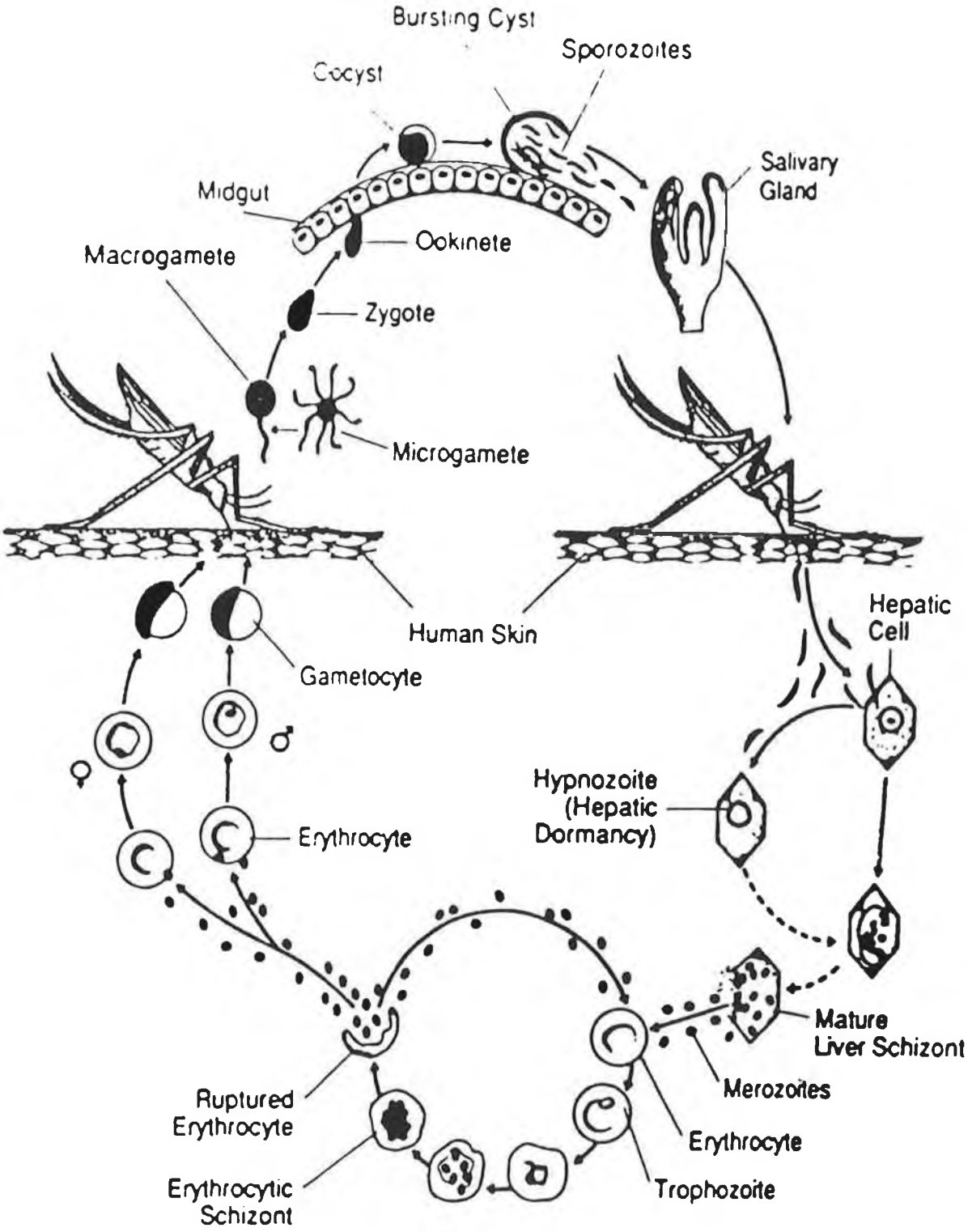


FIG. 1.1. The life cycle of the malaria parasite

new erythrocyte.The process of merozoite formation is known as schizogony and the mature schizont containing individual merozoites is known as a segmentor. These segmentors rupture, releasing the merozoites which
invade new erythrocytes, initiating a new round of the erythrocytic cycle. It is the erythrocytic cycle which is responsible for the clinical manifestations of the disease, including fever, anaemia and spleenomegaly. In addition, *P. falciparum* infected erythrocytes bind to the vascular endothelium and block blood flow in the brain and kidneys, which can prove fatal. Not all invading merozoites proliferate asexually in erythrocytes. Some ring-forms differentiate into sexual forms called gametocytes, which are ingested by mosquitoes during a blood meal. Male gametocytes are termed microgametocytes, and female gametocytes, macrogametocytes. In the mosquito midgut, a microgametocyte fertilises a macrogametocyte, resulting in a motile ookinete which penetrates the mosquito gut wall and multiplies into an enlarging oocyst. Development of the oocyst takes 10-20 days, and results in the production of enormous quantities of threadlike sporozoites. Rupture of the cyst allows release of the sporozoites which migrate to, and accumulate in the salivary gland of the mosquito, thus completing the sexual cycle of the parasite which is referred to as sporogony. In *P. vivax* malaria of man, but not *P. falciparum,* latent EE forms reside in the liver as hypnozoites for months or even years before initiating a relapse clinical infection in the blood.

RODENT MALARIA - *PLASMODIUM BERGHEI*

Plasmodium berghei was first isolated as sporozoites from the *Anopheles dureni* mosquito. The engorged blood In the gut of this mosquito was sourced back to the tree rat *Thamnomys surdaster surdaster* (Vincke & Lips, 1948). The discovery of *P. berghei* has proven to be one of great significance, as it was found possible to transmit this species of malaria to a number of laboratory rodents, enabling much research on malaria. A wide range of laboratory hosts have been tested for susceptibity to *P. berghei.* Rats, mice and hamsters are easily infected, however the response to infection may differ between different mouse strains, some showing 100% death rate while others rarely die (Garnham, 1967). Squirrels are infected

to a lesser degree, while birds and man have proved resistant to infection. The characteristics of *P. berghei* may be summarised as

(a) The exoerythrocytic stages occur in the parenchymal cells of the liver and mature 48 hours after the introduction of sporozoites

(b) The erythrocytic cycle takes 22-24 hours, is asynchronous, and commences with uninucleate rings. The mature schizont contains 10-18 nuclei

(c) The merozoites show a strong preference for reticulocytes. Early in infection, the reticulocytes are parasitised almost exclusively, with increasing parasitism of mature erythrocytes as the reticulocyte numbers drop due to cell destruction (Ott, 1968). Gametocytes seem to develop in mature erythrocytes exclusively.

After the discovery of *P. berghei* , other malarial species - *P. yoelii* (closely related to *P. berghei*), *P. vinckei,* and *P. chabaudi* were isolated in African rodents and adapted to laboratory animals. These rodent malarias differ somewhat in morphology but also in host-erythrocyte preference, and in outcome of infection. *P. berghei* and *P. yoelii* have a preference for invasion of reticulocytes, while reports indicate that *P. vinckei* prefers mature erythrocytes (Garnham, 1967 and Ramakrishnan & Prakash, 1950). McNally *et al.* (1992) demonstrate that *in vitro, P. chabaudi* shows a preference for reticulocytes. The outcome of infection differs between mouse strains, however generally *P. chabaudi* produces an infection which resolves spontaneously while *P. berghei* infection results in a fulminating lethal parasitaemia. In addition two variants of *P. yoelii* have been isolated, one of which is non-lethal (strain 17X); the other, lethal (strain XL) (Yoelii *et al.*, 1975)

Researchers have tried to establish evolutionary relatedness of species of *Plasmodia.* McCutchan *et al.* (1984) examined the base composition of various plasmodial species. Three different rodent parasites displayed a dG-dC content of approximately 18%. In contrast, *P. vivax*, and a number of monkey malarial plasmodia were reported to have a dG-dC content of about 30%. *P. falciparum* however was found to have a base composition more like that of the rodent and avian parasites than the other primate parasites. McCutchan postulated that the rodent malarias bore a closer evolutionary relatedness to *P. falciparum*, than the other primate

malarias did. Therefore research on the rodent malarias would provide valuable insights into the major human malaria. Indeed, in immunological research the extensive immunobiological and genetic knowledge available on inbred strains of mice and rats allows experimental manipulation of the host immune system, and a more detailed understanding of the range of host defence mechanisms. Sacci *et al.* (1992) have reported the growth of mature exoerythrocytic stages of *P. falciparum* in human hepatocytes transplanted into a severe combined immunodeficient (SCID) mouse strain, after intravenous injection of sporozoites. This murine model should facilitate the characterisation of EE-stage antigens, and the assessment of stage specific anti-malarial agents.

VACCINE DEVELOPMENT

Each developmental stage of the malarial life-cycle has adapted so well to its particular environment that they can almost be regarded as separate parasites. As well as having different antigenic determinants, the sporozoite, exoerythrocytic, erythrocytic and sexual forms of the parasite also have different growth requirements and metabolism. Much recent vaccine development work has centred on the exoerythrocytic stage of the life cycle. The aim of such a vaccine would be to prevent the release of blood stage parasites from the liver. The impetus for much of this work came from the observation that solid immunity could be achieved following immunisation with large numbers of radiation attenuated sporozoites via mosquito bites (reviewed in Nussenzweig and Nussenzweig, 1986). However vaccines based on the sporozoite surface protein - the circumsporozoite (CS) protein have failed to provide adequate protection upon challenge (reviewed in Greenwood, 1990). Further definition of the immunoreactive and immunosuppressive epitopes of the CS protein may result in its use as part of a vaccine. However exoerythrocytic immunity is unlikely to be effective in preventing disease as it would theoretically take only one sporozoite or infected hepatocyte to bypass this immunity in order to establish infection.

Research into the sexual stages of the parasite have centred on the genetic signals that initiate the development of macrogametocytes and microgametocytes and on the specific sexual stage antigens of the parasite. Vaccines based on the sexual stages would aim to exert a transmission

blocking effect, that would arrest the development of the parasite in the mosquito and reduce or abolish the transmission of the disease in an endemic area. Development of such vaccines has been facilitated by the observations that antibody alone can mediate functional immunity in mosquito feeding experiments (Carter & Chen, 1976), and that the surface antigens of sexual stages under investigation as vaccine candidates show limited antigenic diversity (Kochen, *et al.*, 1993). The greatest impact of such vaccines is likely to be in regions of low to moderate endemicity, and would probably only be useful in areas of high endemicity when used in conjunction with other control methods.

It is the asexual erythrocytic stage of the parasite that causes the clinical manifestations of the disease. The maximum benefit of vaccines in reducing morbidity and mortality would thus be achieved by an effective immunological response against this stage of the parasite life cycle. The relevance of the erythrocytic stage to the disease and also the general availability of large quantities of erythrocytic parasite material and culturing methods for the erythrocytic stage has facilitated much research into this area of vaccine development. The search for candidate molecules for inclusion in a vaccine has centred on antigenic regions of molecules that are recognised as having a vital role In parasite function and are thus unlikely to be subject to significant variation without affecting the viability of the parasite. The most obvious of these `vital' molecules are those of the merozoite involved in the invasion of the erythrocyte, but equally vital molecules would be those involved in nutrient uptake, and in sequestration which are probably accessible on the infected erythrocyte surface. It is the molecules involved in invasion however that have received the most attention from researchers. The interaction of the merozoite with the erythrocyte is now recognised as a complex multistep process involving specific receptor molecules on the surface of both the erythrocyte and the merozoite. While many of the interactions involved in invasion by *P. falciparum* and *P. knowlesi* have been partly elucidated very little is known about the rodent malarias and this is the focus of our research. We will now review the current understanding of the invasion of erythrocytes by merozoites, beginning with a description of the merozoite, and going on to discuss the molecular interaction involved in invasion.

ERYTHROCYTE INVASION BY MALARIAL MEROZOITES

1. *THE MEROZOITE*

Merozoites of mammalian *Plasmodium* species are small elliptical cells, 2-3μm long in *P. knowlesi* and a little smaller in *P. falciparum*. They have a conical protuberance, the apical prominence, at one end; the other pole containing the nucleus is rounded. A 15-20nm-thick bristly coat covers the plasma membrane, deep into which (except apically) lie two more membranes binding a single flat cisternae, the series of three membranes being termed the pellicle. A single acristate mitochondrion and numerous ribosomes are present in the cytoplasm. Also located in the cytoplasm are three major types of densely granular membranous vesicles. These are : (a) the paired rhoptries which converge on a common rhoptry duct, which ends blindly beneath the apical membrane; (b) several small fusiform micronemes which also appear to connect with the rhoptry duct; (c) up to 20 rounded structures (microspheres or dense granules) present in all parts of the cytoplasm. The existence of dense granules has been reported in *P. knowlesi* merozoites and also in *P. falciparum* merozoites (Bannister *et al.*, 1975 and Langreth *et al.*, 1978)

2. *THE EVENTS IN ERYTHROCYTE INVASION*

Invasion of erythrocytes by *P. knowlesi* merozoites, at the light microscope level, was described by Dvorak (1975). They found that merozoites initially attach to erythrocytes by any part of the merozoite surface. This adherence induces a marked deformation in erythrocyte shape. For penetration to ensue, the more conical, apical end of the merozoite must come into contact with the erythrocyte. The apical contact causes a rapid deformation in the erythrocyte, which lasts about 10 seconds, after which the erythrocyte becomes quiescent, and the merozoite interiorizes in about another 20 seconds. This is followed by intermittent erythrocyte deformations over the next 10-15 minutes until the normal biconcave shape returns. The parasite now proceeds to the ring-stage.

Descriptive ultrastructural studies of invasion using electron microscopy have generally been performed using *P. knowlesi* merozoites, as viable merozoites are relatively easy to obtain from this species. The

process appears to be the same for *P. falciparum* and *P. berghei* (Langreth *et al.*, 1978 and Yan *et al.*, 1986 respectively). The major events are summarised in Fig. 1.2. Merozoites are covered with a thick fuzzy coat, by which they adhere to the erythrocyte at any part of their surface (Fig. 1.2, A). Erythrocytes tend to wrap themselves partly around the merozoite during this first stage of attachment as though maximising the area of attachment between the two (Fig. 1.2, B). For the sequence of invasion to continue, the apical end of the parasite must contact the red cell membrane, leading to the formation of the close junctional zone between the two cells, a step referred to as reorientation (Fig. 1.2, C). Apical contact triggers the secretion of the contents of two sets of membranous vesicles whose ducts open onto the apical prominence of the merozoite, the micronemes and the rhoptries. These secretions lead to the invagination of the red cell surface, thus forming an invasion pit (Fig. 1.2, D), in through which the parasite moves (Fig. 1.2, E). The zone of apical attachment changes from a disc to an annulus through which the parasite passes, finally coalescing once more into a disc posteriorly as the invasion pit closes to isolate the parasite within the parasitophorous vacuole (Fig. 1.2, F) During this process the fuzzy coat of the parasite is lost.

During the final phase of invasion, a set of spheroid membranous vesicles (dense granules or microspheres) move from positions deep in the cytoplasm to the merozoite surface where their contents are discharged into the parasitophorous vacuole (Torii *et al.*, 1989)(Fig. 1.2, G). The parasitophorous vacuole membrane then increases further in surface area to accommodate the rapidly deforming parasite (Fig. 1.2, H). Many merozoitic organelles disappear from the parasite and it elongates, then flattens to a discoidal form, its early ring-stage condition (Fig. 1.2, I).

3. *ADHESION OF MEROZOITES TO ERYTHROCYTES*

There are two major stages in merozoite attachment which can be described as (a) circumferential, distant and reversible; and (b) apical, close and irreversible

(a) *Circumferential attachment*

This is the first to occur, the parasite adhering by any part of its surface to the erythrocyte, at distances between 20 and 40 nm. It is

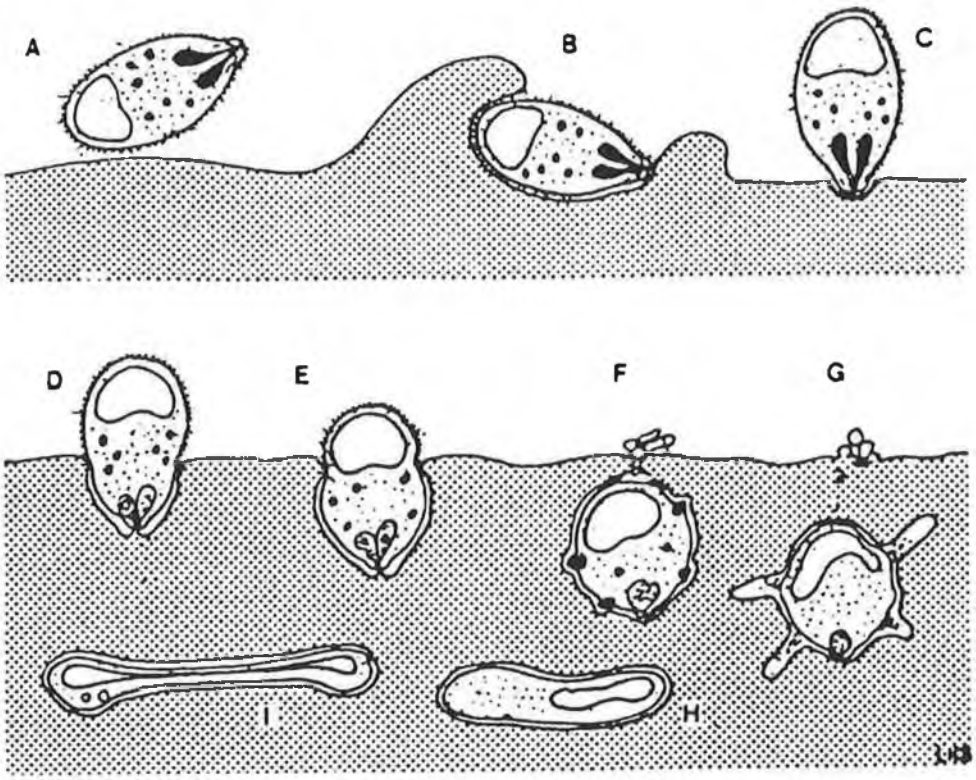


FIG 1.2. Diagram depicting the major stages in merozoite invasion of the erythrocyte

probable that the merozoite filaments making up the merozoite fuzzy coat are responsible for this attachment., as well as longer merozoite surface filaments of up to 40 nm.There are many candidate molecules on the erythrocyte and merozoite surface that are believed to be involved in this attachment for each malaria species. The malarial molecules will be discussed in the next section. It seems likely however that multiple binding sites along both the erythrocyte ligand and the merozoite binding molecule would maximise the adhesion between the erythrocyte and the merozoite. The interactions involved between the molecules are probably charge related, this would allow for a wide range of temporary adhesive interactions.

(b) *Apical adhesion*

This is much closer (<5 nm) and involves the formation of a dense undercoating to the erythrocyte membrane in the region of contact (Bannister et al., 1986). This adhesion step does not occur in the absence of specific erythrocyte ligands. The merozoite components involved in this close apical attachment may be molecules such as the *P. knowlesi* 66kDa molecule or the *P. falciparum* AMA-1, which are both believed to be apically concentrated (Waters *et al.*, 1990). On the erythrocyte surface, `short ' molecules such as band 3 or the Duffy blood group antigen may be the corresponding erythrocyte ligands. Merozoites of *P. knowlesi* will circumferentially attach to Duffy negative erythrocytes but will not form this close apical attachment with formation of the dense undercoating. It is important to consider that the zone of apical adhesion is transformed into an annulus during invasion, and therefore the region of close contact passes back over the merozoite during this process. How this occurs at the molecular level is not understood.

INVESTIGATIONS ON MEROZOITE INVASION OF ERYTHROCYTES

1. *INVASION ASSAYS*

Elucidation of the molecular intricacies of erythrocyte invasion has necessitated the development of methods that allow the examination of the invasion process *in vitro.* Currently two types of *in vitro* erythrocyte invasion

assay exist; (1) those that involve mixing schizont-infected erythrocytes with target erythrocytes; and (2) those that mix free merozoites with target erythrocytes. To examine reagents for their ability to interfere with specific steps in invasion, they are added to these assays and their effects measured by observing the difference in the number of successful invasions compared to appropriate controls.

Invasion assays that use schizont-infected erythrocytes are the simplest to perform. Such invasion assays have been developed for *P. knowlesi, P. falciparum* and *P. vivax* (Miller *et al.*, 1983; Mitchell *et al.*, 1986 and Barnwell *et al.*, 1989 respectively) and are routinely used for investigating inhibitors of invasion, antibody blocking of invasion and the erythrocyte and merozoite molecules required for invasion.Purified schizont-infected erythrocytes are added to target erythrocytes, and after allowing time for rupture, merozoite invasion and development of parasites into visible ring forms, giemsa stained smears are prepared from the assayed erythrocytes. Invasion is estimated microscopically by counting the percentage of erythrocytes containing ring-form parasites. Caution must be exercised when testing certain reagents in invasion assays that use schizont-infected erythrocytes. Some reagents, such as enzyme inhibitors or polysaccharides, may appear to affect the invasion process but may actually inhibit cell rupture (Hadley *et al.*, 1986). Indeed, reagents demonstrated to inhibit invasion in these assays must be shown to be non-toxic to schizonts.

Invasion assays using free merozoites circumvent this problem. However, of the primate malarias, viable free merozoites for these assays can be obtained only for *P. knowlesi.* Merozoites of *P. knowlesi* are prepared by culturing schizont-infected erythrocytes in the presence of leupeptin and chymostatin (which prevent cell rupture), until individual merozoites are easily discernable in the infected erythrocyte. The infected erythrocytes are agglutinated with anti-rhesus serum and then passed several times though a narrow gauge needle, the shear forces rupturing the infected erythrocyte and releasing the merozoites. The released merozoites are then added to the target cells, and after a period of 6 hours smears are prepared and examined.

The difficulty in maintaining rodent malarias *in vitro,* has hampered the development of *in vitro* invasion assays for these species. *P. berghei*

also presents the problems of; (a) the asynchronicity of its asexual stages; (b) the preference of *P. berghei* merozoites for invasion of immature erythrocytes; and (c) erythrocytes containing mature erythrocytes sequester in the deep vasculature. An invasion assay using free *P. berghei* merozoites has been described by Janse *et al.* (1989). A continuous flow culture system was used, with the exit of the chamber covered with a polycarbonate filter, through which only free merozoites could pass. Rupture of the cultured schizont-infected-erythrocytes is caused by the shearing forces of a magnetic bar spinning on the filter. Released merozoites are mixed with rat reticulocytes and invasion recorded as the percentage of ring-stage infected rat reticulocytes. Janse reported invasion rates of up to 13% into rat reticulocytes. Braun Breton *et al.* (1992) have described an invasion assay for the rodent malaria *P. chabaudi*. However their techniques involve the use of flow cytometry, and invasion rates are low.

We sought to develop an *in vitro* invasion assay for *P. berghei* that was simple to perform, that didn't require expensive culturing apparatus or Quantitation equipment and enabled a number of tests to be carried out simultaneously. The invasion assays outlined and discussed in this thesis (also see McNally *et al.*, 1992) meet these requirements and should contribute to the understanding of erythrocyte invasion by *P. berghei* merozoites.

2. *MOLECULAR INTERACTIONS OF INVASION*

The interaction with and entry into erythrocytes by malarial merozoites is a crucial yet potentially vulnerable event for the parasite's survival. The complexity of the interaction has evolved so as to minimise the effect of the immunological reaction of the host. However through the definition of the molecular basis of invasion of erythrocyte by merozoites, it may be possible to discover and exploit weaknesses in the merozoite machinery of invasion and modulate the host immune system to respond to it.

Progress towards the identification of merozoite proteins potentially involved in the invasion of erythrocytes has advanced considerably for *P. falciparum, P. vivax* and *P. knowlesi*. These molecules have been assigned a function in the invasion process for one of the following reasons

(a) They have been shown to bind to erythrocytes. The specificity of binding generally correlates with the erythrocyte invasion preferences of the plasmodial species. It is likely that some of these molecules are involved in the reversible circumferential attachment of merozoite to erythrocytes while other would be involved in the non-reversible apical attachment that precedes invasion.
(b) They have been localised on the merozoite surface or in the rhoptries, micronemes or dense granules by immunocytological studies. In many cases the fate of these molecules during the invasion process has been followed.
(c) Monoclonal antibodies against certain molecules have been found to block or inhibit invasion. Rarely do such antibodies completely block invasion, for the most part their blocking activity is in the range 40-60%. This may be either a result of the inaccessibity of molecules such as those released from the rhoptries or may be due to the existence of several different routes open to the merozoite for attachment and invasion of erythrocytes.

BINDING ASSAYS AND BINDING MOLECULES

The most common technique employed in the identification of malarial molecules that attach to erythrocytes is the Radiolabel Binding Assay. It involves the mixing of erythrocytes with metabolically labelled parasite proteins. Molecules that bind to the erythrocytes are then eluted with high concentrations of NaCl. Using binding assays, three families of erythrocyte binding molecules have been identified for malaria parasites, (1) the EBA/DAP family, (2) the MSP family and (3) the RBP family

1. *THE ERYTHROCYTE BINDING ANTIGEN/DUFFY ADHESION PROTEIN FAMILY*

Camus and Hadley (1985) were the first to report the binding to intact erythrocytes of a *P. falciparum* protein from *in vitro* culture supernatants. They found a 175 kDa Erythrocyte Binding Antigen (EBA-175) whose binding to erythrocytes correlated with the erythrocytes susceptibility to invasion by *P. falciparum* merozoites. It had previously been determined that erythrocyte membrane glycophorin, in particular, its sialic acid, was

required for invasion of erythrocytes by merozoites of *P. falciparum* (Pasvol *et al.*, 1982). Camus & Hadley (1985) and Pasvol *et al.* (1982) have shown that human erythrocytes treated with trypsin, which cleaves glycophorin from the erythrocyte membrane, or neuraminidase, which removes sialic acid, or human TN variant erythrocytes, which lack sialic acid on the o-linked oligosaccharides of glycophorin, did not bind the EBA-175, and were not invaded by *P. falciparum* merozoites. Neither did the EBA-175 bind to erythrocytes of guinea pig or rhesus erythrocytes which are also refractory to invasion. Camus & Hadley (1985) found that the EBA-175 bound to merozoites and they postulated that its role was to act as a bridge between the erythrocyte and the merozoite. This was consistent with the observation that it was released into culture supernatants as schizonts ruptured.

However the presence of the sialic-acid ligand on the erythrocyte does not appear to be an absolute requirement for invasion. Several strains of *P. falciparum* differ in their ability to invade sialic-acid deficient erythrocytes (Mitchell *et al.*, 1986; Hadley *et al.*, 1987). Hadley demonstrated that the *P. falciparum* strain 7G8 could invade neuraminidase treated erythrocytes with 50% of the efficiency that it invaded untreated erythrocytes, while the invasion rates of the Camp strain of *P. falciparum* into neuraminidase treated erythrocytes were <3% of invasion rates into control erythrocytes. Hadley concluded that the 7G8 strain parasites were able to invade by a pathway independent of sialic-acid, and therefore independent of the binding of EBA-175. Recently Dolan *et al.* (1994) reported that the binding of EBA-175 was predominantly to Glycophorin A, while Glycophorin B and an unknown erythrocyte ligand X, were probably the ligands involved in the EBA-175 independent invasion process. Dolan *et al.* (1994) however, was unable to find the merozoite molecule involved in binding to these erythrocyte ligands. This indicates one of the inadequacies of the binding assays, in that it doesn't identify all parasite molecules involved in invasion. Only those molecules that are soluble and binding-competent when released into the culture supernatant are identified.The specificity of binding of the EBA-175 indicates nonetheless that it has a role in the invasion process.

After the discovery of the *P. falciparum* EBA-175, many researchers sought to identify, using binding assays, molecules from other malarial

species that could similarly be linked with the invasion process. In particular, attention turned to *P. vivax* and *P. knowlesi*. It had been well documented that invasion of human erythrocytes by the merozoites of *P. vivax in vivo* and by the merozoites of P. *knowlesi in vitro* was dependent on an erythrocyte membrane glycoprotein serologically defined by the Duffy blood group determinants (Miller *et al.*,1976; Mason *et al.*, 1977). Haynes *et al.* (1988) described a *P. knowlesi* 135 kDa molecule, present in culture supernatants, that bound to Duffy blood group-positive but not to Duffy blood group-negative human erythrocytes. They found that specific anti-Duffy antibodies blocked the binding of the *P. knowlesi* 135 kDa Duffy Adhesion Protein (PkDAP), and that purified PkDAP bound to the 35-45 kDa Duffy glycoprotein on a blot of electrophoretically separated membrane proteins from Duffy-positive erythrocytes. Cleavage of the Duffy determinant by chymotrypsin-treatment of erythrocytes abrogated the binding of the DAP and also inhibited invasion of the erythrocytes by merozoites of *P. knowlesi*. The correlations between binding of the 135 kDa molecule and invasion of erythrocytes led Haynes *et al.* (1988) to conclude that the PkDAP had a role in the invasion of Duffy-positive erythrocytes by *P. knowlesi* merozoites. Dalton *et al.* (1991) demonstrated that the binding of the 135 kDa molecule to the Duffy determinant could be inhibited and reversed by the sulphated polysaccharide fucoidan and the glycoaminoglycan dextran sulphate. These sulphated molecules also inhibited the *in vitro* invasion of susceptible erythrocytes by *P. knowlesi* merozoites. It is likely that it is involved in the apical adhesion events of invasion rather than in the initial circumferential attachment, as *P. knowlesi* merozoites attach to both Duffy-negative erythrocytes and Duffy-positives erythrocytes but only with Duffy-positive erythrocytes does apical junction formation occur and invasion proceed.

Wertheimer & Barnwell (1989) identified a *P. vivax* 135-140 kDa protein that bound with similar specificity as the PkDAP to Duffy-positive erythrocytes. This *P. vivax* Duffy adhesion protein (PvDAP) bound only to Duffy-positive human and *Aotus* monkey erythrocytes which were susceptible to invasion by *P. vivax*, but not to Duffy negative human erythrocytes or erythrocytes from *Macaca* or *Cebus* erythrocytes that are not susceptible to *P. vivax*. As with the PkDAP, treatment of susceptible erythrocytes with chymotrypsin abrogated binding of the PvDAP, and

inhibited invasion of these erythrocytes by *P. vivax* merozoites. The interaction of the PvDAP with Duffy-positive erythrocytes could be specifically inhibited with purified Duffy glycoprotein (Wertheimer & Barnwell, 1989). The Duffy glycoprotein has recently been defined as the erythrocyte receptor for interleukin-8 (IL-8)(Horuk *et al.*, 1993).

While the PvDAP and PkDAP are related in their specificity for the Duffy glycoprotein, their interactions seem to be with different determinants on that glycoprotein (Wertheimer & Barnwell, 1989). Despite this both proteins were shown by Wertheimer to be antigenically related, in that an antiserum raised against *P. knowlesi* proteins which binds to *Saimiri* erythrocytes (including PkDAP), also recognise the *P. vivax* PvDAP. The relatedness of the two proteins however only became apparent after the cloning of their respective genes. Adams *et al.* (1990) reported the cloning of the 3' part of the cDNA of the *P. knowlesi* DAP using antibodies affinity purified on the 135 kDa molecule. Antibodies to multiple regions derived from the the deduced amino acid sequence of the *P. knowlesi* gene immunoprecipited the soluble 135 kDa molecule,indicating that the cloned gene indeed encoded the Duffy receptor. When southern blots of *P. knowlesi* were probed with the cDNA, it hybridised to 3 regions in the genome, indicating the existence of a family of *P. knowlesi* Duffy adhesion proteins. The cloning of the *P. vivax* DAP was reported by Fang *et al.* (1991), using as a probe a cDNA clone from the identified *P. knowlesi* gene. There appeared to be only one locus for this gene in the *P. vivax* genome. Adams *et al.* (1992) reports the completion of this Duffy Adhesion Protein cloning work. He cloned and sequenced all 3 *P. knowlesi* DAP's, and completed the sequencing of the PvDAP gene. When these sequences were compared, the gene structure and predicted amino acid sequence of the cloned PvDAP and PkDAP's were, as expected, very similar, and placed as part of the same gene family. However based on the same criteria, namely gene structure and amino acid homology the gene encoding the *P. falciparum* EBA-175 was placed in the same gene family (Adams *et al.*, 1992). Exon 2 of the *P. knowlesi/P. vivax* genes is known to encode the erythrocyte binding domain of the proteins, and contains a 3' cysteine-rich region and a 5' cysteine-rich region that is highly conserved between these two species. The *P. falciparum* protein differs from the Duffy binding proteins in that it has two copies (F1 and F2) of the 5' cysteine-rich

region. However twelve cysteine residues are present in roughly the same position in the F1 and F2 motifs and in the *P. vivax* and *P. knowlesi* proteins. There is a similar degree of alignment between the tryptophan and tyrosine/phenylalanine residues. In the 3' cysteine-rich region the homology encompasses 104 amino acids, again with alignment of most of the cysteine and tyrosine/phenylalanine residues. The genes for the DAP's and the EBA-175 also have the same intron/exon division. This would indicate a common origin of the genes, as introns are generally rare in malarial genes.

The DAPs and the EBA-175 do not seem to be located on the surface of invasive merozoites, but internally in the micronemes or dense granules (Adams *et al.*, 1990; Sim *et al.*, 1992). Therefore they probably function after initial attachment of the merozoite to the erythrocyte, and apical reorientation.

Similarities in localisation of the DAPs' and EBA-175, and regions of homology between the P. *falciparum* , *P. vivax* and *P. knowlesi* genes are interesting because of the wide evolutionary separation of these species as outlined by Waters *et al.* (1991) and McCutchan *et al.* (1984). Since these molecules are under immune pressure it is likely that the conserved motifs are necessary for function. Whether the regions of homology are conserved for binding function and how they account for the different binding specificities of the DAP's and the EBA-175 however isn't known. The presence of a similar protein in *P. berghei,* which is considered the most evolutionary divergent from *P. falciparum,* would be very significant as it would emphasise the importance of this molecule to the malarial species.

2. *THE MAJOR MEROZOITE SURFACE PROTEIN-1*

The second *P. falciparum* merozoite protein that has been shown to bind in a specific manner to human and certain primate erythrocytes was first identified and it's gene characterised by Holder & Freeman (1982), and since then has been termed gp195, p190, pf200, PMMSA, and more definitively MSP-1 (Merozoite Surface Protein 1) due to its localisation on the merozoite surface. Since its discovery by Holder and Freeman (1982), the *P. falciparum* MSP-1 has been well characterised immunologically (Pirson & Perkins, 1985), and in terms of its primary structure (Holder *et al.*, 1985). However it was not until the report of Perkins and Rocco (1988) that

its binding specificity to human erythrocytes was realised. Like EBA-175, the binding of MSP-1 to human erythrocytes is dependent on the sialic acid of the glycophorin glycoproteins, soluble glycophorin and neuraminidase treatment both inhibit the binding, and it binds only to those cells that are susceptible to infection by *P. falciparum.*

A major question raised by the research carried out on MSP-1 concerns the nature of the actual molecular structure of the MSP-1 on the merozoite surface. Several studies suggest that the large molecular weight form of the MSP-1 is a precursor molecule and that at, or about, the time of merozoite release is proteolytically processed to form three fragments (83 kDa, 38 kDa and 42 kDa) that remain attached to the merozoite membrane presumably as a complex (Holder *et al.*, 1987). Upon invasion the 42 kDa appears to be cleaved to a 33 KDa polypeptide, which is shed as a soluble complex with the other products of MSA-1 processing, and a 19 kDa fragment which is carried on the surface of the merozoite into the new erythrocyte (Blackman *et al.*, 1990). Chappel & Holder (1993) have defined monoclonal antibodies that inhibit *P. falciparum* invasion *in vitro* as reacting with one of two growth factor-like domains in this 19 kDa MSA-1 fragment. However none of the processed fragments have been shown to bind to erythrocytes, the only molecule that does so is the intact MSP-1. This may in itself indicate the limitations of the binding assays as currently performed, but also may indicate that the MSP-1 has indeed a functional role as an intact molecule in the initial attachment of merozoites to erythrocytes, interacting with sialic acid residues of the erythrocyte glycophorin.The fact that intact MSP-1 can be immunoprecipitated from *P. falciparum* culture supernatants indicates that this is the form of the protein on the merozoite surface and the fragments seen may be an effect of the radioiodination procedure used to identify them. This however does not rule out the biological significance of the fragments seen.Several specific proteases have been described for merozoites (Schrevel *et al.*, 1990), one of which may be activated then to cleave MSP-1 after initial attachment to erythrocytes, which may then have a further role in invasion. Szarfman (1988) reported the expression of the MSP-1 in the exoerythrocytic (EE) stages of *P. falciparum.* Therefore a vaccine incorporating this protein could also be effective against the EE-stage parasites.

