Erythrocyte invasion by the rodent malaria *Plasmodium chabaudi chabaudi*.

Thesis

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I hereby declare that this material, which I now submit for assessment on the programme of study leading to the award of PhD 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.

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Date : *13th June 1993*

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Abstract
Erythrocyte invasion is a process essential to the survival of malaria parasites. An understanding of the mechanisms that occur during the invasion event is important, since interruption of any of these events would halt the parasite's asexual cycle and prevent disease. An *in vitro* erythrocyte invasion was developed by first obtaining parasite-infected cells from mice and purifying them using Percoll gradient centrifugation. These erythrocytes were then cultured with target cells, allowed to mature and release daughter parasites which then invaded the target cells. This assay was then used to study the processes involved in invasion. These assays are simple to use and allow a number of tests to be carried simultaneously. Using this system it was demonstrated that *Plasmodium chabaudi chabaudi* shows an *in vitro* preference for immature red blood cells. Whilst RPMI - 1640 is the routinely used medium for the *in vitro* cultivation of malaria parasites, we found that it was not the most suitable for the cultivation of *P. c.chabaudi* parasites. A number of different media were tested by using each medium as the basal medium in an invasion assay. A combination of BME and William's E was found to support the best levels of merozoite invasion and was chosen as the medium of choice for the cultivation of *P. c. chabaudi*.
Target erythrocytes were enzyme treated prior to their addition to invasion assays in order to study the nature of erythrocyte ligands required for parasite invasion. This work led to the proposal that the erythrocyte ligand is a glycoprotein. [^{35}S]methionine-labelled parasites were incubated with murine erythrocytes and a parasite protein of 130 kDa was found to bind specifically to mouse cells. The protein was suggested as a *P. c. chabaudi* receptor for the recognition of erythrocytes. Polyclonal antibodies were raised against various parasite protein preparations. When these antisera were tested in invasion assays, the levels of invasion were reduced significantly. The specificity of the antibodies was also studied using immunoblotting, immunofluorescence and radio-immunoprecipitation and each antiserum was shown to have a characteristic pattern of reactivity. A monospecific antibody against the 130 kDa putative receptor was also obtained by elution of the antibody from nitrocellulose filters containing parasite proteins to which the polyclonal antisera had been bound.

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Abstract
Erythrocyte invasion is a process essential to the survival of malaria parasites. An understanding of the mechanisms that occur during the invasion event is important, since interruption of any of these events would halt the parasite's asexual cycle and prevent disease. An *in vitro* erythrocyte invasion was developed by first obtaining parasite-infected cells from mice and purifying them using Percoll gradient centrifugation. These erythrocytes were then cultured with target cells, allowed to mature and release daughter parasites which then invaded the target cells. This assay was then used to study the processes involved in invasion. These assays are simple to use and allow a number of tests to be carried simultaneously. Using this system it was demonstrated that *Plasmodium chabaudi chabaudi* shows an *in vitro* preference for immature red blood cells. Whilst RPMI - 1640 is the routinely used medium for the *in vitro* cultivation of malaria parasites, we found that it was not the most suitable for the cultivation of *P. c.chabaudi* parasites. A number of different media were tested by using each medium as the basal medium in an invasion assay. A combination of BME and William's E was found to support the best levels of merozoite invasion and was chosen as the medium of choice for the cultivation of *P. c. chabaudi*.
Target erythrocytes were enzyme treated prior to their addition to invasion assays in order to study the nature of erythrocyte ligands required for parasite invasion. This work led to the proposal that the erythrocyte ligand is a glycoprotein. [^{35}S]methionine-labelled parasites were incubated with murine erythrocytes and a parasite protein of 130 kDa was found to bind specifically to mouse cells. The protein was suggested as a *P. c. chabaudi* receptor for the recognition of erythrocytes. Polyclonal antibodies were raised against various parasite protein preparations. When these antisera were tested in invasion assays, the levels of invasion were reduced significantly. The specificity of the antibodies was also studied using immunoblotting, immunofluorescence and radio-immunoprecipitation and each antiserum was shown to have a characteristic pattern of reactivity. A monospecific antibody against the 130 kDa putative receptor was also obtained by elution of the antibody from nitrocellulose filters containing parasite proteins to which the polyclonal antisera had been bound.

ABBREVIATIONS .

BCIP	5 - bromo - 4 - chloro - 3 - indoyl phosphate
Bisacrylamide	*N, N*"-Methylene bisacrylamide
BSA	Bovine serum albumin
CD - 1	Caesarean derived (1959)
DABCO	1, 4 - Diazabicyclo[2, 2, 2]octane
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid disodium salt
ELISA	Enzyme linked immunosorbant assay
E - 64	trans - epoxysuccinyl - L - leucylamido (4 - guanidino) butane
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Giemsa	Azur, eosin methylene blue according to giemsa
HEPES	N - [2 - hydroxyethyl] piperazine - N' [2 - ethanesulfonic acid]
i. p.	intra-peritoneal
NBT	Nitro blue tetrazolium
PAGE	Polyacrylamide gel electrophoresis
PCV	Packed cell volume
PBS	Phosphate buffered saline
PBST	PBS - 0.1 % Tween - 20
PHZ	Phenylhydrazine
PMSF	Phenylmethylsulfonyl fluoride
RPMI	Roswell Park Memorial Institute
RS	Rat Serum
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'- tetramethylethylenediamine
TPCK	N - Tosyl - L - phenylalanine chloromethyl ketone
tris	Tris (hydroxymethyl) aminomethane

Media abbreviations

Medium	Abbreviation
BGJb medium	BGJb
BME - Basal Medium (Eagle)	BME
BME - William's E	BME - WE
CMRL 1066 Medium	CMRL
Dulbecco's Modified Eagle Medium / Nutrient Mix F12 (1:1)	DMEM
McCoy's 5A Medium (modified)	McCoy's
Minimum Essential Medium	MEM
NCTC - 135	NCTC
RPMI - 1640	RPMI-1640
William's Medium E	William's E

Chapter One

Introduction

1. Malaria

Malaria is a disease of humans and animals and is caused by protozoa of the genus *Plasmodium.* All species of *Plasmodium* are by necessity parasites requiring both a vertebrate and an invertebrate (mosquito) host for survival, reproduction and maturation. While the mosquito is unaffected by the presence of the parasite, infection of its vertebrate host results in a disease characterised by symptoms including chills, fevers, headache and other manifestations, and can lead to the death of the host.

Through the disease's direct impact on health, and indirect effects on economic development, migration, military conflict, etc, malaria has played and continues to play an important role in human history. The literature of ancient and modern civilisation contains repeated references to "intermittent" and "malignant" fevers consistent with the diagnosis of malaria. From the writings of Homer, Aristotle, Plato to Chaucer, Pepys and Shakespear, all mention fevers that were undoubtedly malaria related (Bruce-Chwatt, 1988). However it was not until 1846 that Giovanni Rasori proposed that a parasite was responsible for the fever and 1880 when Charles Lavern observed live parasites in the blood of a sick soldier in Algeria. In 1898 transmission of human malaria parasites in *Anopheles claviger* was demonstrated, though there was bitter debate about this until Ross demonstrated the cycle of malaria in a mosquito using an avian model. In 1900 Grassi definitively documented that only *Anopheles* mosquitoes could transmit the human disease. The various developmental blood stages of the parasites were described in the host around this time but it was not until 1948 - 1951 that the liver stage of the parasite was identified and the full life cycle for a range of species elucidated (Garnham, 1966). Malaria has been widely studied in recent decades but due to its complicated life cycle and mode of action, it is not yet fully understood.

It is still prevalent throughout the tropical and sub-tropical areas of the world, although it can exist in any climate that is suitable for the *Anopheles* mosquito. The World Health Organisation (WHO) estimates that there have been in the range of 100 to 300 million cases of malaria over the last decade with one to two million malaria-related deaths annually (Lancet, 1975; Wyler, 1983). These deaths occur mainly

in children under five years of age (New Vaccine Development, 1986). It is estimated that 365 million people live in areas where malaria is endemic and where no specific antimalarial measures are used, while an additional 2.217 billion people live in areas where malaria is endemic but where some measures are used. This implies that nearly one half of the world's population is at some risk (SPRTTD, 1983). Malaria cases among tourists, business travellers, military personnel and migrant workers have been increasing steadily in the last number of years due to increased travel between endemic and non-malarious areas and the now widespread incidence of drug resistant strains of the parasite (Oaks *et al*, 1991). This poses new concerns that the disease will be introduced into currently non-malarious areas.

From the mid - 1940's to the mid - 1950's, the first serious attempts at malaria eradication on an international level were made. These attempts were mainly based on the domestic application of the insecticide DTT (dicophane), which had emerged as the single most effective intervention tactic. In the United States malaria morbidity and mortality decreased to almost zero over a five year period (Bradley, 1966). In 1955 the Eighth World Health Assembly adopted a plan for world wide eradication, based on DTT residual spraying, which would last eight years and consisted of an attack phase of four years and a consolidation phase of four years (WHO, 1955). This would be followed by an indefinite maintenance phase (WHO Expert Committee on Malaria, 1957). While impressive results were obtained in North America and Europe, eradication was not technically or economically feasible in many areas (WHO Expert Committee on Malaria, 1967). The number of cases decreased dramatically in some areas but by the late 1960's malaria was still a serious health threat (Brown *et al*, 1976).

A relatively small amount of research had taken place into malaria until the 1960's when the complexity of the disease and the failure of the eradication programme was realised. Attempts at eradication were still continued but the emphasis now shifted to research and the use of antimalarial drugs. In 1972 DTT was banned due to environmental problems associated with it's use, however the usefulness of insecticides is now doubtful as there are presently 50 mosquito species that have developed resistance to one or more insecticides (Brown,

1983). Resistance to antimalarial drugs such as chloroquinine, mefloquine, halofantrine and Fansidar has become increasingly prevalent. This situation has now been made worse by the emergence of *P. falciparum* strains that have developed multidrug resistance. While the development of new drugs such as artemisinin, as well as agents that reverse chloroquinine resistance continues (Oaks *et al*, 1991), research into vaccine development has become the major hope for prevention of the disease. However no anti-malaria vaccine has been developed to date.

1.1 Malaria, the disease

It is important to distinguish between the "disease" caused by malaria parasites and the frequently asymptomatic "infection" caused by the same parasites. The disease affects individuals who lack certain immunity factors, the specific components of which have yet to be fully determined. There are four species of malaria parasite that cause illness in humans but it is *Plasmodium falciparum* that causes almost all the cases of severe and complicated malaria. Malaria illness frequently comprises of several different, overlapping syndromes, the classical symptoms of which are fever, headache, malaise, cough, nausea, vomiting and diarrhoea. These symptoms are associated with the uncomplicated form of malaria.

Severe malaria has a number of common complications associated with it, among them cerebral malaria, hypoglycemia, anaemia and acute renal failure. Cerebral malaria can be defined as altered consciousness in a patient who has *P. falciparum* parasites in their blood. Current evidence suggests that it is caused by mechanical obstruction of the microcirculation in the brain by sequestering parasitised erythrocytes (MacPherson *et al*, 1985), although tumor necrosis factor (TNF) has also been implicated (Kern *et al*, 1989). Ten to fifty percent of cerebral malaria cases are fatal. Hypoglycemia causes symptoms closely related to cerebral malaria (confusion, coma) and it's coexistence with cerebral malaria in some patients was not suspected until quite recently (White *et al*, 1983). It is especially common in paediatric malaria and is thought to be caused by malaria impairing the liver's capacity to produce glucose. Anaemia is caused by the destruction of erythrocytes

associated with merozoite release. When red blood cell losses are mild, anaemia is well tolerated, but anaemias can quickly become life-threatening in patients with high parasitaemias (Phillips *et al*, 1986; Molyneux *et al*, 1989), while acute renal failure is caused by severe haemolytic anaemia. In *P. vivax* patients, spleenic rupture may occur while *P. malariae* may cause progressive nephritis (Oaks *et al*, 1991).

1.2 Life cycle of the malaria parasite

There are over 100 species of true malaria parasites contained in eleven subgenera of the genus *Plasmodium* (Garnham, 1966), which infect mammals, birds and reptiles (Table 1.1). The species that infect mammals are contained within the three subgenera;

Family	*Plasmodiidae*	
Genus	*Plasmodium*	
Subgenera	*Plasmodium* }	infects all species of
	Laverania }	primates above lemurs
	Vinckeia -	all other species that infect mammals, including rodents.

Despite the large number of species and the range of hosts affected by the malaria parasite, the genus *Plasmodium* has a large number of common characteristics relating to it's life cycle and the basic morphology of it's various developmental stages.

In nature the malaria parasite undergoes over a dozen distinguishable stages of development as it moves from the mosquito vector to it's vertebrate host and back again (Fig 1.1). These stages may be divided into three distinct parts

a) mosquito stage
b) liver stage
c) blood stage

1.2.1 The mosquito stage

The definitive vector for malaria is the female *Anopheles* mosquito of which more than 50 of the 200 known species can transmit malaria (Breuer, 1986). When a mosquito takes a blood meal from an infected

Table 1.1 Different species of malaria parasites that infect a wide range of hosts

HOST	PARASITE
Human	*P. falciparum*
	P. vivax
	P. malariae
	P. ovulae
Monkey	*P. knowlesi*
(Old World)	*P. coatneyi*
	P. fragile
	P. inui
(New World)	*P. brasilianum*
	P. simium
Rodent	*P. berghei*
	P. chabaudi
	P. vinckei
	P. yoelii
Avian	*P. lophurae*
	P. fallax
	P. gallinaceum
	P. retictum
Reptile	*P. maburiae*
	P. vastator
	P. floridense

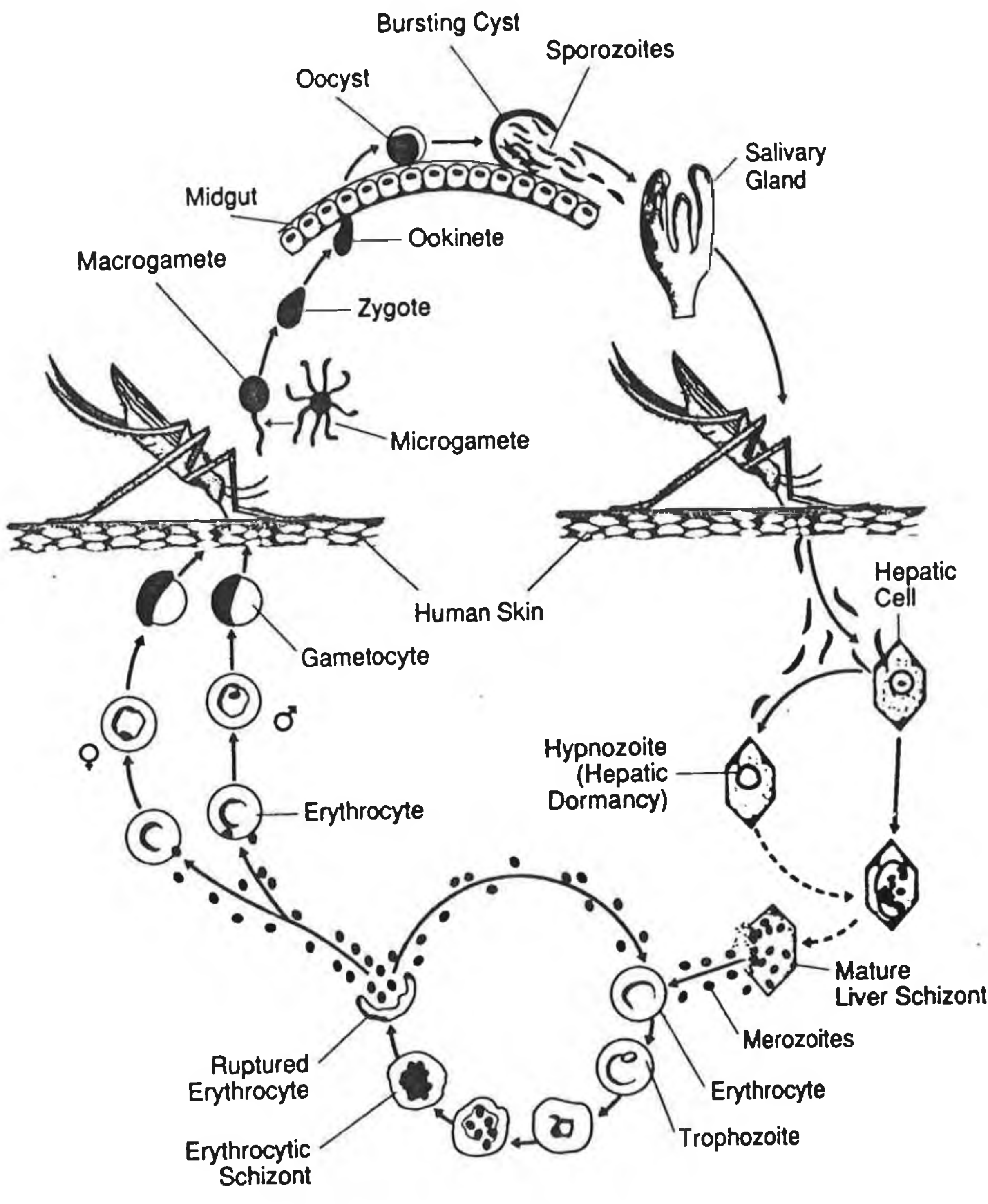


Fig. 1.1 The life cycle of the malaria parasite

individual, it ingests circulating intraerythrocytic sexual stages of the parasite (gametocytes). Within minutes of uptake by the mosquito, the microgametes (male gametocytes) undergo a process of exflagellation and actively move among the erythrocytes ingested by the mosquito in search of a macrogamete (female gametocyte). Fusion of the gametes results in the formation of an "ookinete" which migrates to the wall of the mosquito midgut where it penetrates the peritrophic membrane and epithelium and comes to rest on the external surface of the stomach. Rapid growth of the parasite takes place and a cyst wall develops. Within this "oocyst", motile sporozoites develop, up to 10,000 in a single oocyst while there may be up to 50 oocysts in one mosquito stomach. Ten days to three weeks after the taking of an infected blood meal, the oocyst ruptures and the sporozites enter the mosquito circulation and travel to the salivary glands. There may be as many as 200,000 sporozoites in one mosquito and they assemble in the cells lining the salivary glands. The sporozoites then invade the lumen of the ducts and are discharged with the saliva when the mosquito takes another blood meal.