The discovery of the MSP-1 of *P. falciparum* led to the

characterisation of molecules in other species of malaria that had similar characteristics. They are all synthesised late in schizogony, are localised on the merozoite surface, are of high molecular weight and appear to be processed to smaller fragments. The MSP-1 of *P. yoelii* was identified as a 230 kDa molecule (Holder & Freeman, 1984). When animals were immunised with the purified 230 kDa molecule, they were found to be protected against challenge infection with *P. yoelii*, with fulminating infections being converted to self-limiting reticulocyte-restricted infections (Holder & Freeman, 1981). The protection induced could not be transferred by passive immunisation so it is likely that cell mediated immunity was involved in protection. A polyvalent antiserum raised to the purified *P. yoelii* 230 kDa molecule cross reacted with schizonts of *P. chabaudi, P.vinckei, P. falciparum* and other strains of *P. yoelii* in indirect immunofluoresence tests (Holder & Freeman, 1984). The equivalent MSP-1 in *P. chabaudi* is of size 250 kDa, and monoclonal antibodies recognising this antigen injected into mice reduced the parasitaemia of *P. chabaudi* challenge infections (Bates *et al.*, 1988). David *et al.* (1984) described a MSP type molecule of *P. knowlesi* of 230 kDa, which had processed fragments of 200, 145 and 110 kDa and possible breakdown of these into 75, 57, 50 and 43 kDa fragments. Monoclonal antibodies against the 230 kDa molecule of *P. knowlesi* inhibited *in vitro* invasion of rhesus erythrocytes by *P. knowlesi* merozoites (Epstein *et al.*, 1981) , and its derived 75 kDa fragment, which was found present in the membranes of infected erythrocytes induced partial protection in rhesus monkeys (Schmidt-Ullrich et al., 1983). The *P. vivax* MSP-1 gene was completely sequenced by del Portillo *et al.* (1991) and was found to be a 200 kDa protein. When the MSP-1 genes of *P. yoelii, P.vivax,* and *P. falciparum* were compared, a number of homologous regions were found. The *P. falciparum* and *P. vivax* proteins showed greater similarity in amino acid composition and the overall distribution of the shared amino acid was more highly conserved than when *P. falciparum* and *P. yoelii* were compared (del Portillo *et al.*, 1991). From the work of McCutchan *et al.* (1984), *P. vivax* and *P. falciparum* are predicted to be evolutionary diverse. This is confirmed by the comparisons of the small-subunit ribosomal RNA sequences from various malarial species as determined by Waters *et al.* (1991). That a higher similarity exist in the MSA of these two species, than between the MSP-1 of *P. falciparum* and *P.*

yoelii, which McCutchan described as evolutionarily close may reflect the effect of positive selection in the human host. The conserved regions between the MSP-1 of the two human malaria species are also the regions conserved between the different alleles of the *P. falciparum* MSP-1.

From the data presented, the potential of the MSP-1 as a vaccine candidate was obvious. Not only were its analogous molecules in other malarial species shown to be effective in reducing or preventing infection, but regions of homology between different strains of *P. falciparum* had also been identified. Promising results came from immunisation of Aotus monkeys with preparations of the *P. falciparum* MSP-1, and the complete protection it afforded them (Siddiqui *et al.*, 1987). Peptides derived from the 83 kDa fragment of the *P. falciparum* MSP-1 are constituents in the Spf66 vaccine formulated by Patarroyo and described in the next section.

3. *RETICULOCYTE BINDING PROTEINS OF P. VIVAX*

No erythrocyte binding protein similar to the EBA/DAP or MSP-1 family of proteins have been described in *P. berghei.* Indeed very little is known about the specificity of invasion of *P. berghei* merozoites except that they appear to have a preference for invasion of immature erythrocytes *in vivo* (Ramakrishnan & Prakash, 1950). The work presented by us confirms this observation *in vitro*. *P. vivax,* in addition to being dependent on the Duffy glycoprotein/PvDAP interaction for invasion, is also dependent on the host erythrocyte being immature (reticulocyte) instead of a normocyte. While *in vivo P. berghei* appears to invade both reticulocytes and normocytes, despite its preference for reticulocytes, the *P. vivax* merozoite will exclusively invade only reticulocytes (Kitchen, 1938). It was suggested that this very strong preference for invading reticulocytes may occur because *P. vivax* merozoites are incapable of invading normocytes due to a mature cytosketetal protein network that makes the normocyte membrane less pliable, whereas, the reticulocyte membrane with its less mature cytoskeleton is more readily invaginated by the merozoite during invasion (Mons, 1990). This would imply that the merozoite would go from cell to cell, testing each cell at each contact, until it finds a cell with a membrane pliable enough for it to successfully invade. In a human, reticulocytes make up 0.2-1% of the erythrocyte population (Rapoport, 1986). Such a random invasion strategy would be detrimental to the survival of the parasite, and it is more

likely that *P. vivax* and the other reticulocyte preferring species *P. cynomolgi* (a simian malaria) and *P. berghei* would have a receptor/ligand interaction for identifying reticulocytes.

Galinski *et al.*, (1992) identified and cloned the genes encoding two *P. vivax* merozoite proteins that could be involved in a mechanism for selecting reticulocytes. Two proteins of approximately 250 kDa bind preferentially to reticulocytes of humans and other primates, except for rhesus erythrocytes which are refractory to invasion by *P. vivax* merozoites. These two proteins have been termed reticulocyte binding proteins -1 and -2 (RBP-1 and RBP-2) and their deduced secondary structure seems to be similar. By indirect immunofluoresence assays these two proteins were localised to the apical pole of merozoites of *P. vivax*. Using the same antisera a similar localisation was observed on merozoites of *P. cynomolgi,* but there was no reaction with merozoites of *P. knowlesi* or *P. falciparum.* The exact role, binding domains and reticulocyte ligand for these RBP is not known, but their specificity of binding indicates some interaction with reticulocytes. In southern blot analysis, gene probes from the *P. vivax* RBP hybridised to genomic DNA from *P. cynomolgi,* but not to DNA from *P. knowlesi, P. falciparum* or *P. berghei,* indicating that the reticulocyte preference of *P. berghei* may have its basis in another receptor or in another aspect of the parasite/host relationship (Galinski *et al.*, 1992).

The MSP-1, EBA, DAP and RBP molecules described are all considered to be involved in erythrocyte invasion due to their erythrocyte-binding properties and specificities. There are undoubtably many other molecules involved in invasion, which are not detected by the binding assays, either because of the nature of the binding assays or because the function in some other area of erythrocyte invasion other than direct erythrocyte attachment. The PK66 cloned by Waters *et al.* (1990), which is shed from the merozoite at, or behind the invasion interface, and the ring infected erythrocyte surface antigen (RESA) which is thought to facilitate invasion by interfering with the erythrocyte cytoskeleton (Foley *et al.*, 1990) are examples of two such molecules. Many of the molecules described have been investigated for their potential in asexual stage vaccines against *P. falciparum* (reviewed in Cox, 1991). However the only vaccine developed so far that has had any degree of success in preventing malaria is that of

Patarroyo (Valero *et al.*, 1993).

THE SPF66 VACCINE OF PATARROYO

A pragmatic approach to the development of a malaria vaccine was adopted by M. E. Patarroyo and coworkers at the National University of Colombia. Fractions of mature asexual parasites separated by gel electrophoresis were used to vaccinate *Aotus* monkeys against challenge *P. falciparum* infection. Protein was then purified from the relevant fractions and confirmed as able to give either total or partial protection in the monkeys (Patarroyo *et al.*, 1987). Animals immunised with a 155 kDa or a 55 kDa protein showed delayed onset of parasitaemias by 5 to 7 days compared to controls, suggesting partial protective immunity. Monkeys immunised with a 83 kDa or a 35 kDa fragment were completely protected, showing either no detectable blood infection or very low parasitaemia with spontaneous recovery. The 155 kDa and the 83 kDa proteins were identified as the RESA and the N-terminal processed fragment of the MSP-1, for which the peptide sequences had already been determined (Cowman *et al.*, 1985 and Holder *et al.*, 1985 respectively). Protein sequences were determined for the smaller proteins by N-terminal sequencing. Eighteen peptides were then synthesised corresponding to different fragments of these four proteins and after complexing to bovine serum albumin were used to vaccinate the monkeys, which were subsequently challenged with *P. falciparum* sporozoites. Fifteen elicited antibodies against schizonts, but while no peptide provided complete protection individually, a combination of 3 gave complete or almost complete protection. The most effective combination derived from the 83, 55 and 35 kDa molecules. For a human trial, two polymeric hybrid proteins were synthesised, termed SPf(66)30 and SPf(105)20 (Patarroyo et al., 1988). The SPf(66)30 contained sequences from the 83, 55 and 35 kDa proteins and also the Asn-Ala-Asn-Pro repeat from the *P. falciparum* circumsporozoite protein (CS). These peptide sequences were then polymerised into a 30-mer. SPf(105)30 was a 20-mer polymer of a 58 amino acid peptide which included sequences from the 83 kDa fragment and the CS. Both these peptides were then tested in human volunteers. In half the individuals immunised the SPf(105)20 gave a significant delay in infection after challenge with *P. falciparum* sporozoites,

while the SPf(66)30 gave strong protection to 3 of the 5 volunteers who received it, compared to controls. The three volunteers who were protected with SPf(66)30 had mild infections with steady decrease in parasite counts and total recovery by day 21 after infection. The SPf(66)30 was the first vaccine against to afford this degree of protection in humans. However many scientist were skeptical about this work and when an American team sought to repeat the success of Patarroyos group in immunising Aotus monkeys, they failed to get any protection at all (Ruebush *et al.*, 1990). Other researchers expressed concern that some of the peptide sequences used in SPf(66)30 were from the variable domains of the proteins, and could therefore select very rapidly for variants that would be unaffected by the induced immunity. Patarroyo continued his work, upgrading his production facility to achieve F. D. A. approval and completed a vaccine trial with SPf(66)30 (now termed SPf66) in La Tola, Columbia, involving greater than 1500 volunteers, receiving either 3 doses of the vaccine or the placebo. In a one year follow-up, the vaccine group reported 38.8% fewer clinical episodes of malaria than the placebo group (Valero *et al.*, 1993). Further trials are in progress, with greater numbers of vaccinees both in South America and Africa which should demonstrate the usefulness of the SPf66 vaccine. Advantages of such a chemically synthesised vaccine would be the inexpensiveness of each dose (estimated at 20p) and elimination of the necessity for a cold-chain distribution system.

How exactly the SPf66 works isn't clear. Patarroyo suggests that it blocks merozoite invasion of erythrocytes (BSP, spring 1994). Patarroyo has not been able to establish a correlation between antibodies titres against the SPf66 and the protection it confers. However sera from SPf66-immunised volunteers had a greater capacity to inhibit *in-vitro* parasite growth than control sera, irrespective of the titres of anti-SPf66 IgG antibodies (Salcedo *et al.*, 1991). Molano *et al.* (1992) found that the predominant antibodies in serum from protected individuals were directed against Lysine-Glutamic acid-Lysine (KEK) motifs. The KEK motif is part of the 83 kDa-fragment derived peptide of the vaccine, but is also present in the EBA-175 native protein. Molano *et al.* (1994) suggests that highly charged motifs of this type may be involved in determining the 3-dimensional structure of the erythrocyte binding proteins EBA-175 and MSP-1, and the synthetic peptide may focus the immune response toward

this critical epitope, which may be seldom or only transiently exposed during the natural infection. In addition other serum factors may be responsible for protection, whether these factors are other Ig class antibodies not detected so far, or some other constituent of the immune system isn't known. The most plausible explanation for protection is that there are multiple factors involved in the immune response against the SPf66 vaccine and the *P. falciparum* parasite.

CHAPTER 2

MATERIALS AND METHODS

1. METHODS

Parasites and animals

The *P. berghei* (ANKA strain) line was maintained in 6 to 8 week-old Balb/c mice by passage of infected blood from one animal, with parasitaemia between 15% and 20%, to another animal by intra-peritoneal injection. A cloned line of *P. c. chabaudi* AS was maintained in CD-1 mice and was passaged similarly. These CD-1 mice were adapted to lighting for 7 days prior to, and during infection, artificial daylight beginning at 12 midnight and ending at 12 noon. Parasitaemia was determined by examination of giemsa stained blood smears, (taken from the tail of an infected animal), and recorded as the percentage of erythrocytes which contained parasites.

Induction of reticulocytosis

Solutions of 0.4% phenylhydrazine were prepared, immediately before use in PBS, from a 97% stock solution. Mice were injected intraperitoneally with 100 µl of this solution per 15 g body weight on day 1 and every alternate day up to day 9. Erythrocytes from these mice were used when the reticulocytes number accounted for greater than 50% of the total erythrocyte population, as determined by methylene blue staining.

Preparation of sera for culture media

FCS was bought commercially, aliquoted into 50 ml sterile containers and heated to 56^{o}C for 30 min to inactivate complement The heat inactivated sera was then stored at -20^{o}C until used .

Rat sera was collected from the animal facility in DCU. Adult Wistar rats were bled by cardiac puncture into syringes, which was allowed to clot overnight at 4^{o}C. The clots were then centrifuged at 500 x g for 20 min and the serum (supernatant) removed. The serum was then filter sterilised, through a 0.22 µm filter, before being heat-inactivated at 56^{o}C for 30 min and stored in aliquots at -20^{o}C.

Purification of parasite infected erythrocytes

(a) P. berghei

P. berghei is an asynchronous malaria species, all stages of the erythrocytic cycle except sequestering schizont being present in the blood at any given time. A Percoll density gradient centrifugation step was developed to separate mature-trophozoite infected erythrocytes from immature-trophozoite infected erythrocytes and uninfected erythrocytes. The purified trophozoites were then considered synchronous and could be used in invasion assays. This percoll gradient was termed the *P. berghei* 65-75-85 Percoll Step.

A second Percoll gradient was developed which separated infected erythrocytes from uninfected erythrocytes. The resulting parasite preparation wasn't synchronous, but resulted in a higher recovery of parasite material per ml of infected mouse blood than the 65-75-85 step. This percoll step was termed the *P. berghei* 80 Percoll Step.

1. *P. berghei* 65-75-85 Percoll Step

Approximately 1 ml of blood was removed from a *P. berghei* infected mouse by cardiac puncture into a heparinised syringe. After passage over a 1 ml CF11 column , to remove lymphocytes, the cells were washed in PBS, and resuspended in 0.5 ml PBS. The 65-75-85 stepwise gradient was then prepared by first loading 4 ml of a 65% Percoll solution (see BUFFERS AND SOLUTIONS) into a Corex centrifuge tube. This was then underlaid with 4 ml of the 75% solution which in turn was underlaid with 2 ml of the 85% solution. The resuspended cells were then layered onto the gradient, and it was centrifuged at 5000 x g for 20 min at room temperature. Following centrifugation the very top layer of cells (showing pigmentation), which did not or only slightly penetrated the top Percoll layer, was removed , washed in 10 ml PBS and resuspended in 0.5 ml PBS. In this preparation, >95% of the erythrocytes contained mature-trophozoites.

2. *P. berghei* 80 Percoll Step

Infected blood was collected, washed, and resuspended in 0.5 ml PBS. These cells were then layered onto 10 ml of a 80% percoll solution

in a Corex tube and was centrifuged at 5000 x g for 20 mins at room temperature. The top layer of cells was removed, washed and resuspended in 0.5 ml PBS. This Percoll step resulted in preparations containing >95% parasite-infected erythrocytes.

(b) P. c. chabaudi

As the intra-erythrocytic cycle of *P. c. chabaudi* is synchronous, large quantities of mature-trophozoite infected erythrocytes were obtained by taking blood from an infected animal at the time of day corresponding to that stage of maturation in the cycle (approximately 1pm).The infected erythrocytes were separated from uninfected erythrocytes using a Percoll gradient modified from that described by Wunderlich *et al.* (1985). Cells were collected, washed, and resuspended as before, then layered onto 10 ml of a 74% Percoll solution (see BUFFERS AND SOLUTIONS) in a Corex tube. After centrifugation at 5000 x g for 20 min at room temperature, the cells formed 2 bands in the Percoll. The upper band contained >95% infected erythrocytes, the lower band contained uninfected erythrocytes. This Percoll step was termed the *P. c. chabaudi* 74 Percoll Step.

Preparation of target erythrocytes

Blood from Balb/c, Schofield and CD-1 mice was obtained by cardiac puncture into heparinised syringes, washed in 10 volumes of PBS by centrifugation at 450g for 5 min, and resuspended in 0.5 ml PBS. Erythrocytes from guinea-pigs, rabbits, and humans (supplied by Dublin Transfusion Board) were prepared similarly.

Enzyme treatment of erythrocytes

Erythrocytes were collected from mice by cardiac puncture into heparinised syringes. The cells were washed three times in 10 ml RPMI-1640 and then 1 x 10^9 cells were treated with chymotrypsin (50 U/mg protein), trypsin (10,000 U/mg protein), papain (20U /mg protein) or neuraminidase (1 U/ml, *Vibrio cholera* source) in RPMI 1640, pH 7.4, for 90 min at room temperature. The cells were then washed three times with RPMI 1640, incubated for 10 min at room temperature with a suitable enzyme inhibitor (5mM TPCK for chymotrypsin, 1 mg/ml STI for trypsin, 1mM PMSF for papain), and washed a further three times with RPMI 1640.

Control cells were treated similarly, with the exception that enzymes were omitted.

Invasion assays

(a) P. berghei

There were 3 steps involved in the *P. berghei* invasion assay,

(1) culturing of purified mature-trophozoite infected erythrocytes.

(2) preparation of target erythrocytes.

(3) incubation of parasite infected erythrocytes with target erythrocytes

(1) Culturing of purified mature-trophozoite infected erythrocytes

P. berghei schizont cannot be obtained by bleeding infected mice as this stage of the asexual cycle sequester in the blood vessels of the the liver and spleen. Schizont stages were obtained *in vitro* using culturing methods similar to those described by McLean, Dougall, and Phillips (1986). Synchronous mature-trophozoite infected erythrocytes, obtained using the *P. berghei* 65-75-85 Percoll Step described, were cultured *in vitro* in a 50% FCS/50% RPMI 1640 based media. The cells, at a density of 2.5×10^7 cells/ml were cultured at 37^oC for 5 hours in a tissue culture flask placed in a candle jar. During this time the parasites develop into schizont.

(2) Preparation of target erythrocytes

Immediately before use target erythrocytes or enzyme treated target erythrocytes were prepared.

(3) Incubation of parasite infected erythrocytes with target erythrocytes

A total of 2×10^7 target cells was added to 0.8 ml of the 50% FCS/50% RPMI 1640 media, in wells of a 24-well tissue culture plate. The flask containing the cultured parasitised erythrocytes, was removed from the candle jar after 5 h incubation, and the cells were gently resuspended. Two hundred µl (5×10^6 infected erythrocytes) of this culture were then added to each well containing target erythrocytes. The 24-well plate was then gently agitated and placed into the candle jar which was returned to the 37^oC incubator. Nineteen hours later the contents of each well were removed, dispensed into a microfuge tube and centrifuged at 450 x g for 5

min. The cell pellet was then resuspended in 50 µl FCS, 10 µl of which was used to make a smear on a glass slide. These smears were giemsa stained and examined under a light microscope. The invasion of target erythrocytes was estimated by counting the number of erythrocytes infected with ring-stage parasites and expressing this as a percentage of total erythrocytes.

(b) P. c. chabaudi

The *P. c. chabaudi* invasion assay was carried out in 3 stages, similar to that of the *P. berghei* invasion assay.

(1)Culturing of purified parasite infected erythrocytes

Synchronous mature-trophozoite infected erythrocytes were obtained using the *P. c. chabaudi* 74 Percoll Step described. These cells were then cultured in a RPMI 1640 based media, at 37^{o}C for 5 h in a tissue culture flask in a candle jar, at a density of 2.5 x 10^{7} cells/ml. After this incubation the parasitised cells had developed to schizogony.

(2) Preparation of target erythrocytes

Immediately before use, target erythrocytes or enzyme treated target erythrocytes were prepared.

(3) Invasion assays

The invasion assay was carried out exactly as described for *P. berghei* except that the *P. c. chabaudi* culture medium was used.

Metabolic ^{35}S-labelling of parasite proteins

P. berghei infected mice were bled by cardiac puncture into heparinised syringes when parasitaemias were between 15% - 25%. Following passage over CF11 column, and washing in PBS, mature-trophozoite infected erythrocytes were purified using the P. *berghei* 65-75-85 Percoll Step. The parasites were then cultured at 2.5 x 10^{7} cells/ml in a 50% FCS/50% RPMI 1640 based ^{35}S-labelling media, for 6 hours, at 37^{o}C in a culture flask placed in a candle jar. After this incubation, the culture was centrifuged at 500 x g for 10 min, the supernatant removed and

the cell pellet washed once in 50 ml ice-cold PBS, and 3 times in 1 ml ice-cold PBS. The cells were then extracted by 3 freeze-thaw cycles in 1 ml PBS (containing 1mM iodoacetamide, 1mM PMSF, 1mM EDTA, and 5μM E-64). The extract was centrifuged at 10,000 x g for 30 min at 4^{o}C to remove cell debris and the supernatant brought up to the original culture volume with ice-cold PBS (including protease inhibitors). This extract was termed *P. berghei* Hot Pellet Extract and was stored in 1 ml aliquots at -20^{o}C.

A Hot Pellet Extract, from *P. c. chabaudi* trophozoites that had been purified on the *P. c. chabaudi* 74 Percoll Step and cultured in the *P. c. chabaudi* radiolabelling medium for 6 h was prepared, in exactly the same manner as the*P. berghei* extract. This extract was termed the *P. c. chabaudi* Hot Pellet Extract.

Binding assays

Erythrocytes (1 x 10^{9}) were mixed with 0.4 ml metabolically ^{35}S-labelled Hot Pellet Extract for 30 min at room temperature in a 1.5 ml microfuge tube. The suspension was then layered on 0.5 ml silicone oil and centrifuged for 20s at 12,000 x g. The pellet was resuspended in 1 ml cold RPMI 1640 and centrifuged. The supernatant was removed and 20 μl of 1.5 M NaCl was mixed with the pellet. After 10 min the suspension was centrifuged for 20 s at 12,000 x g. The supernatant was mixed with an equal volume of 2X SDS - PAGE reducing sample buffer, placed in a boiling water bath for 2 min and electrophoresed on 10% reducing SDS polyacrylamide gels in parallel with prestained molecular weight markers. Radioactive bands were visualised by fluorography using Amplify®.

Preparation of P. berghei extract

P. berghei infected blood was collected in heparinised syringes and parasitised erythrocytes were purified using the *P. berghei* 80 Percoll Step. These cells were then cultured at 37^{o}C for 6 h at a cell density of 2.5 x 10^{7} cells/ml. The 50% RPMI 1640/50% FCS media was used and the culture flask was left in a candle jar for the duration of the incubation. The culture was then centrifuged at 500 x g for 10 min and the cell pellet washed twice in a hundred volumes of PBS, and twice in ten volumes of

PBS. The pellet was then resuspended in a volume of PBS (including 1mM PMSF, 1mM EDTA, and 5μM iodacetimide), corresponding to 1% of the initial culture volume. The resuspended cell pellet was then subjected to 3 cycles of freeze-thaw lysis and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was removed, aliquoted and stored at -20°C and used either for immunisations or for separation on SDS-PAGE and blotted to nitrocellulose. This extract was termed C1 Extract.

Production of anti-C1 Extract antibodies

The C1 Extract was used to generate antibodies in a rabbit, the sera produced was termed anti-C1 antisera. Equal volumes of antigen (100 μg protein) and Freunds Complete Adjuvant were mixed and sonicated on ice to form an even emulsion. This was then injected subcutaneously at the base of the neck of a male rabbit. After 3 weeks the rabbit was injected again with an emulsion consisting of equal parts antigen (100 μg protein) and Freunds Incomplete Adjuvant. This injection was repeated 3 times at 3-week intervals. Ten days after the final injection the rabbit was sacrificed and bled by cardiac puncture. Approximately 50 ml of blood was collected and left to clot overnight at 4°C. The serum was drawn off and heat inactivated at 56°C for 30 min, before being aliquoted and stored at -20°C.

Radio-immunoprecipitation

To 200 μl of Hot Pellet Extract was added 5 μl rabbit anti-serum and the mixture was incubated at 4°C overnight. Protein-A agarose (50 μl) was added, and the mixture incubated for one hour at 4°C, with occasional mixing. The precipitates were then washed at 500 x g in this sequence of these buffers-

i) NETT - 0.5% BSA
ii) NETT
iii) NETT - 0.5M NaCl
iv) NETT

The microfuge tube was changed between the third and fourth wash and the fourth wash was spun at 10,000 x g for 5 min. The pellet was resuspended in 40 μl of 1X SDS PAGE reducing sample buffer and was

boiled for 2 min. The sample was then spun at 10,000 x g for 20s to pellet the Protein-A agarose, and the supernatant was analysed by SDS-PAG or stored at -20^{o}C. Immunoprecipitated proteins were visualised by fluorography. Control immunoprecipitations were carried out with pre-immune sera.

If rat sera was used in immunoprecipitations then a rabbit anti-rat antibody was added after the first overnight incubation of sera with labelled proteins, and this was incubated at 4^{o}C for 4 h before proceeding to the Protein -A agarose step.

Fluorography

Electrophoresis gels that were performed for the resolution of radio-labelled proteins, were fixed for 30 min in destain and subsequently incubated in Amplify for 30 min. The gels were then dried, and exposed to X-ray film at -70^{o}C. The film was then developed and fixed according to the manufacturers instructions.

SDS-PAGE

Samples were routinely run on 8% or 10% polyacrylamide gels according to Laemmli (1970). Gels were prepared fresh each day and run at 25mA per gel, with prestained molecular weight markers run in parallel. The gel was removed from the apparatus, fixed in destain for 30 min and then either stained with 0.1% coomassie blue or silver stained. Gels destined for transfer to nitrocellulose were not fixed in destain but incubated for 30 min in transfer buffer.

Semi-dry transfer of protein from PAG to nitrocellulose

An ATTO semi-dry transfer apparatus was used. The gel, nitrocellulose paper, and 16 pieces of filter paper were incubated in transfer buffer for 30 min. The anode was then covered with a thin film of transfer buffer, and 8 pieces of soaked filter paper were layered, one on top of another, onto the wet anode. The nitrocellulose was laid next, followed by the gel, and then the 8 remaining pieces of filter paper completed the sandwich. The cathode in the lid of the apparatus was then lowered onto the top of the sandwich. For the effective transfer of proteins onto the

nitrocellulose the operating current (mA) was calculated as 1.5 times the area of the gel (cm^2). By experience it was found that proteins up to 250 kDa would transfer in 6 h at this current/area ratio. When transfer was complete the current was switched off and the transfer sandwich disassembled. Success of transfer could be gauged by the transfer of the prestained molecular weight markers, and by coomassie staining the gel after transfer.

Western Blotting

After successful transfer of protein to nitrocellulose the blot was washed in TBST for 5 min to remove any acrylamide. This was then followed by incubation in Blocking Buffer (TBST-1% BSA) for 1 h at room temperature or overnight at 4^oC. The membrane was then incubated with shaking in an appropriate dilution of primary antisera in TBST for 1 h, followed by 5 x 10 min washes in TBST. The secondary antibody (alkaline phosphatase conjugated) was then diluted in TBST (1:10,000) and the blot incubated with it for 1 h, followed by washing 5 x 10 min in TBST, to remove unbound antibody. The blot was then incubated in Alkaline Phosphatase Colour Development Solution until reactive areas turned purple. The reaction was stopped by rinsing the membrane in distilled water. The blot was placed on filter paper, air dried and stored in clingfilm.

Construction of cDNA expression library - Preparation

Several precautions were taken to ensure that this work remained RNase free. Micropipette tips and microfuge tubes were washed in chloroform and autoclaved before use. All glassware, non disposable plasticware, and electrophoresis apparatus used for RNA work was kept separately from other labware. Glassware was soaked and rinsed in a solution of 0.02% DEPC, autoclaved, and baked for at least 3 h at 250^oC before use. Solutions to be used with RNA were treated by addition of DEPC to 0.02%, followed by thorough mixing of the solution , incubation at 37^oC overnight and then autoclaving the solution to remove the DEPC. Tris solutions or solutions of substances containing amine or sulfhydryl groups which react with DEPC could not be treated directly, and so were made up in DEPC treated water and autoclaved. Sterile disposable plastic test-tubes

and individually wrapped sterile pipettes were used wherever possible, and disposable gloves were worn at all times and changed regularly.

Isolation of RNA

P. berghei parasitised erythrocytes (2.5×10^9) were purified on the *P. berghei* 80 Percoll Step and cultured for 6 h at 37°C as described. This culturing ensured that the RNA isolated included mRNA from the schizont stage of the life cycle. After washing in PBS, the cell pellet was resuspended in 1 ml PBS (made up in DEPC water). The resuspended pellet was then transferred to a 15 ml corex centrifuge tube and 7 ml of RNAzol B was added. This mix was solubilised by passage up and down a pipette several times. Then 0.1 volume of chloroform was added to the suspension, the corex tube was covered tightly and shaken vigourously for 15s. Subsequently the mix was let sit on ice for 5 min and then centrifuged at 12,000 x g for 15 min at 4°C. The colourless upper aqueous phase (approx. 5 ml), was removed and transferred to a clean Corex tube. An equal volume of cold isopropanol was added and the sample was stored at 4°C for 15 min. Following another centrifugation at 12,000 x g for 15 min at 4°C the RNA had precipitated to form a white-yellow pellet at the bottom of the tube. The supernatant was removed and the pellet was washed once in 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 8 min at 7,500 x g at 4°C. The pellet was then briefly dried in a vacuum drier and resuspended in 100 µl of 1mM EDTA. RNA was spectrophotometrically quantitated and a sample run on an agarose gel. The RNA was considered to be of suitable quality to proceed with mRNA isolation if the ribosomal RNA ran as sharp bands.

Isolation of mRNA from total RNA

The BRL mRNA Isolation System was used to purify mRNA from total isolated RNA. Total RNA was reprecipitated in ethanol and dissolved in the Binding Buffer supplied with the kit. This RNA was passed over a cellulose chromatography matrix containing immobilised deoxythymidine oligomers (oligo dT). The presence of salt in the Binding Buffer promoted hybridisation between the immobilised oligo dT and the poly (A) tails at the 3' termini of

mRNA. After the column was washed to remove unbound nucleic acids, the mRNA was eluted in a buffer containing no NaCl, (Elution Buffer) which destabilised the A-T bonds. NaCl was then added to the Elution Buffer, and the mRNA chromatographed a second time. The second eluate was ethanol precipitated and stored in a 1mM EDTA solution at -70^{o}C until used for cDNA synthesis.

cDNA synthesis

The RiboClone® cDNA Synthesis kit from PROMEGA was used to generate double stranded cDNA from mRNA isolated as described. All procedures were carried out as outlined in the manufacturers instructions. In this method, first strand synthesis was carried out with AMV Reverse Transcriptase using oligo dT primers in the presence of 4mM sodium pyrophosphate, which suppressed the formation of hairpins. Second strand synthesis was then carried out using RNase H to create nicks and gaps in the hybridised mRNA strand, which provides 3' -OH priming sites for DNA synthesis and repair by DNA polymerase 1. The second strand synthesis reaction was allowed to proceed for 4 h to ensure that large size cDNA was synthesised. This reaction was then stopped, and incubated briefly with T4 DNA polymerase to remove any remaining 3' protruding ends. The cDNA was then ready for linker addition.

Ligation of EcoR1 linkers to cDNA

In order to ligate the cDNA into gt11 arms it was first necessary to add EcoR1 linkers to the cDNA. All procedures were carried out using the RiboClone® Linker Ligation Kit according to PROMEGA's instructions. The cDNA was first methylated with an EcoR1 methylase to protect internal EcoR1 restriction sites in the cDNA from EcoR1 treatment later in the procedure. It was important then to eliminate small size DNA's from the linker ligation reaction, and this was done using PROMEGA's Sephacryl ® S-400 spin columns. These columns get rid of double stranded DNA fragments less than 270 bp.The EcoR1 linkers (10 mers) were then ligated to the size fractionated cDNA using T4 DNA Ligase. Subsequently this reaction mix was treated with EcoR1, and the unligated linkers removed by another round of Sephacryl

®S-400 spin column chromatography. The cDNA was then ready for ligation and packaging into lambda.

Ligation and Packaging of cDNA into gt11

The cDNA from the linker ligation step was ligated to alkaline phosphatase treated gt11 arms supplied with the PROMEGA ProtoClone ® gt11 system, using T4 DNA Ligase. This was then packaged into lambda heads using the PROMEGA Packagene® system. A control ligation and packaging was carried out in parallel with just the lambda arms and no insert DNA. This allowed determination of the background level of religated arms, and also provided a supply of wild type gt11. The packaged phage was then titred by infecting LE392 bacteria with dilutions of the phage, and then plating the bacteria out on LB plates in top agar. The Phage stock was then amplified by plating at an appropriate dilution on LB plates.

Preparation and plating of phage competent cells

Two bacterial strains were used.

(a) *E. coli* LE392 - used for amplification, titration, and screening of phage libraries with nucleotide probes. This strain was maintained on LB plates.

(b) *E. coli* Y1090 - used for screening of expression library with antibody probes. Y1090 was always maintained in LB with 100 µg/ml ampicillin and 15 µg/ml tetracyclin.

One colony from a stock plate of the bacteria was used to inoculate 10 ml of LB media (with 0.2% maltose, 10mM $MgSO_4$) which was then cultured overnight at 37°C. Fifty µl of this was then used to inoculate 5 ml of similar media and the cells grown until A_{600nm} was between 0.6 and 0.9.

For each plate to be poured 100 µl of these phage competent cells was mixed with 100 µl of the desired dilution of phage in a microfuge tube and incubated at 37°C for 20 min. This phage/cells mix was then added to 3 ml molten top agar (including 10mM $MgSO_4$) at 48°C and this was mixed by very gentle vortexing before pouring onto prewarmed LB plates. The plates were allowed to harden at room temperature for 10 min, and then incubated inverted at 37°C until plaques reached the desired size.

Titration of phage stock

One hundred µl of phage competent LE392 cells were infected with 100 µl of tenfold increasing dilutions of the packaged phage, by incubation in a microfuge tube for 20 min at 37°C.This mixture was then added to 3 ml molten top agar (with 10 mM $MgSO_4$), and plated out on LB plates. These plates were then incubated overnight at 37°C. The efficiency of packaging was calculated for both the cDNA and the control as the number of Plaque Forming Units (pfu) per µg DNA packaged.

Amplification of phage stock

One hundred µl of phage competent cells was infected with 5000 pfu of the packaged phage for 20 min at 37°C. The entire packaged phage stock was used in this way. The cells were plated out in 3 ml top agar and incubated overnight at 37°C. The top agar was then overlaid with 5 ml SM buffer and rocked at room temperature for 2 h. The SM buffer was then harvested to a centrifuge tube and chloroform added to 5%. This was then mixed gently and centrifuged at 3000 x g for 10 min at 4°C. The supernatant, which contained the amplified phage was removed, leaving cell debris and the chloroform in the tube. The amplified stock was then aliquoted into microfuge tubes, chloroform added to 0.3% and stored at 4°C until used. The stock was regularly titred.

Preabsorption of the antisera

It was important to remove all antibodies reactive with bacterial products from the antisera before commencing screening. This was done by mock screening of wild-type phage plaques with the polyclonal sera. Phage competent *E. coli* Y1090 were prepared, infected with 5000 pfu wild-type phage, and plated out on LB-ampicillin plates as described. The plates were incubated at 42°C until the plaques became visible (2 - 3 h). Nitrocellulose discs (83mm diameter) were soaked in 10 mM IPTG, briefly air-dried, and carefully placed onto the plates to avoid air bubbles. The

plates were then incubated inverted at 42^{o}C for a further 4h. The nitrocellulose discs were then inverted on the plates and the plates were returned to the incubator for another 4 h at 42^{o}C (or incubated overnight at 4^{o}C). The discs were carefully removed from the plates, washed briefly in TBST, and then blocked overnight in TBST-1% BSA at 4^{o}C. The diluted antisera was then incubated with one filter per 4 ml for 4 h at room temperature, the filters were then replaced with another and allowed to incubate with the antisera overnight at 4^{o}C. The second filter was then removed and the preabsorbed antisera stored at 4^{o}C until used.

Preparation of anti-C1 sera for immunoscreening

The antisera against the C1 extract reacted against a very large range of proteins and screening with it would have proved very cumbersome. Therefore it was decided to absorb the antibodies reacting against high molecular weight proteins (>100 kDa) from the sera and to use only this fraction for immunoscreening.

A 10% reducing SDS-PAG was run with 10 lanes of C1 Extract. This was then transferred to nitrocellulose for 6 h at 1.5 mA/cm^{2} . After blotting, the strip of nitrocellulose corresponding to proteins >100 kDa, as determined by the molecular weight markers was cut from the nitrocellulose, and blocked in TBST-1% BSA for 1 h at room temperature. Ten ml of a 1/50 dilution of anti-C1 antisera (which had been absorbed against wild-type plaques) was then added to the nitrocellulose strip and incubated for 1 h at room temperature. Unbound sera was then poured off and the strip washed 5 x 10 min in TBST-0.05% Triton-X to remove non-specifically bound antibody.

The bound antibodies were then removed by incubating the strip in 10 ml glycine buffer pH 2.6 for 2 min. This elution buffer was then poured off the strip and neutralised with a pretitred volume of 2 M Tris base. Azide was added and the anti -C1 (+100 kDa) sera stored at 4^{o}C.

Immunoscreening of lambda library

Twenty plates with ~3000 pfu each were used in primary screening

with each antisera. Phage competent E. *coli* Y1090 were prepared, infected with ~3000 pfu of the library and plated on LB-ampicillin plates as described.The plates were incubated at 42^{o}C until the plaques reached pinprick size (~3 h). Numbered nitrocellulose discs were soaked in 10 mM IPTG, air dried and then placed carefully onto the correspondingly numbered plates, avoiding air bubbles. The plates were then either incubated at 37^{o}C for 4 h or at 4^{o}C overnight. Three asymmetric orientation marks were then made on the filter by stabbing through it into the agar with a needle. The filters were then removed carefully and blocked by incubation in TBST-1% BSA overnight . The plates were wrapped in parafilm, and stored at 4^{o}C until required. The discs were incubated in 4 ml preabsorbed antisera for 2 h at room temperature (The primary antibody was not discarded after use, but was used for subsequent screening) and then washed 3 x 10 min in TBST. They were then incubated in secondary antibody (alkaline phosphatase conjugated) for 2 h at room temperature. After a further 3 x 10 min washes in TBST the discs were developed in Alkaline Phosphatase Developing Solution, until purple rings appeared on the discs. The reaction was stopped by replacing the AP Developing Solution with distilled water. The filters were then air dried.

The marks were then used to orientate the the filters with the original plate. Usually the plaques were too confluent to identify the individual plaques responsible for the positive signal on the filters and therefore the area of the plate corresponding to the positive signal was removed as a agar plug using a sterile pasteur pipette. This plug was transferred to 1 ml of Phage Buffer in a microfuge tube, and was left at 4^{o}C overnight to allow the phage to diffuse out. These were called 'primary positives' .The primary positives were then titred and plated out at ~200 pfu on Y1090 and rescreened as described. As there were less plaques on the plate, it was easier to identify individual positive plaques, and these 'secondary positives' were picked and allowed to diffuse into phage buffer as before. The phage were then rescreened at ~100 pfu per plate and 'tertiary positive' plaques picked. On these plates all the plaques were usually positive. However, if they were not another screening cycle was performed. Purified phage was amplified as described, and stored at 4^{o}C until

required.

Preparation of Lambda lysates and isolation of Lambda DNA

Lambda DNA could be isolated from stocks of amplified phage. However if large amounts (~50 µg) of DNA was required, the phage was amplified using a liquid culture method, and DNA purified from the lysate.

Phage competent E. *coli* Y1090 were prepared as described. Twenty µl of phage stock was added to 500 µl of cells in a microfuge tube, mixed, and incubated at 37°C for 20 min. This was then used to inoculate 100 ml of prewarmed LB (with 10mM $MgSO_4$, 100 µg/ml ampicillin, 15 µg/ml tetracyclin), which was incubated at 37°C in an orbital shaker. The media went cloudy after about 4 h and then clear again after a further hour as the phage lysed all the cells. The culture was then centrifuged at 8000 x g for 10 min to remove cellular debris. The supernatant containing the phage was aliquoted to sterile 50 ml tubes and stored at 4°C until used. As well as being used for preparation of phage DNA, this lysate could also be used as an amplified phage stock.

DNase 1 (1µg/ml) and RNase A (1µg/ml) were added to 80 ml of the lambda lysate supernatant, and it was incubated at 37°C for 30 min. An equal volume of Phage Precipitation Buffer was added to the lysate, mixed a few times by inversion, and incubated overnight in ice-water. The precipitated phage particles were recovered by centrifugation at 10,000 x g for 20 min at 4°C, and the supernatant discarded. The centrifuge tube was allowed to stand inverted on paper towels for at least an hour, to allow the fluid to drain away, before the phage particles were resuspended in 5 ml Phage Buffer. The resuspended phage was then centrifuged at 8000 x g for 2 min to remove debris, and then extracted in turn with one volume of TE-saturated phenol/chloroform, one volume of TEN-saturated phenol/chloroform and finally one volume of chloroform : isoamyl alcohol (24:1). The aqueous phase was mixed with an equal volume of isopropanol in a Corex centrifuge tube and was left at -70°C for 20 min. The solution was then centrifuged at 12,000 x g for 10 min at 4°C, the supernatant carefully drained and the pellet resuspended in 400 µl H_2O and transferred to a microfuge tube. Eight hundred µl ethanol was added, and the tube

placed at -20°C for 1 h. The DNA pellet was recovered by centrifugation at 10,000 x g for 10 min at 4°C, washed with 70% ethanol, and dried under vacuum.

This preparation was often found to have large amounts of RNA and therefore an RNase digestion step necessary. The pellet was resuspended in 200 µl sterile water and RNase A added to 100 µg/ml. Following a 30 min incubation at 37°C, phenol/ chloroform and chloroform extraction, the DNA was precipitated with ethanol, washed in 70% ethanol, vacuum dried, and resuspended in 50 µl sterile H_2O. This procedure routinely yielded between 20 - 50 µg DNA from 80 ml phage lysate. Phage DNA was quantitated spectrophotometrically, and analysed by PCR and EcoR1 digestion.

PCR analysis of lambda DNA

PCR was used to size and isolate the inserts in the phage library. The primers used were derived from the sequence flanking the EcoR1 cloning site of the gt11, and could be used to amplify cloned sequences. In the wild-type lacZ gene the distance between the two primer sites was 39 bp (which includes the EcoR1 site), and since both primers were 24 mers, amplification using the wild-type DNA would be expected to give a PCR product of 87 bp. This was then subtracted from the PCR fragment size generated from phage containing inserts in order to establish the true fragment size.

The PCR conditions were for each reaction

L forward primer (0.5 µg / µl)	1 µl
L reverse primer (0.5 µg / µl)	1 µl
dNTP's (1 mM each)	5 µl
10x PCR buffer	5 µl
$MgCl_2$ (25 mM)	6 µl
Taq Polymerase (5u / µl)	0.5 µl
Lambda DNA (~0.5 µg / µl)	1 µl
Sterile H_2O	30.5 µl

Each 50 µl reaction was overlaid with 50 µl mineral oil and placed in the Hybaid Omnigene PCR machine, and Program no. 1 was executed.

PROGRAM 1

Stage 1 94 ^{o}C 4min.

Stage 2 94 ^{o}C 1min,

55 ^{o}C 2min,

74 ^{o}C 2min.

stage 2 was run for 40 cycles.

Ten µl of each PCR reaction was removed from below the mineral oil, mixed with 2 µl of 6X DNA electrophoresis sample buffer and run on an agarose gel in parallel with size markers.

A 200 bp fragment could be PCR amplified from *P. berghei* genomic DNA using primers derived from the known sequence of the *P. falciparum* 175 kDa Erythrocyte Binding Protein (primer 142 and primer 143). These primers were used to determine whether any of the clones picked from the cDNA library by the immunological screening encoded a gene homologous to the *P. falciparum* that encoded an erythrocyte binding protein. In this PCR reaction, reagents and volumes were as described above, except that primers 142 and 143 were used in place of the Lambda forward and reverse primers and a new program was created.

PROGRAM 2

Stage 1 $94^{o}C$ 4 min.

Stage 2 $94^{o}C$ 30 s,

$42^{o}C$ 2 min,

$74\ ^{o}C$ 1 min.

stage 2 was run for 5 cycles.

Stage 3 $94\ ^{o}C$ 30 s,

$45^{o}C$ 30 s,

$74^{o}C$ 1 min.

stage 3 was run for 25 cycles.

Analysis of PCR products was by agarose gel electrophoresis.

Oligonucleotide probe JA159

A 40 mer oligonucleotide probe (JAI59) was synthesised from the 200 bp sequence that was amplified using P. *berghei* genomic DNA as template and primer 142 and primer 143. An EcoR1 site was engineered into the 5' end as shown (P. Curley, personal communication).

OLIGO JA159

5' -^{OH} AAGGATCCAATAATCTGATATTGAGCAGCATAATTGTTTC -3'

| |

EcoR1

This oligo was first end-labelled with ^{32}P from gamma labelled ATP.

The reaction mix was

JA 159 (700 ng/µl)	0.5 µl
10 x T4 polynucleotide kinase buffer	5 µl
L^{32} ATP (5 µC/µl)	10 µl
T4 polynucleotide kinase (10 u/µl)	1 µl
sterile H_2O	33.5 µl

The kinase and the label were added last and the reaction mix was incubated for 2 h at room temperature. When the reaction was complete, 50 µl of 0.2M EDTA and 100 µl of 20X SSC were added and the reaction tube placed in a boiling water bath for 5 min. The reaction was cooled on ice for 5 min, before addition to the Prehybridisation Solution.

Screening of lambda library with radiolabelled JA 159

Phage competent *E. coli* LE 392 were prepared as described. Twenty LB plates with ~5000 pfu were incubated at 37^{o}C for 6 h. In this time small plaques became clearly visible. Numbered nitrocellulose discs were then placed carefully onto the plates, avoiding the trapping of air bubbles, and left there for 1 min. The discs and plates were then marked with orientation marks by stabbing a needle through the disc and into the agar in several asymmetrically located points. The discs were then removed and washed successively in Denaturing solution, Neutralising solution, and 20 X SSC for 5 min each. This was done by placing the disc on a pool of the solution (made on tinfoil), always keeping the side of the disc which was in contact with the plaques up. The discs were dried on filterpaper for 5 min between each wash. Finally the discs were allowed to dry at room temperature for 20 min and then baked at 80^{o}C for 2 h.

After baking the filters were wet by washing in distilled H_2O for 5 min and then 6 X SSC for 5 min. The filters were then placed into a heat sealable plastic bag, 100 ml of prehybridisation buffer added, the bag sealed and the filters allowed to prehybridise at 68^{o}C for 2 h.The labelling reaction was then added to the prehybridisation solution, and hybridisation carried out at 45^{o}C overnight.

The 200 bp PCR product of the primers 142/143 transferred by Southern transfer onto nitrocellulose served as a positive control. After hybridisation the discs and control were washed in 6 X SSC 0.2% SDS 3 x 10 min at room temperature,1 x 10 min at 50 ^{o}C.

The filters were then touch dried onto filter paper, wrapped in clingfilm and exposed to X-Ray film, in a cartridge containing an intensifier screen, at -70^{o}C overnight. The film was developed and fixed according to

the manufacturers instructions. The area on the film that showed exposure to isotope was aligned with the filter discs, which were then aligned with the original agar plates, to identify the plaques which hybridised with the probe.

The areas of the top agar corresponding to the `primary positive' plaques were placed into 1 ml of Phage buffer in a microfuge tube and the phage allowed to diffuse out overnight. This was then titred and replated at ~200 pfu and a more definite identification of the positive phage made by another round off hybridisation. If any `secondary positive' plaques were found, the hybridisation procedure was repeated until the positive plaques were isolated to purity.

Positive phage were amplified and DNA extracted as described. PCR was used to check for the 200 bp sequence using primers 142 and 143. The insert size was determined using the lambda forward and reverse primers.

Preparation of fusion Protein

Two different methods were used to make fusion protein from lambda clones

(a) Preparation of lysogens

(b) Plate Wash Supernatant method

(a) Preparation of lysogens

Recombinant protein was prepared by creating lysogens of the phage in *E. coli* Y1089. This strain has a mutation which enhances the frequency of phage lysogeny, is relatively resistant to lysis and is deficient in certain protease systems. These qualities allow for the accumulation of large amounts of fusion protein.

Y1089 cells were grown overnight at 37^{o}C in LB medium (supplemented with 0.2% maltose, 10mM $MgCl_2$, and 100 µg/ml ampicillin). The cell concentration was calculated using A_{600} (An A_{600} of 1.0 is equivalent to 8 x 10^8 cells/ml).

One hundred µl of cells were then infected with 100 µl of phage such that the multiplicity of infection (phage:cells) was 10. Generally 10^4 cells were used and the reaction was incubated at 30^{o}C for 20 min. One ml of LB medium (with 10 mM $MgSO_4$) was added to the reaction and the infected

cells plated at a density of 500 cells and 50 cells per plate. These plates were then incubated at 32°C overnight. The next day 50 colonies were picked in duplicate arrays using sterile toothpicks. One plate was grown at 42°C, the other at 30°C. Lysogens were identified by growth at 30°C, but no growth at 42°C. Putative lysogens were tested by another series of growth on duplicate plates at 30°C and 42°C. Positive lysogens were picked, grown in LB-ampicillin at 30°C overnight, frozen stocks made in glycerol, and stored at -70°C until required.

Crude lysates from the lysogens were then prepared. A single colony from a fresh plate of the Y1089 recombinant lysogen was used to inoculate 10 ml of SB-ampicillin, which was grown at 30°C overnight. One ml of this culture was then used to inoculate 50 ml of SB-ampicillin and the culture was incubated shaking at 30°C, until it reached an A_{600} = 0.5. The temperature of the culture was then raised as rapidly as possible to 42°C (this temperature elevation was accelerated by the addition of 5 ml SB media at 55°C). After 20 min incubation at the elevated temperature, IPTG was added to the culture to 10mM. The culture was then incubated at 38°C for up to 2 h. The lysogens sometimes lysed during this incubation. If lysis occured within the first hour of this incubation the procedure was repeated with another isolate of the lysogen (as susceptibility to lysis varies for different lysogens). If lysis occured after the first hour, then the exact time for lysis was determined and the procedure repeated with the same lysogen isolate, with the 38°C incubation allowed to proceed until just short of this lysis time.

When the 38°C culture was complete the cells were centrifuged at 3000 x g for 5 min at 24°C - 37°C, and the cells resuspended in 1 ml TBS buffer (including 1 mM EDTA, 1 mM PMSF, 1 mM iodacetimide) in a microfuge tube. Lysozyme was added to 1 µg/ml and the cells incubated for 20 min, before a freeze/thaw lysis step at -70°C. In most cases this was insufficient to completely lyse the cells, and in these cases sonication was used to complete lysis.

After sonication , the cell preparation had a very gloopy consistency due to the high concentration of DNA. DNase (1 µg/ml) was added and the lysate incubated at room temperature for 30 min on a rotator. This incubation reduced the viscosity of the extract. The extract was then centrifuged at 10,000 x g for 10 min at 4°C to pellet cell debris.

The supernatant should contain the fusion protein sequestered in inclusion bodies. However not all fusion protein are sequestered as such and therefore the lysate pellet and the supernatants from all subsequent wash steps were retained and checked for partitioning of the fusion protein.

The supernatant was transferred to a Corex centrifuge tube and centrifuged at 16,000 x g for 5 min at 4°C. The pellet, containing the inclusion bodies was resuspended in 1 ml cold TBS, 10 mM EDTA, 0.5% Triton-X and was incubated for 5 min at room temperature. The inclusion bodies were then pelleted by centrifugation at 16,000 x g for 5 min at 4°C. The pellet was resuspended, incubated, and centrifuged once more in the TBS-EDTA-Triton-X, followed by resuspension in 100 µl TBS.

An aliquot of this as well as samples of the wash supernatants and the sonicate supernatant were analysed on an 8% SDS-PAG, under reducing conditions and analysed by coomassie staining. If putative fusion proteins were identified, they were checked by western blotting with an anti-ß-galactosidase primary antibody. Wild-type phage lysogen produced ß-galactosidase, purified by the method described, was used as a control.

(b) Plate Wash Supernatant method for production of fusion proteins

Phage competent E. *coli* Y1090 were prepared as described. One hundred µl of cells was infected with ~10,000 pfu of phage for 20 min at 37°C, before pouring onto LB -ampicillin plates in top agar. The plates were then incubated at 42°C for 3 h, by which time plaques had appeared. Five ml of Phage Buffer (including 1 mM EDTA, 1 mM PMSF, 1 mM Iodacetimide, and 10 mM IPTG) was then poured onto the plates and they were placed in a 37°C incubator overnight. There was little bacterial growth left on the plate after this incubation due to the growth of the phage plaques. The phage buffer was transferred to a centrifuge tube to which was added the top agar scraped from the plate. This was vortexed for 20 s before

centrifugation at 10,000 x g for 10 min at 4^{o}C. The supernatant was carefully removed and aliquoted into 1.5 ml microfuge tubes. These tubes were centrifuged at 10,000 x g for 10 min at 4^{o}C, and the supernatant removed to fresh microfuge tubes, which were stored at 4^{o}C until required.

The Plate Wash Supernatants were analysed for presence of fusion protein by reducing SDS-PAGE followed by coomassie or silver staining. Fusion proteins were also analysed by Western blotting using an anti-ß-Galactosidase primary antibody.

Purification of Fusion proteins

The PROMEGA Protosorb® *lacZ* immunoaffinity column was use to purify recombinant fusion proteins. The column matrix consists of a pure, mouse anti-ß-galactosidase monoclonal antibody coupled to cross-linked agarose beads. The affinity of the monoclonal is high enough to allow for specific binding of fusion proteins from crude extracts, yet low enough to allow for efficient elution of the fusion protein under relatively mild conditions. The PROMEGA instructions for operation of the column were followed and fusion protein purified from Plate Wash Supernatants.

Nine ml of a saturated $(NH_4)_2SO_4$ solution was added to 3 ml of Plate Wash Supernatant. This was mixed well and chilled at 4^{o}C for 60 min, before centrifugation at 10,000 x g for 20 min. The supernatant was removed and the pellet resuspended in 1 ml cold TEP buffer. The protein concentration was then determined and the protein sample diluted to 1 mg/ml protein with cold TEP buffer, before loading on the equilibrated column. After extensive washing the fusion protein was eluted with 3 successive 1 ml aliquots of the Elution Buffer, pH 10.8, followed by 1 ml of TBS. The column was then quickly re-equilibrated in TBS.The eluted fractions were analysed by SDS-PAGE and western blots for the presence of the fusion protein.The amount of fusion protein in each aliquot was then determined and then they were stored at 4 ^{o}C until used for immunisations.

Immunisation of rats with fusion protein

Equal volumes of fusion protein and Freunds Complete Adjuvant were sonicated on ice to form an even emulsion. This was then injected

subcutaneously into the base of the neck of male Wistar rats. After 3 weeks the rats were injected with a similarly produced emulsion of fusion protein antigen and Freunds Incomplete Adjuvant. This injection was repeated a further 3 times. The rats were then bled and serum collected as described.

DNA and RNA gel electrophoresis

DNA gels were made up with agarose in 1x TAE buffer and RNA gels were prepared and run as described in Maniatis (1982).

Determination of protein concentration

The PIERCE BCA Protein Assay kit was used for protein determination using BSA as a standard, as outlined by the manufacturers.

2. BUFFERS AND SOLUTIONS

Giemsa stain

The giemsa solution for staining parasites was a solution of 10% giemsa azur-eosin methylene-blue in a solution of 4 mM Na_2HPO_4, 2.5 mM NaH_2PO_4.

PBS

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

qs to 1L and autoclaved

Percoll Solutions

Solution A : 10X PBS-glucose

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1 g
KH_2PO_4	0.2 g
NaH_2PO_4	0.15 g

Glucose monohydrate 22.5 g

qs to 100 ml and sterile filtered

Solution B : 1X PBS - glucose

Tenfold dilution of solution A in sterile H_2O.

Percoll Gradients

%	Percoll		soln A		soln B
65	9	:	1	:	3.8
74	9	:	1	:	2.17
75	9	:	1	:	2
80	9	:	1	:	1.2
85	9	:	1	:	0.58

Media for parasite culture

Three types of media were used for parasite cultivation

(a) *P. berghei* 50% RPMI 1640/50% FCS culture medium

(b) *P. c. chabaudi* culture medium

(c) metabolic radio-labelling medium

(a) *P. berghei* 50% RPMI 1640/50% FCS medium

RPMI 1640 (10X)	10 ml
HEPES	0.96 g
Hypoxanthine	0.5 mg
Neomycin (10 mg/ml)	0.5 ml
Glucose monohydrate	0.2 g

qs to 97 ml with sterile H_2O, pH adjusted to 7.0 , and sterile filtered. Immediately before use, 1 ml of 200 mM glutamine and 2 ml of a 5% sodium bicarbonate solution was added. The required amount of medium was then made up by mixing equal volumes of the RPMI based medium and heat-inactivated FCS

(b) *P. c. chabaudi* culture medium

RPMI-1640 (10X)	10 ml
HEPES	0.46 g
Hypoxanthine	0.5 mg
Neomycin (10 mg / ml)	0.2 ml
Glucose monohydrate	0.2 g

qs to 95 ml with sterile H_2O , pH adjusted to 7.3 , and sterile filtered. Before culturing, 1 ml of 200 mM glutamine and 4.2 ml of a 5% sodium bicarbonate solution was added. The required amount of medium was then made up by mixing 9 parts RPMI based media with 1 part sterile, heat-inactivated rat serum.

(c) metabolic radiolabelling medium

The *P. berghei* radiolabelling media was the same as the 50% FCS/50% RPMI based medium except that incomplete RPMI 1640 was used. This preparation lacked leucine, lysine and methionine. Leucine and lysine were added to 5 mg and 4 mg per 100 ml RPMI 1640, and ^{35}S-methionine added to the required amount of media at 50 μC/ml.

The *P. c. chabaudi* radiolabelling media was the same as the RPMI 1640/10% Rat serum media used for culturing parasites, except that incomplete RPMI 1640 was used. Leucine, lysine and the ^{35}S-methionine were added as for the *P. berghei* radiolabelling culture medium.

Electrophoresis solutions

Running buffer : 25 mM Tris, 192 mM Glycine, pH 8.3.
Fixing/detaining solution : 10% methanol, 10% acetic acid in distilled H_2O.
Staining soln : 0.1% Coomassie blue R-250 in fixing solution.

Electroblotting solutions

Blotting buffer : 25 mM Tris, 192 mM Glycine, 10% Methanol, pH 8.3.

Radio-immunoprecipitation solutions

NETT

NaCl	100 mM
EDTA	1 mM
Tris-HCl pH 8	20 mM
Triton-X	0.1%

Western blot solutions and immunoscreening solutions

TBST

Tris-HCl pH 8.0 10 mM
NaCl 150 mM
Tween 20 0.05%

Blocking solution : 1% BSA in TBST

Alkaline Phosphatase Colour Developing Solution

Tris-HCl pH 9.5 100 mM
NaCl 100 mM
$MgCl_2$ 1 mM
To 10 ml of Developing solution was added,
66 µl NBT (50 mg/ml in 70% DMF),
33 µl BCIP (50 mg/ml in DMF)

mRNA isolation and cDNA synthesis

All reagents for mRNA isolation were supplied with the BRL mRNA Isolation System. All restriction enzymes, buffers and other reagents were supplied with the PROMEGA RiboClone® cDNA synthesis kit except TE buffer.

TE buffer

Tris-HCl pH 8.0 10 mM
EDTA 1 mM

Linker ligation and lambda packaging reagents

All enzymes, buffers and reagents for linker addition were supplied with the PROMEGA RiboClone® Linker Ligation Kit and PROMEGA Sephacryl ® S-400 spin column kit. All enzymes, buffers and reagents for packaging into gt11 arms were supplied with the PROMEGA Packagene® system.

Media, Buffers and Reagents used in bacterial and phage work

LB medium

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g

pH to 7.5 , qs to 1 L , and autoclaved.

LB agar : 1.5% Bacto-agar in LB media. If antibiotics were to be added the autoclaved agar solution was allowed to cool to 55^{o}C before addition of 100 µg / ml ampicillin and 15 µg / ml tetracyclin.

SB medium

Bacto-tryptone	20 g
Bacto-yeast extract	10 g
NaCl	5 g

pH to 7.5 , qs to 1 L , and autoclaved.

Top Agarose

0.6% agarose dissolved in 100 ml LB media and autoclaved.

Phage Buffer

Tris-HCl pH 7.4	20 mM
NaCl	100 mM
$MgSO_4$	10 mM

SM Buffer

This was Phage buffer including 0.01% gelatin.

Phage Precipitation Buffer

NaCl	2 M
PEG 8000	20% (w/v)

Reagents for elution of +100 kD reactive fraction of anti-C1 antisera

Glycine buffer

Glycine	200 mM
NaCl	150 mM
pH 2.6	

Neutralisation buffer

2 M Tris

Reagents for labelling of oligonucleotide probe and library screening

The T4 Polynucleotide kinase was supplied by PROMEGA along with a kinase 10X buffer which contained,

Tris-HCl pH7.6	700 mM
$MgCl_2$	100 mM
DTT	50 mM

Denaturing solution

NaCl	1.5 M
NaOH	0.5 M

Neutralising solution

NaCl	1.5 M
Tris-HCl pH 6.8	0.5 M

20X SSC

NaCl	3.0 M
$C_6H_5Na_3O_7$ (sodium citrate)	0.3 M
pH to 7.0	

Prehybridisation solution

30 ml 20 X SSC

10 ml 500 mM Na_2HPO_4

10 ml 50 X Denhardts solution

0.5 ml SDS

1 ml Salmon sperm DNA (1 mg/ml)

qs to 100 ml.

50X Denhardts solution

10 mg/ml Ficoll, 10 mg/ml Polyvinylpyrrolidine, 10 mg/ml BSA.

Solutions used in purification of Fusion proteins

TEP Buffer

Tris-HCl pH 7.4	100 mM
EDTA	10 mM
PMSF	1 mM

Tris Buffer

Tris-HCl pH 7.3	50 mM

TN Buffer

Tris buffer with 0.2% NP-40.

High pH Elution Buffer

50 ml 0.1 M $NaHCO_3$

50 ml 0.1 M Na_2CO_3

adjust pH of solution to 10.8 with NaOH.

TBS Buffer

Tris-HCl pH 7.3	50 mM
NaCl	150 mM

Buffer for DNA gel electrophoresis

50X TAE

Tris	2 M
EDTA (pH 8)	50 mM
Glacial acetic acid	57.1 ml/L

PCR reagents

A 10X PCR reaction buffer and 25 mM $MgCl_2$ were supplied with the Taq Polymerase by PROMEGA.

10X PCR buffer

KCl	500 mM
Tris-HCl pH 9.0	100 mM
Triton - X	1%

A 10X stock of dNTP's was made such that the concentration of each dNTP was 1 mM, giving a working concentration of 0.1 mM.

3. MATERIALS' SUPPLIERS

Aldrich Chemical Company
Phenylhydrazine-HCl, Triton-X.

Amersham
Amplify.

AMS Biotechnology ltd
RNAzol B.

Becton - Dickinson
All needles and syringes.

Biological laboratories
CD - 1 mice.

Biotrin research
Protein - A agarose.

Boehringer Mannheim
Neuraminidase.

Dupont
ATP $^{32}P_{Gamma}$.

Flow Laboratories
FCS, L - glutamine.

General Electric Company
Silicone oil.

Gibco -BRL
RPMI 1640, mRNA isolation kit.

Griener

All tissue culture flasks, and sterile disposable plasticware.

ICN

Trans ^{35}S - methionine.

Kodak

X - Omat X-ray film, FX -40 liquid fixer, LX - 24 developer.

Labscan

Methanol, propanol, Ethanol, Chloroform.

Merck

Acetic acid.

Nunc

24 - well tissue culture plates.

Oxoid

Bacto-tryptone, Bacto-yeast extract, Bacto-agar no. 3.

Promega

RiboClone® cDNA Synthesis System, RiboClone® Linker Ligation System, Sephacryl® S - 400 Spin Columns, Packagene® Lgt11 Packaging System, Taq Polymerase and 10X reaction buffer, T4 Polynucleotide Kinase and 10 X reaction buffer, EcoR1 and 10 X buffer (H), Kpn 1 and Sac 1 and 10 X buffer (J),
Mouse anti-ß-galactosidase monoclonal, Alkaline Phosphatase linked secondary antibodies, Protosorb® *lacZ* Immunoaffinity column dNTP's, Agarose.

Reidel-de-Haen

Ammonium persulphate, Bromophenol blue, DMF, Azur-eosin-methylene blue,EDTA Glucose monohydrate, Glycerol, Glycine, Hydrochloric acid, Magnesium chloride-6-hydrate, Potassium dihydrogen phosphate, Sodium chloride, Sodiun hydroxide, Sodium dihydrogen phosphate, Sodium hydrogen phosphate, TEMED.

Schleicher and Schull

Nitrocellulose paper 0.45 μm.

Sigma

Acrylamide, Ammonium sulphate, Bis-acrylamide, BCIP, BSA, Chymotrypsin, E-64, Freunds adjuvants, Gentamycin, Neomycin, Heparin, Leupeptin, HEPES, Hypoxanthine, Leucine, Lysine, Magnesium sulphate, Maltose, NBT, Papain, Percoll, PMSF, RPMI 1640 (incomplete), Sodium azide, Sodium bicarbonate solution, Sodium bicarbonate, Sodium carbonate, Sodium citrate, SDS, SDS-PAGE molecular weight markers, TPCK, Trypsin, Trypsin inhibitor, Tween - 20.

Trinity College Dublin

New Zealand white rabbits.

Whatman

CF11, 3MM filter paper.

CHAPTER 3

RESULTS

Development of Plasmodium berghei erythrocyte invasion assays

To perform *P. berghei* invasion assays, specific techniques for purification and culturing the parasites were developed.

(a) Percoll purification of parasites

The first requirement of *P. berghei* invasion assays was synchronous mature-trophozoite-infected erythrocytes free from other parasitised erythrocytes and uninfected erythrocytes. For *P. berghei* a Percoll density gradient centrifugation step, the `*P. berghei* 65-75-85 Percoll', was developed. This gradient routinely gave cell preparations containing >95% mature-trophozoite infected erythrocytes from a preparation of asynchronous infected erythrocytes. (Fig. 3.1A and 3.1B).

While this percoll step yielded cells containing the most mature trophozoites, they represented only 1-2% of the total number of parasitised erythrocytes. A second Percoll centrifugation step was developed therefore which yielded much greater amounts of parasite material. This `*P. berghei* 80 Percoll' step resulted in cell preparations that contained >95% asynchronous parasitised erythrocytes and yielded enough parasite material for procedures such as RNA preparation.

(b) Culturing of parasites

Successful cultivation of *P. berghei* was a requirement not only of invasion assays, but for RNA preparation and protein extract preparation as it was necessary to cultivate the parasites in order to obtain schizonts. A culture medium was developed consisting of 50% RPMI 1640/50% FCS. When purified mature-trophozoite infected erythrocytes were cultured for 6 h in this medium, they continued their development to schizogony (Fig 3.1C). On being added to target erythrocytes in fresh medium, the parasites ruptured their erythrocytes and released merozoites which invaded the new erythrocytes. Nineteen hours after adding the parasitised cells to the uninfected cells the percentage of newly invaded erythrocytes could be estimated by counting the number of erythrocytes containing ring-stage or young trophozoite parasites (Fig. 3.1D). In all experiments, invasion into erythrocytes from rabbits or humans acted as controls for background invasion. Any parasites observed in these controls were mature

trophozoites and were therefore introduced with the infected mouse erythrocytes as ring-stage parasites.

(a) Invasion of reticulocytes and mature erythrocytes by P. berghei merozoites

The preferential invasion of reticulocytes by *P. berghei* merozoites both *in vivo* and *in vitro* is well documented (Ramakrishnan & Prakash, 1950; Janse *et al.*, 1984, 1989; Suhrbier *et al.*, 1987). The results from our invasion assays, presented in Fig. 3.2, confirm these observations. In the *in vitro* invasion assay using target erythrocytes from 8 to 10-week-old mice an invasion rate of 0.5-2% was routinely observed (Fig. 3.2, Exp. 2 and 3). Reticulocyte counts in these mice were usually 0.5-1.5%. Only in one case was a higher invasion level observed using these erythrocytes (Fig. 3.2, Exp. 1), however the reticulocyte count in the blood of the animal was 11%, which was unusually high for an animal of that age.

In an attempt to improve invasion levels, erythrocyte preparations from very young mice were used. These preparations contain higher reticulocyte counts than preparations from older animals. In two experiments shown (Fig. 3.2, Exp. 1 and 2), the reticulocyte counts of the 2-week-old mice were 29% and 17.7% and the respective invasion levels were 3.9% and 5.1%, indicating a greater invasion level for reticulocyte-rich preparations. The lower level of invasion in Exp. 1 compared to Exp. 2 was attributed to variation between experiments.

Phenylhydrazine treatment was used to obtain erythrocyte preparations with even higher reticulocyte counts. The reticulocyte count was >50% on days 6, 8 and 10 during phenylhydrazine treatment, using the protocol outlined. When these cell preparations were used in invasion assays, the observed invasion levels increased greater than 7-fold over that obtained with normal erythrocytes (Fig. 3.2, Exp 1, 2 and 3).

(b) P. berghei merozoite invasion into erythrocytes obtained from various murine strains.