1.2.2 The liver stage

The second stage begins when an infected anopheline mosquito takes a blood meal on a vertebrate host. The motile sporozoite stage of the parasite is transmitted from mosquito to host via the mosquito's salivary glands. In mammalian hosts, the sporozoites are cleared from the circulation within one to two hours and are localised in hepatic parenchymal cells. Invasion of these liver cells (hepatocytes) can occur within a few minutes. These extra-erythrocytic forms undergo a process of asexual reproduction (schizogony) and after a variable period of time (five to 40 days depending on the species [Breuer, 1986]), the infected liver cells rupture and each discharges several thousand daughter cells (merozoites) into the bloodstream where they invade red blood cells (erythrocytes). Experimental work carried out on *in vitro* invasion with *P. vivax* sporozoites suggests that while human malaria may infect a variety of cell types, only functionally intact hepatocytes may provide the proper conditions for complete extra-erythrocytic development of *P. vivax* (Mazier *et al*, 1984).

Except in highly endemic areas, the number of parasites inoculated by a single mosquito is small, probably fewer than 100 (Rosenberg *et al*, 1990; Beier *et al*, 1991). The efficiency of infection and the uniqueness of the target cell suggest that this invasion is receptor-mediated. However the nature of the ligands and receptors is unknown. It is also unclear whether the parasites enter the hepatocytes directly from the circulation (Shin *et al*, 1982) or whether they are initially recognised by Kupffer cells (Meis *et al*, 1983) or endothelial cells prior to invading hepatocytes.
Schizogony in the liver was thought to proceed without pathological manifestations but recent evidence points to the existence of inflammatory mechanisms and cellular infiltration, including cytotoxic T cells, all of which may be immunologically important (Deans & Cohen, 1983).

1.2.3 The blood stage

The asexual blood stage begins when merozoites are released from the liver into the bloodstream. Merozoites are not motile and come into contact with target erythrocytes via random motion in the bloodstream. Invasion occurs when the merozoite attaches to the erythrocyte membrane via it's apical end which contains various micro-organelles. The erythrocyte membrane undergoes rapid deformation and invagination of the membrane at the point where the parasite is attached, followed by the subsequent envelopment of the merozoite and resealing of the erythrocyte membrane around the parasite (Aikawa *et al*, 1978; Hadley *et al*, 1986; Perkins, 1989; Bannister & Dluzewski, 1990; Wilson, 1990).
After invasion, the parasite lies within a membranous parasitophorous vacuole. The earliest form of the parasite seen in the vacuole is the "ring-stage". It appears as a ring with a vacuolated area in the centre of the parasite surrounded by a delicate ring of cytoplasm and a small nucleus at one side. As the parasite develops it becomes larger and the vacuolated centre is lost, forming the "trophozoite-stage". As the parasite grows it synthesises nucleic acids, proteins, lipids, mitochondria and ribosomes which are assembled into new merozoites. The nucleus divides into two, then four, etc and as maturity approaches, the nuclei

take up peripheral positions around the "schizont" and a small amount of cytoplasm concentrates around each nucleus. These merozoites eventually break free from their host erythrocytes after a process of vesiculation of the erythrocyte membrane and distortion of the erythrocyte due to movement of the intracellular merozoites. The cell swells and eventually ruptures, releasing the merozoites with explosive suddenness (Dvorak *et al*, 1975). The number of merozoites released per infected cell is species specific e.g. 12 - 24 in *P. vivax* and six to eight in *P. chabaudi*, and the entire erythrocytic cycle from invasion to erythrocyte rupture takes between 24 and 72 hours, also depending on the species of the parasite.

Within some of the newly invaded erythrocytes, the parasite does not develop into schizonts, but differentiates into either micro- or macro-gametes. The factors that trigger gametocyte development or sexual differentiation are not well known, though gametocytes do not appear until after a few generations of schizonts have been produced. The parasite grows more slowly (ten days for *P. vivax*) and develops into a large uni-nucleated gametocyte. These are taken up by the mosquito during a blood meal and the sexual cycle begins.

1.3 Cultivation of malaria parasites

During the last 30 years interest has increased into the cultivation of all stages of malaria parasites. There are many reasons for this interest including the fact that many biological, biochemical, chemotherapeutic and immunological problems associated with malaria research can be answered adequately only by the use of reliable cultivation techniques (both long- and short-term). Experimental vaccines have been produced from various stages of the parasite that produce some protection in animals and humans and continuous *in vitro* cultivation of specific stages of the parasite would provide a possible source of antigen for such a vaccine.

Since Bass and Johns (1912) first attempted to culture *P. falciparum* and *P. knowlesi* using a test tube culturing method, only one or at best a few cycles of development were achieved. However in 1976, Trager & Jensen first successfully cultured *P. falciparum in vitro* in continuous culture. Haynes *et al*, (1976) also cultured *P. falciparum* continuously *in*

vitro at this time using slightly different conditions. However the method of Trager & Jensen (1976) became the universally accepted method for the cultivation of *P. falciparum.* Because of this advance many strains of *P. falciparum* are now routinely maintained in laboratories (Trager, 1982) and a number of other species of malaria have been successfully maintained in continuous culture; *P. fragile* (Chin *et al*, 1979),*P. knowlesi* (Butcher, 1979; Wickham *et al*, 1980), *P. cynomolgi* (Nyugen-Dinh *et al*, 1981) and *P. berghei* (Janse *et al*, 1984). *Plasmodium vivax* and *P. chabaudi* have not been cultured continuously although *P. vivax* has been cultured through a couple of cycles of development with a five-fold multiplication rate during the first cycle (Mons *et al*, 1988b). There are many important differences between the earlier attempts of Geiman *et al*, (1946), Trigg and Gutteridge (1971) and Phillips *et al*, (1972) and the successful culturing of *P. falciparum* by Trager & Jensen (1976). These differences include improvements in the areas of the medium used to support parasite growth, the buffering capacity of the culture systems and the nutritional requirements of the parasites and the host erythrocytes, among others.

1.3.1 Medium

The first advance in *in vitro* culturing was made by Geiman *et al*, (1946) with the development of the Harvard medium for culturing *P. knowlesi* parasites through one cycle of development with a four-fold multiplication rate. This medium was modified and adapted over the following years for various malaria species, including *P. falciparum* (Manwell *et al*, 1950; Siddiqui *et al*, 1969; Butcher & Cohen, 1971). In 1976 Trager compared the modified Harvard medium (Trager, 1971) to then available commercial media and found that RPMI - 1640, a medium developed for the culture of human leucocytes (Moore *et al*, 1967) supported prolonged cultivation of *P. coatneyi* and *P. falciparum* . RPMI - 1640 is presently the routinely used medium for the cultivation of all species of malaria parasites.

1.3.2 Buffering

There is a high lactic acid production by parasites in culture, due to an increase in glucose utilisation by infected cells (Homewood, 1977),

which can lower the pH of the medium. The pH of the medium should be maintained at between 7.3 and 7.6 (Geiman *et al*, 1966). In both successful *P. falciparum* culturing systems (Trager & Jensen, 1976; Haynes *et al*, 1976), the cultures were initiated at low densities to reduce lactate production. Although Trager & Jensen (1976) and Haynes *et al*, (1976) used two different media (RPMI - 1640 and Medium 199), they both used two buffering systems. The first system consisted of a zwitterionic buffer (Trager & Jensen used HEPES and Haynes used TES) which improved the overall buffering capacity of the medium and ensured the stability of the pH when the cultures were outside the CO_2-rich atmosphere used for culturing parasites. Both groups also used a bicarbonate / CO_2 system which requires adequate exchange between gaseous and liquid phases, thus the thin layer of cells provided by low density cultures, covered by a shallow layer of medium improved buffering. Trager (1971) found that the thickness of the cell and medium layer was crucial for the development of *P. falciparum* in short-term cultures.

1.3.3 The gas phase

The gas phase is important in the growth of asexual erythrocytic stages as high oxygen concentrations are inhibitory to parasite growth and development. However oxygen does appear to be necessary since complete maturation to the schizont stage of *P. knowlesi* does not occur under anaerobic conditions (Trigg, 1969). Trager & Jensen (1976) found that 1 % O_2 was better than 5 % (CO_2 at 7 %). This low oxygen tension can be provided via either a slow current of the gas mixture being passed over the culture or carrying out the culturing in a candle jar. This is a well known biological method which results in low oxygen and high carbon dioxide levels.

1.3.4 Removal of leucocytes

As far back as 1912 Bass and Johns stressed the removal of leucocytes as essential to parasite cultivation, but no further work was carried out on this aspect of parasite cultivation. Haynes *et al*, (1976) removed leucocytes by passing the parasitised blood over a CF11 column, and while Trager & Jensen (1976) did not use any specific

procedure to remove leucocytes, they did remove plasma and the buffy coat. This procedure would also have removed a large proportion of the leucocytes. Leucocyte removal is now a routine step when culturing *P. berghei* (Janse *et al*, 1984) and during attempts to culture *P. vivax* (Mons *et al*, 1988a). It is possible that leucocytes may damage parasites by their phagocytic activities and their metabolism may lead to nutritional deficiencies and accumulation of toxic products in the medium. Between 75 and 99 % of leucocytes are removed by the use of a CF11 column (Richards & Williams, 1973). To date the effect of leucocyte removal on levels of merozoite invasion has not been investigated.

1.3.5 Serum

Serum is routinely added to cultures at a rate of 10 to 15 %, although Jensen & Trager (1977) found no advantage when 15 % human serum was used instead of 10 % for the cultivation of *P. falciparum*. McNally *et al*, (1992) however found improved levels of invasion in short-term cultures of *P. berghei* cultured with 50 % foetal calf serum (FCS). Serum should always be stored at - 20 ^{0}C and should not be subjected to repeated cycles of freezing and thawing, as this can adversely affect the quality of the serum and inhibit parasite development *in vitro* . Previously human parasites were routinely cultured in human serum, while monkey parasites were cultured in monkey serum. These parasites have now been cultured in other types of serum with no deleterious effects after an initial adjustment period. *Plasmodium falciparum* has been cultured in FCS (Haynes *et al*, 1976) and rabbit serum (Sax & Rieckmann, 1980), while *P. berghei* and *P. chabaudi* are cultured in FCS (Janse *et al*, 1984; Gilks *et al*, 1990). Mouse serum however will actually cause severe inhibition of the asexual development of *P. berghei* (Janse *et al*, 1989).

1.3.6 Nutritional requirements

Various additives to RPMI - 1640 culture medium are said to enhance parasite growth *in vitro*. Osisanya *et al*, (1981) added an extra 2.0 g glucose per litre of culture medium based on the fact that parasitised erythrocytes utilise glucose very rapidly (Homewood, 1977). Geary *et al*,

(1985) later showed that glucose could not be replaced by other sugars. Exogenous purine is required by the parasites (malaria parasites can produce their own pyrimidines) and hypoxanthine is the key purine source (Sherman, 1979; Geary *et al,* 1985). It was reported by Zolg *et al,* (1982) that the addition of hypoxanthine up to 50 µg / ml increased the growth rate of *P. falciparum.*

RPMI - 1640 is a nutritionally complex medium which supports parasite growth but the components which are actually essential to parasite development and re-invasion were not studied prior to it's adoption as the basal medium for malaria parasites. Divo *et al,* (1985) investigated the effects by omission of vitamins and amino acids in the medium and found that pantothenate, isoleucine, methionine, cystine, proline, tyrosine and glutamate were essential for growth. The complete omission of either all of the vitamins or amino acids contained in RPMI - 1640, also resulted in a marked reduction in growth as determined by [^{3}H]hypoxanthine uptake. It is difficult to study the requirements for vitamins and amino acids based on their omission from the culture medium, as red cells and serum would contain most known vitamins and amino acids in sufficient quantities to sustain parasites through one cycle of development. An example of this is the fact that Divo *et al,* (1985), using omission studies, showed that a deficiency of *p* - aminobenzoic acid (PABA) does not affect re-invasion *in vitro*. However PABA has been found to be essential to growth and development of parasites *in vivo* (Jacobs, 1964; Ferone, 1977). This requirement has been indicated by a reduction in parasitaemias in hosts with dietary PABA deficiencies.

With the development of long-term, continuous cultures of *P. falciparum* and other malaria parasites, the culturing process has been upscaled and automated (Trager, 1982), as the petri dish method described by Trager & Jensen (1976) is very labour intensive and produces only limited amounts of parasite material. These semi-automated systems have been developed for static cultures (Trager, 1979) and suspension systems (Ponnudurai *et al,* 1986) in which parasites have been shown to multiply faster and reach higher densities than when in static cultures (Butcher, 1981). Janse *et al,* (1984) however found that the long-term culture of *P. berghei,* using a suspension system, could be

achieved by switching from the fragile mouse cells to more stable rat cells which were not prematurely ruptured by mechanical stirring. Despite advances in the technology involved in continuous culture, the use of the petri dish and candle jar is still a viable method for continuous culture. While the achievement of long term *in vitro* cultivation of malaria is essential to the production of malarial antigens, the elimination of the necessity for animals for parasite maintenance and for the study of the parasites themselves, short-term cultures are also essential. Single cycle cultures were used to investigate the nutritional requirements and culture conditions for malaria parasites described above. Single cycle cultures or invasion assays can also be used to study biological, immunological and chemotherapeutic aspects of malaria parasites.

1.4 Invasion assays

Elucidation of the molecular intricacies of erythrocyte invasion involves the development of methods, *in vitro*, that allow examination of each event in the invasion process (Dalton *et al*, 1993). There are two types of invasion assay available to researchers at the moment. Both are based on culturing parasites through a single cycle of development *in vitro* or through a segment of a single cycle. The two types of assay are:

(i) assays that involve mixing schizont-infected erythrocytes with target erythrocytes

(ii) assays that mix free merozoites with target erythrocytes

1.4.1 Primate invasion assays

Invasion assays that use schizont-infected erythrocytes are simple to perform and are routinely used to study inhibitors of invasion and the erythrocyte ligands required for invasion, as well as antibody blocking of invasion. Assays have been developed for a number of *Plasmodium* species including *P. knowlesi* (Miller *et al*, 1983), *P falciparum* (Mitchell *et al*, 1986; Clark, 1989) and *P. vivax* (Barnwell *et al*, 1989). Schizont invasion assays are basically short-term cultures where schizont-infected erythrocyte are cultured in the presence of target

erythrocytes under conditions suitable for the rupture of the schizonts and the invasion of target erythrocytes by the released merozoites. Time is then allowed for the development of parasites to the visible ring-stage forms. The period of time required for these events *in vitro* varies, usually six hours for *P. knowlesi* assays, overnight for *P. falciparum* and six to eight hours for *P. vivax* assays. The erythrocytes are then removed from culture and washed once in FCS, spread in a thin smear and giemsa stained. Invasion into erythrocytes is estimated microscopically by calculating the percentage erythrocytes containing one or more ring-stage parasites.