Three strains of mice (Balb/c, Schofield and CD-1) were treated similarly phenylhydrazine and their erythrocytes tested in invasion assays and compared to normal erythrocytes of the same strain. In Exp. 3 (Fig. 3.2) invasion levels into Balb/c was higher than into the other two strains, this

was attributed to experimental variation, as in Exp. 2 (Fig. 3.2) the invasion levels into Balb/c and Schofield mice were almost identical.

(c) P. berghei merozoite invasion into enzyme treated murine erythrocytes

The effect of enzyme treatment of the target erythrocyte preparations on the *P. berghei* merozoite invasion was examined. As *P. berghei* invasion levels into normal erythrocyte preparations were too low to allow any significant conclusions to be made, these studies were carried out using reticulocyte-rich preparations. As can be seen in Table 1, treatment of the erythrocytes with the proteinases chymotrypsin, trypsin and papain (100 µg/ml) resulted in significant reduction in invasion levels compared to untreated cells; merozoite invasion was 42%, 8% and 28% of controls, respectively.

Neuraminidase treatment of target erythrocytes resulted in a slight (82% of control), but not significant reduction in *P. berghei* merozoite invasion compared to untreated cells (Table 1).

TABLE 1.

Invasion of P. berghei merozoites into enzyme treated murine erythrocytes in vitro

Treatment		Invasion %*
Control (no treatment)		100
Chymotrypsin	100 µg/ml	42
-(50U/mg)	10 µg/ml	66
Trypsin	100 µg/ml	8
-(10,000 U/mg)	10 µg/ml	21
Papain	100 µg/ml	28
-(20 U/mg)	10 µg/ml	77
Neuraminidase	50 mU/ml	82

* Invasion is expressed as a percentage of merozoite invasion into controls (untreated murine erythrocytes). Typically these controls had invasion levels of between 5% and 8% (ring-stage parasitised erythrocytes expressed as a percentage of total erythrocytes). Erythrocyte preparations used in these invasion assays contained >50% reticulocytes.

Binding of radiolabelled *P. berghei* proteins to murine erythrocytes

The binding of *P. berghei* metabolically labelled proteins to erythrocytes was investigated by mixing *P. berghei* ^{35}S-labelled proteins with erythrocytes. Molecules that bound to erythrocytes were eluted with NaCl and analysed by reducing SDS-PAGE and fluorography.

P. berghei incorporated ^{35}S-methionine into many of its proteins during the 6 h labelling culture (Fig. 3.3, lane 1). When these labelled proteins were mixed with murine erythrocytes a 130 kDa molecule selectively bound to the cells and could be eluted with 1.5 M NaCl (Fig. 3.3, lane 2). Erythrocytes from rats, rabbits, or guinea pigs failed to bind any *P. berghei* labelled protein (Fig. 3.3, lane 3, 4 and 5). A similarly sized molecule, which also bound selectively to murine erythrocytes had previously been shown to be ^{35}S-labelled *in vitro* by *P. c. chabaudi* (O' Donovan, S., PhD Thesis and Fig. 3.4A, lane 7).

Binding assays were carried out comparing the binding of the *P. berghei* 130 kDa molecule and the *P. c. chabaudi* 130 kDa to enzyme treated murine erythrocytes (Fig. 3.4A). Neither molecule bound to murine erythrocytes treated with the proteinases chymotrypsin (100 μg/ml), trypsin (100 μg/ml) or papain (100 μg/ml), (Fig. 3.4A, lanes 3-5 and 8-10). Neuraminidase treatment of erythrocytes didn't affect the binding of the 130 kDa molecule from either species.The effect of enzyme treatment on the binding of the *P. berghei* 130 kDa was the same with both reticulocyte rich and normal erythrocyte preparations.

Binding assays were also carried out examining the binding of the *P. berghei* 130 kDa to erythrocyte preparations containing varying amounts of reticulocytes (Fig. 3.4B). Erythrocytes from a phenylhydrazine treated mouse were mixed with erythrocytes from a similarly aged and sexed untreated-mouse in varying proportions to give 3 preparations of cells with differing reticulocyte populations. Preparations A, B and C contained 3.8%, 23% and 46% reticulocytes respectively. From densitometric analysis of the autoradiograph, the amount of the 130 kDa molecule in prep. B (Fig. 3.4B, lanes 3) and prep.C (Fig. 3.4B, lane 4) is only 60% and 32% respectively of

the amount in prep. A (Fig. 3.4B, lane 2). This indicated an inverse relationship between the % of reticulocytes in the erythrocyte preparation and the binding of the 130 kDa *P. berghei* molecule.

Immunoprecipitation of ^{35}S-labelled *P. berghei* proteins with various antisera

The antisera used in radio-immunoprecipitations were as follows
(1) Anti-C1 : Rabbit antiserum generated against `C1 Extract ' as described in METHODS.
(2) Balb/c 4.3 antiserum : Serum collected from balb/c mouse that had cleared *P. berghei* infection.
(3) Anti-TCS : Antiserum raised by S. O' Donovan using spent culture media from a *P. c. chabaudi* culture as antigen (S. O' D., PhD Thesis).

The pattern of the proteins immunoprecipitated by the antisera are shown in Fig. 3.5. A sample of the Hot Pellet Extract used in the immunoprecipitation and the 130 kDa eluted from murine erythrocytes in a binding assay are included on lanes 1 and 8 respectively.

As expected the anti-C1 antisera precipitated a large number of labelled proteins (lane 5), the most prominent of which were the pair at ~42 kDa, the single protein at ~55 kDa and a doublet at ~119 kDa.

The balb/c 4.3 antiserum also precipitated a range of proteins (lane 6), particularly strongly the ~55kDa protein as well as molecules of ~90 kDa and 110 kDa. The most interesting molecules precipitated by this sera however were the doublet at ~130 kDa. The lower band of this doublet comigrated to the 130 kDa eluted in binding assays (lane 8). A similar doublet appeared in the anti-C1 immunoprecipitated proteins (lane 5), although at a much lower intensity. The arced protein bands at ~55 kDa in lanes 5 and 6 are probably due to presence of large amounts of immunoglobulin at this position in the gel.

The anti-TCS sera precipitates a protein of ~42 kDa, as well as a slightly smaller protein (lane 7). There is a faint doublet, precipitated with the anti-TCS, comigrating with the 130 kDa band of the binding assay (compare lane 7 and 8). As with the doublet precipitated with balb/c 4.3 antiserum, the lower band of this doublet corresponds with the 130 kDa molecule eluted in the binding assay. No *P. berghei* protein was precipitated by any of the control sera (Fig. 3.5, lanes 2, 3 and 4).

Preparation of cDNA library

(a) mRNA isolation and cDNA synthesis

The RNAzol B method for RNA isolation yielded approximately 500 µg RNA from 10^{10} cultured parasitised erythrocytes. The integrity of the RNA was assessed by running a sample on a denaturing RNA gel (Fig 3.6A). The markers used were *E. coli* rRNA, which contains 16S and 23S rRNA (lane 1). This analysis indicates that the *P. berghei* rRNA bands are the 5S, 18S and 28S typical of eukaryotic rRNA (lanes 2 and 3). The ribosomal RNA bands of the *P. berghei* RNA were seen as sharp bands indicating no RNase activity (lanes 2 and 3). There was a diffuse background of mRNA spread throughout the entire gel. The $A_{260/280}$ of this RNA was 1.9.

Passage of all 500 µg RNA over the oligo dT column yielded 5 µg poly(A)$^+$ mRNA. This was used in first and second strand synthesis. A fraction of the reaction from the completed second strand synthesis was run on an agarose gel alongside ØX174 x HaeIII markers (Fig. 3.6B, lanes 3 and 1 respectively). A very diffuse smear of cDNA, up to approximately 1500 bp, can be seen in lane 3. Larger cDNA may have been synthesised, but was too diffuse to visualise.

This cDNA was also analysed by PCR, using primers 142 and 143, which were constructed from the known sequence of the *P. falciparum* EBA-175 (Fig 3.6C). Both *P. berghei* genomic DNA and the synthesised cDNA gave a 200 bp PCR product when amplified using primers 142 and 143 (lanes 5 and 3 respectively).

(b) Ligation and packaging of cDNA into gt11 arms

After methylation and linker addition, 200 ng of cDNA was ligated to 500 ng gt11 arms and packaged. The efficiency of packaging for the cDNA

library was calculated as 1 x 10^5 recombinants per µg packaged DNA. A control ligation reaction was carried out with just the gt11 arms and no insert DNA. This was packaged and the efficiency of packaging calculated as 1 x 10^2 recombinants per µg DNA. The cDNA library was amplified to 10^{10} pfu/ml and the control recombinants were used as a source of wild-type gt11.

(c) Screening of cDNA library with ^{32}P-labelled JA159

The probe used was a 40-mer oligonucleotide synthesised from the known internal sequence of the PCR product amplified from *P. berghei* genomic DNA using primers 142 and 143. The oligo was then end labelled with ^{32}P using T4 polynucleotide kinase.Several attempts were made at screening the library with the hot probe. Each time approximately 50,000 recombinants were screened. While the labelled JA159 was found to hybridise strongly to the 200 bp PCR product on southern blots, it failed to pick out true positives from the library. Any `primary positives' that were picked failed to give `secondary positives' and were thus assumed to be false.

The stringency of the post-hybridisation washing was reduced by washing with 6X SSC, 0.02% SDS and by reducing the temperature of the final wash down to 45^{o}C. These measures however didn't result in the identification of any true positives.

(d) Immunoscreening of cDNA library

Two different antisera were used in the immunoscreening of the cDNA library,

(i) Anti-TCS antisera prepared by Susan O'Donovan (S. O'D., PhD. Thesis).

(ii) Anti-C1(+100 kDa) antisera

(i) ANTI - TCS

The anti-TCS antisera was raised against spent media from a *P. c. chabaudi* culture but was nonetheless reactive against a range of *P. berghei* proteins, as can be seen from the immunoprecipitations (Fig. 3.5, lane 7).

Twenty plates with ~3000 pfu each were used in the primary screening with a 1/500 dilution of preabsorbed anti-TCS. After 2 rounds of purification a total of 20 `tertiary positives' were chosen for further investigation. Each of the 20 positives were amplified and DNA was extracted. The size of the insert in each positive clone was then determined by PCR, using the lambda forward and reverse primers (Fig 3.7A).The clones were then classified on the basis of this size of the PCR product.

Group 1	PCR product ~1400 bp	Clones 4A1, 4A2, 17, 20.2,14.3.
Group 2	PCR product ~1300 bp	Clones 14.2.
Group 3	PCR product ~2000 bp	Clones 14.1, 19.1, 19.2, 19.3.
Group 4	PCR product ~300 bp	Clones 16.1.

The remainder of the 20 ` tertiary positives ' didn't produce a PCR product and were disregarded. Clone 20.2 produced 2 PCR fragments, the largest of which corresponded to the 1400 bp PCR product of group 1.

Grouping of the clones into 4 classes was confirmed by EcoR1 digestion of the lambda DNA, followed by gel analysis (Fig. 3.7B). The insert size determined by EcoR1 digestion was ~100 bp less than the size of the corresponding PCR product as was expected. It was probable that there wasn't sufficient DNA from clone 16.1 digested to enable the 200 bp insert to be seen. Clone 14.2 had an insert of ~1200 bp similar to it's PCR product.The inserts corresponding to the PCR product of 2 Kb (group 3), had an internal restriction site for EcoR1, as it produced fragments of ~1400 bp and ~600 bp upon digestion (Fig. 3.7B, lanes 21-24). The clones from group 1 also contained an insert with an internal restriction site producing restriction fragments of ~1000 bp and ~300 bp (Fig. 3.7B, lanes 4-8). There was insufficient DNA from clones 20.2, 4A1 and 4A2 digested to see the smaller restriction fragment (Fig. 3.7B lanes 5,7 and 8).After elimination of clone 20.2, because of the multiple PCR products, 10 clones remained for further investigation.

(ii) ANTI-C1 (+100 kDa)

The anti-C1 antisera was reactive against a range of *P. berghei* proteins as can be seen from its immunoprecipitation of labelled proteins in Fig. 3.5 (lane 5).

The antisera used in this screening was a fraction of the anti-C1 antisera that had been absorbed to and eluted from C1 proteins >100 kDa bound to nitrocellulose. This antisera should preferentially select for recombinants whose native protein is >100 kDa.

Twelve plates with ~3000 pfu each were screened with a 1/5 dilution of the anti-C1 sera fraction prepared as described. Twenty-five `primary positives' were reduced to 16 positives after 4 rounds of purification. DNA was purified from all 16 clones and the insert size determined by PCR using the Lambda forward and reverse primers (Fig. 3.8A and 3.8B). Clones K, Q and U amplified fragments of 1800 bp, 2000 bp and 650 bp respectively. All the other clones gave PCR products of between 1300 bp and 1500 bp, except O and G which gave no PCR product and were discarded. Clone D was the largest of the 1300-1500 bp group followed by clones C, H, L, M, N, P, S and V which all appeared to have the same size insert. The clone J insert was slightly smaller than this group's, clone A having the smallest insert at approximately 1300 bp. These remaining 14 clones were further investigated.

DNA from the 10 clones isolated by anti-TCS antisera and the 14 isolated by the anti-C1(+100 kDa) antisera were used in PCR with primers 142 and 143. These primers were constructed from the known sequence of the 3' cysteine rich region of the *P. falciparum* EBA-175. No clone gave any PCR product. When DNA was extracted from the total phage library and used in this PCR, no fragment was amplified.

Preparation of fusion proteins

The following methods were used to make fusion proteins from the phage clones which had been selected from the *P. berghei* cDNA library by immunoscreening,

(a) Preparation of lysogens

Lysogens of many of the immunoselected phage clones and wild-type phage were generated in *E. coli* Y1089 as described. After induction of the lytic cycle and fusion protein production, extracts of these lysogens were separated by reducing SDS-PAGE, transferred to nitrocellulose and probed with anti-ß-galactosidase antibody. Fig. 3.9 contains an immunoblot

of 2 extracts of *E. coli* infected with wild-type, clone K and clone 14.2 (lanes 1&2, 4&5 and 6&7 respectively). The antibodies were reactive with the ß-galactosidase band in the extracts of *E. coli* infected with wild-type phage (Fig. 3.9, lane 1 and 2). Many other proteins were also visualised with the sera. The sera did not detect any protein in the extracts of *E. coli* infected with recombinant phage that was unique to them i.e. all bands observed were also present in the wild-type infected *E. coli* extracts.

(b) Plate Wash Supernatant method

Fig. 3.10 shows a coomassie stained gel of Plate Wash Supernatants of several immunoselected clones. The ß-galactosidase protein is abundant in wash supernatants of *E. coli* infected with wild-type phage (Fig. 3.10, lanes 2 and 8). The wash supernatants from the immunoselected clones did not contain this ß-galactosidase (Fig. 3.10, lanes 3-7, 9 and 10). No protein was detected in the wash supernatants of *E. coli* infected with the immunoselected clones that wasn't present in the wash supernatant of the *E. coli* infected with wild-type phage.Similar results were obtained for all the clones selected by anti-TCS or anti-C1(+100 kDa).

Analysis of recombinant clones isolated from *P. falciparum* gametocyte cDNA library

Lambda clones R_t and R_g were selected from a *P. falciparum* gametocyte cDNA library in gt11 by immunoscreening with an antiserum fraction that was specifically reactive against *P. falciparum* sexual stage antigens.

(a) DNA analysis

The clones R_t and R_g were first titred and plated on phage competent cells at ~100 pfu per plate. Single plaque plugs were picked, the phage amplified and DNA extracted. The DNA was then used in PCR with the lambda forward and reverse primers. The R_g clone amplified multiple fragments, the strongest of which were fragments of ~290 bp and ~600 bp, while the R_t clone amplified a fragment of ~350 bp (Fig 3.11, lanes 1-3 and lane 4 respectively). Multiple fragments were amplified from every phage isolate of R_g.

Attempts to EcoR1 digest the R_t lambda DNA in order to confirm the size of the insert proved unsuccessful. One of the EcoR1 sites may have been damaged.The R_t DNA was therefore digested with Kpn1 and Sac1 (Fig. 3.12A). These two enzymes have restriction sites on either side of the cloning EcoR1 site, and produce a 2.1 Kb fragment when used on wild-type gt11 DNA (Fig. 3.12A, lane 1).This fragment still contains the EcoR1 cloning site of gt11. When R_t DNA was restricted with these Kpn1 and Sac1, a fragment migrating at 2.4-2.5 Kb was observed, indicating an insert of 300-400 bp (Fig. 3.12A, lane 2).The fragment excised with Kpn1 and Sac1 from both wild type DNA (fragment L) and R_t DNA (fragment U) were cut from the gel and PCR amplified with the lambda forward and reverse primers. R_t phage DNA and wild-type phage DNA were similarly PCR amplified as a positive and negative control.The PCR reactions were analysed by agarose gel electrophoresis (Fig. 3.12B). A 350 bp fragment was amplified from fragment U and from the R_t phage DNA (Fig. 3.12B, lanes 3 and 5 respectively).This indicates that the Kpn1/Sac1 restriction fragment of R_t DNA contains an insert in the EcoR1 cloning site. No product was amplified from the L band or from wild-type phage DNA (Fig. 3.12B, lane 2 and 4 respectively).

(b) Production of fusion protein

Lysogens of phage R_t were generated in *E. coli* Y1089. Preparation of fusion protein from these lysogens proved unsuccessful as the cells always ruptured within 20 min of the induction of the lytic cycle. If the culture was stopped short of this time the cells ruptured during the first centrifugation step no matter what temperature it was carried out at.

The Plate Wash Supernatant method was then used to produce fusion protein. When the wash supernatant from both R_t and R_g plates were analysed by reducing SDS-PAGE, a large protein not found in the wild-type wash supernatant was present in the R_t supernatant. Fig. 3.13 shows an immunoblot of plate wash supernatants of R_t , R_g and wild-type probed with anti-ß-galactosidase (Fig. 3.13, lanes 1, 2 and 3 respectively). A protein from clone R_t (Fig. 3.13, lane 1) which was larger (~150 kDa) than wild-type ß-galactosidase (Fig. 3.13, lane 3) was reactive with anti-ß-galactosidase. The antibody did not recognise any proteins in the R_g wash supernatant

that was unique to it, i.e. all reactive proteins were also present in the wild-type wash supernatant (compare lanes 2 and 3).

(c) Purification of R_t recombinant protein.

An anti-ß-galactosidase immunoaffinity column was used to prepare fusion protein from the R_t and wild-type plate wash supernatants.The wild-type plate wash supernatant was passed over the column and the ß-galactosidase eluted. The column was then reequilibrated and the fusion protein purified from the R_t wash supernatant. The elution was performed with 3 x 1 ml high-pH (10.2) elution buffer, followed by 1 ml TBS. A sample of each of these elution fractions was analysed on reducing SDS-PAGE (Fig. 3.14A), transferred to nitrocellulose, and probed with an anti-ß-galactosidase antibody (Fig. 3.14B). Most of the wild-type ß-galactosidase and fusion protein was present in their respective second and third eluted 1 ml fractions (Fig. 3.14B, lanes 2&3 and 8&9 respectively). There appeared to be ß-galactosidase remaining on the column after the first series of elutions which was subsequently eluted with the R_t fusion protein.

Fractions 2 and 3 of the eluted R_t fusion protein were combined and their protein concentration calculated as 10 µg/ml. Fractions 2 and 3 of the wild-type ß-galactosidase were combined and their protein concentration calculated as 20 µg/ml. Five µg of each preparation was then used in each rat immunisation.

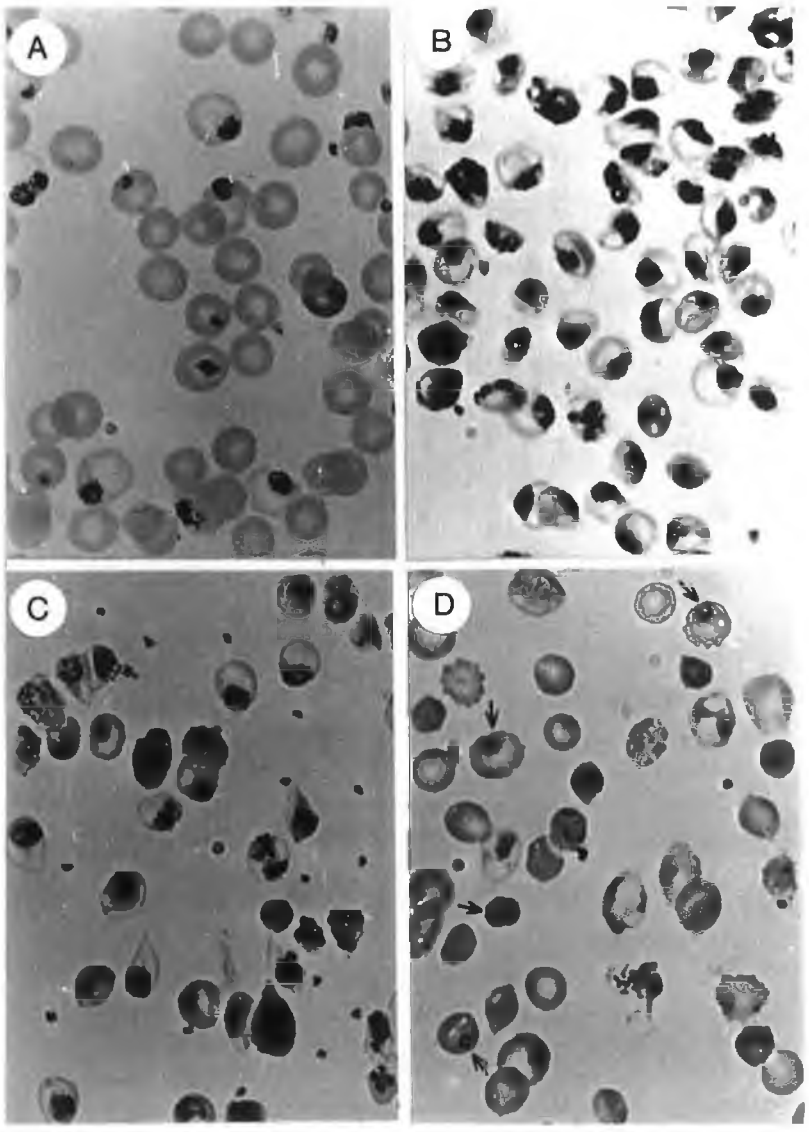


FIG. 3.1. Purification of *Plasmodium berghei* mature trophozoite-infected erythrocytes, *in vitro* culturing, and reinvasion. Blood from an infected Balb/c mouse (A) was layered onto a step-wise Percoll gradient as described (METHODS). After centrifugation the erythrocytes infected with mature trophozoites banded on the top (B) and were removed, washed and maintained *in vitro* for 6 h (C) before adding to target erythrocytes. Reinvasion was assessed by estimating the percentage ring-stage parasites in erythrocytes 19 h later (D, arrowed). Panel D shows reinvasion into erythrocytes obtained from phenylhydrazine-treated mice.

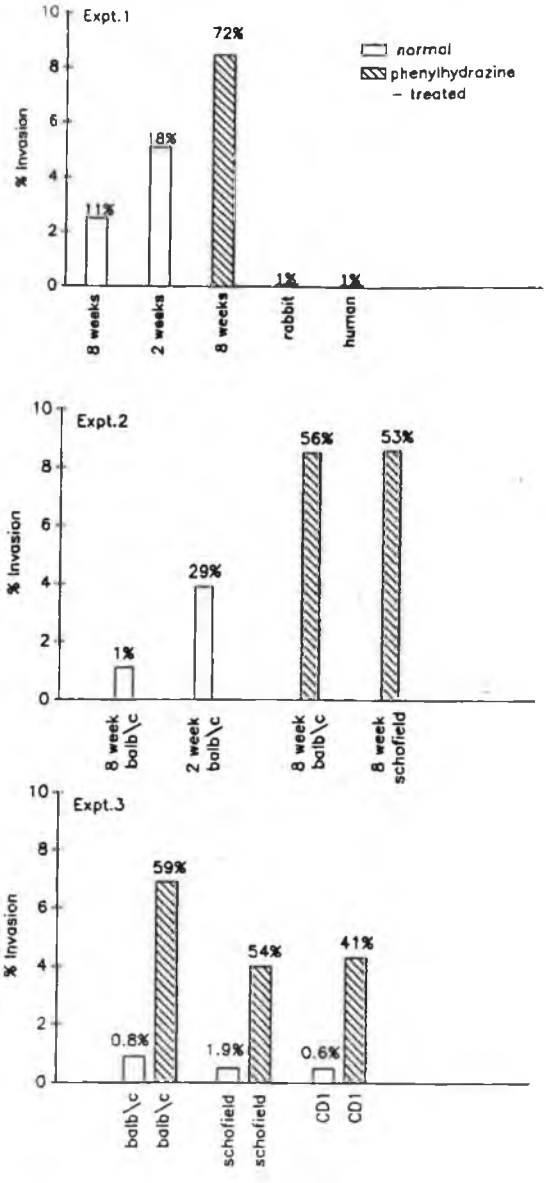


FIG. 3.2. Invasion of erythrocytes *in vitro* by *Plasmodium berghei*.
Exp. 1, comparison of *P. berghei* invasion rates into erythrocytes obtained from 8-week-old mice, 2-week-old mice, phenylhydrazine-treated 8-week-old mice (all Balb/c), rabbit and human.
Exp.2, comparison of invasion rates into erythrocytes of 8-week-old Balb/c mice, 2-week-old Balb/c mice and phenylhydrazine treated 8-week-old Balb/c and Schofield mice.
Exp. 3, comparison of invasion rates into erythrocytes of normal and phenylhydrazine-treated 8-week-old Balb/c, CD-1 and Schofield mice.
Percentages at the top of columns represent reticulocyte counts of each particular target erythrocyte preparation. Shaded columns refer to cell preparations from phenylhydrazine-treated mice.

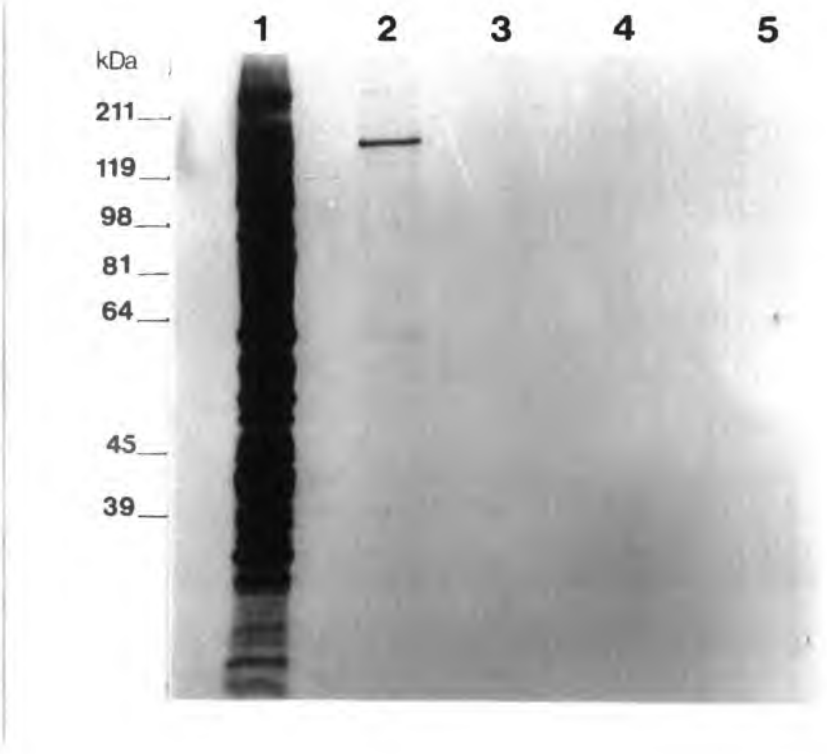


FIG. 3.3. Binding of the *Plasmodium berghei* 130 kDa molecule to erythrocytes obtained from various mammals. Binding assays were carried out by mixing ^{35}S-labelled Hot Pellet Extract of *P. berghei* (lane 1) and erythrocytes from Balb/c mouse (lane 2), rat (lane 3), rabbit (lane 4) and guinea pig (lane 5). Molecules that bound to erythrocytes were eluted with 1.5M NaCl and visualised by reducing SDS-PAGE and fluorography.

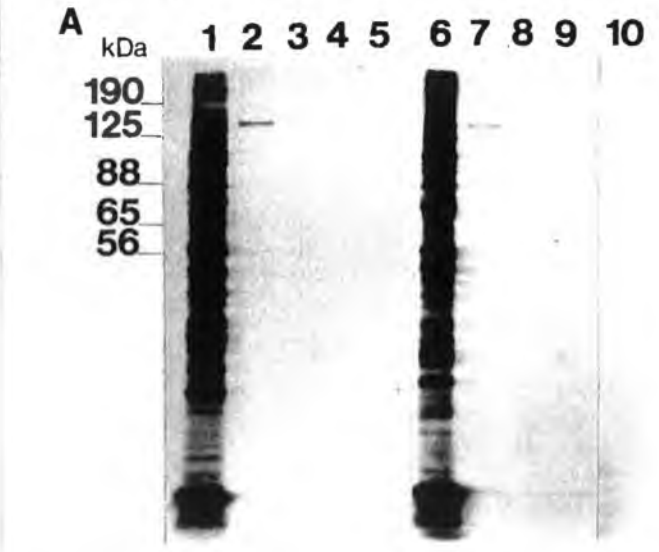


FIG. 3.4A. Binding of the *Plasmodium berghei* and *Plasmodium chabaudi chabaudi* 130 kDa molecule to enzyme-treated murine erythrocytes. ^{35}S-labelled Hot Pellet Extract of *P. berghei* (lane 1) and *P. c. chabaudi* (lane 6) were mixed with murine erythrocytes (lanes 2-5 and 7-10 respectively). The erythrocytes were untreated (lanes 2 and 7), treated with 100 µg/ml chymotrypsin (lanes 3 and 8),100 µg/ml trypsin (lanes 4 and 9) or 100 µg/ml papain (lanes 5 and 10). Molecules bound to erythrocytes were eluted with 1.5M NaCl and visualised by reducing SDS-PAGE and fluorography.

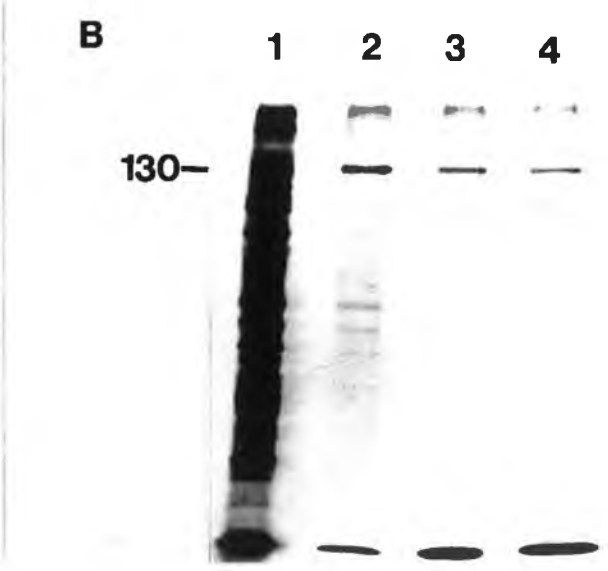


FIG. 3.4B. Binding of the *Plasmodium berghei* 130 kDa molecule to cell preparations containing varying amounts of reticulocytes. *P. berghei* ^{35}S-labelled Hot Pellet Extract (lane 1) was used in binding assays with 3 erythrocyte preparations (A,B and C), which contained 3.8% (lane 2), 23% (lane 3) and 46% (lane 4) reticulocytes respectively. Bound molecules were eluted with 1.5M NaCl and analysed by reducing SDS-PAGE and fluorography.

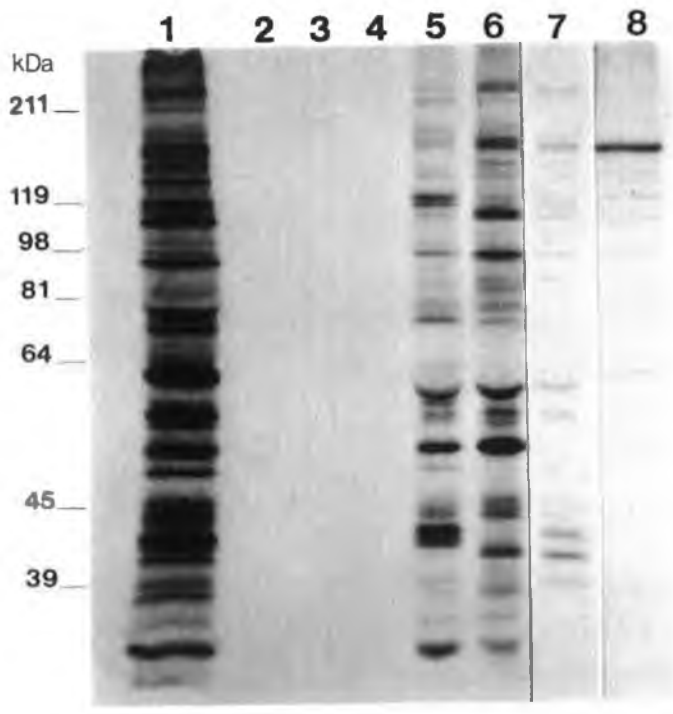


FIG. 3.5. Immunoprecipitation of ^{35}S-labelled *Plasmodium berghei* proteins. Hot Pellet Extract of *P. berghei* (lane 1) was used in immunoprecipitation reactions with anti-C1 antiserum (lane 5), Balb/c 4.3 serum (lane 6), and anti-TCS antiserum (lane 7). Precipitation reactions using control rabbit, mouse and rat serum were also carried out (lanes 2, 3 and 4 respectively). The *P. berghei* 130 kDa erythrocyte binding protein is shown in lane 8.

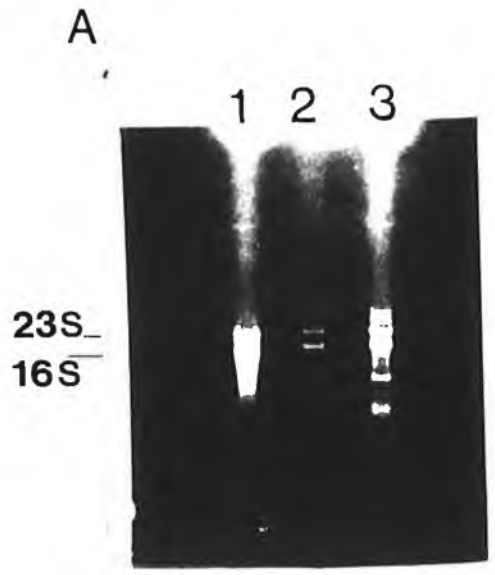


FIG. 3.6A. Purification of *Plasmodium berghei* RNA using RNAzol B. Two different samples of *P. berghei* RNA prepared by the RNAzol method were analysed on a 0.8% reducing agarose gel (lanes 2 and 3), in parallel with *E. coli* RNA standards (lane 1).

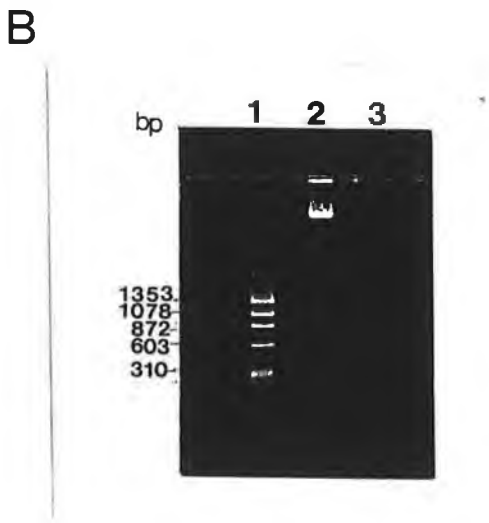


FIG. 3.6B. *Plasmodium berghei* cDNA synthesis. A sample of the completed second strand cDNA synthesis reaction was analysed on a 2% agarose gel (lane 3). ØX174 x HaeIII markers and undigested gt11 and were run in parallel (lanes 1 and 2 respectively).

C

FIG. 3.6C. PCR Amplification of 200 bp sequence using primers 142 and 143. Samples of PCR reactions using synthesised *P. berghei* cDNA and *P. berghei* genomic DNA as template were run on a 2% agarose gel (lane 3 and 5 respectively). Lanes 1, 2 and 4 contained negative controls (no template, no dNTP's and no polymerase respectively) and ØX174 x HaeIII were run in parallel (lane 6) as markers.

A

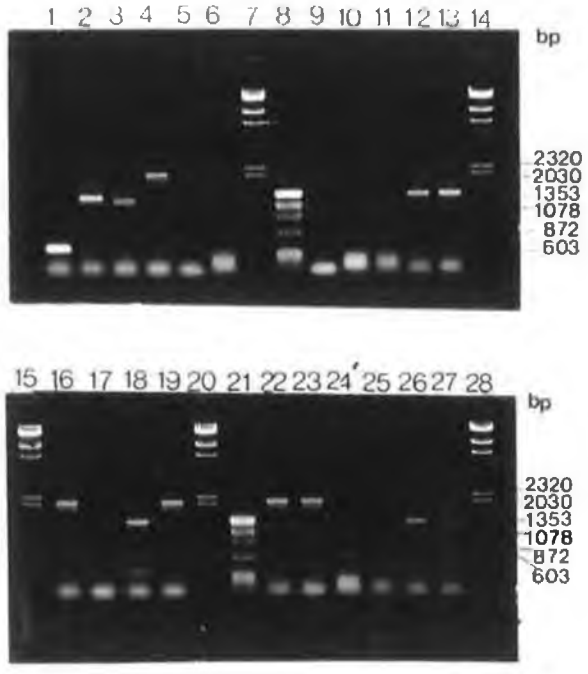


FIG. 3.7A. PCR Amplification of inserts in gt11 clones (immunoselected by anti-TCS antiserum) using lambda forward and reverse primers. A sample of each PCR reaction was analysed on a 2% agarose gel.

lanes 1-6 clones 16.1, 14.3, 14.2, 14.1, 13.1 and 12.2.

lanes 9-13 clones 11.4, 11.3, 11.2, 4A2 and 4A1.

lanes 16-19 clone 19.3, negative control, clones 20.2, 19.3.

lanes 22-27 clones 19.2, 19.1, 18.4, 18.3, 17 and 16.2.

lanes 7, 14, 15, 20 and 28 x HinDIII

lanes 8 and 21 ØX174 x HaeIII

B

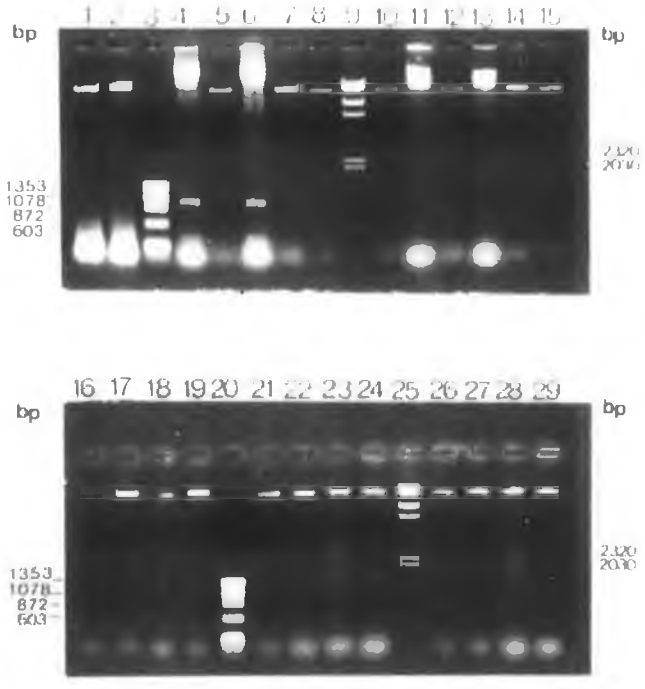


FIG. 3.7B. EcoR1 digestion of DNA from gt11 clones immunoselected by anti-TCS antiserum. A sample of EcoR1 digested and undigested DNA from each clone was run on a 2% agarose gel

Group 1	14.3, 20.2, 17, 4A2, 4A1.	lanes 4-8 cut, lanes 11-15 uncut.
Group 2	14.2.	lane 1 cut, lane 2 uncut.
Group 3	19.3, 19.2, 19.1, 14.1.	lanes 21-24 cut, lanes 26-29 uncut.
Group 4	16.1.	lane 17 cut, lane 19 uncut.
	clone 16.2.	lane 16 cut, lane 18 uncut.
	x HinDIII	lanes 9,25.
	ØX174 x HaeIII	lanes 3, 20.

A

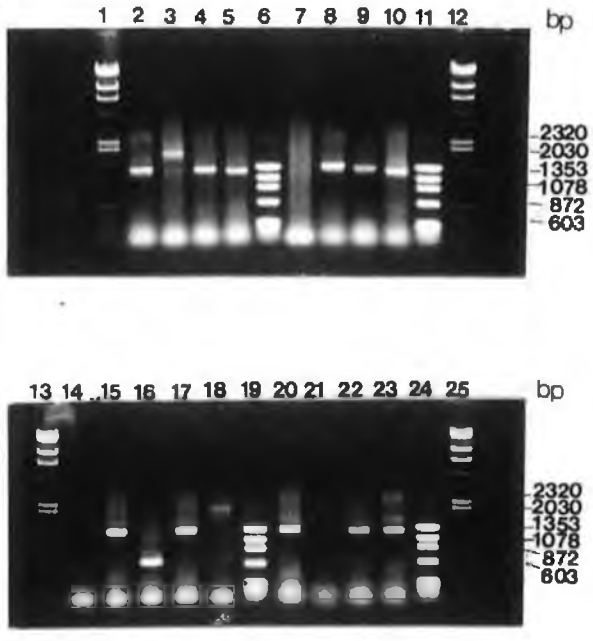


FIG. 3.8A. PCR amplification of inserts of gt11 clones immunoselected by anti-C1(+100 kDa) antiserum using lambda forward and reverse primers. Samples of each PCR reaction were analysed on a 2% agarose gel.

lanes 2-5. clones L, K, J, H.

lanes 7-10. clones G, D, C, A.

lanes 15-18. clones V, U, S, Q.

lanes 20-23. clones P, O, N, M.

lane 14 PCR negative control.

lanes 1, 12, 13, 25. x HinDIII.

lanes 6, 11, 19, 24. ØX174 x HaeIII.

B

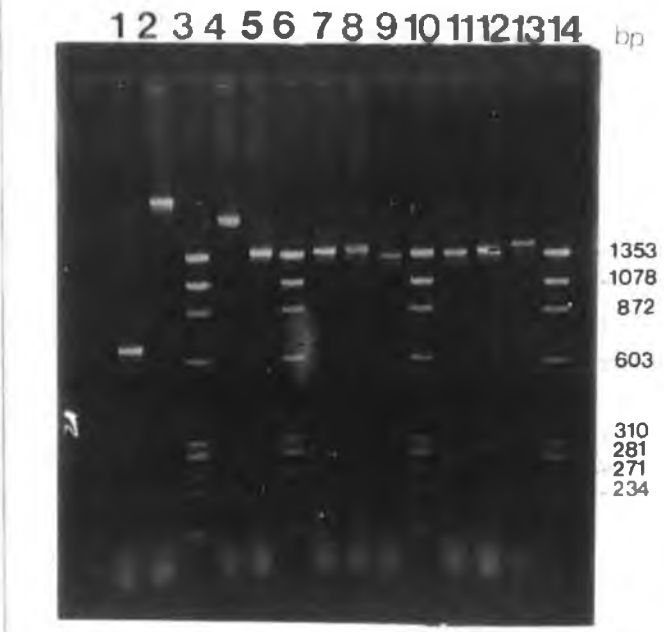


FIG. 3.8B. PCR amplification of inserts of gt11 clones immunoselected by anti-C1(+100 kDa) antiserum, using lambda forward and reverse primers. Samples of PCR reactions were analysed on a 3% agarose gel

lanes 1, 2, 4, 5. clones U, Q, K, C.

lanes 7, 8, 9. clones L, M, A.

lanes 11, 12, 13. clones J, H, D.

lanes 3, 6, 10, 14. ØX174 x HaeIII.

FIG. 3.9. Western blot of cell extracts of lysogens prepared from immunoselected phage clones. Cell extracts of wild-type (lanes 1&2), clone K (lanes 4&5) and clone 14.2 (lane 6&7). lysogens were separated by 10% reducing SDS-PAGE, transferred to nitrocellulose and probed with an anti-ß-galactosidase antibody. The cell extracts examined were samples of the inclusion bodies pellet (lanes 2, 5 and 7), and the TBS-EDTA-TRITON-X washes of the inclusion bodies' pellet (lanes 1, 4 and 6) prepared as described in METHODS.

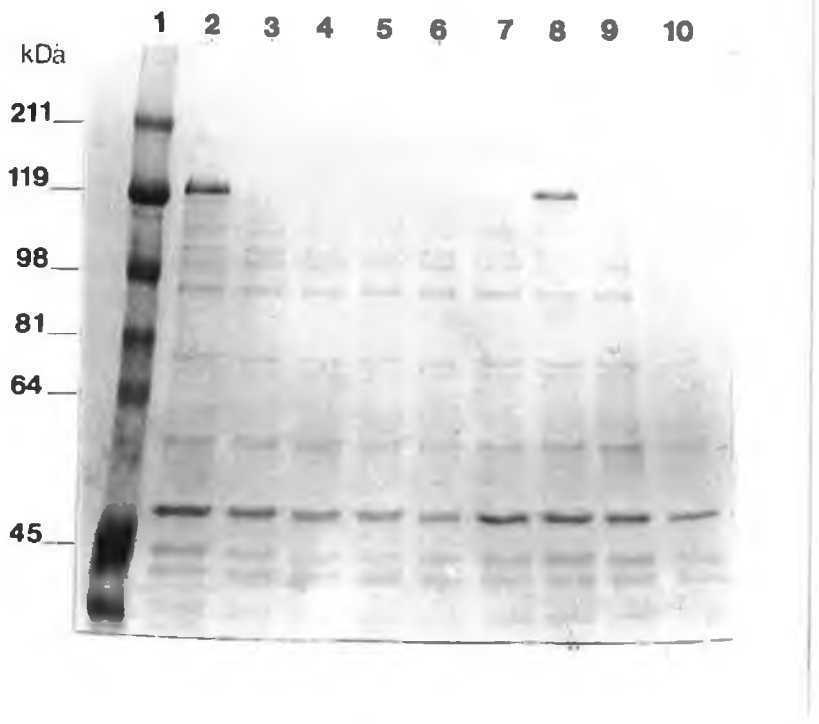


FIG. 3.10. Production of fusion proteins by Plate Wash Supernatant method. Fifty µl of Plate Wash Supernatants from clones immunoselected by anti-TCS antiserum or anti-C1(+100 kDa) antiserum were analysed by 10% reducing SDS-PAGE.

lane 1 prestained molecular weight markers.

lanes 2, 8 wild-type.

lanes 3-7 clones 19.3, 19.2, 16.1, 14.3, 14.2.

lanes 9,10 clones C, A.

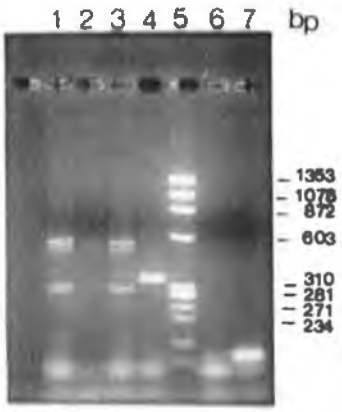


FIG. 3.11. PCR amplification of inserts in gt11 clones R_t and R_g using lambda forward and reverse primers. Samples of the PCR amplification reaction of 3 distinct isolates of clone R_g (lanes 1-3) and of clone R_t (lane 4) were analysed on a 2% agarose gel in parallel with ØX174 x HaeIII markers (lane 5). The negative control for PCR was a reaction without template DNA (lane 7) and gt11 DNA as template was used as a positive control (lane 6).

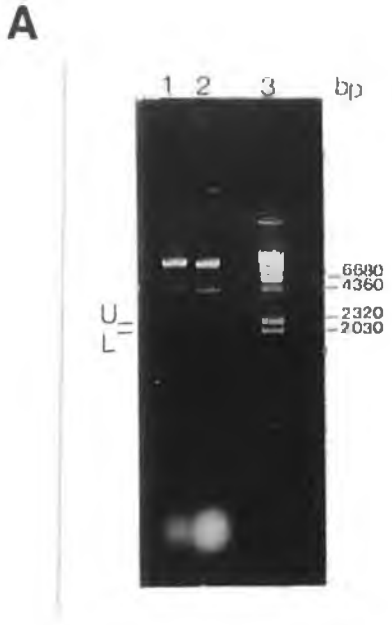


FIG 3.12A. Digestion of phage DNA from wild-type gt11 and clone R_t using KpnI and SacI. Five µg of wild-type and R_t DNA was digested with 10 U of KpnI and SacI each and the reaction products analysed on a 2% agarose gel (lanes 1 and 2 respectively). Lane 3 contained x HinDIII markers. Bands L and U refer to the KpnI/SacI restriction fragment from wild-type and R_t DNA which contains the EcoRI restriction site or insert DNA.

B

FIG. 3.12B. PCR amplification of inserts from fragments U and L using lambda forward and reverse primers. Restriction fragments U and L were cut from a gel similar to that in Fig. 3.12A and the DNA separated from the agarose by centrifugation through glass wool (10,000 x g, 10s).These DNA preparations were then used as template in PCR amplification with lambda forward and reverse primers. Samples of the PCR amplification reactions of bands L and U were analysed on a 2% agarose gel (lanes 2 and 3 respectively) alongside gt11 x HinDIII markers (lane 1). The negative control for PCR was a reaction without template (lane 4) and the positive control used R_t DNA as template (lane 5).

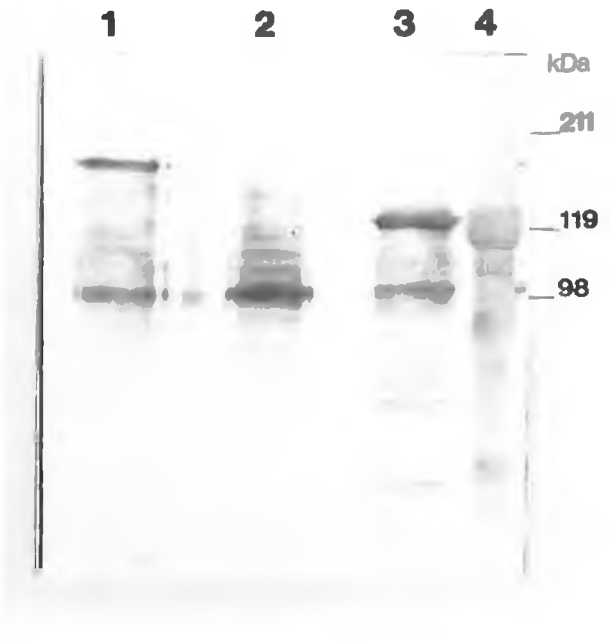


FIG. 3.13. Analysis of Plate Wash Supernatants of clones R_t and R_g for fusion protein. Samples of R_t, R_g and wild-type phage Plate Wash Supernatant were analysed by 10% reducing SDS-PAG (lanes 1, 2 and 3 respectively), in parallel with prestained molecular weight markers. The gel was transferred to nitrocellulose and immunoblotted using anti-ß-galactosidase antibody.

A

FIG. 3.14A. Purification of fusion protein from Plate Wash Supernatants of clone R_t and wild-type phage. Ammonium sulphate precipitates of the Plate Wash Supernatants of wild-type and R_t phage infected *E. coli* were applied to the immunoaffinity column, and eluted in 1 ml fractions. These fractions were analysed by 10% reducing SDS-PAG. Lanes 1-4 contain samples of the fractions consecutively eluted from the purification from the wild-type batch. Lanes 7-9 contain samples of the fractions eluted from the R_t batch. A sample of the ammonium sulphate precipitate of the R_t supernatant (lane 6) and prestained molecular weight markers (lane 5) were included on the gel. The protein band labelled R_t refers to the R_t fusion protein.

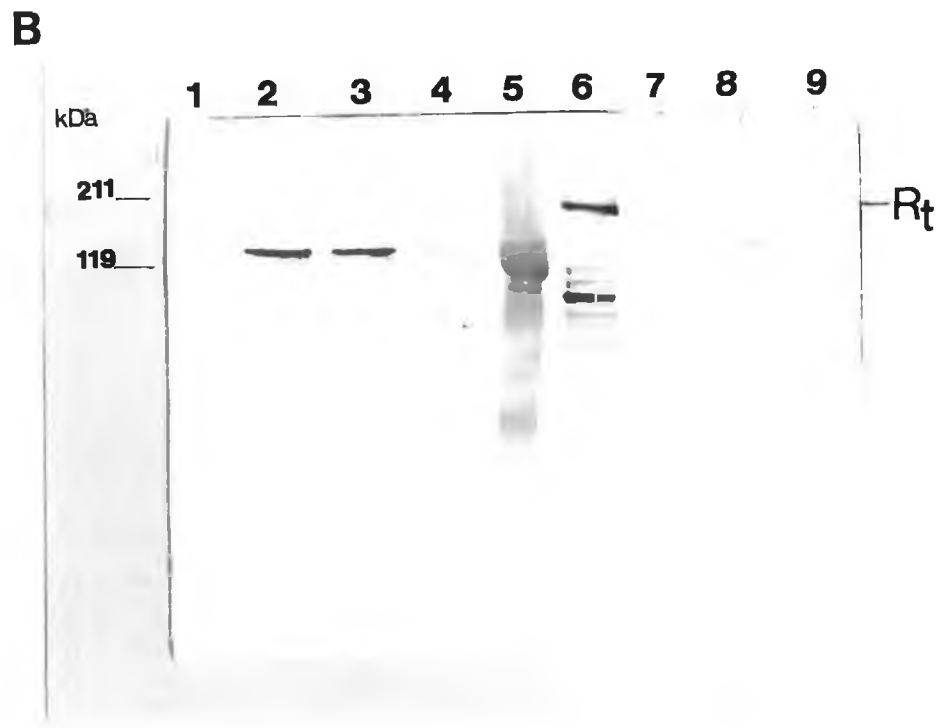


FIG. 3.14B. Immunoblot of fusion proteins purified on immunoaffinity column. A gel as shown in Fig. 3.14A was transferred to nitrocellulose, and probed with anti-ß-galactosidase antibody. Lanes 1-4 contain samples of the fractions consecutively eluted from the purification from the wild-type batch. Lanes 7-9 contain samples of the fractions eluted from the R_t batch. Lane 6 contains a sample of the ammonium sulphate precipitate of the R_t plate wash supernatant. The reactive band labelled R_t refers to the R_t fusion protein.

CHAPTER 4

DISCUSSION

1.Development of erythrocyte invasion assays for Plasmodium berghei

Invasion assays have been developed for several *Plasmodium* species, including *P. knowlesi*, *P. falciparum*, and *P. vivax* (Mason *et al.*, 1977; Miller *et al.*, 1977; and Barnwell *et al.*, 1989 respectively). These assays are essential for investigating the molecular intricacies of erythrocyte invasion by merozoites either at the ultrastructural level using electron microscopy, or at the molecular level using antibodies or other reagents which promote or hinder invasion. The invasion assay that we have developed for *P. berghei* is similar to those described for the primate species that use schizont infected erythrocytes as a source of merozoites, but involves additional steps due to the nature of this particular rodent malaria.

The first requirement for the development of a *P. berghei* invasion assay was to find a media that would support the growth of all stages of the parasite.The most successful reports of *in vitro* cultivation of the asexual stages of *P. berghei* were those of Mons *et al.* (1983) and Janse *et al.* (1984) using a novel suspension system. They achieved continuous cultivation of the parasite for up to 90 days, parasitaemias were maintained at 7-8% and the average multiplication rate was between 3-3.4 (Janse *et al.*, 1984). The length of the schizogonic cycle was similar to that *in vivo*. However they found that continuous agitation of the culture was necessary. Furthermore, the culture required the use of rat reticulocytes to maintain high invasion rates. RPMI based preparations are the most commonly used media for malarial work since the report of continuous cultivation of *P. falciparum* by Trager and Jensen (1976). The media used by Mons *et al.* (1983) was essentially that of Trager and Jensen (1976) except that heat-inactivated FCS and neomycin replaced the human serum and gentamycin components of Trager and Jensen's media.

We wished to examine *P. berghei* intra-erythrocytic growth in mouse erythrocytes using simple static cultures. Initially we assessed the affect of various types and concentrations of serum in serum/RPMI based media on *P. berghei* intra-erythrocytic development by setting up short-term low-density cultures of parasitised blood. We found that 50% heat-inactivated FCS in RPMI supported the development of trophozoites to segmentors.

Merozoites subsequently ruptured the erythrocytes and invaded fresh erythrocytes. In *in vivo P. berghei* infections, schizont and segmentor forms of the parasite sequester in the deep vasculature and are not seen in, nor can be obtained from the circulation. Schizonts are a necessary requirement for invasion assays, and their development in our media, and the production of invasive merozoites was a good indication of its suitability for *P. berghei*.

The high percentage of FCS in our media probably served to supply some of the complex nutritional requirements of the parasite, as well as buffering the media. Also In our hands, murine erythrocytes, whether infected or not, were quite fragile and prone to lysis, and another necessity for such a FCS-rich media may have been related to this fragility. FCS would have increased the viscosity of the media which may have helped to stabilise the extracellular merozoite and the intraerythrocytic parasite. Lanners (1992) reports the use of methylcellulose in a media developed for the similar malaria *P. vivax* in order to increase the viscosity of the media and increase the stability of the cells.

The schizonts used in invasion assays had also to be highly purified and synchronous. Purification of schizont-infected erythrocytes from non-infected erythrocytes ensures that scored invasions are only as a result of invasion into the intended target cells. Synchronisation ensures that scored invasions occur within the time-scale of the assay and are not carry-over ring-stages from the introduced parasite population. The *P. berghei* 65-75-85 Percoll Step was developed from the procedure and reagents described by Wunderlich *et al.* (1985) and served to both purify and synchronise the mature trophozoites from an asynchronous preparation of infected blood. This density gradient centrifugation step was rapid and simple to perform and the percoll was non-toxic to the parasites. Yields of >95% pure, synchronous trophozoite-infected erythrocytes were routinely achieved. This proved to be a much simpler method for obtaining synchronous parasites to that described by Mons *et al.* (1985) which involved *in vitro* culturing followed by *in vivo* passage through rats and which resulted in preparations of very low purity.

The invasion assays involve an initial culturing of the pure, synchronous trophozoite-infected erythrocytes at a density of 2.5 x 10^7cells/ml for 5-6h prior to transferring 5 x 10^6 (0.2 ml) of these cells into

fresh media (0.8 ml) containing 2 x 10^7 target erythrocytes. The cell concentrations were kept relatively low and the media change was found quite necessary as even in our well buffered media (50% FCS and 20mM HEPES) the pH dropped to below 7.0 during the preliminary 5h culturing period. This pH drop was due to the production of lactic acid as a consequence of the exhaustive growth of the parasites in developing to schizogony. *P. berghei* may require a better buffered media than *P. falciparum* as its schizogonic cycle is 24 h compared to 48 h for *P. falciparum* which would suggest a more intensive metabolism for *P. berghei* and hence a more rapid exhaustion of the media.

Centrifugation washes were avoided as schizont-infected erythrocytes have a tendency to clump and become difficult to resuspend. Because of the fragility of murine erythrocytes and reticulocytes, manipulation of these cells was reduced to a minimum and single washes with large volumes were used in preference to multiple washes.

The total culturing time for the invasion assay was 24 h. As the assay involves development from schizont to visible ring-stage infected-erythrocyte, this indicates that parasite development in our system was slower than the *in vivo* development of *P. berghei* in which the whole erythrocytic cycle takes 24 h. Mons *et al.* (1983) reported a normal erythrocytic cycle for *P. berghei* in the suspension culture system. It is probable also that development in static cultures is slower than development in suspension cultures due to the effects of nutrient gradients.

Erythrocyte invasion preferences of *P. berghei* merozoites in our *in vitro* system correlated well with its known *in vivo* preferences. Erythrocytes of rabbits, guinea pigs and humans were not invaded *in vitro* while mouse erythrocytes preparations were. It is well known that *P. berghei* merozoites preferentially invade reticulocytes (Ramakrishnan & Prakash, 1950; Janse *et al.*, 1984, 1989; Suhrbier *et al.*, 1987). Using the *P. berghei in vitro* invasion assay, we observed a direct correlation between *P. berghei* merozoite invasion and the presence of reticulocytes. This correlation held whether the reticulocytes were `natural' i.e. obtained from very young mice, or `unnatural' i.e. obtained from phenylhydrazine-treated mice. Therefore phenylhydrazine alone was not the cause of the increased susceptibility of such cells to invasion. Greater invasion into reticulocyte-rich preparation was also shown by Mons *et al.* (1985) indicating that the greater invasion

into reticulocyte rich preparation was not just an artifact of static cultures. Invasion rates were similar into erythrocytes from all murine strains tested.

P. berghei invasion assays were carried out using enzyme-treated erythrocytes as target cells. To provide statistically significant data, invasion into enzyme-treated reticulocyte-rich preparations were examined. Treatment of these cell preparations with the enzymes trypsin, chymotrypsin or papain reduced the level of merozoite invasion while neuraminidase treatment had little significant effect. The effect of the trypsin on invasion was so marked (92% reduction in invasion compared to controls) that it is reasonable to assume that its action completely destroyed reticulocyte ligands for merozoite invasion, while the chymotrypsin and papain may have affected both reticulocytes and normocytes less severely.The lack of effect of the *Vibrio cholera* neuraminidase was not surprising as this enzyme is known to be relatively inactive against sialic acids on murine sialoglycoproteins which are o-aceytlated (Sam-Yellowe & Perkins, 1990).

A clearer understanding of the reticulocyte preference of *P. berghei* merozoites could have been obtained if the relative invasion rates into reticulocytes and normocytes could have been calculated. This was not possible, however, due to the maturation of the reticulocytes during the course of the assay. Reticulocytes *in vivo* mature to normocytes within 24-36h after release from the bone marrow into the circulation. (Gronowicz, Swift & Steck, 1984). Reticulocyte maturation in our *in vitro* system was close to the *in vivo* situation and hence many reticulocytes had matured into normocytes during the assay making it impossible to determine whether the ring-forms were as a result of newly invaded reticulocytes or normocytes.

The reticulocyte preference of *P. berghei* is not as marked as that observed for *P. vivax* (a human malaria) or *P. cynomolgi* (a simian malaria), which almost exclusively invade reticulocytes, and are as such, limited in their multiplication potential (Kitchen, 1938 and Warren, 1966 respectively). *P. berghei* has the capacity to invade mature erythrocytes, very few reticulocytes are seen throughout *P. berghei* infection *in vivo*, possibly due to early parasite invasion and subsequent cell destruction. Ott (1968) found that if reticulocyte numbers in rats were boosted prior to infection by *P. berghei*, the reticulocytes were parasitised to the near exclusion of mature erythrocytes. Whether the basis of the reticulocyte preference of *P. berghei* is due to an increased structural predisposition of reticulocytes to invasion

or to a specific receptor-ligand interaction is not known. Both these factors may contribute to the reticulocyte preference of *P. berghei*. Reticulocytes are known to possess a spectrin based cytoskeletal network that is incompletely packed, and therefore would be more pliable or deformable and so more susceptible to invasion. Janse *et al.* (1984) reported that in some continuous cultivation experiments he found rat reticulocytes containing up to 15 invaded parasites of *P. berghei*. This could represent a reticulocyte sub-population that was particularly susceptible to invasion.

2. Erythrocyte binding assays using metabolically labelled *P. berghei* proteins

The specificity of merozoite attachment to and invasion of erythrocytes is mediated by interactions between surface molecules of the erythrocyte and merozoite molecules. In the case of *P. falciparum* , the human-erythrocyte ligand has been shown to be the sialic acid residue of glycophorins, and for *P. knowlesi* and *P. vivax* , a component of the Duffy blood group system was shown to be involved (reviewed by Hadley *et al.*, 1986). Erythrocyte binding molecules have been discovered for these three malarial species and much work has focused on trying to correlate their specificities of binding to erythrocytes with the known requirements of the particular malarial species for erythrocyte invasion. The *P. falciparum* molecules, EBA-175 and MSP-1 have been shown to bind to susceptible erythrocytes in a sialic-acid dependent manner (Camus & Hadley, 1985 and Perkins & Rocco, 1988 respectively). A similar correlation between the binding of a 135 kDa molecule of *P. knowlesi*, PkDAP-1, or the binding of a *P. vivax* 140 kDa molecule, PvDAP-1, and the availability of components of the Duffy blood group system on susceptible cells indicated an important role for these erythrocyte binding molecules in erythrocyte invasion (Haynes *et al.*, 1988 and Wertheimer & Barnwell, 1989)

Applying similar methods to those described by Haynes *et al.* (1988), we succeeded in radiolabelling *P. berghei* proteins and identifying an erythrocyte binding protein of *P. berghei*. We estimated the molecular weight of this protein to be 130 kDa as it migrated slightly faster than the PkDAP-1 (135 kDa) on reducing SDS-PAGE (J. Dalton, personal communication). This *Plasmodium berghei* erythrocyte binding protein was

termed PbEBP-130. When *P. berghei* trophozoites were cultured to schizogony this erythrocyte binding protein, PbEBP-130 was released into the media, and also could be recovered from freeze-thaw extracts of the schizonts. This indicates that the PbEBP-130 was probably merozoite associated, and was either released into the culture media with the merozoites as the erythrocyte ruptured or dissociated from the merozoite as the merozoite deteriorated. There was no difference in molecular weight between the culture media PbEBP and the extracted PbEBP, and no smaller labelled proteins bound to erythrocytes, ruling out the possibility of processing of the protein as was observed with the *P. falciparum* MSP-1 (Holder and Freeman, 1982, 1984).

The binding of the PbEBP-130 could not be correlated with invasion specificities of *P. berghei*. Merozoites of *P. berghei* invade rat erythrocytes both *in vivo* and *in vitro* (Janse *et al.*, 1984), however the PbEBP-130 failed to bind to rat erythrocytes. Neither did the PbEBP-130 bind to erythrocytes from rabbits or guinea pigs which are not invaded *in vivo*. Enzymatic digestion of murine erythrocytes by trypsin, chymotrypsin or papain abrogated binding of the PbEBP-130. This correlated with the effect of protease treatment on merozoite invasion of erythrocytes . However it cannot be concluded that it was solely the loss of the PbEBP-130-specific ligand on the erythrocyte that resulted in the reduced invasion.

No correlation was found between the binding of PbEBP-130 and the preference of *P. berghei* merozoites for reticulocytes. The amount of the PbEBP-130 binding decreased as the percentage of reticulocytes in the cell preparation used in the binding assay increased. This would indicate that the molecule we have detected binding to mouse erythrocytes is not the *P. berghei* equivalent of the *P. vivax* reticulocyte binding proteins PvRBP-1 and PvRBP-2. These reticulocyte binding proteins (RBP) described by Galinski *et al.* (1992) adhere specifically to reticulocytes of humans and other *P. vivax* susceptible species regardless of their Duffy phenotype. Since these molecules were localised on the apical pole of merozoites, Galinski *et al.* (1992) postulated that the PvRBPs were responsible for the attachment and/or re-orientation of merozoites to reticulocytes. This interaction would then provide the signal for junction formation and invasion involving the interaction of the released PvDAP-1 and the Duffy determinant. If *P vivax* invasion took place by trial and error probing, many

abortive invasion would occur as reticulocytes represent only ~1% of the erythrocyte population, therefore the RBP provides the merozoite with a means to discriminate between cell types. However Galinski *et al.* (1992) was unable to detect genes homologous to the PvRBPs in *P. knowlesi*, *P. falciparum* or *P. berghei* when southern blots of genomic DNA were probed with PvRBP sequences, indicating that the reticulocyte preference in other malarial species may have a different basis to that of *P. vivax*. Certainly the reticulocyte preference of *P. berghei* (Ramakrishnan & Prakash, 1950) or *P. falciparum* (Pasvol *et al.*, 1980) is not as pronounced as that of *P. vivax* (Kitchen, 1938) and this may reflect a different basis for reticulocyte preference between these plasmodial species.

Galinski *et al.* (1992) presents binding assays using metabolically labelled *P. vivax* culture supernatant and Duffy blood group positive erythrocytes in which he detects the binding of the 250 kDa RBP-1 and -2 to erythrocyte population with elevated reticulocyte populations. It appears from the autoradiographs published in this work, that the amount of PvDAP that bound to the reticulocyte-rich preparation is less than the amount that bound to the normal erythrocyte preparation, even though the number of erythrocytes used each time was kept constant. The reduced binding of the PvDAP to human reticulocytes is similar to the reduced binding of the PbEBP-130 to murine reticulocytes observed in the present study,and may indicate a common function for the two molecules.

It is known that *P. falciparum* invades mouse erythrocytes with low efficiency (Klotz *et al.*, 1987). Although ring stage parasites form in the mouse erythrocytes, further development does not occur, either due to parasite extrusion from the erythrocyte or erythrocyte lysis (Klotz *et al.*, 1987). Klotz *et al.* (1987) was able to detect an identical distribution of the *P. falciparum* RESA molecule in invaded mouse erythrocytes as is found in invaded human erythrocytes, indicating a similar invasion mechanism. Investigations into the binding of *P. falciparum* molecules to mouse erythrocytes were described by Sam-Yellowe & Perkins (1990). They found that mouse erythrocytes failed to bind the EBA-175 or MSA-1 molecules already identified as having a role in sialic-acid dependent invasion by *P. falciparum* merozoites of human erythrocytes, but did bind a 140/130/110 kDa complex of proteins that were located to the rhoptries. Trypsin or chymotrypsin treatment of murine erythrocytes reduced invasion of mouse

erythrocytes by merozoites of *P. falciparum* by up to 80% and also abrogated the binding of the 140/130/110 complex. Neuraminidase had little effect either on invasion, or on binding of the rhoptry complex to mouse erythrocytes. This data compares with the binding of the PbEBP-130 to, and the invasion of enzyme-treated murine erythrocytes by *P. berghei.* When Sam-Yellowe & Perkins (1990) investigated the invasion of murine erythrocytes by different strains of *P. falciparum*, they found that the sialic-acid independent strain 7G8 invaded mouse cells 3-4 times better than the sialic-acid dependent strain FCR-3. Binding of the rhoptry complex of strain 7G8 was also stronger than that of strain FCR-3 to mouse erythrocytes. This may indicate that the mouse erythrocytes are invaded by a process similar to the sialic-acid independent invasion process of *P. falciparum.*

Sam-Yellowe & Perkins (1990) also observed that 50% of the murine erythrocytes invaded by either strain of *P. falciparum* were reticulocytes. Anti mouse-glycophorin antibody blocked both the binding of the rhoptry complex to mouse erythrocytes and the invasion of erythrocytes, but had no effect on the rhoptry complex binding to reticulocytes, or the invasion of these cells by merozoites (Sam-Yellowe & Perkins, 1990). The preferential invasion of mouse reticulocytes by merozoites of *P. falciparum* could therefore be related to the presence of distinct but antigenically related sialoglycoproteins on each erythropoetic stage of the murine erythrocyte. Whether the reticulocyte preference of *P. falciparum* or *P. berghei in vivo* is related to the expression of such sialoglycoproteins is not known.