When investigating any process connected with invasion, it is important to add only schizont-infected cells to the target erythrocytes, especially when studying the erythrocyte ligands required for invasion. An example of this would be enzyme treatment of target cells where the addition of untreated erythrocytes with the schizont-infected cells may obscure true invasion preferences. To overcome this problem infected erythrocytes are removed from the host or from culture when the parasites have reached approximately the six-merozoite infected cell stage and the schizont-infected cells are purified by density gradient centrifugation. For *P. falciparum* assays schizont-infected cells can be purified by centrifugation through narrow glass tubes (Miller *et al*, 1983) or by using Percoll-sorbitol gradients (Aley *et al*, 1984), while *P. vivax* schizont-infected cells are purified on Nycodenz gradients (Mons *et al*, 1988b). For *P. vivax* invasion assays the percentage of immature erythrocytes (reticulocytes) in the target cell preparation is increased by passing the cells through Percoll or Nycodenz cushions (Barnwell *et al*, 1989; Mons *et al*, 1988b). The schizont to target cell ratio is generally 1 : 5 or 1 : 10 with the number of target cells normally in the region of 1×10^7 -> 1×10^8 / ml. At these ratios sufficient merozoites to allow good statistical analysis of the data are added to the 24-well tissue culture plates, or microtitre wells, which contain previously prepared target erythrocytes. The cells are also at a low enough density for the development and re-invasion of parasites as stressed by Trager (1971). The plates are then placed in a CO_2 incubator or a candle jar at 37 ^oC. Alternatively, assays may be performed in Eppendorfs (cheaper microcentrifuge tubes may leach chemicals that

are toxic to parasites and / or erythrocytes) which are gassed (6 % CO_2, 3 % O_2, 91 % N_2) and placed on a rotating wheel at 37 ^{0}C. In both types of invasion assay the percentage of erythrocytes invaded is between five and 15 % and there is complete rupture of all schizont-infected cells. This implies that many of the released merozoites do not successfully re-invade, a situation that has not been investigated. Invasion assays using free viable merozoites are technically more difficult to perform due to a rapid loss of merozoite viability (Johnson *et al*, 1980) and the requirement for synchronous schizonts. The advantage of the merozoite invasion assay over the schizont invasion assay is that the assay concentrates specifically on the merozoite invasion of the uninfected cell during the assay period. Thus when testing reagents (using a merozoite invasion assay) that appear to have an effect on invasion, they may be found to inhibit rupture and not invasion as determined by a schizont invasion assay (Hadley *et al*, 1983).

Unfortunately among the primate malarias, free viable merozoites can only be obtained from *P. knowlesi* and *P. falciparum*. The most simple method is the syringe release method (Miller *et al*, 1983). Schizont-infected erythrocytes are maintained *in vitro* in the presence of the proteinase inhibitors, leupeptin and chymostatin, which inhibit rupture of schizonts and merozoite release but not schizont development (Hadley *et al*, 1983). After approximately three hours when individual merozoites are easily discernible, the cells are resuspended in one millilitre of RPMI -1640 containing ten microlitres of anti-rhesus serum to agglutinate the cells. The merozoites are released by passing the cells ten times through a 25-gauge needle. The suspension is passed over two 4 x 0.5 cm protein A -Sepharose CL-4B columns to remove cell debris and unruptured cells. Aliquots of the supernatant are added immediately to target cells in Eppendorfs, gassed and incubated for six hours. Thin smears are made and examined as before. The percentage erythrocytes containing new rings is routinely calculated to be between 5 and 10 %. *Plasmodium knowlesi* merozoites can also be isolated using a polycarbonate sieve in the base of a perspex culture chamber. The chamber contains medium with schizont-infected erythrocytes and a stirring bar which aids in the rupture of the schizonts. The released

merozoites are forced though the sieve with medium and are relatively free of contaminating cell debris as determined by electron microscopy (Dennis *et al*, 1975). Johnson *et al*, (1980) showed that at 37 ^{0}C, *P. knowlesi* merozoites had an invasive half-life of approximately four minutes, which could be extended to 15 - 30 minutes by handling the merozoites at 23 ^{0}C. Merozoites from *P. falciparum* are also obtained using a sieve apparatus (Mrema *et al*, 1982).
To study merozoite attachment as a separate event cytochalasin B is added to free merozoites at a final concentration of 10 μg / ml. Cytochalasin B-treated merozoites are able to attach to erythrocytes but are unable to complete the invasion process (Miller *et al*, 1979). After the addition of treated merozoites to target cells, the Eppendorfs are continuously agitated in a 37 ^{0}C water bath for ten minutes, after which the cells are fixed in a glutaraldehyde / saline solution and the attached merozoites are counted by interference microscopy.

1.4.2 Rodent invasion assays

Until recently the development of rodent invasion assays was hampered by the difficulty in maintaining rodent malaria species *in vitro*. Some rodent malarias such as *P. berghei* are asynchronous and *vivax*-like in their preference for reticulocytes (Hingst, 1938; Kitchen 1938; Ramakrishnan & Prakash, 1950). In addition, erythrocytes containing mature parasites sequester in the deep vasulature. However McNally *et al*, (1992) have developed an invasion assay similar to those for primate malarias using schizont-infected cells. Mice are bled before sequestration begins and the parasites are purified by Percoll density centrifugation and cultured for six hours to allow schizonts to develop. The schizonts are added to target erythrocytes and cultured for a further 19 hours to allow rupture, re-invasion and development of the parasites to visible ring forms. As *P. berghei* has a preference for reticulocytes, mice are treated with phenylhydrazine to raise the number of reticulocytes in the target cell preparation (> 50 % reticulocytes). The percentage invasion is calculated as before and 5 to 15 % invasion is routinely achieved.
An invasion assay using free *P. berghei* merozoites has been recently described by Janse *et al*, (1989) but it has not yet been adapted to a

merozoite attachment assay. The method for obtaining merozoites is similar to the sieve method used for *P. knowlesi* (Dennis *et al*, 1975). Free merozoites are isolated from a culture chamber in which parasite-infected erythrocytes are maintained and through which medium is continuously passed. Rupture is caused by the shearing forces of a stirrer spinning on the membrane. The freed merozoites are passed through the membrane with the medium and the merozoites are immediately put into culture with target cells. The cells are incubated for 20 hours and after this time up to 13 % of the target cells may be invaded.

David *et al*, (1978) obtained free merozoites by binding *P. chabaudi* schizont-infected erythrocytes to an immobilised Concanavalin-A column and eluting the released merozoites (the merozoites do not bind to the column). The yield of merozoites is poor with this method due in part to low merozoite viability and in part to re-invasion occurring on the the column. However no invasion assay is available to utilise these merozoites. Braun-Breton *et al*, (1992) recently described a method for performing attachment and invasion assays with *P. chabaudi* parasites. Leucocyte-free erythrocytes, obtained from infected mice, are incubated in the presence of the DNA-binding bisbenzimdazole dye, Hoechst 333342. The cells are maintained in serum free culture medium for 60 - 90 minutes and merozoite release is followed by flow cytometry. Freed merozoites are washed to remove any contaminating cells and cell debris, and are then mixed with target erythrocytes. After one hour the cells are removed and the attached merozoites quantitated by flow cytometry. If the assay is allowed to proceed for six to seven hours the invasion of erythrocytes is quantitated by Giemsa staining of smears. Using murine erythrocytes as target cells, under the assay conditions described, 10 - 20 % of erythrocytes bind merozoites, but only 0.7 - 2 % are invaded.

1.4.3 Uses of invasion assays

Both types of invasion assay have been widely used to study *in vitro* a variety of processes and to correlate results obtained *in vitro* with *in vivo* observations, in order to present more complete picture of the actual process. The ability to obtain viable *P. knowlesi* merozoites and

the development of an invasion assay allowed detailed study of the mechanism of erythrocyte invasion by merozoites (Dvorak *et al*, 1975; Aikawa *et al*, 1978). This mechanism has since been shown to apply to other malaria species such as *P. berghei* (Janse *et al*, 1989) and *P. falciparum* (Langreth *et al*, 1978).

Invasion assays have also been used to study the susceptibility of various host cells and to study possible erythrocyte receptor molecules based on enzyme and chemical treatment of the cells (Miller *et al*, 1973; Miller et *al*, 1977a). The effect of polyclonal antiserum to parasite proteins and monoclonal antibodies to specific parasite molecules were also studied for their possible blocking effects *in vitro* (Miller *et al*, 1983).

1.5 Invasion of red blood cells

The ability to invade new erythrocytes is crucial to the survival of malaria parasites and if parasite penetration of erythrocytes could be interrupted, the erythrocytic cycle of the parasite would be broken and the infection terminated. The stages of the invasion sequence can be simply categorised as recognition, attachment, junction formation and entry of the parasite. Both the resistance and susceptibility of animal species to particular malaria parasites correlate with surface interactions between the blood cell and the parasite i.e. recognition and attachment. More specifically Miller *et al*, (1977a) have shown that there is an association between "receptors" for malaria parasites and the erythrocyte surface "ligands".

Merozoites of *P. knowlesi*, the most studied species, are ellipsoidal in shape with an anterior apical protuberance. The nucleus is situated at the posterior end, surrounded by a typical nuclear envelope, and mitochondria and ribosomes have been observed in the cytoplasm. Of the various membrane-bound organelles, the most prominent is a pair of organelles with electron-dense interiors called the rhoptries, and their associated microbodies called micronemes. The presence of rhoptry bodies with ducts leading to the apical end of the merozoite is a characteristic feature of invasive forms of all Sporozoa (Bannister, 1979). In addition to a plasma membrane with the usual unit structure, the merozoite is covered by an electron-dense surface coat consisting

of outward-projecting filaments. Due to availability of sufficient quantities of viable *P. knowlesi* merozoites, this species has been used for the majority of research into invasion. However from observations with *P. berghei*, *P. gallinaceum* (Ladda *et al*, 1969) and *P. falciparum* (Langreth *et al*, 1978) the general morphology of merozoites and the fine structure of invasion are very similar for all malaria species. Merozoites are non-motile, however as the merozoites are surrounded by erythrocytes in the bloodstream they do not require a high degree of mobility to contact target erythrocytes. Contact and attachment can occur between any surface of the merozoite and the erythrocyte (Dvorak *et al*, 1975; Ladda *et al*, 1969), however only if recognition of specific receptors occurs can invasion proceed. If as a result of a random contact, the merozoites initial contact is between the side of the merozoite and the erythrocyte, the merozoite appears to reorient itself until it's apex becomes opposed to the surface of the target erythrocyte (Miller, 1977).
In 1969 Ladda *et al*, reported the invasion of erythrocytes by merozoites of *P. berghei* and *P. gallinaceum* and established that merozoites enter within an invagination of the erythrocyte membrane rather than by penetrating it (Fig. 1.2). After contact, a junction forms between the merozoite and the host cell (Aikawa *et al*, 1978).The junction is characterised by an electron dense area beneath the bilayer of the erythrocyte membrane which extends only to regions of the erythrocyte in direct opposition with the merozoite. It appears to be a circumferential attachment at the orifice of the parasitophorous vacuole. The region of contact between the erythrocyte and the parasite is characterised by a very close opposition of the two membranes (approximately ten nanometres). Correct orientation results in a rapid and marked deformation of the erythrocyte for a period of five to ten seconds (Dvorak *et al*, 1975). The merozoite remains attached to the erythrocyte but does not enter the cell during this initial deformation. Although in thin sections, analysed by transmission electron microscopy, the junction at the neck of the invagination is perceived as a limited region of membrane contact, it is thought to delineate a ring which encircles the merozoite throughout it's entry (Breuer, 1986). In fact merozoites of several species have been repeatedly observed to be

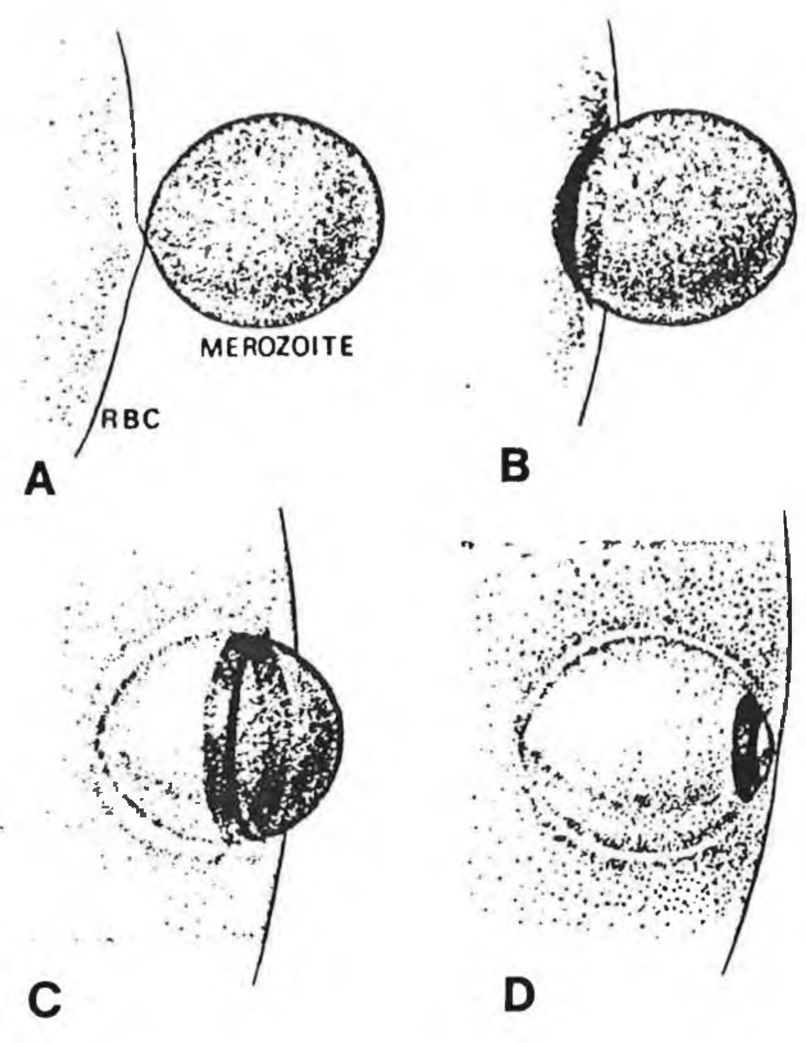


Fig. 1.2 A schematic representation of invasion. The merozoite attaches via it's apical end (A) and a junction is formed (B, arrowed). The junction moves from the apical end (B) to the posterior end (C), bringing the merozoite into the parasitohorous vacuole (D).

distorted as they pass through the junction ring, apparently squeezing through an inflexible opening (Bannister *et al*, 1975; Aikawa *et al*, 1978). As invasion proceeds, the junction moves as a circumferential band from the apical to the posterior end of the merozoite and the merozoite is slowly brought within the invaginated erythrocyte membrane. The erythrocyte and the parasite only remain in contact at the junction and the apical region. The junction fuses at the posterior end of the merozoite, enclosing the parasite within a vacuole originating from the inverted erythrocyte membrane. The merozoite membrane still remains in close contact with the thickened erythrocyte membrane at the point of final closure (Aikawa *et al*, 1978). During the invasion process the distribution of the surface coat alters with the rough surface coat of the merozoite disappearing during entry while the face of the invaginated membrane which forms the parasitophorous vacuole has a greatly reduced number of intramembranous particles compared to the intact membrane (Wilson, 1982).The internalisation of the parasite is an active process on the part of the parasite as the erythrocytes are non-phagocytic (Jensen & Hammond, 1974). It is thought that the release of the contents of the rhoptries during the invasion process is connected with the widespread deformation that occurs prior to parasite entry and vacuole formation as these processes are not due to physical pressure from the merozoite (Breuer, 1986).
Following internalisation, the erythrocyte is again deformed. This second wave of deformation continues intermittently for ten to 15 minutes, after which the parasite becomes quiescent and the erythrocyte resumes its biconcave shape accompanied by a slight swelling. The parasite is still exposed to the external milieu through the orifice of the invagination. The orifice and membrane lesion permit sodium, water, etc to enter at a rate in excess of it's normal exchange capacity, while preventing the movement of larger molecules (Dvorak *et al*, 1975).

1.5.1 Species specificity of invasion

The individual species of malaria parasites are quite limited in the range of hosts they infect in nature and in the laboratory (Garnham,1966). This is illustrated in Table 1.2, which provides a partial list of some of the commonly studied *Plasmodium* species and their

Table 1.2 Commonly studied malaria species

Plasmodium species	Natural host	Studies on RBC invasion	Maintenance in the laboratory
P. falciparum	Man	+	Aotus monkey,chimpa -nzee,continuous culture *in vitro* in erythrocytes
P. malariae	Man, chimpanzee	-	Aotus monkey
P. vivax	Man	+	Aotus monkey,chimpa -nzee
P. ovale	Man	-	Chimpanzee
P. knowlesi	Old World monkeys	+	Rhesus monkey, continuous culture *in vito* in erythrocytes
P. cynomolgi	Kra,Rhesus and other monkeys	+	Rhesus monkey, continuous culture *in vitro* in erythrocyt
P. berghei	Forest rodents	+	Mice,rats,continuous culture *in vitro* in erythrocytes
P. yoelii	Forest rodents	+	Mice
P. vinckei	Forest rodents	-	Mice,rats,hamsters
P. chabaudi	Forest rodents	+	Mice
P. lophurae	Fire backed pheasant	+	Ducks,chicks,culture *in vitro* in and out of erythrocytes
P. gallinaceum	Jungle fowl and domestic hens in Asia	+	Ducks,chicks

hosts. As the growth and propagation of malaria parasites *in vivo* is governed by a multiplicity of factors, evidence of host specificity at the level of red blood cell invasion has been derived from *in vitro* studies. The first demonstration of specificity for host erythrocytes was provided by McGhee (1953), who showed that *P. lophurae* had a preference for duck erythrocytes over chick erythrocytes. Butcher *et al*, (1973) found that erythrocytes from Rhesus, Kra and Aotus monkeys as well as cells from man were susceptible to invasion *in vitro* by *P. knowlesi*, whereas erythrocytes from bush baby, hamster, rabbit, guinea pig, mouse and pig were completely resistant to invasion. Parallel results were obtained by measuring merozoite attachment to erythrocytes, and these in turn, agreed with *in vivo* studies of infectivity (Breuer, 1986). These observations confirmed the observations of Miller *et al*, (1973) and Mason *et al*, (1977). *Plasmodium falciparum* will not invade Rhesus monkey or subprimate erythrocytes but will penetrate into human, chimpanzee and Aotus monkey erythrocytes (Miller *et al*, 1977b). These *in vitro* observations closely parallel *in vivo* susceptibility. Thus the ability of malarias to infect specific hosts is determined at least in part, by the host erythrocytes.

1.5.2 Erythrocyte age specificity of invasion

Some *Plasmodium* species have been observed to preferentially invade red blood cells of different ages within the same host. *Plasmodium ovale, P. vivax, P. berghei, P. yoelii* and *P. cynomologi* display a particular preference for reticulocytes, while *P. malariae* infects mature cells and *P. falciparum* invades both mature and immature blood cells (Garnham, 1966). A virulent strain of *P. yoelii* has been described which is distinguished by it's ability to invade both mature erythrocytes and reticulocytes, unlike the avirulent strain which is restricted to reticulocytes (Howard & Miller, 1981).

1.6 Erythrocyte ligand molecules

The malarial merozoite has the unique ability to distinguish between host and non-host erythrocytes. The basis for this selectivity is the recognition by the parasite of specific ligands on the erythrocyte cell surface. Only for three species of *Plasmodium; P. falciparum, P. vivax*

and *P. knowlesi,* have the erythrocyte ligands been defined in any detail (Table 1.3) (Perkins, 1989).

1.6.1 The Duffy blood group antigen

Miller *et al*, (1973) observed a variation in *P. knowlesi* susceptibility of invasion into red blood cells from different species, implying the presence on the erythrocyte cell surface of a specific ligand which mediates invasion by malarial merozoites. When human erythrocytes were treated with chymotrypsin and Pronase, the cells were no longer susceptible to invasion while trypsin and neuraminidase had no effect on invasion. These results implied that a protein ligand existed on erythrocytes for *P. knowlesi.*

In 1975, Miller *et al*, discovered that *P. knowlesi,* a parasite that normally invades human erythrocytes (Miller *et al*, 1973) and infects man failed to successfully invade human erythrocytes lacking the Duffy blood group determinant (a glycoprotein of molecular weight 35 - 43,000 dalton [Hadley *et al*, 1984]). The Duffy negative genotype however is present in approximately 90 % of West Africans (Sanger *et al*, 1955), the Duffy-negative genotype being rare in other groups, and it was observed *in vivo* that this group was resistant to *P. vivax* infection (Boyd & Stratman-Thomas, 1933; Young *et al*, 1955). As it was not possible to study *P. vivax* invasion *in vitro,* Miller *et al*, (1973) used *P. knowlesi* to study this phenomenon *in vitro.* By treatment of Duffy-positive erythrocytes with chymotrypsin and Pronase, the Duffy blood group determinant was removed and *P. knowlesi* merozoites were prevented from invading the cells. These observations mirrored earlier results (Miller *et al*, 1973) when a protein ligand was suggested for the parasite. Invasion by merozoites was also prevented by incubation of homozygous Duffy cells (Fy^a or Fy^b) with anti-Fy^a or anti-Fy^b antibody (Howard & Miller, 1981).

Using interference microscopy *P. knowlesi* was shown to attach to and cause widespread deformation of Duffy-negative erythrocytes but invasion did not follow (Miller *et al*, 1975b). Eventually the merozoite detached from the erythrocyte but failed to attach to any other erythrocytes. Miller *et al*, (1979) revealed that the apical attachment of merozoites and partial invagination of the erythrocyte did occur but that

Table 1.3 Properties of erythrocyte receptors for *P. falciparum, P. knowlesi* and *P. vivax*

	P. falciparum	*P. knowlesi*	*P. vivax*
Major receptor	Glycophorin A,B,C (GPA,GPB,GPC)	Duffy glycoprotein (Fy)	Duffy glycoprotein
MW of receptor	90 kDa (GPA),40 kDa (GPC), 20 kDa (GPB)	35 - 45 kDa	35 - 45 kDa
Evidence for 2^y receptor	Sialic acid independent invasion in some strains	Chymotrypsin insensitive site on rhesus erythrocytes	Not characterised
Erythrocytes invaded	Human,aotus,siamiri, mouse	Human,rhesus saimiri,aotus	Human,saimiri, aotus

junction formation failed to occur. One explanation for this is that two possible ligands, one for attachment and one for internalisation, may be required for the complete invasion process with the Duffy blood group determinants necessary for internalisation.
However in the case of *P. knowlesi* invasion (Miller *et al*, 1977a; Mason *et al*, 1977), it was demonstrated that *P. knowlesi* could invade trypsin and neuraminidase treated Duffy-negative cells, New World monkey erythrocytes and chymotrypsinised chimpanzee and Kra monkey erythrocytes, implying that the actual junction ligand for *P. knowlesi* is somehow associated with but distinct from the Duffy antigens and becomes masked by a third molecule in the absence of Fy^a or Fy^b determinants.
Barnwell *et al*, (1989) carried out *in vitro* invasion assays with *P. vivax* to test the *in vivo* observations with respect to the Duffy blood group determinants as erythrocyte receptors for *P. vivax*. He found that Duffy-negative cells were refractory to invasion as were chymotrypsin and Pronase treated Duffy-positive cells. Invasion by *P. vivax* and *P. knowlesi* was also inhibited by the presence of monoclonal antibodies to the Fy^6 epitope (susceptibility to *P. vivax* merozoite penetration is dependent on the presence of the Fy^6 epitope [Nichols *et al*, 1987]).
Plasmodium vivax has a preference for reticulocytes even though the Duffy glycoprotein is present on reticulocytes and mature erythrocytes. Thus *P. vivax* may also require two ligands for invasion. The Duffy glycoprotein for junction formation and a separate component characteristic of reticulocytes for efficient invasion of human erythrocytes.

1.6.2 The glycophorins

Plasmodium falciparum merozoites invade both Duffy- positive and -negative erythrocytes as well as chymotrypsin- and Pronase-treated cells. They do not invade trypsin or neuraminidase treated cells implying that there are different ligand molecules involved in invasion by *P. falciparum* merozoites compared to invasion by *P. knowlesi* and *P. vivax* merozoites (Miller *et al*, 1977a). The major erythrocyte ligands in *P. falciparum* invasion have been identified as the glycophorins, A, B and C (Miller *et al*, 1977a; Perkins, 1981; Perkins, 1984; Perkins & Holt,

1988 and Pasvol *et al*, 1982). Miller *et al*, (1977b) showed that the reduced invasion into human erythrocytes which lacked glycophorin A by *P. falciparum* parasites implied that glycophorin is not the sole receptor. If it was, there would have been complete inhibition of invasion. En (a-) erythrocytes which lack glycophorin A, contain some glycophorin B and the invasion that does occur may be due to the presence of this other glycophorin, as glycophorins A and B are homologous for the 28 N-terminal amino acids and both contain many O-linked oligosaccarides (Antsee, 1981). Sialic acid on the erythrocyte membrane has been shown to be a requirement for optimal invasion by *P. falciparum* strains reported on to date (Perkins, 1981; Pasvol *et al*, 1982; Breuer *et al*, 1983). As glycophorins A and B are oligosaccarides with O-linked terminal sialic acid residues, this may explain the importance in invasion. It was observed by Mitchell *et al*, (1986) that the degree of dependence on erythrocyte sialic acid varied between strains of *P. falciparum*, based on invasion levels into cells that had been treated with neuraminidase. Neuraminidase is a sialase which removes N-acetylneuraminic acid (NeuNAc), the terminal sugar residues on the glycophorins (Perkins & Holt, 1988). Hadley *et al*, (1987) trypsin and neuraminidase treated M^kM^k cells, which lack glycophorin A and B. Trypsin removes an N-terminal segment of glycophorin A and C, leaving the cells completely lacking in glycophorin and he found that different strains of *P. falciparum* differ in their ligand requirements for invasion. One of these ligands being a trypsin sensitive ligand which is distinct from the glycophorins that is required by *P. falciparum* strain 7G8. Hadley *et al*, (1987) results confirmed the findings of Mitchell *et al*, (1986) about the existence of a glycophorin independent pathway for invasion. Through the study of the effects of various treatments on different cell types, Hadley *et al*, (1987) confirmed the existence of parasite receptor heterogeneity.

These results combined with work based on competitive inhibition of the merozoite surface receptors through the addition of isolated glycophorin A or B, red blood cell extracts containing sialoglycoproteins, distinct carbohydrate structures (glycoproteins), monoclonal antibodies to portions of glycophorin A, etc, lead to a tentative two-step model for invasion (Hermentin, 1987). This model suggested that the initial

attachment of the merozoite surface coat to the erythrocyte may reflect a lectin-like interaction, in which the parasite binds to a specific manner to a cluster of oligosaccharides present on glycophorin A or B (or both). Once attachment has occurred and the apical end of the merozoite has orientated to the membrane, invagination may be triggered, possibly by rhoptry proteins. However the interactions involved are far from clear still.

1.6.3 Other erythrocyte ligands

Band 3 (a major membrane spanning protein in human erythrocyte membranes) has also been suggested as a possible ligand for *P. knowlesi* as monoclonal antibody to Rhesus erythrocyte Band 3 can prevent invasion by this parasite in invasion assays (Miller *et al*, 1983). Meanwhile two recent studies have provided strong evidence that Band 3 could be a ligand during erythrocyte invasion by *P. falciparum* (Okoye & Bennett, 1985; Friedman *et al*, 1985). Friedman *et al*, (1985) have suggested that erythroglycan, the polylactosamine moiety of Band 3 may be the primary binding site during invasion. Hence there may be some common receptor molecules shared by different parasite species.

1. 7. Parasite molecules involved in erythrocyte recognition and invasion

Erythrocyte invasion by the malarial merozoite is a multistep process initiated by the receptor-mediated binding of the parasite to it's host cell. While specific ligands are required on erythrocytes for invasion to occur, the species specificity of receptor recognition implies that there must be present on the merozoite surface, a receptor protein or proteins that bind to the erythrocyte. The study of these receptor molecules can greatly aid our knowledge of the process by which merozoites identify host and non-host erythrocytes and to understand the invasion process at a molecular level.

Protection against malaria has been transferred to nonimmune individuals by immune sera in human, simian and rodent infections (Coggeshell & Kim, 1937; Cohen *et al*, 1961; Sadun *et al*, 1966; Diggs & Osler, 1969; Wilson & Phillips, 1976). The protection was associated

with IgG (Cohen *et al*, 1961) and was possibly due to the inhibition of invasion by merozoites (Cohen *et al*, 1969; Miller*et al*, 1975b; Chulay *et al*, 1981). Inhibition of invasion by antibodies *in vitro* suggests that merozoite surface antigens can Induce protective antibodies. Target antigens are being identified as potential immunogens for vaccine development. The parasite receptor molecules are also potential immunogens, since antibodies to them should block invasion. However it is possible that receptors are not natural immunogens but they may be effective if presented to the host as purified molecules in the absence of other malarial antigens. Care must be taken as proteins that bind to the erythrocyte surface and that elicit an antibody response, may not be involved in erythrocyte recognition and invasion, eg. a 130 kDa soluble protein that binds to soluble glycophorin and erythrocyte membranes but is not involved with the invasion process (Perkins, 1988).

1.7.1 ***Plasmodium falciparum*** **erythrocyte receptor molecules**

A number of molecules have been identified as possible receptors for *P. falciparum*. It has already been shown that *P. falciparum* parasites bind to erythrocytes which have sialic acid-containing molecules in their membranes. A soluble molecule of 175 kDa (EBA - 175) was identified in culture supernatents by Camus & Hadley (1985), which binds to sialic acid-containing determinants. It was found to bind to susceptible erythrocytes but not to trypsin or neuraminidase treated erythrocytes (treatments that render human erythrocytes resistant to *P. falciparum* invasion). Binding of EBA - 175 did not occur in human Tn cells. These cells, which resist invasion, lack sialic acid on the O -linked tetrasaccharides of the glycophorins (Camus & Hadley, 1985; Hadley *et al*, 1988). Merozoites were incubated with radio-labelled *P. falciparum* culture supernatant and the protein was found to bind to merozoites. Based on the evidence that EBA - 175 binds to both merozoites and erythrocytes, it has been postulated that the protein has two domains, one for the merozoite and one for the erythrocyte, so that the protein forms a bridge between the two. Orlandi *et al*, (1990) found that there was a great similarity, both biochemically and immunologically between

EBA - 175 from different strains of *P. falciparum.* The protein is first synthesised during schizogony and is a membrane-bound precursor that is concentrated apically in merozoites. It is not known when release of the protein begins or terminates, though some protein is detectable on the surface of released merozoites. Sim *et al,* (1990) developed antibodies against a synthetic peptide comprising of amino acid residues 1062 - 1103 (EBA - peptide 4 whose nucleotide sequence is conserved among a number of strains of *P. falciparum*). This antibody blocked red blood cell binding and inhibited invasion. The gene encoding EBA - 175 was cloned and characterised and it was found that the protein's primary structure is distinct from other proteins implicated in erythrocyte invasion, such as rhoptry proteins (Jungery *et al,* 1983; Perkins, 1984; Sam-Yellowe & Perkins, 1990).