The binding of the rhoptry complex to reticulocyte-rich populations rules out the possibility that the PbEBP-130 is related to the *P. falciparum* rhoptry complex, as the PbEBP-130 does not bind to reticulocytes. However the invasion of mouse erythrocytes, in particular the preferential invasion of reticulocytes by *P. falciparum*, indicates a possible similarity in the molecular interactions involved in invasion between this malaria and *P. berghei.* As it is the sialic-acid independent strains of *P. falciparum* that invade murine erythrocytes most efficiently, the mechanism of murine erythrocyte invasion by *P. falciparum* may be related to the sialic-acid independent pathway for invasion.

The precise role played by the PbEBP-130 in invasion of erythrocytes by *P. berghei* is unclear. Because of its binding specificities it

cannot be regarded as being similar to the reticulocyte binding proteins of *P. vivax*. It is unlikely to belong to the MSP group of proteins first described by Holder and Freeman (1982), as it has a smaller molecular size and doesn't appear to be processed to smaller fragments as the MSP-1 of *P. falciparum*.

The PbEBP-130 may be related to a group of erythrocyte binding proteins described by Adams *et al.* (1992). Based on gene structure and amino acid homology, Adams *et al.* (1992) have proposed that the *P. vivax* Duffy binding protein PvDAP-1, the three *P. Knowlesi* Duffy binding proteins and the *P. falciparum* EBA-175 are members of the same gene family. These three species of malaria are quite evolutionary diverse and therefore it is reasonable to assume that *P. berghei* would possess a candidate molecule in this family.

The PbEBP-130 is interesting in itself; not only was it shown to bind to erythrocytes, and hence has a possible role in erythrocyte invasion by *P. berghei* merozoites, but it was also immunoprecipitated from an extract of metabolically labelled *P. berghei* schizonts by serum from a Balb/c mouse that had cleared a *P. berghei* infection. This indicate a possible role for this protein in evoking a successful immune response to *P. berghei* infection.

3. Construction of a gt11 cDNA expression library from erythrocytic stages of *P. berghei*

The cloning and sequencing of many malarial genes has now been reported, most of which encode antigens, from the erythrocytic stage. The technique used most frequently for cloning antigen genes has been to screen cDNA or genomic DNA expression libraries with antibody preparations ranging from monoclonals to total human or monkey immune serum. A number of different *E. coli* expression vectors have been used, but the phage vector gt11 has been the most popular.

Many malarial proteins show stage specific synthesis, and similarly mRNAs have been shown to change drastically in concentration between different stages. Deans *et al.* (1983), examined the stage specific protein synthesis by erythrocytic *P. falciparum*. They detected major changes in the type of protein synthesised by the parasite at the onset of schizogony, and they concluded that the new proteins synthesised in schizogony were

involved in the events of nuclear division or were merozoite associated proteins (Deans *et al.* 1983). David *et al.* (1984) examined the appearance of the MSP-1 of *P. knowlesi* by immunoprecipitation and found that it was synthesised late in schizogony. Hyde *et al.* (1984) were the first to report the construction of a cDNA library from the erythrocytic stages of *P. falciparum,* and also performed *in vitro* translation using mRNA from different stages of the erythrocytic parasite, and concluded that a library from schizonts would be most suitable for identifying and cloning the *P. falciparum* MSP-1 gene.

In the metabolic labelling of *P. berghei,* we cultured mature schizonts, and found that the PbEBP-130 was present both in the culture supernatant and also in the extracted parasite.The protein that we have identified as PbEBP-130 was therefore synthesised during schizogony. We therefore used cultured schizonts, prepared in the same manner as for the metabolic labelling, for the extraction of mRNA. A cDNA library was constructed from this mRNA. This library would be expected to contain gene sequences, encoding proteins expressed late in the erythrocytic cycle, such as the PbEBP-130.

We wished to construct a cDNA library that could be screened both as a expression library with an antibody probe and also with an oligonucleotide probe. The phage vector gt11 was considered the most suitable for these purposes. A phage vector was chosen for construction of the cDNA library, as large numbers of recombinants can be screened easily, and phage libraries tend to produce cleaner and less ambiguous positives than plasmid libraries (Hyde, 1990). Phage vectors in general give more transformants, from a given amount of starting DNA than plasmid vectors, as *in vitro* packaged phage particles enter cells much more efficiently than naked pieces of DNA. As we had only very small amount of starting mRNA, this was a major consideration in attempting to construct a comprehensive library (Hyde, 1990).

gt11 contains an EcoR1 cloning site located within the *lacZ* gene, upstream from the ß-galactosidase termination codon. If the inserted DNA has an open reading frame in register with the remainder of the ß-galactosidase gene, a hybrid or fusion protein is expressed when IPTG is used to inactivate the repressor (*LacI*) of *lacZ* directed expression. This vector also produces a temperature sensitive repressor, c1857, which is inactive at $42^{o}C$, and allows control of the lytic cycle of phage reproduction.

The vector gt11 is also defective in cell lysis, and when used with a bacterial strain that is lon^- (protease deficient), can produce large amount of recombinant protein.

An essential part of the strategy to isolate a particular clone from an expression library is the antibody preparation used to screen the library. Screening libraries with a monoclonal antibody is often unsuccessful, as the single epitope that it recognises may not be encoded by any of the gene fragments expressed or may not be in the correct conformation for detection by the monoclonal. Polyclonal antisera are generally more successful as they recognise all possible epitopes. One pitfall of screening expression libraries with antibodies is that multiple clones containing unrelated DNA sequences, but expressing cross-reacting epitopes are often selected. Several cross-reacting proteins were selected from a gt11 expression library using monoclonal antibodies to the *P. falciparum* circumsporozoite (CS) protein by Dame *et al.* (1984) before the true CS protein was selected.

Adams *et al.* (1990) constructed a cDNA expression library in gt11, from the mRNA from the late schizont stages of *P. knowlesi,* and cloned two genes of the Duffy receptor family. The sera used to screen the library was a fraction of immune rhesus monkey serum eluted from a nitrocellulose strip onto which the PvDAP had been transferred.

We were able to identify metabolically labelled PbEBP-130 in culture supernatant of *P. berghei* schizonts and in extracts of cultured schizonts. Attempts to purify large amounts of PbEBP-130 antigen from unlabelled culture supernatants, by affinity purification from erythrocytes, and subsequent immunisation of rats with the eluted proteins proved unsuccessful. Attempts were also made to cut the PbEBP-130 band from SDS-PAGE gels of total schizont extracts; the acrylamide slice then used in immunisations. This approach also failed to induce a polyclonal antisera in rats that reacted with the PbEBP-130. The sera that were finally used in the library screening were both polyspecific. One was raised against a culture supernatant of *P. c. chabaudi.* The other was reactive against schizont extract proteins >100 kDa. Both these sera were considered suitable for screening the library in order to clone the PbEBP-130 gene as they both immunoprecipitated the labelled PbEBP-130 from labelled extracts of *P. berghei* schizonts.

Extraction of RNA from the cultured schizonts was performed using the commercial preparation RNAzol B. This method of RNA extraction was much simpler than the published methods of Comczynski & Sacchi (1987) or Hyde *et al.* (1981), which involved acid guanidinium thiocyanate-phenol-chloroform extractions. The manufacturers instructions for use of the RNAzol B were followed. Recently Certa (1994) reported use of RNAzol B to extract cellular RNA from *P. berghei*. Certa used 1 ml of infected mouse blood with a parasitaemia of 80% and resuspended the washed cells in the RNAzol B, followed by a 3 h incubation of this mixture on ice. We used a CF11 column, before culturing the cells, to remove leucocytes and incubated the RNAzol B/cells mixture on ice for 5 minutes. It is likely that the RNA extracted by Certa (1992) contains mouse RNA as leucocytes were not removed from the blood preparation, and there would be a large population of leucocytes in the blood of a mouse that had a parasitaemia of 80%.

4. Screening of cDNA library with an oligonucleotide probe

The cDNA library was screened with the oligonucleotide probe JA159. A schematic representation of the source of oligo JA159 is shown in Fig. 4.1, and the nucleotide sequence of JA159 and of primers 142 and 143 is presented in Fig. 4.2.

Primer 142 and 143 were synthesised from the known DNA sequence of a section of the 3' cysteine-rich region of the gene for the EBA-175 of *P. falciparum* (Sim *et al.*, 1990). Adams *et al.* (1992) demonstrated that the 3' cysteine rich region of *P. falciparum* was highly homologous to the 3'cysteine rich regions of the Duffy adhesion proteins of *P. vivax* and *P. knowlesi* and based on the amino acid homology and gene structure of the 3' cysteine-rich region and other regions placed the EBA-175 gene and the DAP genes as part of the same gene family. If a member of the EBA/DAP gene family existed in *P. berghei*, it was likely that it also would possess an homologous 3' cysteine-rich region. A 200 bp fragment was PCR amplified with primer 142 and 143 when either *P. berghei* genomic DNA, or the prepared cDNA were used as template. The 40-mer oligonucleotide JA159 was synthesised from the internal sequence of the *P. berghei* 200 bp PCR

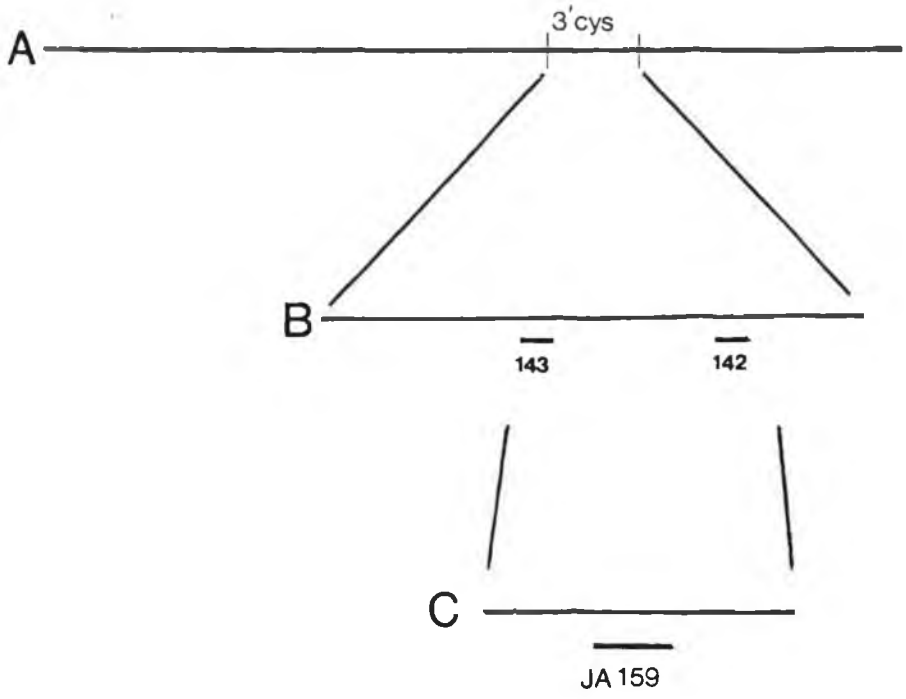
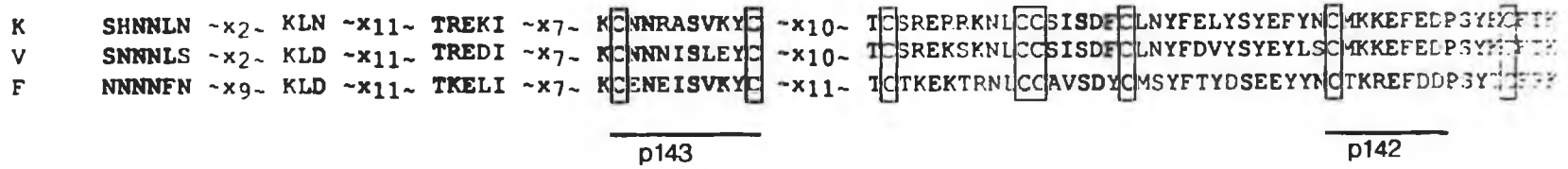


Fig 4.1

Primer 142 and primer 143 were a 25-mer and a 29-mer respectively, synthesised from the known sequence of the 3' cysteine rich region (B) of the *P. falciparum* EBA 175 gene (A). When Primers 142 and 143 were used in PCR with *P. berghei* genomic DNA as template, a 200 bp fragment (C) was amplified. The 200 bp PCR product was sequenced and a 40-mer oligonucleotide, JA159, was synthesised from the DNA sequence.

A 3' Cysteine-rich Region



B

Primer 142 3' GG ATC ATC AAA TTC CCT TTT CGT AC

Primer 143 3' TGT GAG AAC GAA ATT TCT GTA AAA TAT TG

Oligo 159 5' AAG GAT CCA ATA ATC TGA TAT TGA GCA GCA TAA TTG TTT C

EcoR1

Fig 4.2.

Panel A. Conserved 3' cysteine-rich regions of the erythrocyte binding proteins of *P. knowlesi* (K); *P. vivax* (V); *P. falciparum* (F). Conserved cysteine residues are boxed; X_n, the number (n) of intervening residues. Protein sequence encoded by the nucleotide sequences of primer 142 (p142) and primer 143 (p143) are underlined.

Panel B. Nucleotide sequences of primer 142, primer 143, and probe JA159. An EcoR1 restriction site was engineered into the 5' end of JA159.

product (P. Curley, personal communication).

This 200 bp fragment is slightly larger than the 178 bp fragment PCR amplified from genomic DNA from *P. falciparum* using primers 142 and 143. However it does indicate that an homologous gene sequence to that encoding the EBA-175 may exist in *P. berghei* genomic DNA and in the cDNA that we prepared.

The T_m (melting temperature) of JA159 was estimated at over 90^{o}C even though an EcoR1 site had been engineered into the 5' end. JA159 was end-labelled with ^{32}P, and was found to hybridise well to southern blots of the *P. berghei* 200 bp PCR product. We were unable, however, to identify any clones from the gt11 cDNA library hybridising to JA159, despite using very low stringency hybridising and washing conditions. Any primary positives that were identified failed to give secondary positives and were thus assumed to be false. When DNA was extracted from the total phage library, and used in PCR with primer 142 and 143, no fragment was amplified indicating that our gt11 cDNA library did not contain a sequence corresponding to the sequence in the EBA/DAP genes encoding the 3' cysteine rich region.

5. Immunoscreening the gt11 cDNA expression library

Immunoscreening of the gt11 expression library was performed with two separate sera. The first antisera, termed anti-TCS, had been raised to culture supernatant of *P. c. chabaudi* (a rodent malaria). It nevertheless immunoprecipitated a metabolically labelled *P. berghei* protein corresponding in size to the PbEBP-130, and on this basis was used for immunoscreening. The second antisera, termed anti-C1, used to immunoscreen the expression library was raised to proteins extracted from cultured schizonts of *P. berghei* (termed C1 proteins). It also immunoprecipitated a metabolically labelled *P. berghei* protein that corresponded in size to the PbEBP-130. As the anti-C1 sera immunoprecipited such a large number of *P. berghei* proteins, the immunoscreening was performed with a fraction of the sera that should react only with recombinants whose native protein is >100 kDa.

The immunoscreening with anti-TCS sera yielded 10 clones, which

were placed into 4 groups based on the size of the inserts they contained. The insert sizes were determined by PCR amplification of the phage DNA using primers constructed from sequence on either side of the EcoR1 cloning site. EcoR1 digestion of the phage DNA confirmed the sizing of the inserts and the group classification, each group having identical EcoR1 restriction fragments. A further indication that 4 groups of similar clones had been selected was given by the antibody reactivities of each selected clone with the anti-TCS sera; clones in each group reacted similarly with the immunoselection sera.

Immunoscreening with the anti-C1 sera fraction resulted in the identification of 14 clones, most of which had inserts of 1300-1500 bp as determined by PCR amplification using primers from either side of the EcoR1 cloning site.

When phage DNA from any of the 24 immunoselected clones was used in PCR with primers 142 and 143, no fragment was amplified. This indicates that the immunoscreening failed to identify a clone whose insert corresponded to that encoding the EBA/DAP 3' cysteine-rich region. This was expected as phage DNA extracted from the total library failed to amplify any fragment using primers 142 and 143.

The ability to amplify a 200 bp fragment from *P. berghei* genomic DNA, and from the prepared cDNA using primers 142 and 143, but not from total phage library DNA under the same conditions, indicates that the cDNA library generated may not have been representative of the cDNA synthesised from the schizont-stage mRNA. The transformation efficiency of the packaging reaction was 10^5 transformants per µg packaged DNA. This is lower than the transformation efficiency of 10^6-10^7 transformants/µg that the manufacturers of the packaging mix indicated should be expected. We suspect that the EBA/DAP-like protein would be synthesised in large amounts during schizogony, and therefore its particular mRNA should be quite abundant, and would be represented in our library despite the low transformation efficiency. Dalton *et al.* (1987) reported the cloning of a surface antigen of *Schistosoma mansoni* from a gt11 cDNA library with a transformation efficiency of 2.3×10^5 recombinants/µg packaged DNA.

We did not determine the average insert size of clones in the cDNA library. Assuming the insert sizes of the immunoselected clones are

representative, our library appears to contain inserts of 200-2000 bp. While these insert sizes are smaller than the manufacturers indicated should be expected, they are in practice, the size range usually found in gt11 libraries. Although the EBA-175 gene is ~4.5 kb, Sim *et al.* (1990) isolated only a 2.5 kb fragment of this gene from their gt11 cDNA library. Similarly, the gene encoding the DAP of *P. knowlesi* is ~4 kb, and only 2.6 kb was found in the gt11 expression cDNA expression library of Adams *et al.* (1990). It is possible therefore that only part of the homologous *P. berghei* gene was packaged, and this part of the gene did not contain the sequence identifiable by JA159 or amplifiable using primers 142 and 143.

It is also possible that the cDNA synthesis did not result in the synthesis of cDNA encoding the EBA/DAP-like protein. This could be due either to the lack of the corresponding mRNA molecule in the mRNA prepared or to the incomplete synthesis of the first or second cDNA strand. If the latter was the case then another probe, perhaps to a region of homology in the 5' cysteine-rich region may select a clone encoding part of the EBA/DAP-like protein. The 200 bp PCR product amplified from the cDNA preparation could in fact have been amplified from contaminating genomic DNA in the prepared cDNA. Although Hyde *et al.* (1984) used two rounds of oligo-dT affinity chromatography to purify mRNA from total *P. falciparum* RNA for use in preparation of a cDNA library, they found contaminating genomic DNA in the fraction eluted from the column. The binding of *P. falciparum* genomic DNA to the column was probably a result of the high A+T content of that DNA, a feature shared by the DNA of *P. berghei* (McCutchan *et al.*, 1984).

From the data obtained, it was evident that we would be unable to identify a clone containing an homologous region to the EBA/DAP 3' cysteine-rich region. We decided therefore to analyse the immunoselected clones and to determine whether any of the inserts in these clones encoded part or all of the PbEBP-130. The strategy would be to produce antisera to the fusion proteins of these clones, which could then be used in immunoprecipitations of metabolically labelled *P. berghei* proteins. The antisera generated could also be used to identify molecules involved in merozoite invasion of erythrocytes by their ability to block invasion in *in vitro* invasion assays The fusion proteins could also be tested for their

ability to elicit a protective immune response against *P. berghei* in mice. However the inability to produce fusion protein either from plate wash supernatants of *E. coli* infected with the immunoselected clones, or from *E. coli* lysogens of the immunoselected clones, precluded any of these investigations.

No fusion protein seemed to be present in the wash supernatants, indicating that the fusion proteins may have been present in an insoluble form. When lysogens were generated they proved extremely difficult to handle, lysing very soon after the induction of the lytic cycle. Inclusion bodies were prepared from some of the lysogens, but they proved very difficult to extract. Inclusion bodies are insoluble precipitates within the *E. coli* lysogen containing the overproduced fusion protein in a reduced and denatured form. They are thought to form because the protein is forced to adopt an unnatural pathway of folding when synthesised at high concentrations in a foreign environment. The only phage clone that produced fusion protein in *E. coli* lysogens or in infected *E. coli* plate wash supernatants was the wild type phage.

The inability to produce fusion proteins from the immunoselected clones could also have been due to damage done to the cloning site during ligation of the inserts to the vector arms. Alternatively the cloned cDNA may not have been in the correct orientation, or may have been in the incorrect codon frame for expression as a fusion protein. Some of the lysogen infected *E. coli* produced ß-galactosidase, indicating that transcription initiated from the ß-galactosidase start codon was functional, but that it was interrupted before transcription of the insert. Alternatively translation could have been similarly interrupted. In cloning the circumsporozoite protein of *P. falciparum,* Dame *et al.* (1984), were unable to produce fusion protein from 5 of the 6 clones immunoselected from a gt11 library. They discovered that the clones that did not express fusion proteins as *E. coli* lysogens had inserts that were not in frame with the ß-galactosidase gene or were in an opposite direction to the ß-galactosidase gene (Dame *et al.*, 1984). The immunoselection of these clones was made possible probably due to transcription from a lambda promoter rather than from the *lac* promoter. The ability to detect antigen produced by *E. coli* lysogenised clones whose coding sequence was out of frame was first observed by Young and Davis (1983) in their report outlining the construction and use of gt11 as an

expression vector. Detection of antigen when the insert was orientated opposite to that of the ß-galactosidase was also reported by Goto & Wang (1984).

6. Investigations on gt11 clones R_t and R_g

Lambda clones R_t and R_g were immunoselected from a *P. falciparum* gametocyte cDNA library in gt11 using an antisera that was specifically reactive against *P. falciparum* sexual stage antigens (J. Dalton, personal communication).

We were unable to establish the size of the insert in clone R_g. PCR amplification using R_g phage DNA as template and lambda forward and reverse primers always resulted in the production of multiple fragments. This clone also failed to produce a detectable fusion protein in plate wash supernatants of infected *E. coli*.

DNA from clone R_t PCR amplified a fragment of ~350 bp using lambda forward and reverse primers. As one of the EcoR1 sites flanking the insert appeared to be damaged, the insert size was confirmed by Kpn1/Sac1 digestion of phage DNA. A fusion protein of ~150 kDa was detected in plate wash supernatants of R_t infected *E. coli*. As ß-galactosidase is ~120 kDa this would imply that the native protein encoded for by the insert in clone R_t was ~30 kDa. A protein of this size is twice as large as could be encoded by an insert of ~300 bp. The anomalous migration of this fusion protein under reducing conditions may indicate a highly charged amino acid content or a repeat amino acid motif in the native R_t protein. In cloning the circumsporozoite (CS) protein of *P. falciparum*, from a gt11 expression library, Dame *et al.* (1984) noted similar anomalities in the migration of the native CS protein and a ß-galactosidase/CS fusion protein which they believed to be related to the repeat regions of the CS protein.

Fusion protein purified from plate wash supernatants of phage R_t infected *E. coli* was used to immunise rats. If the anti-ß-galactosidase reactivity could be absorbed from the sera, the remaining fraction reactive against the native R_t protein could be used in further analysis of the R_t protein. Such a sera fraction could be first used to determine the size of the native R_t protein by immunoprecipitation of metabolically labelled sexual stage proteins, and then used to screen the gt11 cDNA expression library

again or a genomic expression library in order to isolate the full coding sequence of the native protein. The R_t -protein reactive fraction of the sera could also be used to localise the native R_t protein in *P. falciparum* sexual stages either by immunofluoresence or immuno-electron microscopy. This would provide an indication of the function or activity of the native protein. If it were localised on the exterior of the gametocyte, it may function in the molecular reaction involved in gametocyte fertilisation. If however it were localised in the nucleus of gametocytes, this may indicate a role in the gene regulation of gametocytogenesis. Very little is known about regulation of malaria genes. The complex life cycle of a malaria parasite is matched by an equally intricate system of gene regulation. The genetic switches that send merozoites into the developmental pathways leading to rings, macrogametocytes or microgametocytes are likely to be as fascinating to the developmental biologist as they are to the vaccinologist.

A 200 bp DNA sequence was determined from the 350 bp PCR fragment amplified from the R_t phage DNA using lambda forward and reverse primers. The determined sequence is shown in Fig. 4.3. The sequence was determined for one strand only using the lambda reverse primer as the initiation primer for chain elongation. Computer search analysis at the DNA and protein level were performed on the 200 bp DNA sequence determined. The protein sequence search found greatest similarity with the sequence of the *Trypanosoma brucei* CR4 protein, whose mRNA is trans-edited in the kinetoplast of this protozoan (Stuart, 1991). Over a stretch of 19 amino acids, there were 9 matches with amino acids encoded by one codon frame of the R_t sequence.

The nucleotide sequence search found greatest similarity with a mouse mRNA for the NF1-B protein with 63% homology over 71 nucleotides. The NF1-B (nuclear factor 1-B) protein is a DNA binding protein found in mouse brain cells and is necessary for the activation of transcription from several promoters, and also found to inactivate transcription from other promoters (Inoue *et al.*, 1990). The homology between the R_t insert sequence and the sequence of this NF1-B DNA binding-protein involved in brain-specific gene regulation is interesting as it may indicate a similar role as a DNA binding protein involved in regulation of transcription for the native protein encoded (partially) for, by the insert sequence in the R_t clone. No definite conclusion or comparisons can be

drawn from the 200 bp sequence as it is too short for a definitive protein sequence or nucleotide sequence similarity search. Significantly however, no similarity was found between the sequence of the insert in the R_t clone and any previously published gene sequence of *P. falciparum,* including those of the sexual stage antigens Pfs 25/28, Pfs 48/45 or Pfs 15 (Kaslow *et al.*, 1987; Kochen *et al.*, 1993; Langsley *et al.*, 1992), indicating that we have cloned a novel protein of *P. falciparum* sexual stages.

Future work on the R_t clone insert should involve confirmation of the DNA sequence of the R_t clone insert by sequencing the complementary strand. The PCR fragment itself or an oligonucleotide probe could then be used to determine the size of the primary transcript of the native R_t protein in northern hybridisations with sexual stage mRNA from *P. falciparum,* and in screening a cDNA library or an genomic library if the 300 bp insert in clone R_t does not represent the full gene, in order to obtain the full coding sequence.

GAACTAATAGAAGAAGATATACAAAAAGATATAGGAAGAAGATATACAAAAAGATATAGT
ACCAAGATTTACAAAAAGTTATGGTNCAAGATATAGAACAAGATATAGANCAAGATNTAC
AAGATGATGTGCAAGAAGANGATTTACTTGTAGGAACCCCTTTCCACAAAAGGACCTGCC
ACATTTTCTANAACCTCAGC

Fig 4.3.
200 bp DNA sequence of the cloned insert of phage R_t (N = nucleotide at this position not determined).

CONCLUSION

We have developed a method for the purification of *Plasmodium berghei* infected erythrocytes, that results in preparations containing greater than 95% purified parasites. This purification technique and the development of a media that supported *in vitro* growth of *P. berghei* allowed investigations into the invasion of erythrocytes by merozoites of this parasite using *in vitro* invasion assays and erythrocyte binding assays.

The *in vivo* erythrocyte invasion specificities of *P. berghei* merozoites were confirmed *in vitro* in our erythrocyte invasion assays. In particular we observed *in vitro,* the preference of *P. berghei* merozoites for invasion of reticulocytes. Using the erythrocyte binding assay, we identified a 130 kDa molecule, termed PbEBP-130, that was synthesised late in the erythrocytic cycle by *P. berghei* and that bound exclusively to murine erythrocytes. Sera from a mouse that had cleared *P. berghei* infection reacted with the PbEBP-130 molecule in immunoprecipitations, indicating a role for PbEBP-130 in inducing immunity to infection.

Polyclonal antisera were raised to protein preparations of *P. berghei.* These sera immunoprecipitated the PbEBP-130 from labelled protein extracts of *P. berghei,* and were used to screen a cDNA expression library that was constructed in gt11. Immunoselected clones failed to produce fusion protein. The cDNA library was screened with a labelled oligonucleotide probe, synthesised from known DNA sequence of the gene for the erythrocyte binding protein of the human malaria, *Plasmodium falciparum.* No clone was selected by the labelled oligonucleotide.

Two gt11 clones, immunoselected from a cDNA library constructed from the sexual stages of *P. falciparum* were investigated. Fusion protein was prepared from one of the clones and used to immunise rats, from which a polyclonal antisera reactive with *P. falciparum* sexual stages was collected.

CHAPTER 5

REFERENCES

Adams, J. H., Hudson, D. E., Torii, M., Ward, G. E., Wellems, T. E., Aikawa, M. & Miller, L. H. 1990. The Duffy receptor family of *Plasmodium knowlesi* is located in the micronemes of invasive malaria merozoites. *Cell* **63**, 141-153.

Adams, J. H., Kim Lee Sim, B., Dolan, S. A., Fang, X., Kaslow, D. C. & Miller, L. H. 1992. A family of erythrocyte binding proteins of malaria parasites. *P. N. A. S.* (USA) **89**, 7085-7089.

Bannister, L. H., Butcher, G. A., Dennis, E. D. & Mitchell, G. H. 1975. Invasive behaviour of *Plasmodium knowlesi* merozoites *in vitro*. *Parasitology* **71**, 483-491.

Bannister, L. H., Mitchell, G. H., Butcher, G. A. & Dennis, G. A. 1986. Lamellar membranes associated with rhoptries in erythrocyte merozoites of *Plasmodium knowlesi*. *Parasitology* **92**, 291-303.

Barnwell, J. W., Nichols, M. E. & Rubinstein, P. 1989. *In vitro* evaluation of the role of the Duffy blood group in erythrocyte invasion by *Plasmodium vivax*. *J. Exp. Med.***169**, 1795-1802.

Bates, M. D., Newbold, C. I., Jarra, W. & Brown, K. N. 1988. Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi chabaudi* in CBA/Ca. III. Protective and suppressive responses induced by immunisation with purified antigens. *Parasite Immunology* **10**, 1-15.

Braun-Breton, C., Blisnick, T., Jouin, H., Barale, J. C., Rabilloud, T., Langsley, G. & da Silva, L. P. 1992. *Plasmodium chabaudi* p68 serine protease activity required for merozoite entry into mouse erythrocytes. *P. N. A. S.* (USA) **89**, 9647-9651.

Blackman, M. J., Heidrich, H. G., Donachie, S., McBride, J. S. & Holder, A. A. 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J. Exp. Med.* **172**, 379-382.

Camus, D. & Hadley, T. J. 1985. A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. *Science* **230**, 553-556.

Carter, R. & Chen, D. H. 1976. Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature* **263**, 57-60.

Certa, U. 1994. Regular initiation of translation of *Plasmodium berghei* aldolase-2 after pre-mRNA splicing. *Mol. Biochem. Parasitol.* **63**, 291-297.

Chappel, J. A. & Holder, A. A. 1993. Monoclonal antibodies that inhibit *Plasmodium falciparum* invasion *in vitro* recognise the first growth factor-like domain of merozoite surface protein-1. *Mol. Biochem. Parasitol.* **60**, 303-312.

Chomczynski, P. & Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Cowman, A. F., Coppel, R. L., Saint, R. B., Favarolo, J., Crewther, P. E., Stahl, H. D., Bianco, A. E., Brown, G. V., Ander, R. F. & Kemp, D. J. 1985. The ring-infected erythrocyte surface antigen (RESA) polypeptide of *Plasmodium falciparum* contains two separate blocks of tandem repeats encoding antigenic epitopes that are naturally immunogenic in man. *Mol. Biol. Med.* **2**, 207-211.

Cox, F. E. G. 1991. Malaria vaccines- progress and problems. *TIBTech* **9**, 389-394.

Dalton, J. P., Tom, T. & Strand, M. 1987. Cloning of a cDNA encoding a surface antigen of *Schistosoma mansoni* schistosomula recognised by sera of vaccinated mice. *P. N. A. S.* (USA) **84**, 4268-4272.

Dalton, J. P., Hudson, D., Adams, J. H. & Miller, L. H. 1991. Blocking of the receptor-mediated invasion of erythrocytes by *Plasmodium knowlesi* malaria with sulphated polysaccharides and glycoaminoglycans. *Eur. J. Biochem.* **195**, 789-794.

Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L., Wirtz, R. A., Hockmeyer, W. T., Maloy, W. L., Haynes, J. D., Schneider, I., Roberts, D., Sanders, G. S., Reddy, E. P., Diggs, C. L. & Miller, L. H. 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum. Science* **225**, 593-599.

David, P. H., Hadley, T. J., Aikawa, M. & Miller, L. H. 1984. Processing of a major parasite surface glycoprotein during the ultimate stages of differentiation in *Plasmodium knowlesi. Mol. Biochem. Parasitol.* **11**, 267-282.

Deans, J. A., Thomas, A. W., Inge, P. M. G. & Cohen, S. 1983. Stage-specific protein synthesis by asexual blood stage parasites of *Plasmodium falciparum. Mol. Biochem. Parasitol.* **8**, 45-51.

del Portillo, H. A., Longacre, S., Khouri, E. & David, P. H. 1991. Primary structure of the merozoite surface antigen-1 of *Plasmodium vivax* reveals sequences conserved between different Plasmodium species. *P. N. A. S.* (USA) **88**, 4030-4034.

Dolan, S. A., Proctor, J. L., Alling, D. W., Okubu, Y., Wellems, T. E. & Miller L. H. 1994. Glycophorin B as an EBA-175 independant *Plasmodium falciparum* receptor of human erythrocytes. *Mol. Biochem. Parasitol.* **64**, 55-63.

Dvorak, J. A., Miller, L. H., Whitehouse, W. C. & Shiroishi, T. 1975. Invasion of erythrocytes by malaria merozoites. *Science* **187**, 748-749.

Epstein, N., Miller, L. H., Kaushel, D. C., Udeinya, I. J., Rener, J., Howard, R. J., Asofsky, R., Aikawa, M. & Hess, R. L. 1981. Monoclonal antibodies against a specific determinant on malarial (*Plasmodium knowlesi*) merozoites block erythrocyte invasion. *J. Immunology* **127**,212-217.

Fang, X., Kaslow, D. C., Adams, J. H. & Miller, L. H. 1991. Cloning of the *Plasmodium vivax* Duffy receptor. *Mol. Biochem. Parasitol.* **44**, 125-132.

Foley, M., Murray, L. J. & Anders, R. F. 1990. The ring-infected erythrocyte surface antigen protein of *Plasmodium falciparum* is phosphorylated upon association with the host cell membrane. *Mol. Biochem. Parasitol.* **38**, 69-76.

Gronowicz, G., Swift, H. & Steck, T. L. 1984. Maturation of the reticulocyte *in vitro* . *J. Cell Sci.* **71**, 177-179.

Galinski, M. R., Corredor Medina, C., Ingravallo, P. & Barnwell, J. W. 1992. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. *Cell* **69**, 1213-1226.

Garnham, P. C. C. 1967. Malaria in mammals excluding man. *Advances in Parasitology*, **5**, 139-204.

Goto, T. & Wang, J. C. 1984. Yeast DNA topoisomerase II is encoded by a single copy, essential gene. *Cell* **36**, 1073-1080.

Greenwood, B. M. 1990. Anti-sporozoite vaccines. *Bull. W. H. O.* **68** (suppl.), 184-190

Hadley, T. J., McGinniss, M. H., Klotz, F. W. & Miller, L. H. 1986. Blood group antigens and invasion of erythrocytes by malaria parasites. *Red cell antigens and antibodies.* American Association of Blood Banks, Arlington, VA. Garratty, G. ed.

Hadley, T. J., Klotz, F. W., Pasvol, G., Haynes, J. D., McGinniss, M. H., Okubo, Y. & Miller, L. H. 1987. Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (M^kM^k). *J. Clin. Invest.* **80**, 1190-1193.

Haynes, J. D., Dalton, J. P., Klotz, F. W., McGinniss, M. H., Hadley, T. J., Hudson, D.E. & Miller, L. H. 1988. Receptor-like specificity of a *Plasmodium knowlesi* malarial protein that binds to Duffy antigen ligands on erythrocytes. *J. Exp. Med.* **167**, 1873-1881.

Holder, A. A. & Freeman, R. R. 1981. Immunisation against blood stage rodent malaria using purified parasite antigens. *Nature* **294**, 361-364.

Holder, A. A. & Freeman, R. R. 1982. Biosynthesis and processing of a *Plasmodium falciparum* shizont antigen recognised by immune serum and a monoclonal antibody. *J. Exp. Med.* **156**, 1528-1538.

Holder, A. A. & Freeman, R. R. 1984. The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. *J. Exp. Med.* **160**, 624-629.

Holder, A. A., Lockyer, M. J., Odink, K. G., Sandhu, J. S., Riveros-Moreno, V., Nicholls, S. C., Hillman, Y., Davey, L. S., Tizard, M. L. V., Schwarz, R. T. & Freeman, R. R. 1985. *Nature* **319**, 270-273.

Holder, A. A., Sandhu, J. S., Hillman, Y., Davey, L. S., Nicholls, S. C., Cooper, H. & Lockyer, M. J. 1987. Processing of the precursor to the major merozoite surface antigen of *Plasmodium falciparum. Parasitology* **94**, 199-208.

Horuk, R., Chitnis, C. E., Darbonne, W. C., Colby, T. J., Rybicki, A., Hadley, T. J. & Miller L. H. 1993. A receptor for the malaria parasite *Plasmodium vivax*: The erythrocyte chemokine receptor. *Science* **261**, 1182-1184.

Hyde, J. E., Zolg, J. W. & Scaife, J. G. 1981. Isolation and characterisation of ribosomal RNA from the human malaria parasite *Plasmodium falciparum. Mol. Biochem. Parasitol.* **4**, 283-290.

Hyde, J. E., Goman, M., Hall, R., Osland, A., Hope, I. A., Langsley, G., Zolg, J. W. & Scaife, J. G. 1984. Characterisation and translation studies of messenger RNA from the human malaria parasite *Plasmodium falciparum* and the construction of a cDNA library. *Mol. Boichem. Parasitol.* **10**, 269-285.

Hyde, J. E. 1990. Molecular Parasitology, Open University Press, Milton Keynes, U. K.

Inoue, T., Tamura, T., Furuichi, T. & Mikoshiba, K. 1990. Isolationof complementary DNAs encoding a cerebellum enriched nuclear factor I family that activates transcription from the mouse myelin basic protein promoter. *J. Biol. Chem.* **265**(31), 19065-19070.

Janse, C. J., Mons, B., Croon, J. J. A. B. & van der Kaay, H. J. 1984.Long-term *in vitro* cultures of *Plasmodium berghei* and preliminary observation on gametocytogenesis. *Int. J. Parasitol.* **14** (3), 317-320.

Janse, C. J., Boorsma, E. G., Ramesar, J., Grobbee, M. J. & Mons, B. 1989. Host cell specificity and shizogony of *Plasmodium berghei* under different *in vitro* conditions. *Int. J. Parasitol.***19**, 509-514.

Kaslow, D. C., Quakyi, I. A., Syin, C., Raum, M. G., Keister, D. G., Coligan, J. E., McCutchan, T. F. & Miller, L. H. 1987. A vaccine candidate from the sexual stage of human malaria that contain EGF-like domains. *Nature* **333**, 74-76.

Kitchen, S. F. 1938. The infection of reticulocytes by *Plasmodium vivax*. *Am. J. Trop. Med.* **18**, 347-359.

Klotz, F. W. & Chulay, J. D. 1987. Invasion of mouse erythrocytes by the human malarial parasite *Plasmodium falciparum*. *J. Exp. Med.* **165**, 1713-1715.

Kochen, C. H. M., Jansen, J., Kaan, A. M., Beckers, P. J. A., Ponnudurai, T., Kaslow, D. C., Konings, R. N. H., Schoenmakers, J. G. G. 1993. Cloning and expression of the gene coding for the transmission blocking target antigen Pfs48/45 of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **61**, 59-68.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Langreth, S. G., Jensen, J. B., Reese, R. T. & Trager, W. 1978. Fine structure of malaria *in vitro*. *J. Protozoology.* **25**, 443-452.

Langsley, G., Kaslow, D. C., Barbot, P., Blisnick, T., Ponnudurai, T., Barale, J. C. & Braun-Breton, C. 1992. A*Plasmodium falciparum* gene coding for a 15 kilodalton antigen expressed in asexual stage parasites, gametocytes and gametes. *Mol. Biol. Parasitol.* **55**, 221-224.

Lanners, H. N. 1992. Prolonged *in vitro* cultivation of *Plasmodium vivax* using Trager's continuous flow method. *Parasitol. Res.* **78**, 699-701.

Maniatis, T., Fritsch, E. F. & Sambrook, J. 1982. *Molecular Cloning. A Laboratory Manual.*Cold Spring Harbor Laboratory, Cold Spring Harbor.

Mason, S. J., Miller, L. H., Shiroishi, T., Dvorak, J. A. & McGinniss, M. H. 1977. The Duffy blood group determinants: Their role in the susceptibility of human and animal erythrocytes to *Plasmodium knowlesi* malaria. *Brit. J. Haemt.* **36**, 327-335.

McCutchan, T. F., Dame, J. B., Miller, L. H. & Barnwell, J. 1984. Evolutionary relatedness of *Plasmodium* species as determined by the structure of DNA. *Science* **225**, 808-811.

McLean, S. A., MacDougall, L. & Phillips, R. S. 1986. An indirect fluorescent antibody test which recognises antigenic variants of *Plasmodium chabaudi*. *IRCS Medical Science* **14**, 896-897.

McNally, J., O'Donovan, S. M. & Dalton, J. P. 1992. *Plasmodium berghei* and *Plasmodium chabaudi chabaudi* : development of simple *in vitro* invasion assays. *Parasitology* **105**, 355-362.

Miller, L. H., & Carter, R. 1976. A review: Innate resistance in malaria. *Experimental Parasitology* **40**, 132-146.

Miller, L. H., Haynes, J. D., McAuliffe, F. M., Shiroishi, T., Durocher, J. R. & McGinnis, M.H. 1977. Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. *J. Exp. Med.* **146**, 277-281.

Miller, L. H., Hudson, D., Rener, J., Taylor, D., Hadley, T. J. & Zilberstein, D. 1983. A monoclonal antibody to Rhesus erythrocyte Band 3 inhibits invasion by malaria. *J. Clin. Invest* **72**, 1357-1364

Mitchell, G. H., Hadley, T. J., McGinniss, M. H., Klotz, F. W. & Miller, L. H. 1986. Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: Evidence for receptor heterogenecity and two receptors. *Blood* **67**, 1519-1521.

Molano, A., Segura, C., Guzman, F., Lozada, D. & Patarroyo, M. E. 1992. In human malaria protective antibodies are directed mainly against the LYS-GLU ion pair within the LYS-GLU-LYS motif of the synthetic vaccine. *Parasite Immunology* **14**, 111-126

Mons, B., Janse, C. J., Croon, J. J. A. B. & van der Kaay, H. J. 1983. *In vitro* culture of *Plasmodium berghei* using a new suspension system. *Int. J. Para.* **13** (2), 213-217.

Mons, B., Janse, C. J., Boorsma, E. G. & van der Kaay, H. J. 1985. Synchronised erythrocytic schizogony and gametocytogenesis of *Plasmodium berghei in vivo* and *in vitro. Parasitology* **91**, 423-430.

Mons, B. (1990). Preferential invasion of malarial merozoites into young red blood cells. *Blood cells* **16**, 299-312.

Nussenzweig, V. & Nussenzweig, R. S. 1986. Development of a sporozoite malaria vaccine. *Am. J. Trop. Med. Hyg.* **35**, 678-688.

Ott, K. J. 1968. Influence of reticulocytosis on the course of infection of *Plasmodium chabaudi* and *Plasmodium berghei. J. Protozool.* **15** (2), 365-369.

Pasvol, G., Weatherall, D. J. & Wilson, R. J. M. 1980. The increased susceptibility of young red blood cells to invasion by the malarial parasite *Plasmodium falciparum. Brit. J. Haematol.* **45**, 285-295.

Pasvol, G., Jungery, M., Weatheral, D. J., Parsons, S. F., Anstee, D. J. & Tanner, M. J. A. 1982. Glycophorin as a possible receptor for *Plasmodium falciparum. Lancet* **II**, 947-950.

Patarroyo, M. E., Romero, P., Torres, M. L., Clavijo, P., Moreno, A., Martinez, A., Rodriguez, R., Guzman, F. & Cabezas, E. 1987. Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature* **328**, 629-632.

Patarroyo, M. E., Amador, R., Clavijo, P., Moreno, A., Tascon, R., Franco, M. A., Muriollo, L., Ponton, G. & Trujillo, G. 1988. A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. *Nature* **332**, 158-160.

Perkins, M. E. & Rocco, L. J. 1988. Sialic acid-dependant binding of *Plasmodium falciparum* merozoite surface antigen, Pf200, to human erythrocytes. *J. Immun.* **141** (9), 3190-3196.

Perkins, M. E. 1989. Erythrocyte invasion by malaria merozoites: Recent advances. *Exp. Parasitol.* **69**, 94-99.

Pirson, P. J. & Perkins, M. E. 1985. Characterisation with monoclonal antibodies of a surface antigen of *Plasmodium falciparum* merozoites. *J. Immunology* **134**, 1946-1951.

Ramakrishnan, S. P. & Prakash, S. J. 1950. Studies on *Plasmodium berghei* n. sp. Vinke and Lips 1948. Morphology, periodicity and pathogenicity in blood induced infection in mice rats and garden squirrels. *Ind. J. Mal.* **4**, 369-375.

Rapoport, S. M. 1986. The Reticulocyte. CRC Press, Inc. Boca Raton, Florida, USA

Ruebush, T. K., Campbell, G. H., Moreno, A., Patarroyo, M. E. & Collins, W. E. 1990. Immunisation of owl monkeys with a combination of *Plasmodium falciparum* asexual blood-stage synthetic peptide antigens. *Am. J. Trop. Med. Hyg.* **43**(4), 355-366

Sacci, J. B., Schriefer, M. E., Resau, J. H., Wirtz, R. A., Detolla, L. J., Markham, R. B. & Azad, A. F. 1992. Mouse model for exoerythrocytic stages of *Plasmodium falciparum* malaria parasite. *P N. A. S.* (USA) **89**, 3701-3705.

Salcedo, M., Barreto, L., Rojas, M., Moya, R., Cote, J. & Patarroyo, M. E. 1991. Studies on the humoral immune response to a synthetic vaccine against *Plasmodium falciparum* malaria. *Clin. Exp. Immunol.* **84**, 122-128.

Sam-Yellowe, T. Y. & Perkins, M. E. 1990. Binding of *Plasmodium falciparum* rhoptry antigens to mouse erythrocytes and their possible role in invasion. *Mol. Biochem. Parasitol.* **39**, 91-100.

Schmidt-Ullrich, R., Lightholder, J. & Monroe, T. M. 1983. Protective *Plasmodium knowlesi* M_r 74000 antigen in membrane of shizont-infected rhesus erythrocytes. *J. Exp. Med.* **158**, 146-158.

Schrevel, J., Deguercy, A., Mayer, R. & Monsigney, M. 1990. Proteases in malaria- infected red blood cells. *Blood Cells* **16**, 563-584.

Siddiqui, W. A., Tam, L. Q., Kramer, K. H., Hui, G. S. N., Case, S. E., Yamaga, K. M., Chang, S. P., Chan E. B. T. & Kan, S-C. 1987. Merozoite surface coat precursor protects *Aotus* monkeys against *Plasmodium falciparum* malaria. *P N. A. S.* (USA) **84**, 3014-3018.

Sim, B. K. L., Orlandi, P. A., Haynes, J. D., Klotz, F. W., Carter, J. M., Camus, D., Zegans, M. E. & Chulay, J. D. 1990. Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. *J. Cell Biology*, **111**,1877-1884.

Sim, B. K. L., Toyoshima, T., Haynes, J. D. & Aikawa, M. 1992. Localisation of the 175 kiloDalton erythrocyte binding antigen in micronemes of *Plasmodium knowlesi* merozoites. *Mol. Biochem. Parasitol.* **51**, 157-160.

Stuart, K. 1991. RNA editing in kinetoplastid protozoa. *Curr. Opin. Gen. Devel.* **1**, 412-416.

Suhrbier, A., Janse, C. J., Mons, B., Fleck, S. L., Nicholas, J., Davies, C. S. & Sinden, R. E. 1987. The complete development *in vitro* of the vertebrate phase of the mammalian malaria parasite *Plasmodium berghei. Trans. Roy. Soc. Trop. Med. Hyg.* **81**, 907-909.

Szarfman, A., Walliker, D., McBride, J. S., Lyon, J. A., Quakyi, I. A. & Carter, R. 1988. Allelic forms of gp195, a major blood stage antigen of *Plasmodium falciparum*, are expressed in the liver stages. *J. Exp. Med.* **167**, 231-236.

Torii, M., Adams, J. H., Miller, L. H. & Aikawa, M. 1989. Release of merozoite dense granules during erythrocyte invasion by *Plasmodium knowlesi. Infect. and Immun.* **57**(10), 3230-3233

Trager, W. & Jensen, J. B. 1976. Human malaria parasites in continuous culture. *Science* **193**, 674-675.

Valero, M. V., Amador, L. R., Galindo, C., Figeuroa, J., Bello, M. S., Ortega, N. M., Rosas, J. E., Alonso, P. L. & Patarroyo, M. E. 1993. Vaccination with SPf66, a chemically synthesised vaccine, against *Plasmodium falciparum* malaria in Columbia. *Lancet* **341**, 705-710.

Vincke, I. H. & Lips, M. 1948. Un nouveau plasmodium d'un rongeur sauvage du congo, *Plasmodium berghei* N. sp. *Annales de la societe Belge de medecine tropicale,* **28**, 97-104.

Warren, M., Skinner, J. C. & Guinn, E. 1966. Biology of the simian malarias of South East Asia. I. Host cell preferences of young trophozoites of four species of *Plasmodium. J. Parasitol.* **52**, 14-16.

Waters, A. P., Thomas, A. W., Deans, J. A., Mitchell, G. H., Hudson, D. E., Miller, L. H., McCutchan, T. F. & Cohen, S. 1990. A merozoite receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium. J. Biol. Chem.* **265**, 17974-17979.

Waters, A. P., Higgins, D. G. & McCutchan T. F. 1991. *Plasmodium falciparum* appears to have arisen as a result of lateral transfer between avian and human hosts. *P. N. A. S.* (USA) **88**, 3140-3144

Wertheimer, S. P. & Barnwell, J. W. 1989. *Plasmodium vivax* interaction with the human Duffy blood group glycoprotein: Identification of a parasite receptor-like protein. *Exp. Para.* **69**, 340-350.

Wunderlich, F., Schillinger, G. & Helwig, M. 1985. Fractionation of *Plasmodium chabaudi* infected erythrocytes into parasites and ghosts. *Zeitschrift fur parasitenkunde* **7 1**, 545-551.

Yan, G. H. & Wang, G. J. 1986. Electron microscopic study on the invasion of erythrocytes by *Plasmodium berghei* merozoites. *J. Parasitol. Parasit. Dis.* **4**, 25-27.

Yoeli, M.,Hargreaves, B., Carter, R. & Walliker, D. 1975. Sudden increase in virulence in a strain of *Plasmodium berghei yoelii. Ann. Trop. Med. Parasitol.* **6 9**, 173-178.

Young, R. A. & Davis, R. W. 1983. Efficient isolation of genes by using antibody probes. *P. N. A. S.* (USA) **8 0**, 1194-1198.

CHAPTER 6

APPENDICES

Plasmodium berghei and *Plasmodium chabaudi chabaudi*: development of simple *in vitro* erythrocyte invasion assays

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SUMMARY

Erythrocyte invasion assays are described for two species of rodent malaria, namely *Plasmodium berghei* and *P. c. chabaudi*. These invasion assays are simple, are carried out using a candle jar and allow a number of assays to be performed simultaneously. Our results demonstrate that both rodent malaria species show an *in vitro* preference for reticulocytes although the preference of *P. c. chabaudi* for these cells is not as marked as that of *P. berghei*. The details of our invasion assays and our results obtained are discussed.

Key words: *Plasmodium berghei*, *Plasmodium chabaudi chabaudi*, malaria, rodent, reticulocytes, erythrocytes, invasion assays.

INTRODUCTION

Erythrocyte invasion is a process essential to the survival of malaria parasites. An understanding of all the molecular mechanisms and interactions that take place during the invasion event is important since interruption of any of these would halt the parasite's asexual cycle and hence prevent disease. The majority of our knowledge on the molecular interactions that occur during erythrocyte invasion has come from studies on the human malaria, *Plasmodium falciparum* and the monkey malaria, *P. knowlesi* (Hadley *et al.* 1986). Erythrocyte invasion by both of these malarias can be examined relatively easily because of the availability of *in vitro* invasion assays. Thus the requirement for particular erythrocyte ligands, such as sialic acid by certain isolates of *P. falciparum* (Hadley *et al.* 1987) and the Duffy blood group antigen by *P. knowlesi* (Miller *et al.* 1975), was clearly demonstrated. Furthermore, these assays were essential to the isolation of antibodies capable of blocking erythrocyte invasion by *P. knowlesi* merozoites and the subsequent characterization of the target antigens (reviewed by Hudson, Wellems & Miller, 1988). More recently, invasion assays were used to correlate the binding of *P. knowlesi* merozoite receptor molecules to the Duffy blood group antigen on primate erythrocytes with the ability of merozoites to invade these cells (Haynes *et al.* 1988; Dalton *et al.* 1991).

Invasion assays similar to those performed with *P. falciparum* and *P. knowlesi* have not been developed for the rodent malarias, such as *P. berghei*, *P. chabaudi* and *P. yoelii*. This is unfortunate since these models are more accessible and relatively inexpensive compared to the primate malaria models. Erythrocyte invasion assays for the rodent malarias could provide easy systems for the identification of merozoite molecules susceptible to blocking antibodies. The homologous molecules could then be characterized in the more important human malarias. In the present report we detail methods for invasion assays for two rodent malarias namely, *P. berghei* and *P. chabaudi chabaudi*. Our primary goal is to provide invasion assays that are simple to use, requiring no elaborate equipment and that allow a number of tests to be carried out simultaneously.

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MATERIALS AND METHODS

Parasites and animals

The *P. berghei* (ANKA strain) line was maintained in 6 to 8-week-old Balb/c mice by passing blood from one animal with parasitaemia between 15 and 20% to the next. A cloned line of *P. c. chabaudi* AS was maintained in CD-1 mice and was passaged similarly. These CD-1 mice were adapted to reversed lighting for 7 days before infection, the artificial daylight beginning at 12 midnight and ending at 12 noon. Balb/c and Schofield mice were bred in our own animal facility in Dublin City University and CD-1 mice were purchased from Biological Laboratories, Ballina, Ireland.

Preparation of target erythrocytes

Blood from Balb/c, Schofield and CD-1 mice was obtained by cardiac puncture into heparinized syringes. Ten ml of phosphate-buffered saline (PBS) was added to 1 ml of blood and the cells were centrifuged at 450 ***g*** for 5 min. The pelleted cells were then

resuspended in 0·5 ml of PBS. Erythrocytes from guinea-pigs, rabbits and humans (supplied by Dublin Transfusion Board) were prepared similarly.

Induction of reticulocytosis using phenylhydrazine

Solutions of 0·4 % phenylhydrazine were prepared in PBS from a 97 % stock solution (Aldrich Chemicals, Milwaukee, WI, USA) immediately before use. Mice were injected intraperitoneally with 100 μl of this solution/15 g body weight on day 1 and then every alternate day up to day 9. Erythrocytes from these mice were used when the reticulocyte count reached above 50 % as determined by methylene blue staining.

Plasmodium berghei *invasion assay*

The assay was performed in 4 sequential steps.

(*a*) *Purification of mature* P. berghei *trophozoites by Percoll gradient. P. berghei* is an 'asynchronous' malaria species, all stages of the erythrocytic cycle, except sequestered schizonts, being present in the blood at any given time. We developed a stepwise Percoll gradient method for separating mature trophozoite-infected erythrocytes from immature trophozoite and ring-infected erythrocytes. These purified mature trophozoite-infected erythrocytes were then cultured *in vitro* to allow the parasites to develop to segmentors which were used in our invasion assay. Approximately 1 ml of blood was removed from *P. berghei* infected Balb/c mice with parasitaemias between 15 and 25 % by cardiac puncture into a heparinized syringe. After passing over a 1 ml CF11 column (Whatman International Ltd, Kent, UK) the volume was adjusted to 10 ml with PBS and the cells were centrifuged at 450 ***g*** for 5 min. The pelleted cells were resuspended in 0·5 ml of PBS and carefully layered onto a stepwise Percoll (density 1·13 g/l, Sigma Chemical Company, Poole, Dorset, UK) gradient. To prepare the stepwise gradient 4 ml of the 65 % Percoll solution was loaded into a Corex (Corning Glasswork, NY, USA) centrifuge tube first and this was underlaid with 4 ml of the 75 % solution which in turn was underlaid with 2 ml of the 85 % solution. After the washed cells from the infected mice were layered on top of the gradient they were centrifuged at 5000 ***g*** for 20 min at room temperature. Following centrifugation, the very top layer of cells (showing pigmentation), which did not or only slightly penetrated the top Percoll layer, was removed, added to 10 ml of PBS, centrifuged at 450 ***g*** and the pelleted cells resuspended in 0·25 ml of PBS. This preparation routinely consisted of > 95 % mature parasite-infected erythrocytes. This preparation we refer to as synchronous mature trophozoites.

The 65 % Percoll was prepared by mixing 4·5 ml of Percoll, 1·9 ml of solution A (PBS containing 2·25 % monohydrate glucose) and 0·5 ml of solution B (10 × solution A). The 75 % Percoll was prepared by mixing 4·5 ml of Percoll, 1·0 ml of solution A and 0·5 ml of solution B and the 85 % Percoll by mixing 4·5 ml, 0·29 ml and 0·5 ml of the respective solutions.

(*b*) *Culturing of purified mature trophozoite-infected erythrocytes.* Since *P. berghei* schizonts cannot be obtained by bleeding infected mice, because they sequester in the blood vessels of the liver and spleen, we obtained these *in vitro* using culturing methods similar to those described by McLean, MacDougall & Phillips (1986). Synchronous mature trophozoite-infected erythrocytes, obtained as described above, were cultured *in vitro* in 50 % heat-inactivated foetal calf serum (FCS, Gibco Laboratories, Paisley, Scotland) and 50 % RPMI-1640 (Gibco Laboratories: to which was added 4 ml of 1 M HEPES, 0·5 mg hypoxanthine, 0·2 g glucose, 400 μl of neomycin, 1 ml of 200 mM glutamine and 2 ml of a 5 % sodium bicarbonate solution/100 ml). These cells were maintained at a density of $2{\cdot}5 \times 10^7$ cells/ml in a T25 tissue-culture flask (Nunc, Kamstrup, Denmark) for 5 h at 37 °C in a candle jar (Trager & Jensen, 1976) in which time the parasites had developed into schizonts.

(*c*) *Target erythrocytes.* Target erythrocytes were prepared immediately before use as described above.

(*d*) *Invasion assay.* A total of 2×10^7 target cells was added to 0·8 ml of the 50 % FCS/50 % RPMI-1640 medium, prepared as above, in wells of a 24-well tissue-culture plate. The T25 flask containing the parasitized erythrocytes was removed from the candle jar (after the 5 h culture period) and the cells gently resuspended. Two hundred μl (5×10^6 infected erythrocytes) of this culture were then added to each well containing target cells. The 24-well plate was then gently agitated and placed into the candle jar which was then returned to the 37 °C incubator. Nineteen hours later the contents of each well were removed, dispensed into a 1 ml centrifuge tube and centrifuged at 450 ***g*** for 5 min. The cells were resuspended in 50 μl of FCS, centrifuged again and the pellet resuspended in 20 μl of FCS. Smears were made on glass slides which were Giemsa stained and examined under a light microscope. The invasion of target erythrocytes was estimated by counting the number of erythrocytes infected with ring-stage parasites and expressing this number as a percentage.

Plasmodium chabaudi chabaudi *erythrocyte invasion assay*

P. c. chabaudi erythrocyte invasion assays were carried out in 4 steps similar to those of the *P. berghei* invasion assay but with some necessary differences.

(*a*) *Purification of parasite-infected erythrocytes.* As the intra-erythrocytic cycle of *P. c. chabaudi* is synchronous there is only a requirement to separate infected from non-infected erythrocytes. A single 74% Percoll solution, adapted from the method of Wunderlich *et al.* (1985), was prepared by mixing 9 ml of Percoll, 2·17 ml of solution A and 1·0 ml of solution B. Blood (1 ml) taken from a *P. c. chabaudi* infected CD-1 mouse with a parasitaemia between 15 and 25% was passed over a 1 ml CF-11 column and the volume brought to 10 ml with PBS. The cells were centrifuged at 450 *g* and resuspended in 0·5 ml of PBS. This 0·5 ml was carefully layered on 12·17 ml of the above Percoll solution in a Corex tube and centrifuged at 5000 *g* for 20 min at room temperature. The cells divided into 2 layers, infected erythrocytes (> 95% infected) band at the very top whilst uninfected cells band at the bottom. The top layer was removed and washed once in 10 ml of PBS.

(*b*) *Culturing of parasite-infected erythrocytes.* Parasitized cells were then maintained *in vitro* for 4 h in RPMI-1640 (to which was added 2 ml of 1 M HEPES, 0·5 mg hypoxanthine, 0·2 g glucose, 200 μl of neomycin, 1 ml of 200 mM glutamine and 4·2 ml of 5% sodium bicarbonate solution/100 ml) containing 10% heat-inactivated rat serum (DCU animal room facility).

(*c*) *Target erythrocytes.* The preparation of target erythrocytes was as for the *P. berghei* invasion assay.

(*d*) *Invasion assay.* The invasion assay was carried out exactly as described for *P. berghei* except that the *P. c. chabaudi* culture medium was used.

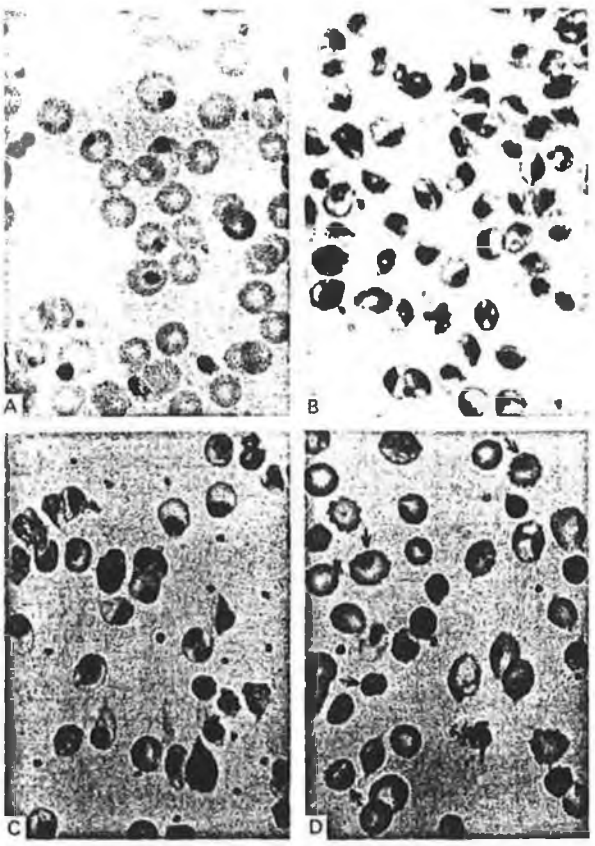

Fig. 1. Purification of *Plasmodium berghei* mature trophozoite-infected erythrocytes, *in vitro* culturing and reinvasion. Blood from an infected Balb/c mouse (A) was layered on a step-wise Percoll gradient as described in the Materials and Methods section. After centrifugation the erythrocytes infected with mature trophozoites banded on the top (B) and were removed, washed and maintained *in vitro* for 5 h (C) before adding to target erythrocytes. Reinvasion was assessed by estimating the percentage ring-stage parasites in erythrocytes 19 h later (D, arrowed). Panel D shows reinvasion into erythrocytes obtained from phenylhydrazine-treated mice.

RESULTS

P. berghei *invasion assays*

The Percoll procedure outlined in the Materials and Methods section can be used for the efficient purification of parasite-infected erythrocytes. This procedure has the added advantage in that the purified *P. berghei* parasites are predominantly mature trophozoites in contrast to the blood obtained from infected mice which also contained ring-form and young trophozoites (Fig. 1A and B). These synchronous mature trophozoites developed to segmentors within 5 h when maintained *in vitro*, although a few had already become schizonts and others had developed further and had ruptured their host erythrocyte (Fig. 1C). When segmented parasites were added to culture wells containing fresh erythrocytes they continued their development, ruptured the erythrocyte and released merozoites that invaded these new erythrocytes. Nineteen hours after adding the parasitized cells to the uninfected cells the percentage of newly infected erythrocytes could be estimated by counting the number of erythrocytes harbouring ring-stage or young trophozoite parasites (Fig. 1D). No invasion was observed into erythrocytes of rabbits or humans. In all experiments erythrocytes of rabbits or humans acted as controls for background invasion. Parasites observed in these controls were usually mature trophozoites and were therefore considered as being ring-stage parasites introduced with the infected mouse erythrocytes.

Using our *in vitro* invasion assay we have shown a preference for reticulocytes directly; Fig. 2 details several of our experiments. In our invasion assay using erythrocytes obtained from 8 to 10-week-old mice we routinely observed invasion to a level of 0·5–2%. Reticulocyte counts in these mice were usually 0·5–1·5%. Only in one case (Fig. 2, Exp. 1) did we observe a higher invasion (2·5%) using

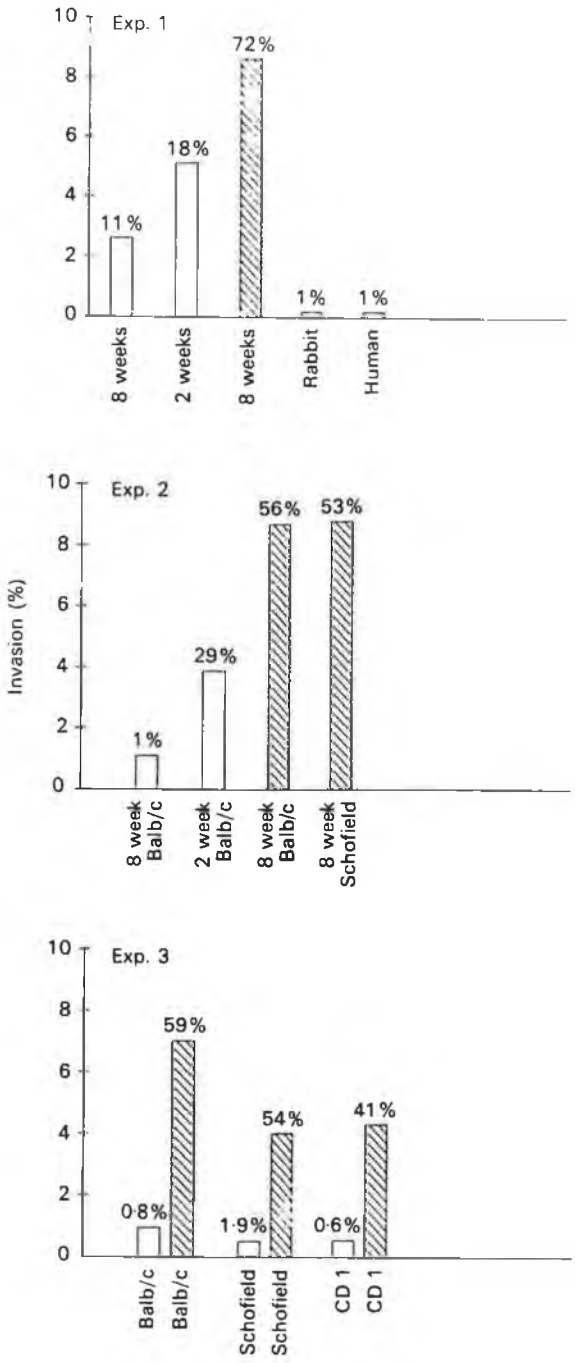

Fig. 2. Invasion of erythrocytes *in vitro* by *Plasmodium berghei*. Exp. 1, comparison of *P. berghei* invasion rates into erythrocytes obtained from 2-week-old mice, 8-week-old mice, phenylhydrazine-treated 8-week-old mice (all Balb/c), rabbit and human. Exp. 2, comparison of invasion rates into erythrocytes of 2-week-old Balb/c mice, 8-week-old Balb/c mice and phenylhydrazine-treated 8-week-old Balb/c and Schofield mice. Exp. 3, comparison of invasion rates into erythrocytes of normal and phenylhydrazine-treated 8-week-old Balb/c, CD-1 and Schofield mice. Percentages at the top of columns represent reticulocyte counts of each particular target erythrocyte preparation. (□) Normal; (▨) phenylhydrazine treated.

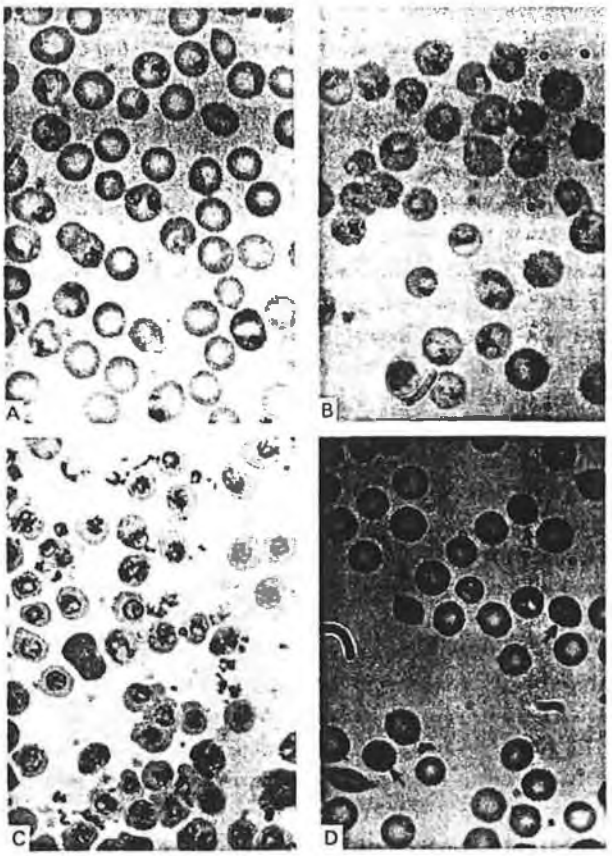

Fig. 3. Purification of *Plasmodium c. chabaudi*-infected erythrocytes, *in vitro* culturing and reinvasion. Blood obtained from infected CD-1 mice (A) was layered onto a 74 % Percoll gradient prepared as described the Materials and Methods section. After centrifugation the parasite-infected erythrocytes banded at the top of the Percoll (B) and were removed, washed and maintained *in vitro* for 4 h (C) before adding to target erythrocytes. Reinvasion was assessed by estimating the number of ring-stage parasites in erythrocytes 19 h later (D, arrowed). (D) Shows reinvasion into erythrocytes obtained from 8-week-old Balb/c mice.

erythrocytes from 8 to 10-week-old mice; however, the reticulocyte count in the blood of the animal used in this experiment was unusually high (11 %). In an attempt to improve our invasion assays we used erythrocyte preparations from very young mice as these animals contain higher reticulocyte numbers in their blood. In the two experiments shown (Fig. 2, Exps 1 and 2) the reticulocyte counts of the 2-week-old mice used were 29 and 17·7 % and the respective percentage invasions were 3·9 and 5·1; hence the invasion of erythrocytes by *P. berghei* merozoites improved. We attribute the lower level of invasion in Exp. 1 as compared to Exp. 2 simply to variation between experiments.