A second sialic acid-dependant receptor (Pf200, also known as gp 195, PMMSA and MSA 1) has been identified which is a major surface antigen of *P. falciparum* schizonts and merozoites and which is processed to generate three merozoite surface antigens of 83, 42 - 45 and 19 - 25 kDa (Perkins & Rocco, 1988). A role for Pf 200 in merozoite invasion was initially suggested as binding is inhibited by soluble glycophorin and the protein does not bind to neuraminidas-treated cells (Perkins, 1981) while it's role in the initial recognition of human erythrocytes is indicated by the fact that the antigen only binds to erythrocytes susceptible to infection by *P. falciparum* (Perkins & Rocco, 1988). The immunological importance of Pf200 has been shown from experiments in which immunisation of saimiri (Perrin *et al*, 1984), aotus monkeys (Hail *et al,* 1984; Siddiqui *et al*, 1987) and humans (Patarroyo *et al*, 1988) with purified Pf200 and synthetic peptides of Pf200 was found to be protective against subsequent challenge with *P. falciparum* infection.

The rhoptries and their associated organelles, the micronemes, which are located at the apical end of the merozoite, play a critical role in the steps of invasion following receptor binding. The rhoptry proteins (80, 60, 42 /40 kDa - low molecular weight complex; 150, 135 and 110 kDa - high molecular weight complex; 225 and 55 kDa) have had no specific function associated with them though the high molecular weight complex does bind to erythrocyte membranes (Brown & Coppel, 1991),

while enzyme treatment does not affect binding (Perkins, 1989). However antibodies against rhoptry molecules are protective in active immunisation studies and passive transfer experiments *in vivo* and they inhibit parasite growth *in vitro* (Holder & Freeman, 1981; Schofield *et al*, 1986).

1.7.2 *Plasmodium knowlesi* merozoite receptors

A 135 kDa soluble protein was found to be released into the supernatant of *P. knowlesi* (a monkey malaria) cultures during merozoite release and reinvasion (Haynes *et al*, 1988). This molecule binds specifically to Duffy-positive cells and anti-Duffy antibodies can block this binding. Dalton *et al*, (1990) found that the receptor binding correlates to the parasite's ability to invade enzyme-treated erythrocytes. The receptor binding was found to be blocked by sulphated sugars which also blocked *in vitro* invasion of human erythrocytes by *P. knowlesi* merozoites, implying that the binding site on the Duffy molecule is a negatively charged ligand. This is analogous to the sialic acid requiring receptor of *P. falciparum.* Antibodies prepared against the 135 kDa Duffy binding protein were used in immunolocalisation studies and revealed that the receptor is located in the micronemes of the merozoite (Adams *et al*, 1990).

A second Duffy binding protein of 155 kDa has been identified (Miller *et al*, 1988) which bound to malaria susceptible New World monkeys cells but not to the refractory cells of New World monkeys, implying that the protein bound to a Duffy determinant independent of Fy6.

A *P. knowlesi* 230 kDa has also been identified which selectively binds to erythrocytes, although the binding is not dependent on the presence of the Duffy glycoprotein (Epstein *et al*, 1988; Perkins, 1989)

1.7.3 *Plasmodium vivax* receptor molecules

Plasmodium vivax, a human malaria, also invades Duffy-positive erythrocytes. Wertheimer & Barnwell (1989) demonstrated that the Duffy-receptor binding protein is a polypeptide of 135 kDa, whose binding can be blocked by the presence of a monoclonal antibody directed against the Duffy determinant by purified Duffy glycoprotein. Adams *et al*, (1992) cloned and sequenced the P. *knowlesi* 135 kDa, the *P. vivax*

135 kDa and the *P. falciparum* 175 kDa receptor genes and found that they all had similar structures. On the basis of similar gene structure and amino acid homology, it was shown that the Duffy binding proteins of *P. knowlesi* and *P. vivax* and the sialic acid binding protein (EBA - 175) of *P. falciparum* are members of the same gene family.

1.8 *Plasmodium chabaudi*

Plasmodium chabaudi was first discovered and described in thicket rats from the Central African Republic by Landau (1965) as a rodent malaria species similar to *P. vinckei*. It was further characterised by Carter & Walliker (1975) who attributed it with a number of characteristics.

i) synchronous development
ii) erythrocytic cycle of 24 hours
iii) rings have a thin circle of cytoplasm with one or two chromatin dots
iv) strong preference for mature erythrocytes
v) schizonts contain 4 - 8 merozoites and usually do not occupy more than half of the cell
vi) trophozoites are amoeboid and may cause reddening of the host cell.

Of the above initial characteristics of the parasites, only the preference for mature erythrocytes has been challenged. Ott (1968) described the preference for mature erythrocytes by analysis of parasitaemias in infected mice that have had reticulocytosis induced by phenylhydrazine -treatment of the mice. Ott (1968) found that the course of the *P. c. chabaudi* infection was altered, in that lower parasitaemias were reached in mice that were phenylhydrazine-treated prior to infection. Carter & Walliker (1975) found that the reticulocytes were invaded by *P. chabaudi* parasites when the circulating normocyte population had been depleted by rupturing parasites. However Jarra & Brown (1989), again using phenylhydrazine-treatment of infected mice, found that the invasive behaviour of the parasites *in vivo* reflected the availability of erythrocytes of different ages, with no preference for either mature

erythrocytes or reticulocytes.
Since the initial discovery of *P. chabaudi* a number of wild strains have been isolated (Table 1.4) and an even larger number of clones created. The strain *P. chabaudi chabaudi* AS is a cloned line. Following infection of NIH and CBA / Ca mice it results in an acute primary parasitaemia lasting 12 to 20 days, followed by one or two patent recrudescences. Within two months of infection most of the mice achieve a self-cure. The 24 - hour asexual cycle is synchronous, with schizogony occurring around midnight. This can be altered by modifying the daylight times to which the mice are exposed.
Some strains of *P. chabaudi* show at least partial sequestration in the last two to three hours of the asexual erythrocytic cycle. *Plasmodium c. chabaudi* AS strain is one such strain, therefore in order to obtain mature trophozoite / schizonts, parasites have to be cultured for five to six hours *in vitro* (McLean *et al*, 1986). Cox *et al*, (1987) reported that the major site of *P. chabaudi* sequestration was the hepatic sinusoids. These workers also provided the first evidence that *P. chabaudi* could provide a rodent model of sequestration as seen in *P. falciparum*, although under the electron microscope *P. chabaudi* does not show "knobs" (the erythrocyte surface antigens which mediate binding to endothelial cells). Sequestration is not a feature of all *Plasmodiae*, though *P. falciparum* is the only human parasite to sequester, undergoing deep vascular schizogony, a significant proportion of which takes place in the post-capillary venular endothelium of the brain. This withdrawal of parasite-infected erythrocytes to the brain, where they could cause an obstruction in the cappillaries, can result in cerebral malaria (McPhearson *et al*, 1985).
McLean *et al*, (1982 a & b) demonstrated that the emergence of recrudescences in *P. chabaudi* infections was both associated with a decline in the effector arm of the immune response and also with the ability of the parasite to undergo antigenic variation. Of 25 recrudescences tested using a passive transfer system, in all but two cases the recrudescences were antigenically different from the parent populations. In 1986, McLean *et al*, developed a triple layered indirect immunofluorescent antibody test which demonstrated that a variable antigen is a parasite-derived antigen on the surface of intact infected

Table 1.4 ***P. c. chabaudi*** **subspecies**

Species	Isolate	Host species	Region of capture	Date of capture
P. c. chabaudi	54 X	*T. rutilans* [1]	CAR[2]	1965
	864VD	*T. rutilans*	CAR	1970
	3AC	*T. rutilans*	CAR	1969
	2AD	*T. rutilans*	CAR	1969
	16AF	*T. rutilans*	CAR	1969
	AJ	*T. rutilans*	CAR	1969
	1AL	*T. rutilans*	CAR	1969
	1AS	*T. rutilans*	CAR	1969

1 *T. rutilans, Thamnomys rutilans*

2 CAR, Central African Republic

erythrocytes. Recrudescences that occur in *P. c. chabaudi* infected mice arise from erythrocytic parasites which have survived when the host's immune response has removed the majority of the parasites from the preceding parasitaemia. The kinetics of the appearance of recrudescent variants have been investigated and it has been found that the variants are already detectable during remission of the primary patent parasitemia (McLean *et al*, 1990).

Plasmodium falciparum, the parasite that is the major cause of fatal cases of malarial is characterised by two main features;

i) antigenic variation

ii) sequestration.

Phenotypic antigenic variation during the course of a malarial infection, as a means of avoiding the host's immune response, was first described by Brown & Brown (1965) in *P. knowlesi* and has since been described in *P. berghei* (Cox, 1962), *P. cynomolgi* (Voller & Rosan, 1969) and *P. falciparum* (Hommel *et al*, 1983). Changes occur in the parasite-derived red cell surface antigens which are expressed late in the asexual cycle.

As has already been stated sequestration is a feature of relatively few of the malarias. Unfortunately it has proved difficult to examine both sequestration and antigenic variation in one model and where *in vivo* systems are described for either phenomenon, they usually involve primate models. *Plasmodium chabaudi* however could be used to study both systems *in vivo*, in the one model system. Gilks *et al*, (1990) confirmed that both limited sequestration and antigenic variation are characteristics of *P. chabaudi* and showed that they were intimately linked and suggested that the ability to express variant surface antigen is a prerequisite for the development of a chronic infection.

Due to the absence of invasion assays for rodent malaria parasites, little is known about the receptors involved in merozoite invasion of mouse erythrocytes. Gabriel *et al*, (1986) showed the existence of a 105 kDa (Pch105) soluble, heat-stable protein which is present on the erythrocyte membrane of *P. chabaudi*-infected erythrocytes. The antigen binds to glycophorin of either mouse or human erythrocytes and is susceptible to Pronase treatment. Pch105 shares these characteristics with a *P. falciparum* antigen of 155 kDa (Pf155), which is deposited on

the erythrocyte membrane on invasion (Perlmann *et al*, 1984). Wanidworanun *et al*, (1987) achieved partial protection against *P. chabaudi* in mice immunised with a purified protein from the membrane of *P. chabaudi*-infected erythrocytes. This antigen had a similar molecular weight (Pc96) and was reactive with a monoclonal antibody to Pch105. A monoclonal antibody to this antigen recognised a 155 kDa antigen on immunoblots of extracts of *P. falciparum*-infected erythrocytes and therefore Pch105 could be a *P. chabaudi* analogue of Pf155. Rodriguez *et al*, (1987) described three P. chabaudi proteins that bound to the glycophorin GP2.1. The proteins were of 126, 105 (possibly Pch105) and 72 kDa, and recognised glycophorins on the surface of mouse erythrocytes, however the relationship between the three proteins during binding is unknown.

Plasmodium chabaudi can also be used as a model to study antigens that may be used as possible vaccine candidates for *P. falciparum*. Two antigens (MSA -1, MSA-2) that are associated with the asexual erythrocytic forms of falciparum malaria have been studied as possible vaccines. MSA-1(185,000 - 195,000 daltons) has been used to immunise monkeys and has shown some degree of protection (Hall *et al*, 1984; Siddiqui *et al*, 1987) while antibodies to MSA-2 (46,000 daltons) have inhibited *in vitro* parasite growth. In *P. chabaudi* a protein similar to MSA-1 of size 250,000 daltons has been identified (Boyle *et al*, 1982) and monoclonal antibodies have been shown to reduce the parasitaemia of subsequent challenge infections (Lew *et al*, 1989).The genetic mechanisms such as recombination, by which malaria parasites generate novel antigenic forms, are of obvious importance to the development of antimalarial vaccines. For example the serological diversity seen in *P. falciparum* MSA-1 appears to be as extensive in *P. chabaudi* MSA-1. Comparisons between *P. falciparum* MSA-1 gene and the MSA-1 gene of *P. chabaudi* (strain IP-IPC1), reveal a similar structure between the two genes (McLean *et al*, 1991). *Plasmodium chabaudi* could this be used to study the generation of these novel antigenic forms of parasite antigens.

To date *P. chabaudi* has been used mainly as an *in vivo* model for the study of various processes involved in the life cycle of malaria and malaria infections due to the absence of an *in vitro* assay system. The aims of this project involved the development a reliable and reproducible *in vitro* invasion assay for *P. chabaudi,* which was simple to use and that allowed a number of assays to be carried out simultaneously. The assay system was then to be used to gain information on the erythrocyte ligands used by *P. c. chabaudi* for invasion of murine erythrocytes. Furthermore, antisera prepared against various parasite extracts were examined for their ability to block merozoite invasion. We also carried out studies to identify *P. c. chabaudi* molecules that bind to murine erythrocyte ligands and hence may be parasite receptors for these.

Chapter Two

Materials and Methods

MATERIALS

Aldrich Chemical Company
Phenylhydrazine - HCl, Triton-X.

Amersham
Amplify, L-[4,5-^{3}H]isoleucine.

Becton - Dickinson
21 G needles, 18 G needles, 1 ml, 2 ml and 10 ml syringes.

Biological Ladoratories
CD - 1 mice.

Bio-Rad Laboratories
Bradford reagent.

Biotrin Research
Protein-A Agarose.

Boehringer Mannheim GmbH
Neuraminidase.

British Drug House
Acetic acid, acrylamide, mercaptoethanol, bisacrylamide, sodium azide, sodium dodecyl sulphate.

Dakopatts
FITC-conjugated rabbit anti-mouse immunoglobulin, FITC-conjugated rabbit anti-rat immunoglobulin, immunohistochemistry pen.

Flow Laboratories
Foetal calf serum, L - glutamine (200 mM).

General Electric Company

Silicone oil.

Gibco

BGJb medium, BME - Basal Medium (Eagle), CMRL 1066 Medium, Dulbecco's Modified Eagle Medium / Nutrient Mix F12 (1:1), McCoy's 5A Medium (modified), Minimum Essential Medium, NCTC - 135, RPMI - 1640, William's Medium E .

Greiner

T25 and T75 tissue culture flasks.

ICN

Ecolite scintillation fluid, trans[^{35}S]methionine.

Kodak

X-ray film.

Labscan

Acetone, methanol, propan-2-ol.

Nikon

Light microscope, ultra-violet microscope.

Nunc

24-well tissue culture plates, 96-multiwell plates.

Pharmacia

Heparin-Sepharose CL-6B.

Menzel

Frosted microscope slides.

Reidel-de-Haen

Ammonium persulphate, bromophenol blue, DMF, giemsa stain, glucose, glycerol, glycine, hydrochloric acid, magnesium chloride-6-hydrate,

potassium dihydrogen chloride, sodium chloride, sodium EDTA, sodium dihydrogen phosphate, sodium hydrogen phosphate, TEMED, tris-(hydroxymethyl)-aminomethane .

Sartorius
Minisart sterile filters (0.2 µm).

Schleicher & Schull
Nitrocellulose paper.

Sigma
BCIP, ß-lactoglobulin, BSA, chymotrypsin, DABCO, DMSO, E - 64, Freund's adjuvant (complete), Freund's adjuvant (incomplete), gentamicin (10 mg / ml), heparin, leupeptin, HEPES, hypoxanthine, leucine, lysine, methylene blue, molecular weight markers, NBT, papain, Percoll, p-nitrophenyl disodium phosphate, PMSF, Pronase, rabbit anti-mouse alkaline phosphate-labelled antibody, rabbit anti-rat alkaline phosphate-labelled antibody, rabbit anti-rat IgG, RPMI - 1640 (incomplete), saponin, sodium azide, sodium bicarbonate solution, sodium m-periodate, TPCK, tri-sodium citrate, trypsin, trypsin inhibitor, Tween-20, zinc chloride.

University College Cork
Guinea pigs, New Zealand White rabbits.

Whatman
CF11, Whatman chromography paper 3MM, Whatman No. 1 filter paper.

Methods

2.1 Animals

Male CD - 1 mice (Biological Laboratories, Ballina), were used for the maintenance of rodent malaria parasites. Prior to infection the mice were adjusted to an artificial light cycle for a minimum of five days. This cycle consisted of 12 hours of daylight beginning at 12 noon (12.00) and ending at 12 midnight (24.00). Male Balb / c and Schofield mice were bred in the animal facility in Dublin City University (DCU), as were female Wistar rats. New Zealand White rabbits and guinea pigs were imported from the animal facility at University College Cork (UCC).