Phenylhydrazine treatment is commonly used to induce reticulocytosis in mice. Using our protocol of 100 μl of 0·4 % phenylhydrazine/15 g body weight given on days 1, 3, 5, 7 and 9 the reticulocyte counts in treated mice would be > 50 % on days 6, 8 and 10.

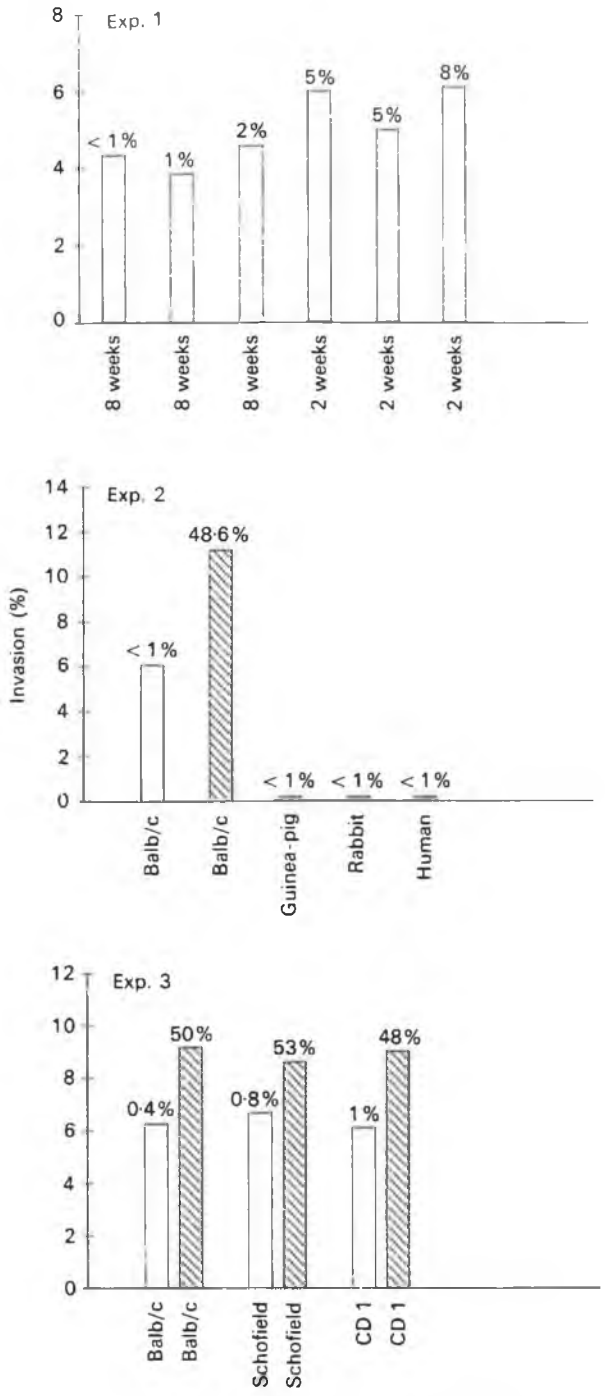

Fig. 4. Invasion of erythrocytes *in vitro* by *Plasmodium c. chabaudi*. Exp. 1, comparison of *P. c. chabaudi* invasion rates into erythrocytes obtained from 2-week-old and 8-week-old Balb/c mice. Exp 2, comparison of invasion rates into erythrocytes obtained from normal and phenylhydrazine-treated 8-week-old Balb/c mice. Human, rabbit and guinea-pig erythrocytes were also tested. Exp 3, comparison of invasion rates into erythrocytes of normal and phenylhydrazine-treated 8-week-old Balb/c, CD-1 and Schofield mice. Percentages at the top of columns represent the reticulocyte counts in each particular target erythrocyte preparation. (□) Normal; (▨) phenylhydrazine treated.

Erythrocyte preparations with high reticulocyte counts were tested in the invasion assay. Inclusion of these cells improved the invasion of *P. berghei* merozoites better than 7-fold over normal erythrocytes (Fig. 2, Exps 1, 2 and 3). Three strains of mice (Balb/c, CD-1 and Schofield mice) were similarly phenylhydrazine treated and their erythrocytes tested in invasion assays and compared to normal erythrocytes of the same strains. Although in Exp. 3 invasion into Balb/c cells was higher than the other two strains tested we attribute this to experimental variation, because in Exp. 2 the invasion into Balb/c and Schofield mice was almost identical. We conclude therefore, that *P. berghei* invades equally the red cells of all mouse strains tested (Fig. 2, Exps 2 and 3).

P. c. chabaudi *invasion assays*

When *P. c. chabaudi* was maintained in CD-1 mice, acclimatized to artificial daylight from 24.00 to 12.00 h, sequestration began at approximately 14.00 h. For the invasion assays mice were bled approximately 1 h before sequestration began, usually at 13.00 h. A simple continuous gradient Percoll step was then used to purify parasite-infected erythrocytes to a level of $> 95\%$ from uninfected erythrocytes (Fig. 3A and B). When these infected erythrocytes were cultured *in vitro* for 4 h the parasites developed into segmentors (Fig. 3C) which, when added to fresh erythrocytes, developed further, ruptured the red cell and the released merozoites invaded target erythrocytes (Fig. 3D).

In our invasion assay *P. c. chabaudi* invaded erythrocytes obtained from 8 to 10-week-old mice at a level of $> 4\%$ (Fig. 4, Exp 1); no invasion occurred into guinea-pig, rabbit or human erythrocytes (Fig. 4, Exp. 2). We also carried out *P. c. chabaudi* invasion assays using erythrocytes obtained from 2-week-old mice and surprisingly found consistently higher invasion levels into these erythrocyte preparations (Fig. 4, Exp. 1). We believed that these results indicated that *P. c. chabaudi* preferably invades young red cells and therefore carried out invasion assays using erythrocytes from phenylhydrazine-treated mice. *P. c. chabaudi* merozoites consistently showed an increased invasion into erythrocyte preparations obtained from phenylhydrazine-treated mice, which in each case examined contained almost an equal ratio of normal erythrocytes to reticulocytes, as compared to erythrocytes from non-phenylhydrazine-treated mice (Fig. 4, Exps 2 and 3).

P. c. chabaudi invasion assays were performed using erythrocytes obtained from phenylhydrazine-treated and non-treated Balb/c, CD-1 or Schofield mice. The results showed that *P. c. chabaudi* invades erythrocytes of different mouse strains at a similar level (Fig. 4, Exp. 3).

Development of reticulocytes in vitro

Erythrocytes from phenylhydrazine-treated mice were maintained in culture for 19 h at a cell density and under similar culturing conditions as in the invasion assays. Reticulocyte counts were performed using methylene blue before and after the culture period. The reticulocyte count, determined by methylene blue staining, decreased from 56·56 % to 1 % during this time period indicating that the reticulocytes were maturing into normocytes within the period of our invasion assay (data not shown). Because of this reticulocyte maturation we were unable to compare the relative invasion of *P. berghei*, or *P. c. chabaudi* into reticulocytes and erythrocytes.

DISCUSSION

In the present report we detail methods for performing erythrocyte invasion assays with two rodent malarias, *P. berghei* and *P. c. chabaudi*. Several experiments are also presented, which are the culmination of many that tested every parameter involved in these assays, and which validate our assays as the results correlate with previously reported *in vivo* and *in vitro* reports.

Necessary requirements for the development of useful invasion assays include highly purified and synchronized parasitized erythrocytes. Purification is necessary as scored invasions must have occurred into the introduced target cells only and synchronization ensures that these invasions happened during the course of the assay. The Percoll centrifugation steps described in the present study for the purification of *P. berghei* and *P. c. chabaudi*-infected erythrocytes are rapid and simple to perform and yields of > 95 % are routinely achieved. This is a much simpler and improved method for preparing synchronous parasites to that described by Mons *et al.* (1985) which involved *in vitro* culturing and *in vivo* passage through rats and resulted in preparations of very low purity.

The invasion assays include an initial culturing of *P. berghei* and *P. c. chabaudi*-infected erythrocytes at a density of $2{\cdot}5 \times 10^7$/ml for 4–5 h prior to transferring 5×10^6 (200 μl) of these cells in fresh medium (0·8 ml) containing the target erythrocytes. Within this period the parasites develop into schizonts and will rupture within several hours of the transfer. This step essentially acts as a medium change and reduces the amount of manipulation of the parasites to a minimum. We have eliminated centrifugation washes as schizont-infected erythrocytes of both malaria species have a tendency to clump and become difficult to resuspend (data not shown). We have also observed that mouse erythrocytes and reticulocytes are very fragile and hence we have reduced manipulation of these cells to a minimum preferring single washes with large volumes to multiple washes. McLean *et al.* (1986) also cultured *P. c. chabaudi*-parasitized erythrocytes for 5–6 h, in RPMI-1640 medium containing 5–10 % FCS to obtain trophozoites/schizonts for immunofluorescence assays. After testing various serum types and concentrations we found that 50 % FCS (heat inactivated) provided the best growth rate for *P. berghei* and 10 % rat serum (heat inactivated) was optimum for *P. c. chabaudi*.

It is well known that *P. berghei* merozoites preferentially invade reticulocytes (Ramakrishnan & Prakash, 1950; Janse *et al.* 1984, 1989; Suhrbier *et al.* 1987). Using our *in vitro* assays we observed a direct correlation between *P. berghei* merozoite invasion and the presence of reticulocytes. Invasion levels into erythrocytes obtained from 8 to 10-week-old-mice which contained 0·5–1·0 % reticulocytes were between 0·5 and 2 %. Only in one case did we observe a higher invasion (2·5 %) into erythrocytes of these mice and this correlated with an unusually high reticulocyte count (11 %). Since reticulocyte levels in the blood of newborns are higher than those in mature animals (Rapoport, 1986) by using erythrocytes preparations from 2-week-old mice (reticulocyte counts 17·7 and 29 %) we improved invasion to 5·1 and 3·9 %. Best invasion levels, however, were achieved using erythrocytes from phenylhydrazine-treated mice as much higher reticulocyte levels (> 50 %) could be obtained. Invasion levels were routinely above 4 % and often above 8 %; in all experiments the invasion into these cells was 7-fold greater than into normal erythrocytes. The variation observed in these experiments, we believe, is due to the difficulty in obtaining identical preparations of reticulocytes in terms of number of reticulocytes, stage of maturation and health. For the purpose of studies on erythrocyte invasion, for example anti-merozoite antibody blocking studies, erythrocyte preparations from 2-week-old or phenylhydrazine-treated mice would give acceptable invasion rates to ensure statistically significant results and although the former preparations result in lower invasion rates they may be simpler to obtain.

Erythrocyte invasion assays for *P. c. chabaudi* are easier to perform when compared to those of *P. berghei* because synchronous parasites are easily obtained and the provision of erythrocyte preparations containing high levels of reticulocytes is not necessary. Invasion levels of 5–6 % were routinely obtained using erythrocytes of 8 to 10-week-old mice. A detailed study of the course of *P. c. chabaudi* (AS strain) parasitaemias in CBA/Ca mice showed that this parasite shows no preference for reticulocytes. When the *in vivo* reticulocyte level was enhanced to equal that of mature erythrocytes (normocytes) by phenylhydrazine treatment prior to infection the invasion frequency of *P. c. chabaudi* into each cell type was similar (Jarra & Brown, 1989). This study supported earlier *in vivo* studies (Carter

& Walliker, 1975). The results presented in this study are interesting to compare to the *in vivo* study of Jarra & Brown (1989) as we show that when *P. c. chabaudi* (AS strain) are presented *in vitro* with erythrocyte preparations with almost equal reticulocyte and normocyte levels invasion is consistently better than into erythrocyte preparations with $< 1\%$ reticulocytes. Our results therefore indicate that *P. c. chabaudi* does show a preference for reticulocytes although this preference is not as marked as in *P. berghei*. This is the first study in which erythrocyte invasion by these two malaria parasites is compared directly and simultaneously. We examined erythrocyte and reticulocyte preparations from three mouse strains and observed similar results indicating that availability of erythrocyte ligands does not influence the course of parasitaemias in different mouse strains. A host genetic factor, mediated through the availability of reticulocytes, may control the course of *P. berghei* parasitaemias *in vivo* (see review by Miller & Carter, 1976). The enhanced invasion of *P. c. chabaudi* into reticulocytes may indicate that this species spontaneously arose from a line that had a preference for reticulocytes similar to *P. berghei* and *P. yoelii*. An example of such a spontaneous divergence already exists for the latter parasite (Yoeli *et al.* 1975).

A clearer understanding of *P. berghei* and *P. c. chabaudi* merozoite invasion into reticulocytes and normocytes would have been obtained if counts of newly invaded parasites into each cell type could have been performed. Reticulocytes *in vivo* mature into normocytes within 24–36 h after release from the bone-marrow into the circulation (Gronowicz, Swift & Steck, 1984). In our *in vitro* conditions we found maturation to be close to the *in vivo* situation; hence by the end of our invasion assays we observed that reticulocytes had matured, losing their ribonucleic acid and could not be visualized with methylene blue or other stains and therefore making it impossible to determine if newly invaded parasites had invaded reticulocytes or normocytes. This observation was important since if reticulocyte-specific ligands for malaria invasion are also lost in such a short time period it is crucial, in order to obtain high levels of invasion, to provide the reticulocytes as close to the time of *P. berghei* merozoite emergence as possible. Therefore, the culturing of *P. berghei*-infected erythrocytes prior to addition to target erythrocytes functions not only in maturing the parasites to segmentors/schizonts but also synchronizes their rupture before much reticulocyte maturation can take place.

In conclusion, we have developed simple assays for the study of erythrocyte invasion by *P. berghei* and *P. c. chabaudi*. As these types of assays were only previously available for the monkey malaria, *P. knowlesi*, and the human malaria, *P. falciparum*, studies on the molecular mechanisms of invasion have been largely confined to these parasites. Undoubtedly, the rodent malarias can contribute immensely to our knowledge of erythrocyte invasion, and because of their ease of maintenance in the laboratory and the availability of the invasion assays detailed here, future information should be more easily forthcoming.

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REFERENCES

CARTER, R. & WALKER, D. (1975). New observations on the malaria parasites of the Central African Republic, *Plasmodium vinckei petteri* subsp. nov and *P. chabaudi* Landau (1965). *Annals of Tropical Medicine and Parasitology* **69**, 187–96.

DALTON, J. P., HUDSON, D., ADAMS, J. H. & MILLER, L. H. (1991). Blocking of the receptor-mediated invasion of erythrocytes by *Plasmodium knowlesi* malaria with sulfated polysaccharides and glycosaminoglycans. *European Journal of Biochemistry* **195**, 789–94.

GRONOWICZ, G., SWIFT, H. & STECK, T. L. (1984). Maturation of the reticulocyte *in vitro*. *Journal of Cell Science* **71**, 177–97.

HADLEY, T. J., KLOTZ, F. W., PASVOL, G., HAYNES, J. D., MCGINNISS, M. H., OKUBO, Y. & MILLER, L. H. (1987). Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B ($M^k M^k$). *Journal of Clinical Investigations* **80**, 1190–3.

HADLEY, T. J., MCGINNISS, M. H., KLOTZ, F. W. & MILLER, L. H. (1986). Blood group antigens and invasion of erythrocytes by malaria parasites. In *Red Cell Antigens and Antibodies* (ed. Garratty, G.), pp. 17–33. Arlington, VA, USA. American Association of Blood Banks.

HAYNES, J. D., DALTON, J. P., KLOTZ, F. W., MCGINNISS, M. H., HADLEY, T. J., HUDSON, D. E. & MILLER, L. H. (1988). Receptor-like specificity of a *Plasmodium knowlesi* malaria protein that binds to Duffy antigen ligands on erythrocytes. *Journal of Experimental Medicine* **167**, 1873–81.

HUDSON, D. E., WELLEMS, T. E. & MILLER, L. H. (1988). Molecular basis for mutation in a surface protein expressed by malaria parasites. *Journal of Molecular Biology* **203**, 707–14.

JANSE, C. J., BOORSMA, E. G., RAMESAR, J., GROBBEE, M. J. & MONS, B. (1989). Host cell specificity and schizogony of *Plasmodium berghei* under different *in vitro* conditions. *International Journal for Parasitology* **19**, 509–14.

JANSE, C. J., MONS, B., CROON, J. J. A. B. & VAN DER KAAY, H. J. (1984). Long-term *in vitro* cultures of *Plasmodium berghei* and preliminary observations on gametocytogenesis. *International Journal for Parasitology* **14**, 317–20.

JARRA, W. & BROWN, K. N. (1989). Invasion of mature and immature erythrocytes of CBA/Ca mice by a cloned line of *Plasmodium chabaudi chabaudi*. *Parasitology* **99**, 157–63.

MCLEAN, S. A., MACDOUGALL, L. & PHILLIPS, R. S. (1986). An indirect fluorescent antibody test which recognizes antigenic variants of *Plasmodium chabaudi*. *IRCS Medical Science* **14**, 896–7.

MILLER, L. H. & CARTER, R. (1976). A review. Innate resistance in malaria. *Experimental Parasitology* **40**, 132–46.

MILLER, L. H., MASON, S. J., CLYDE, D. F., MCGINNISS, M. H. & ROTHMAN, I. K. (1975). Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science* **189**, 561–3.

MONS, B., JANSE, C. J., BOORSMA, E. G. & VAN DER KAAY, H. J. (1985). Synchronized erythrocytic schizogony and gametocytogenesis of *Plasmodium berghei in vivo* and *in vitro*. *Parasitology* **91**, 423–30.

RAMAKRISHNAN, S. P. & PRAKASH, S. (1950). Studies on *Plasmodium berghei* n. sp. Vinke and Lips 1948. 11. Morphology, periodicity and pathogeneity in blood induced infection in mice, rats and garden squirrels. *Indian Journal of Malariology* **4**, 369–75.

RAPOPORT, S. M. (1986). *The Reticulocyte*. Boca Raton, Florida: CRC Press.

SCHRBIER, A., JANSE, C., MONS, B., FLECK, S. L., NICHOL J., DAVIES, C. S. & SINDEN, R. E. (1987). The complete development *in vitro* of the vertebrate phase of the mammalian malaria parasite *Plasmodium berghei*. *Transactions of the Royal Society of Tropical Medici and Hygiene* **81**, 907–9.

TRAGER, W. & JENSEN, J. B. (1976). Human malaria parasites in continuous culture. *Science* **193**, 673–5.

WUNDERLICH, F., SCHILLINGER, G. & HELWIG, M. (1985 Fractionation of *Plasmodium chabaudi*-infected erythrocytes into parasites and ghosts. *Zeitschrift fü Parasitenkunde* **71**, 545–51.

YOELI, M., HARGREAVES, B., CARTER, R. & WALLIKER, D. (1975). Sudden increase in virulence in a strain of *Plasmodium berghei yoelii*. *Annals of Tropical Medic and Parasitology* **69**, 173–8.

Techniques

In Vitro Assays for the Study of Erythrocyte Invasion by Malarial Parasites

J.P. Dalton, J. McNally and S.M. O'Donovan

In vitro assays for the study of erythrocyte invasion by merozoites are available for several primate and rodent malarial species. These assays are essential means by which potential anti-merozoite vaccine candidates are identified. John Dalton, John McNally and Susan O'Donovan describe the various types of invasion assays that are in current use, outline the procedures for performing these assays and add some pointers on interpretation of data derived from them.

The clinical manifestations of malaria are caused by the asexual erythrocytic stages of the parasite. Invasion of erythrocytes by merozoites involves recognition, attachment, re-orientation, junction formation and invagination of the erythrocyte membrane around the merozoite. Inhibition of any of these events would prevent disease; hence identification and characterization of merozoite molecules involved in all of these events is important, as each represents a target to which protective humoral immunity could be directed.

Elucidation of the molecular intricacies of erythrocyte invasion will necessitate the development of methods, *in vitro*, that allow examination of each event in the invasion process. Currently, two types of *in vitro* erythrocyte-invasion assays exist: (1) those that involve mixing schizont-infected erythrocytes with target erythrocytes; and (2) those that mix free merozoites with target erythrocytes. In these assays the number of parasites that invade the target cells is quantified. To examine reagents for their ability to interfere with specific steps in invasion, they are added to these assays and their effects measured by observing the difference in the number of successful invasions compared to appropriate controls. However, these assays do not allow us to dissect the various events of interaction between the merozoite and the erythrocyte surface as the assays are terminated when newly invaded parasites have reached a size within the target erythrocyte whereby they are easily recorded. The rapidity with which merozoites invade erythrocytes makes it difficult for us to halt their movements at will. There is only one step in the erythrocyte invasion process that can be readily impeded, that is, between junction formation and invagination, by the addition of cytochalasin B to the assay. In the presence of cytochalasin B, merozoites will re-orientate and will form a junction, but will proceed no further[1]. This 'freezing' of the invasion process has permitted the development of merozoite attachment assays that can be useful in determining whether a reagent exerts its effect on invasion before or after junction formation[2].

Primate Malarias

Invasion assays that use schizont-infected erythrocytes are the simplest to perform. These assays are used routinely for investigating inhibitors of invasion, antibody blocking of invasion and erythrocyte ligands required for invasion and have been developed for several *Plasmodium* species, including *P. knowlesi*[2], *P. falciparum*[3,4] and *P. vivax*[5]. For these assays it is necessary to obtain synchronous schizont-infected erythrocytes free of uninfected erythrocytes. In cases where erythrocyte ligands required for invasion are being investigated (eg. by enzyme treatment of target cells), any uninfected cells introduced with the schizonts to the assays may obscure true invasion preferences. Infected erythrocytes are removed from the host or from culture when the parasites have reached approximately the six merozoite-infected cell stage, and schizont-infected cells are purified by density gradient centrifugation[2–5]. The purified parasite-infected cells are added to 24-well tissue culture plates, or microtitre plate wells, containing the previously prepared target erythrocytes. The plates are placed in a CO_2 incubator c candle jar at 37° C. For *P. vivax* invasio assays, the percentage of immatur erythrocytes in the target cells increased by processing cells over pe coll or Nycodenz cushions[5,7]. Th schizont : target cell ratio is general 1 : 5 or 1 : 10 with the number c target cells typically 2–5 x 10^7. At thes ratios, sufficient numbers of merc zoites, that allow good statistical analy sis of data, will invade. Alternativel assays can be performed in Eppendor (cheaper microcentrifuge tubes ma leach chemicals that are toxic to para sites) which are then gased (6% CO 3% O_2, 91% N_2) and placed on a rotat ing wheel at 37° C. After allowing tim for rupture, re-invasion and develop ment of parasites to visible ring form (usually six hours for *P. knowlesi* assays overnight for *P. falciparum* assays, an six to eight hours for *P. vivax*), the eryth rocytes are removed from the wel and washed once with foetal ca serum before preparing smears whic are then Giemsa stained. Invasion c erythrocytes is estimated microscop cally by calculating the percentage c erythrocytes containing one or mor ring-form parasites. These percentage usually range from 5 to 12%. It is clear therefore, considering the parasitaemi at which the assay is initiated, tha many merozoites do not successfull re-invade. At which stage of invasior the difficulty for merozoites occurs i not known; unruptured cells are rarel seen.

The assays described above are use ful for testing or searching for erythro cyte invasion-blocking antibodies. The mechanism by which an antibod blocks invasion can be either a specific inhibition of the activity of merozoite molecules, such as enzymes or mol ecules that interact with erythrocyte ligands, or by an agglutination of mero-zoites. These two means of blocking can be differentiated by preparing Fab fragments. Fab fragments of antibodies that block by agglutination will have nc

blocking activity in these invasion assays, whereas those of specific blocking antibodies will. Furthermore, if free merozoites can be obtained, agglutination of these can be tested directly. The level of blocking of invasion by antibody will obviously depend on its concentration, affinity for its epitope and its isotype. Antibody-blocking assays carried out in tissue-culture wells and Eppendorfs may render different results, as the length of time that merozoites are exposed to antibody may differ. In tissue-culture wells, since the cells are static, merozoites invade the erythrocytes immediately surrounding the ruptured cell, suggesting that the distance a merozoite must travel in these assays may be the width of a cell membrane[8].

Caution must be exercised when testing certain reagents in invasion assays that use schizont-infected erythrocytes. Some reagents, such as enzyme inhibitors or polysaccharides[9], may appear to affect the invasion process but may actually inhibit cell rupture. Indeed, reagents demonstrated to inhibit invasion in these assays must be shown to be non-toxic to schizonts. Invasion assays using free merozoites circumvent this problem. However, of the primate malarias, viable free merozoites for these assays can be obtained only for *P. knowlesi*. These merozoites are most simply prepared by the syringe-release method[2]. Schizont-infected erythrocytes are maintained, *in vitro*, in the presence of the proteinase inhibitors, leupeptin and chymostatin. These inhibitors prevent the rupture of the cell and the release of merozoites[10]. The development of the parasites *in vitro* is observed by removing samples from the culture at hourly intervals and examining them microscopically following Giemsa staining. After approximately three hours, when individual merozoites are easily discerned, the cells are then removed from the culture and the proteinase inhibitors washed out by centrifugation. The cells are resuspended in 1 ml medium, 10 µl anti-rhesus erythrocyte serum is added to agglutinate the cells and then the merozoites are released by passing (five times) the cells vigorously through a 25-gauge needle attached to a 1 ml syringe. The suspension is passed over a 4 x 0.5 cm protein A-Sepharose column to remove cell debris and unruptured cells. As free *P. knowlesi* merozoites are viable for only a short time, aliquots of the eluant containing merozoites are added immediately to prepared target cells in Eppendorfs that have been placed in a 37°C waterbath. The tubes are gased and moved to a rotating wheel placed at 37°C. After a period of six hours, smears are prepared and examined as for the previously described invasion assays. The percentage of erythrocytes containing one or more newly invaded parasites is routinely between 5 and 10%. Because of the urgency in combining the free merozoites with the target cells, the exact number of viable merozoites added can not be estimated correctly. However, 2×10^8 schizont-infected cells should release enough merozoites for 5–10 invasion assays[2]. For invasion-blocking experiments, reagents are mixed with the target cells before the addition of merozoites.

To study merozoite attachment, cytochalasin B is added to freed merozoites to a final concentration of 10 µg ml^{-1}. Following addition of the merozoites to the target cells, the tubes are continuously agitated in the waterbath at 37°C for 10 min, after which the cells are fixed by the addition of an equal volume of 4% glutaraldehyde in physiological saline. Attached merozoites are counted under interference microscopy. Samples from each assay are pipetted in the triangular space created by placing one coverslip partially over another. Attached merozoites are scored while tapping the microscope slide in order to jounce the fixed erythrocytes so that all sides are observed and to ensure that the merozoites are indeed attached[1].

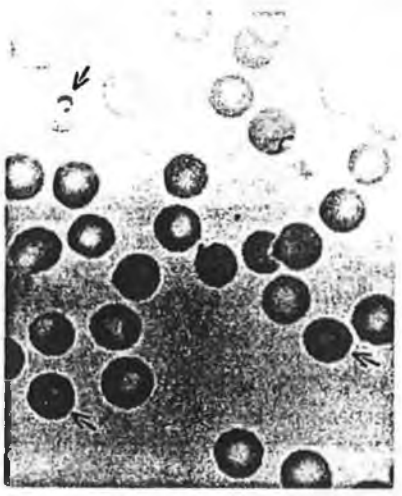

Fig. 1. Photograph of Giemsa-stained smears on a microscope slide, showing newly invaded ring-stage parasites (arrows) of Plasmodium chabaudi chabaudi in target (mouse) erythrocytes 19 h after initiation of the invasion assay, in vitro.

Rodent Malarias

The difficulty in maintaining rodent malarias, *in vitro*, has hampered the development of *in vitro* erythrocyte invasion assays for these species. Some rodent malaria species, such as *P berghei*, are asynchronous, and invade only immature erythrocytes. In addition, erythrocytes containing mature parasites sequester in the deep vasculature.

Recently, erythrocyte-invasion assays have been developed for rodent malaria species *P. berghei* and *P chabaudi chabaudi*[11]. These assays are similar to those described for the primate species using schizont-infected erythrocytes. Infected mice are bled before parasitaemias reach 25%: at higher parasitaemias, multiply infected cells appear and parasites within these cells do not develop to schizonts. Mice infected with *P. c. chabaudi* are bled before sequestration commences. Parasite-infected erythrocytes are purified to >95% by percoll gradients. In the case of *P. berghei* these gradients also provide preparations of erythrocytes infected with late trophozoite forms that are very synchronous. The parasite-infected erythrocytes are maintained, *in vitro*, at 2×10^7 cells per millilitre for six hours to allow the parasites to develop to schizonts. Two hundred microlitres of cell suspension is then removed and added to the target cells in 24-well plates. For *P. berghei*, immature erythrocytes are provided. These cells are obtained by treating mice with phenylhydrazine until the reticulocyte count is above 50%. It takes 19 hours for parasites to rupture from the cells, invade and reach visible ring forms (see Fig. 1). The percentage of erythrocytes containing newly invaded parasites is routinely between 5 and 12%.

An invasion assay using free *P. berghei* merozoites has been described by Janse *et al.*[12], but has not yet been adapted to establish a merozoite attachment assay. Free merozoites are isolated from a culture chamber in which parasite-infected erythrocytes are maintained, and through which medium is continuously passing. The exit of the chamber is covered with a polycarbonate filter, which allows the passage of only free merozoites. Rupture is caused by shearing forces of a magnetic bar which is spinning on the membrane. Freed merozoites are mixed with erythrocyte preparations containing high reticulocyte counts, obtained from phenylhydrazine-treated rats, and cultured for 20 hours. After

this time period, as many as 13% of the target rodent erythrocytes are invaded.

Recently, Braun Breton *et al.*[13] described a method for performing attachment and invasion assays with *P. chabaudi*. Leucocyte-free erythrocytes, obtained from infected mice, are incubated in the presence of the DNA-binding bisbenzimidazole dye, Hoechst 333342. The cells are then maintained, in serum-free culture medium, for 60–90 minutes, and merozoite release followed by flow cytometry. Freed merozoites are washed (to remove contaminating cells and cell debris) and are then mixed with target erythrocytes. After one hour the cells are removed, and attached merozoites quantified by flow cytometry. If the assay is allowed to proceed for six to seven hours, invasion of erythrocytes is quantified by Giemsa staining of smears. Using murine erythrocytes as target cells, under the assay conditions described, 10–20% erythrocytes bind merozoites and 0.7–2% are invaded. To test reagents for their ability to inhibit invasion, freed merozoites are incubated with these for 10 minutes, then washed extensively before adding to target cells.

Conclusion

In vitro erythrocyte invasion assays have contributed greatly to our understanding of the interactions between merozoite and erythrocyte at a molecular level[7]. It is clear that there is still a requirement for assays that permit the study of each step in the invasion process, and it is also obvious that, for many malaria species, invasion assays are not yet available. No doubt these will be forthcoming, and with them will come the identification of more anti-merozoite vaccine candidates.

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References
1 Miller, L.H. *et al.* (1979) *J. Exp. Med.* 149, 172–184
2 Miller, L.H. *et al.* (1983) *J. Clin. Invest.* 72, 1357–1364
3 Mitchell, G.H. *et al.* (1986) *Blood* 67, 1519–1521
4 Clark, J.T. *et al.* (1989) *Mol. Biochem. Parasitol.* 32, 15–24
5 Barnwell, J.W., Nichols, M.E. and Rubinstein, P. (1989) *J. Exp. Med.* 169, 1795–1802
6 Mons, B. *et al.* (1988) *Int. J. Parasitol.* 18, 307–311
7 Mons, B. *et al.* (1988) *Exp. Parasitol.* 66, 183–188
8 Bruce, M.C. *et al.* (1990) *Parasitology* 100, 191–200
9 Hadley, T.H., Klotz, F.W. and Miller, L.H. (1986) *Annu. Rev. Microbiol.* 40, 451–477
10 Hadley, T.H., Aikawa, M. and Miller, L.H. (1983) *Exp. Parasitol.* 55, 306–311
11 McNally, J., O'Donovan, S.M. and Dalton, J.P. (1992) *Parasitology* 105, 355–362
12 Janse, C.J. *et al.* (1989) *Int. J. Parasitol.* 19, 509–514
13 Braun Breton, C. *et al.* (1992) *Proc. Natl Acad. Sci. USA* 89, 9647–9651

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Books

Entomological Field Techniques for Malaria Control

Part 1: Learner's Guide; World Health Organization Publications, 1992. US$13.50/Sw.fr.15.00 (Sw.fr.10.50 in developing countries) (77 pages) ISBN 92 4 154439 2
Part 2: Tutor's Guide; World Health Organization Publications, 1992. US$10.80/Sw.fr12.00 (Sw.fr.8.40 in developing countries) (54 pages) ISBN 92 4 154440 6
NB Both parts are available in English and will be available also in French and Spanish.

This training manual is in two parts: a *Learner's Guide* intended for use by the trainees, and a *Tutor's Guide* to be used by the course instructor. It is designed for the training of public health workers engaged in entomological field work for malaria control, and it can also be incorporated into a longer, more comprehensive training programme on malaria. This is the second module on malaria control to be produced by the World Health Organization, as *Basic Malaria Microscopy* was published in 1991 (see Ref. 1).

The *Learner's Guide* is divided into 13 Learning Units, each containing a series of objectives. Students are expected to be thoroughly conversant with each unit before moving on to the next. The training activities take place in the classroom, the laboratory and the field. The Learning Units contain all the essential information needed to apply a variety of entomological techniques, including: the role of entomological work in malaria control, recognition of anopheline mosquitoes, collection of mosquitoes indoors and outdoors (in exit traps and by using bait), and collecting larvae and pupae. Two additional techniques which perhaps could have been included are the slide-mounting of larvae and pupae, and insecticide susceptibility testing of adult mosquitoes. The text is easy to understand and is complemented with clear, simple line drawings. At the back of the manual, explanations of terms used are included. The activities are nearly all field-based with instructions on equipment required, and on methods of transport for material collected. For some activities, students are encouraged to work in pairs, with each taking a turn to comment on the performance of the other. The importance of good record-keeping is stressed throughout.

The *Tutor's Guide* gives suggestions for the organization of the training programme and sets out the teaching methods that are appropriate for each of the 13 Learning Units. A number of these contain very useful performance-appraisal forms for use in assessing students' progress. Great care is taken during each field activity to ensure the local community is fully aware of the reasons why the work is being carried out. Reference is made in the *Tutor's Guide* to the two techniques which are not covered in the *Learner's Guide*, ie. the pinning of adult mosquitoes and precipitin testing of bloodmeals. Perhaps details of these methods should have been included. Two useful annexes are to be found in the *Tutor's Guide*. The first lists various teaching methods (such as: brainstorming, buzz-groups, case discussion, demonstrations, video tapes and role playing) and their purposes. These permit variation in the way subject matter is relayed to the students. The second annex is a questionnaire to be completed by course participants for evaluation of the training, included with this is a method of analysing their responses.

Considerable thought has been incorporated in these guides to ensure the style of teaching is appropriate to situations that exist in those developing countries where malaria is endemic.

Reference
1 Guy, M.W. (1992) *Parasitology Today* 8, 319

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