2.2 Maintenance of parasite strains

Two strains of the rodent malaria, *Plasmodium chabaudi* were received as frozen stabilates. *Plasmodium chabaudi chabaudi* AS strain was kindly donated by Dr. K. N. Brown (NIMR, Mill Hill, England), while clone F of *P. chabaudi* strain IP - IPC1 was a gift from Dr. G. Langsley (Institut Pasteur, Paris). Both strains were maintained in eight to 12 week old mice, by passage, via intra-peritoneal injection (i. p.), of infected blood.

2.3 Preparation of frozen stabilates

Frozen stabilates were made on a regular basis for the preservation of the strains. Blood was obtained from an animal with a parasitaemia of greater than 6 % when the parasites were at the ring-stage of development, as determined by giemsa staining. Using a heparinised syringe, blood was withdrawn, via cardiac puncture, and centrifuged for five minutes at 450 x g. The supernatant was then removed and the packed cell volume (PCV) was mixed in a ratio of five parts glycerolising solution to three parts PCV in the following manner; one fifth of the required volume of the glycerol was added dropwise to the PCV, mixed gently and the solution left to equilibrate at room temperature for five minutes. The remainder of the glycerolising solution was then added dropwise while gently vortexing and the cell suspension stored at - 70 ^{0}C. For long-term storage, the cells were stored under liquid nitrogen.

2.4 Initiation of infection using frozen stabilates

When frozen parasites were required for infection of animals (the animals were infected with frozen stock every two months), a frozen vial was thawed slowly on ice (to minimise the amount of lysis of erythrocytes) and immediately injected i. p. into the recipient mice. The infection was normally detectable within five to seven days following injection.

2.5 Staining procedures

Parasite staining: Thin blood smears were taken from the tails of infected mice and air dried. The slides were fixed in methanol and stained using a 10 % giemsa stain for ten minutes. The slides were then washed, air dried and examined using light microscopy.
Reticulocyte staining: Equal small volumes (50 µl) of fresh blood and a 2 % methylene blue staining solution were mixed and left at room temperature for ten minutes. Thin smears were then made using approximately 3 µl of cell suspension. The slides were air dried and examined using light microscopy.

2.6 Cell culture procedures

All culturing work was carried out in a Gelaire BSB4 laminar flow, using standard cell culture techniques. It is essential to use ultrapure water in cell culture and water for the preparation of all growth media and reagents was passed through an Elgastat Ultra High Purity Unit. In this unit deionised water is passed through a number of purification steps, resulting in water of reagent grade which was regularly monitored by an on-line resistivity meter (10 - 18 megohms / cm was the acceptable resistivity). Where possible sterile, disposable items were used in the culturing of parasites and cells. Glassware was washed thoroughly before being rinsed in ultrapure water, dried and autoclaved at 120 ^{0}C for 20 minutes. Water and temperature resistant solutions were also autoclaved, while temperature labile chemicals (e.g. enzymes, amino acids, etc) were filter sterilised through 0.22 µm sterile, disposable filters.

2.7 Growth media

Nine different media were used during the course of this work, with seven of the media being bought in as 1X stock solutions and two as 10X stock solutions. Each medium was prepared according to Table 2.1, via a number of stepwise procedures. These procedures varied only slightly between the 1X and 10X stock solutions. Initially the glucose, HEPES, gentamicin and hypoxanthine were added to each 1X stock medium before it's pH was adjusted to 7.3. This solution was then filter sterilised and dispensed into cell culture flasks or plates. Sterile 5 % sodium bicarbonate solution, L - glutamine and serum were then added immediately prior to the addition of the parasites and target cells. Where a 10X stock medium was used, the glucose, HEPES, gentamicin and hypoxanthine were added to 10 ml of medium and the medium was made up to 95 ml with ultrapure water. It can be seen from Table 2.1 that as the compositions of the various media were different, the additives that were used to supplement each medium varied between the different media.

Complete RPMI - 1640 was made up as follows;

RPMI - 1640	10 mls
Glucose	0.2 g
Hypoxanthine	0.5 mg
Gentamicin	250 µl
HEPES (1 M)	2 ml

The medium was made up to 95 ml with ultrapure water and the pH adjusted to 7.3, before filter sterilisation.

Incomplete RPMI - 1640, which lacks a number of amino acids, was used for radio-isotope incorporation experiments, where the isotope to be incorporated was [^{35}S]methionine. This media was prepared as follows:

RPMI - 1640	1 g
Glucose	0.2 g
Hypoxanthine	0.5 mg
Lysine	4 mg
Leucine	5 mg

Table 2.1 Composition of media used for culturing erythrocytic stages of *P. chabaudi chabaudi*

Medium	Volume of medium (ml)	HEPES (ml)	Bicarbonate (ml)	L - glutamine (ml)	H_2O (ml)
BGJb	98	2	-	-	-
BME	99	-	-	1	-
CMRL	97	2	-	1	-
DMEM	100	-	-	-	-
McCoy's	98	2	-	-	-
MEM	10	2	4.2	1	82.8
NCTC	98	2	-	-	-
RPMI - 1640	10	2	4.2	1	82.8
William's E	97	2	-	1	-

* In all 100 ml of media there was 5 mg hypoxanthine, 2 g glucose and 2.5 mg gentamicin.

Gentamicin 250 μl
HEPES (1 M) 2 ml

It was made up to 95 ml and the pH adjusted to 7.3 and filter sterilised.

2.8 Serum preparation

Groups of ten adult Wistar rats were bled into non-heparinised syringes and the blood was left to clot overnight at 4 ^{0}C. The clots were spun out at 1,000 x g for 20 minutes and the serum removed. The serum was then filter sterilised through a 0.22 μm filter, before being heat - inactivated at 56 ^{0}C for 20 minutes and stored in aliquots at - 20 ^{0}C.

2.9 Cell counting

A sample of a cell suspension was applied to a Hausser Hy-lite ultra plane Neubauer haemocytometer so that it was held in the counting area between the slide and a cover slip, showing a series of grided lines through a 40 X microscope lens. A representative square from each of the 16 squares in the corner grids was counted and the average found (X). This value was used to calculate the number of cells per ml of the cell suspension as follows :-

$$(X) \times 16 \times \text{dilution factor} \times 10^4$$

The dilution factor was normally 1 in 100 followed by 1 in 20.

2.10 Purification of parasite-infected erythrocytes

The intraerythrocytic cycle of *P. c. chabaudi* is synchronous, therefore a single-step gradient is sufficient to separate trophozoite-infected erythrocytes from uninfected cells. A modified version of the gradient developed by Wunderlich (Wunderlich *et al*, 1985) was used. Approximately 1 ml of blood was removed from *P. c. chabaudi*-infected CD - 1 mice when the parasitaemia was between 15 - 25 %, by cardiac puncture into a heparinised syringe. The blood was passed over a RPMI - 1640 equilibrated CF11 column, the volume brought up to 10 mls and centrifuged at 450 x g for five minutes. The pelleted cells were then resuspended in 0.5 mls of medium and layered onto 12.17

ml of a single-step, 74 % Percoll solution prepared as follows;

solution	volume
Percoll	9.0 ml
solution A	1.0 ml
solution B	2.17 ml

After centrifugation at 5,000 x g for 20 minutes, at room temperature, the infected cells (> 98 % pure and showing brown pigmentation) had only just penetrated the top of the Percoll while the uninfected cells were banded close to the bottom. The top layer was removed and washed in 10 mls of medium and the pellet resuspended in a further 0.5 ml of medium.

2.11 Culturing of infected erythrocytes

The parasitised erythrocytes obtained by Percoll purification were cultured *in vitro* to obtain mature trophozoites / schizonts at the stage just prior to rupture. The culturing medium used was a 3 : 1 ratio of BME and William's E (BME - WE). The BME and William's E media were combined as 1X stocks, with the nutritional supplements added (per 100 mls medium) as follows:-

BME	66.4 ml
William's E	22.1 ml
glucose	0.2 g
HEPES (1 M)	0.5 ml
gentamicin	250 µg
L - glutamine	1 ml
hypoxanthine	0.5 mg
rat serum	10 ml

This medium was filter sterilised before the addition of the rat serum and the L - glutamine. The parasites were cultured at a concentration of not greater than 1 x 10^8 cells / ml in either a T25 (5 mls of medium) or a T75 (10 mls of medium) culture flask. These cells were

maintained in a candle jar to achieve a low oxygen tension (approximately 5 %), at 37 ^{0}C (Trager and Jensen, 1976).

2.12 Preparation of target erythrocytes

Target cells were collected using heparised syringes. The blood was then passed over a medium equilibrated CF11 column before being washed with 10 mls of medium. The pelleted cells were then resuspended in 0.5 mls of medium. The cells of the mice, rats, rabbits and guinea pigs were used immediately due to their fragility while the human erythrocytes (supplied by the Dublin Blood Transfusion Board) could be stored for a number of days in heparin, at 4 ^{0}C, before being washed twice in medium and resuspended in 0.5 mls BME - WE.

2.13 Invasion assay

Parasitised erythrocytes were purified as described in Section 2.10 and were then cultured in BME - WE for four hours. The late trophozoites / schizonts were gently resuspended in the culture medium, removed from the culture flask and 8×10^6 of these parasitised erythrocytes were added to 4×10^7 target erythrocytes (Section 2.12). The cells were gently mixed in 800 µl BME - WE and 100 µl rat serum per well of a 24-well tissue culture plate. The plate was returned to the candle jar and incubated at 37 ^{0}C for 19 hours. The contents of each well were then transferred to eppendorfs and centrifuged at 450 x g for five minutes. The pelleted cells were resuspended in 50 µl FCS and centrifuged again. These cells were resuspended in a further 20 µl of FCS and used to make thin smears on glass slides. The smears were giemsa stained and the percentage of erythrocytes infected with ring-stage or young trohozoite-stage parasites was then calculated using light microscopy.

2.14 Induction of reticulocytosis by phenylhydrazine treatment

Solutions of 0.4 % phenylhydrazine were prepared in PBS from a 97 % stock solution immediately before use. Mice were injected i. p. with 100 µl of this solution per 15 g body weight on Day 1 and on

every alternate day until Day 9. The erythrocytes from these mice were used on days when the reticulocyte count was approximately 50 %, as determined by methylene blue staining.

2.15 [^{35}S]methionine and [^{3}H]isoleucine incorporation by parasites cultured *in vitro* in various media

Parasitised erythrocytes were obtained, purified and washed as before (Section 2.10). These cells (1×10^7 cells) were then added to 900 µl of BME - WE and 100 µl of RS in the wells of a 24-well tissue culture plate. Parasite proteins were radio-labelled by adding [^{3}H]isoleucine or [^{35}S]methionine to wells and culturing the cells in a candle jar at 37 ^{0}C. Ten microcuries of isoleucine or 2.5 µCi of methionine was added to each well. After one, two and four hours of incubation, the cells were removed from duplicate wells, washed three times in PBS and then lysed in 1 ml PBS containing 1 % Triton-X. The soluble proteins were precipitated by the addition of TCA to a final concentration of 10 %, followed by centrifugation at 10,000 x g. The pellets were washed with cold acetone, re-centrifuged and resuspended in 1 ml 0.5 % Triton-X. The suspensions were sonicated and 50 µl aliquots were mixed with 4 mls Ecosint, for the measurement of incorporated radioactivity by liquid scintillation counting.

2.16 Enzyme treatment of target erythrocytes.

Target cells were collected as before (Section 2.12), passed over an RPMI - 1640 equilibrated CF11 column and washed in RPMI - 1640. One hundred microlitres PCV was used for each digest. Following digestion of the cells, cell counts were carried out and 4×10^7 cells were used for invasion assays. The enzyme solutions were prepared immediately before use and were all prepared in RPMI - 1640.

a) *Protease treatment of erythrocytes*

The target erythrocytes were incubated for two hours in 900 µl of enzyme solution at 37 ^{0}C, with occassional agitation. The concentration of the enzymes were as follows;

Trypsin	10,000 U / mg
Chymotrypsin	5 U / mg
Papain	13 U / mg
Pronase	4 U / mg

The cells were washed three times in RPMI - 1640 before being incubated for five minutes in 900 µl of medium containing 1 mg TPCK and 1 mg trypsin inhibitor. The cells were then washed twice, resuspended to a final volume of 1 ml and counted.

b) *Neuraminidase treatment of erythrocytes*

The target erythrocytes were incubated for two hours, at 37 ^{0}C in 900 µl of a neuraminidase solution in PBS (1 U / ml neuraminidase) with occassional shaking. After digestion the cells were washed four times to remove all traces of the enzyme before the cells were used for assaying.

2.17 Sodium periodate treatment of erythrocytes

The erythrocytes were incubated for five minutes with 100 µl of sodium periodate solution made in PBS at room temperature. The cells were washed once with PBS before being further incubated for 30 minutes in 1 ml of a 1 % glycine (made in PBS) at room temperature (Woodward *et al*, 1985; Perkins & Holt, 1988). The cells were then washed twice and resuspended to 1 ml with RPMI - 1640 and counted.

2.18 Heparin-Sepharose CL-6B chromatography

Commercially obtained Heparin-Sepharose CL-6B was swollen in PBS and used to construct a 100 µl column, using a 1 ml syringe plugged with glass wool. The column was equilibrated with 2 ml PBS before 200 µl of protein extracted from parasites that had been cultured for six hours was passed over the column three times. The column was washed with 2 mls of PBS and proteins were eluted with 800 µl of 1.5 M NaCl. The fractions from the column were collected in eppendorfs coated with ß-lactoglobulin to prevent the proteins from

coating the eppendorfs. The solutions were then dialysed against dH_2O to remove salt and lyophilised.

2.19 Antigen preparation for raising polyclonal antibodies

A range of polyclonal antibodies was prepared using a variety of antigen preparations. These preparations were as follows :

a) total culture supernatant (TCS)
b) Triton-X extracted proteins (T-XP)
c) erythrocyte binding proteins (EBP)
d) total schizont protein (TSP)
e) antigen purified using a heparin column (HEP)

a) *TCS antigen preparation*

TCS proteins were prepared according to Fig. 2.1. Purified parasite-infected erythrocytes were obtained in the usual manner (Section 2.10) and were cultured overnight in BME - WE, at 37 ^{0}C, in a candle jar. A 50 ml culture was used containing 1.4×10^7 cells / ml medium. The culture medium which contained mainly merozoites and parasite proteins (exoantigens), was centrifuged at 6,700 x g for 20 minutes and the supernatent decanted. Some cell debris was also pelleted. The culture supernatent containing exoantigens was concentrated down to 5 ml using an Amicon ultrafiltration apparatus. This concentrate was then lyophilised, resuspended in PBS, dialysed overnight to desalt it and stored at -20 ^{0}C until use.

b) *T-XP antigen preparation*

The pellet of merozoites and possibly some cell debris that was the result of centrifugation of an overnight culture, was used to provide the proteins for T-XP antigen preparation. The pellet was resuspended in PBS and soluble parasite proteins extracted using Triton-X detergent. The mixture was vortexed vigorously before being spun to pellet all non-soluble proteins. The soluble proteins in the supernatant were then stored frozen until immunisation.

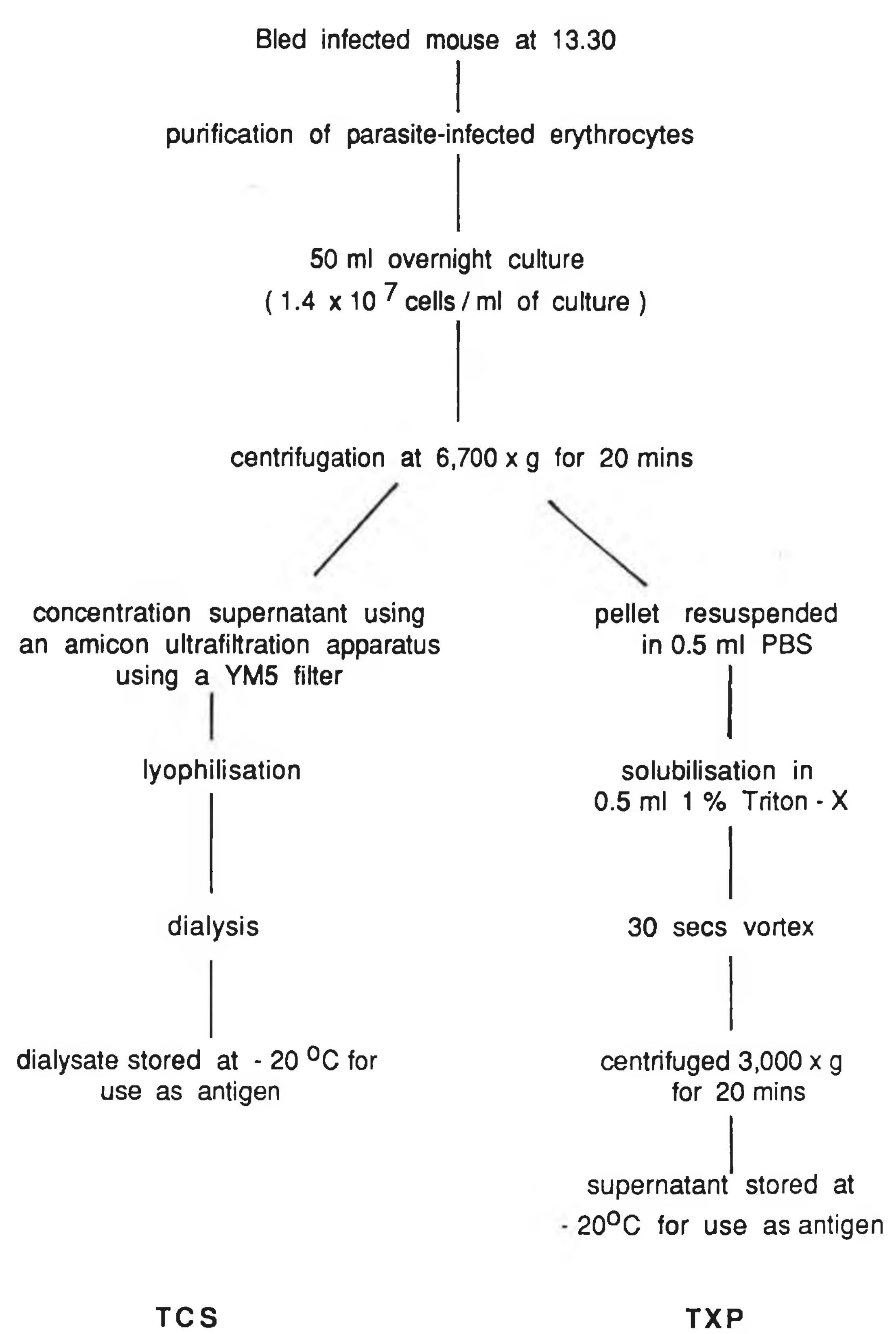


Fig. 2.1 **Flow chart of the method used to obtain the antigens TCS and TXP**

c) *EBP antigen preparation*

Total culture supernatant was obtained as detailed in Fig. 2.1. The 50 mls of culture supernatant containing parasite exoantigens was mixed gently and thoroughly with 6 mls PCV of rat blood cells which had been prepared in the same manner as target erythrocytes for invasion assays (Section 2.12). The cell suspension was incubated at room temperature for 30 minutes with occassional shaking, before centrifugation at 900 x g for 5 minutes. The pelleted cells were washed in cold PBS, spun at 6,700 x g for 30 seconds and further incubated at room temperature for ten minutes in 6 mls of 1 M NaCl. The cells were then centrifuged at 6,700 x g for 30 seconds. The supernatant was removed and freeze-dried. The resultant pellet was resuspended to a volume of 1 ml with PBS and dialysed overnight, to remove any excess salts. The preparation was stored at - 20 ^{0}C until use.

d) *TSP antigen preparation*

The parasite-infected cells were purified and cultured for a period of six hours as described above (Sections 2.10 & 2.11). The parasites were then centrifuged for five minutes at 450 x g. The packed cells were made up to 0.5 ml with PBS and lysed with the addition of 0.5 ml of a 0.1 % saponin solution in PBS. The lysed cells were immediately centrifuged at 450 x g for five minutes, the supernatant removed and the pellet washed a further three times to remove any saponin, haemoglobin and erythrocyte debris. The pellet of mostly intact parasites was resuspended in 500 μl of PBS and was subjected to three cycles of freeze-thaw lysis to lyse the parasites and release parasite proteins. Following the final thawing, the protein solution was spun for five minutes at 3,000 x g at 4 ^{0}C to pellet parasite membranes and any proteins bound to them, from the protein solution. The proteins from a number of preparations were pooled and diluted with PBS containing enzyme inhibitors, to final concentration; E - 64 (5 μg ml), leupeptin (5 μg / ml), EDTA (2.5 μg / ml) and PMSF (1 mM). This suspension was aliquoted into the volumes required for each immunisation and stored at - 20 ^{0}C.

e) *HEP antigen preparation*

The parasites were purified, cultured for six hours, saponin lysed and freeze-thaw lysed as before.The resulting protein preparation was passed over a heparin column (Section 2.18) and the column washed with PBS before the proteins were eluted off using 1.5 M NaCl. The eluate was dialysed against dH_2O to remove excess salts and lyophilised. The dried proteins were then resuspended in dH_2O for use in immunisations.

2.20 Production of antibodies

When the antigen had been prepared, the protein concentration of each preparation was calculated using a micro-Bradford assay. Aliquots were prepared in such a way that each immunisation with a specific antigen preparation contained the same concentration of antigen. The antigen concentrations, however, varied between different antigen preparations. The concentrations were as follows;

TCS	100 µg / injection
T-XP	40 µg / injection
EBP	20 µg / injection
TSP	100 µg / injection
HEP	50 µg / injection

Equal volumes of the antigen and Freund's complete adjuvant were sonicated on ice to form an even emulsion. This was then injected subcutaneously at the base of the neck of female Wistar rats to produce antibodies. After three weeks the rats were injected again with an emulsion consisting of equal volumes of antigen and Freund's incomplete adjuvant. This injection was repeated a further three times, at three week intervals. Ten days after the final injection the animals were sacrificed and their blood collected. The blood was left to clot overnight at 4 ^{0}C, the serum drawn off and heat-inactivated at 56 ^{0}C for 20 minutes. This was then stored at - 70 ^{0}C.

2.21 Protein estimation

Protein was estimated using a micro-Bradford assay. Using a 96-well microtitre plate, standards and samples were assayed by mixing 200 µl of 1 X Bradford (commercially obtained) reagent with 10 µl of test solution. The absorbance was read at 595 nm after a ten minute incubation at room temperature and the protein values of the samples extrapolated from the graph of the protein values of the standards.

2.22 Pretreatment of antisera prior to use

Prior to carrying out experiments using antisera generated in rats, the heat-inactivated serum was adsorbed to remove any possible antibodies that had been developed against components of murine erythrocytes. One millilitre of antiserum was incubated with 50 µl PCV of washed mouse erythrocytes for 30 minutes. The cells were pelleted by centrifugation at 400 x g for five minutes. The anti-serum was then removed and incubated for 30 minutes with a further 50 µl PCV of erythrocytes before centrifugation. This process was carried out five times in total. The antiserum was then aliquoted and stored at - 70 ^{0}C.

2.23 Preparation of antigen for SDS - PAGE

After the antigen had been extracted, 50 µl (20 - 50 µg protein) of this protein solution was solubilised in 5X sample buffer containing 12.5 % SDS, 50 % glycerol, 12.5 % mercaptoethanol and 0.005 % bromophenol blue in 0.3 M Tris - HCl, pH 6.8. This was boiled for two minutes before loading the samples into the wells of polyacrylamide gels.

2.24 SDS - PAGE

Samples were routinely run on 10 % SDS polyacrylamide gels, according to Laemmli (1970). The gels were prepared as in Section 2.33 and were routinely run at 25 mA. Gels were removed from the apparatus when the dye front was within 1 cm of the bottom of the gel (electrophoresed for approximately four hours), stained with 0.1 % Coomassie Blue and then destained.

2.25 Molecular weight estimation

Molecular weights were determined from a standard curve, obtained from commercial pre-stained molecular weight markers, run in parallel with samples. The mobility of the following seven protein standards were plotted against the logarithm of their molecular weights; ∂_2 - macroglobulin, 211,000; ß - galactosidase, 119,000; fructose - 6 - phosphatase kinase, 98,000; pyruvate kinase, 80,600; fumarase, 64,400; lactic dehydrogenase, 44,600 and triosephosphate isomerase, 38,900 Daltons.

2.26 Radio-labeling of parasite proteins

Purified parasites were obtained as described in Section 2.10. Incomplete RPMI - 1640 was prepared as detailed in Section 2.7. Nine millilitres of this 1 X stock was supplemented with 1 ml RS, 130 µl of 5 % bicarbonate solution,100 µl of 200 mM L - glutamine and 100 µl of trans[^{35}S]methionine. The parasitised cells were added at a concentration of 2.5 x 10^7 / ml of culture. The parasites were cultured overnight so that rupture of parasitised erythrocytes occurred. The culture was then centrifuged at 450 x g for five minutes, the supernatant removed and used in erythrocyte binding assays.

A second set of radio-labelled parasite proteins was prepared for use in radio-immunoprecipitation experiments. The cells were added to [^{35}S]methionine-containing medium and cultured for six hours to allow the parasites to develop into mature trophozoites / schizonts. The parasitised cells were pelleted by centrifugation at 450 x g for five minutes and the supernatant removed. The pelleted cells were resuspended in PBS and stored at -20 ^{0}C. These pellets were then lysed by means of three cycles of freeze-thaw lysis and centrifuged at 3,000 x g for five minutes. The radio-labelled protein extract was made up to the initial culture volume of 10 ml with PBS, allquoted and stored at - 70 ^{0}C.

2.27 Erythrocyte binding assay

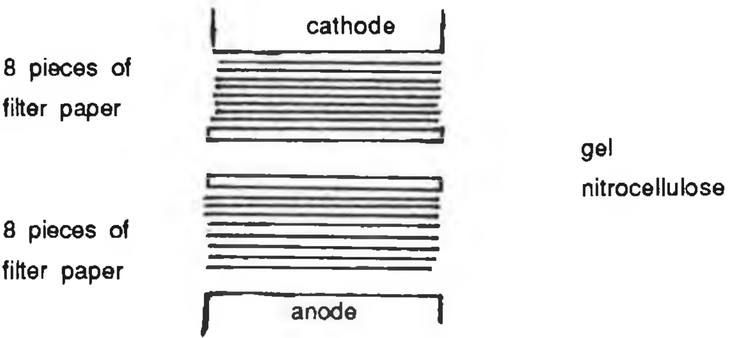
One hundred and fifty microlitres PCV of washed erythrocytes was incubated with 300 µl of [^{35}S]methionine-labelled culture supernatant (overnight culture) at room temperature for 30 minutes with occasional shaking. The solution was layered onto 400 µl of silicone oil and centrifuged at 3,000 x g for ten seconds. The eppendorf was punctured and the pelleted cells were collected dropwise into 1 ml of cold PBS. These cells were pelleted by centrifugation at 3,000 x g for ten seconds. The supernatant was discarded and the cells were resuspended in 20 µl of 1.5 M NaCl and left at room temperature for ten minutes with occasional shaking. The suspension was centrifuged at 3,000 x g for ten seconds and the supernatant was removed and analysed by SDS electrophoresis and fluorography.

2.28 ELISA

The wells of a 96-well microtitre plate were coated with 50 µl of antigen (200 µg / ml) and incubated at 37 ^{0}C overnight. The excess protein binding sites on the wells were blocked by incubating with 200 µl PBST (0.1 % Tween-20 in PBS) - 0.1 % FCS for one hour at 37 ^{0}C. The wells were then washed three times for five minutes in cold PBST. Primary antibody dilutions (50 µl volumes, diluted with PBST), were dispensed into each well and incubated for one hour at 37 ^{o}C. The wells were then washed three times as before and 50 µl of secondary antibody (1 / 7,000 dilution of alkaline phosphatase labelled anti-rat IgG) was added to detect the primary antibody. The sample wells were incubated for another hour at 37 ^{0}C. The wells were washed again with cold buffer and 100 µl of a solution of 1 mg / ml p - nitrophenyl disodium phosphate solution in ELISA substrate buffer was added to each well. After ten minutes the optical densities of each well were read in an EL - 307 ELISA reader (BIO - TEK Ltd.) using a 405 nm filter. The titre of antibody in each serum sample was taken to be that dilution of serum which gave a discernible reading above controls of similar dilutions on the ELISA reader.

2.29 Immunoblotting

Following electrophoresis, the SDS gel was equilibrated in transfer buffer for 30 minutes. A piece of nitrocellulose paper and 16 pieces of filter paper (Whatman chromatography paper 3MM) cut to the same size as the gel to be blotted, were similarly equilibrated for 30 minutes in transfer buffer. An Atto semi-dry blotting apparatus was used for the electroblotting procedure. Firstly the anode was covered with a film of transfer buffer and the gel / membrane sandwich constructed upon it according to the diagram ;



The cathode in the lid of the apparatus was then lowered to rest on top of the sandwich and thus complete the connection. For the effective transfer of proteins onto the nitrocellulose, the current used was calculated according to the formula

area of the gel x 0.8 mA x 0.5

The proteins were blotted for a period of approximately three hours (the higher the molecular weight of the protein to be transferred, the longer the electroblotting period). When the transfer of proteins was completed, the current was tuned off and the gel / membrane sandwich was disassembled. The gel was stained to check that protein transfer had occurred and the nitrocellulose filter was washed 5 x 5 minutes in PBST. In order to saturate non-specific protein binding sites, the membrane was blocked overnight, at room temperature, in PBST containing 0.1 % BSA. The membrane was then washed 5 x 5 minutes before being incubated at room temperature for three hours in a 1 / 500 dilution of primary antibody diluted in PBST. To remove unbound antibody the membrane was washed 5 x 5 minutes in PBST.

The membrane was then transferred to a 1/7,500 dilution of rabbit anti-rat (whole molecule) alkaline phosphatase conjugate and incubated for a further two hours. After washing the membrane as before, the alkaline phosphatase colour reaction was carried out. Sixty-six microlitres of NBT and 50 µl BCIP were added to 15 mls of substrate buffer and the membrane was incubated in this solution until reactive areas turned purple. The reaction was stopped by rinsing the membrane in water. The blott was then air dried and stored in cling-film.

2.30 Isolation of a monospecific antibody

Antigen from parasites cultured for six hours were run on a 10 % SDS polyacrylamide gel. The proteins were then electroblotted onto nitrocellulose and blocked with BSA. One lane was incubated in anti - TCS and developed in the normal way (Section 2.25) to detect the band of interest. This lane was then aligned with lanes that had been incubated in anti - TCS and a 0.5 cm length of the lane corresponding to the position of the desired protein was cut out and the antibody eluted off the protein in 0.5 ml of 0.1 M glycine (pH 2.8) containing 100µg BSA for five minutes according to a modification of the method used by Adams *et al*, (1990) to elute a monospecific antibody for screening a library. This was then neutralised with 0.5 ml of 0.1 M Tris (pH 9.2). The eluted antibody was used to probe an identical lane and the blot was developed as before.

2.31 Radio-immunoprecipitation

To 200 µl of radio-labelled supernatant was added 5 µl of anti-serum and the mixture was incubated overnight at 4 ^{0}C. Five microlitres of rabbit anti-rat was added to this solution, mixed thoroughly and incubated for a further four hours at 4 ^{0}C. Protein A-agarose (50 µl) was then added and the mixture incubated for one hour at 4 ^{0}C. The precipitates were then washed a number of times, at 450 x g for five minutes according to the following procedure:

i) NETT - 0.5 % BSA

ii) NETT

iii) NETT - 0.5 M NaCl

iv) NETT

The eppendorf was changed between the 3rd and 4th wash. The final wash was spun at 3,000 x g for five minutes and all of the supernatant was removed. The pellet was resuspended in 40 µl of 1X sample buffer and boiled for two minutes. The sample was centrifuged at 3,000 x g for 30 seconds to pellet the Protein A-agarose and the supernatant (sample) was removed and stored at - 20 ^{0}C. The samples were run using 10 % SDS - PAGE and analysed using fluorography.

2.32 Fluorography

Electrophoresis gels that were run for the separation of radio-labelled proteins were fixed for 30 minutes in destain and subsequently incubated in Amplify for 30 minutes to enhance the radioactive emissions from labelled proteins. The gels were dried down and exposed to X-ray film at - 70 ^{0}C. After a sufficient exposure time, the X-ray film was developed and the results examined.

2.33 Immunofluoresence

Plasmodium c. chabaudi parasite-infected erythrocytes, either from infected blood or cultured erythrocytes, were washed three times in excess PBS and once in FCS before being resuspended in FCS. This cell suspension was used to make thin smears. The slides were air dried (hot air should not be used to dry the slides as this lowers the level of antigenicity of the slides) and fixed with 100 % methanol for two minutes, the excess methanol shaken off and slides left to air dry. The slides were individually wrapped in tissue and stored in polythene bags containing dessicant before being stored at - 70 ^{0}C. Upon removal from the freezer, the slides were left to thaw on the bench for a minimum of 30 minutes and allowed to air dry. Wells were made on these antigen slides by scraping a ring around the test area and filling the ring outline in with an immunohistochemistry pen. This was allowed to dry. The test antibodies were diluted with PBS containing 0.1 % BSA and 0.1 % sodium azide. After careful labelling of the slides, 5 µl of the diluted test antibodies were added

to each well. The slides were incubated for one hour in a humidified chamber. The slides were rinsed with PBS before being washed six times (five minutes per wash) in immunofluorescence washing buffer. Five microlitres of FITC-labelled secondary antibody was then added to each well (pre-diluted in PBS containing BSA and sodium azide). The slides were incubated for a further one hour in a humidified chamber before being rinsed and washed as before. While the slides were still wet (they must not dry out), they were mounted using DABCO mountant and coverslips and sealed using nail polish. The antigen slides were stored in the dark at 4 ^{0}C for 15 to 30 minutes and examined using a fluorescence microscope. All fluorescent fields were also examined under Direct Interference Contrast (DIC) microscopy to visualise the erythrocytes and parasites without fluorescent labelling.

Plasmodium falciparum antigen slides were prepared from cultured erythrocytes and were methanol fixed for immunofluorescence procedures.

A mouse that was immune to *P. c. chabaudi* AS was used as a positive control for immunofluorescence. The mouse was infected with the parasite and the infection was allowed to proceed through the initial peak of infection and one recrudescence. The animal was then Infected again and when the parasitaemia had fallen to zero, the mouse was reinfected. No infection resulted from this injection, nor from one further injection. The mouse was now considered to be immune to *P. c. chabaudi* AS.

2.33 Buffers and solutions

Glycerolising solution for storage of parasites at - 70 ^{0}C

A pyrogen-free container was used when making up 750 ml of a 10 % tri-sodium citrate solution. One litre of glycerol was added to this solution, followed by 750 ml distilled water. The container was then closed and the contents mixed thoroughly by shaking (The Blood Transfusion Service Board , personal communication).

10 % Giemsa stain for visualising parasites

10 ml concentrated giemsa stain

6.1 ml M / 15 Na_2HPO_4

3.9 ml M / 15 NaH_2PO_4

The solution was made up to 100 ml with distilled water and filtered through Whatman No. 1 filter paper.

Phosphate buffered saline (PBS).

NaCl	8 g
KCl	2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
H_2O to	1 litre

The pH was adjusted to 7.3.

Percoll solutions for P. c. chabaudi purification .

Solution A (10 X PBS - glucose)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1 g
NaH_2PO_4	0.15 g
KH_2PO_4	0.2 g
glucose - H_2O	22.5 g
H_2O to	100 ml

Solution B (1 X PBS / glucose)

Tenfold dilution of 10 X PBS / glucose stock.

Solutions for SDS - gel electrophoresis

Bisacrylamide solution

Acrylamide	30 g
Bisacrylamide	0.8 g
H_2O to	100 ml

The solution was stored in the dark, at 4 ^{0}C.

Running buffer

Tris	3.2 g
Glycine	15.5 g
SDS	1 g
H_2O to	1 litre

Separation gel :-

Bisacrylamide solution	10 ml
1 M Tris - HCl pH 8.8	11.2 ml
dH_2O	8.7 ml
10 % SDS	300 μl
10 % Ammonium persulphate	100 μl
TEMED	20 μl

Stacking gel :-

Bisacrylamide solution	1.25 ml
1 M Tris - HCL pH 6.8	1.25 ml
dH_2O	7.5 ml
10 % SDS	100 μl
10 % Ammonium persuphate	100 μl
TEMED	25 μl

Coomassie stain

Coomassie R	1 g
Methanol	500 ml
Acetic acid	10 ml
H_2O to	1 litre

Destain solution

Methanol	100 ml
Acetic acid	100 ml
H_2O to	1 litre

Solutions for immunoblotting

Electroblotting buffer

Methanol	100 ml

Tris	2.42 g
Glycine	7.5 g
H_2O to	1 litre

The pH of the solution was adjusted to 8.3.

Substrate buffer

Tris	1.21 g
NaCl	0.58 g
$MgCl_2.6H_2O$	1 g
H_2O to	100 ml

The pH of the buffer was adjusted to 9.5.

NBT
75 mg in 1 ml 70 % DMF.

BCIP
50 mg in 1 ml DMF.

Substrate buffer for ELISA

Glycine	0.75 g
$MgCl_2.6H_2O$	0.02 g
$ZnCl_2$	0.02 g
H_2O to	100 ml

The pH was adjusted to 10.4.

NETT (for radio-immunoprecipitation)

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

The solutions were made up as 10X stock solutions and the NETT buffer was made up immediately before use.

Solutions for immunofluorescence

Washing buffer

PBS	1 L
Tween-20	500 µl

DABCO mountant

Glycerol	90 ml
PBS	10 ml
DABCO	2.5 g

2.34 Statistical analysis of data

The bars in each histogram represents the average value for invasion levels obtained from a number of invasion assays. The error bars represent the standard deviations about these values, due to variation within and between experiments. A pooled estimator was then used to achieve a better estimate of the variance between the control values for invasion and the test values. The value Sp was calculated as follows;

$$Sp = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

where $$s^2 = \frac{\Sigma(x - \bar{x})^2}{n - 1}$$

These figures were then analysed using a one-tailed student t-test (where $n < 30$) to compare the statistical significance of the difference between two levels of invasion e.g. control and enzyme-treated.

$$t_0 = \frac{\bar{x}_1 - \bar{x}_2}{Sp\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Differences were said not to be significant where $t_0 < t_{0.05}$ (at $p \leqslant 0.05$) where t is obtained from a table of t values (Appendix 1, Murdoch & Barnes, 1983).

Chapter Three

Results

3.1 Maintenance of *P.c.chabaudi* AS strain

Plasmodium chabaudi chabaudi (AS strain) is a rodent malaria whose course of infection in laboratory mice is characterised by an acute but seldom fatal primary parasitaemia usually lasting between ten to 20 days. The subpatent period that follows the primary parasitaemia is accompanied by at least one low level recrudescence before the mouse becomes aparasitic (McDonald & Sherman, 1980; Gilks *et al*, 1990).

The AS strain of the parasite was established in the animal facility in D.C.U. in CD - 1 mice, from a frozen stabilate. Once the infection had been established in the mice, the course of infection was monitored daily. Thin smears were made using blood from infected animals, the slides giemsa stained and the number of infected erythrocytes in 10 - 20 fields of view counted using light microscopy. The percentage parasitaemia was then calculated.

From the results shown in Fig. 3.1, the parasitaemia was seen to reach a peak of > 50 % infected erythrocytes by Day 10, falling to subpatency by Day 15. A low parasitaemia was detected on Day 29, which lasted until Day 34 and never reached above 2.5 %. This represented the recrudescent period and the overall course of infection indicated that *P. c. chabaudi* AS behaved in the expected manner.

The condition of both parasitised and non-parasitised erythrocytes in the blood smears was examined and it was noted that although levels of > 50 % infected erythrocytes were achieved, at levels greater than 25 %, both infected and non-infected erythrocytes, as well as the parasites themselves, appeared less healthy.

For the purpose of making frozen stabilates animals with a parasitaemia of between 6 and 25 % were used. The animals were normally bled when the parasites were at the ring-stage of development; erythrocytes infected with this parasite stage are less prone to lysis during freezing and thawing of the stabilate.

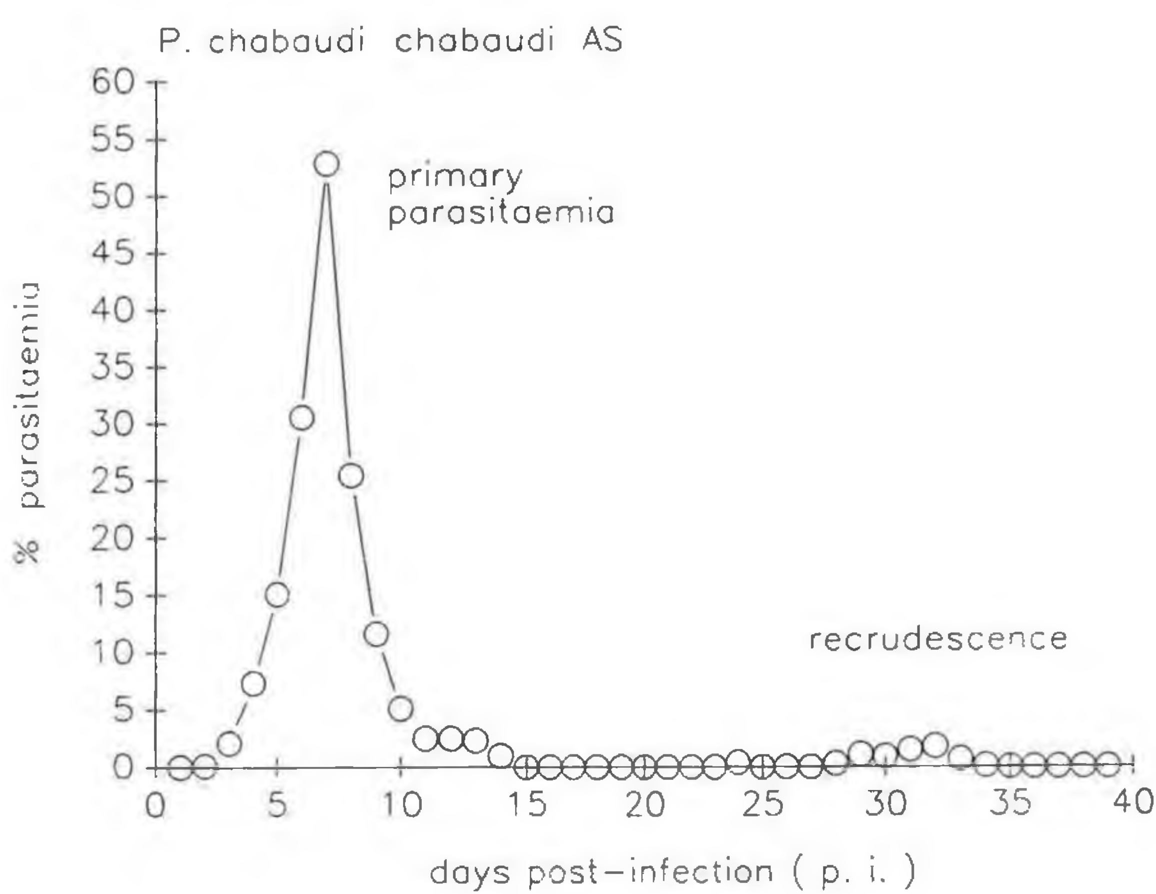


Fig. 3.1 Course of infection of *P. c. chabaudi* AS in CD -1 mice

3.2 Asexual erythrocytic cycle of *P.c.chabaudi* AS

Plasmodium c. chabaudi AS is a malaria parasite that undergoes a full cycle of asexual development in a 24 hour period (Carter & Walliker, 1975). Ring-stage parasites develop through the trophozoite-stage and onto segmentors (schizogony). It was this stage just prior to the rupture of the parasites that was required for study; however, in *P. c. chabaudi* AS, the parasites withdraw to the endothelial cells from the peripheral circulation (Gilks *et al*, 1990) before schizogony (sequestration). Hence the recovery of segmenting parasites directly from infected animals was not possible. The most mature stage of the parasites that were recovered from the peripheral circulation were middle- to late-stage trophozoites. The parasite follows a 24 hour cycle with schizogony commencing around 24.00 hours and continuing into the early hours of the morning (Carter & Walliker, 1975). This time scale was not conducive to research, therefore the animals were kept in a 24.00 to 12.00 daylight cycle (Section 2.1).

Blood smears were made during the course of the erythrocytic cycle to determine at which time in this adjusted light cycle the different developmental stages of the parasite were present in the peripheral circulation and at what time sequestration occurred (Table 3.1). The parasites matured to middle - late trophozoites in the latter period of daylight hours (9.30 - 14.30), while sequestration began after 13.30, with no parasites detectable in the peripheral circulation between 16.30 and 18.30. Very immature ring-stage parasites began to appear at this stage. Based on these results it was decided to bleed animals between 13.00 and 13.30 for the purpose of obtaining mature parasites prior to sequestration. Only animals with a parasitaemia of between 15 and 25 % were bled; above this parasitaemia parasites appeared unhealthy (Section 3.1).

Table 3.1 Asexual erythrocytic cycle of *P.c.chabaudi* AS strain

Time	Rings	Trophozoites			Schizonts	Parasitaemia
		Early	Middle	Late		(% age)
9.30	-	+	+++	-	-	12.2
10.30	-	-	+++	-	-	12.2
11.30	-	-	+++	+	-	12.3
12.30	-	-	++	++	-	12.2
13.30	-	-	+	+++	-	12.1
14.30	-	-	-	+++	-	8.1
15.30	-	-	-	+++	-	< 0.1
16.30	-	-	-	-	-	0
17.30	-	-	-	-	-	0
18.30	-	-	-	-	-	0
19.30	+++	-	-	-	-	1.1
20.30	+++	-	-	-	-	9.8
21.30	+++	-	-	-	-	23.7

- none present
+ small proportion of overall parasite
++ equal proportions of two stages
+++ all one stage

3.3 Culturing of malaria parasites to schizogony

Mice with a parasitaemia of approximately 25 % were bled at 13.00 - 13.30 and the erythrocytes were washed a number of times in PBS to remove heparin. The cells were maintained at a concentration of approximately 2.5 x 10^7 cells / ml in complete RPMI - 1640 medium (Section 2.8) and containing 10 % heat-inactivated FCS, in accordance with accepted procedures (Trager & Jensen, 1976; Gilks *et al*, 1990). Every hour aliquots of cultured cells were taken, washed with FCS, smeared and stained and the condition of the infected erythrocytes noted. It was found that the parasites survived and remained in a healthy condition during the short-term culturing and that after a period of six hours, the majority of the parasites had entered schizogony, and a small number had already ruptured to release merozoites into the medium.

A number of cultures were left overnight and the contents were examined the following morning. Two results were obtained from this experiment. On examination of the culture smears it was found that all the erythrocytes that contained parasites on initiation of the culture had matured through schizogony and ruptured their host cells, releasing merozoites into the culture medium. Also a number of very immature ring-stage parasites were detected in erythrocytes. The presence of ring-stage parasites indicated that invasion had occurred into uninfected erythrocytes during the overnight culture.

3.4 Steps in the development of an *in vitro* erythrocyte invasion assay for *P.c.chabaudi* AS

3.4.1 Parasite purification by Percoll

P. c. chabaudi-infected erythrocytes were separated from non-infected erythrocytes using density gradient centrifugation. PBS-washed blood from an infected animal was layered onto 12 .17 ml of a 74 % Percoll solution. After centrifugation two layers were formed, one at the top of the Percoll and one towards the bottom of the gradient (Fig. 3.2). On analysis of the layers by giemsa staining, it was found that the top layer consisted of cells enriched with parasite-infected cells to a level of 95 - 99 % of erythrocytes (Fig. 3.3b). The bottom layer contained no parasitised cells.

It was observed that if the parasitaemia of the animal was > 25 %, the banding pattern in the Percoll smeared and the purity of the enriched band was decreased due to contamination with reticulocytes (immature erythrocytes). Reticulocytes are less dense than mature erythrocytes (Rapperport, 1986) and are therefore separated from mature erythrocytes by density centrifugation. The increase in the number of reticulocytes in the blood of infected animals is due to the destruction of red blood cells. This is caused by the release of parasites by rupturing cells at high parasitaemias and the resultant increased rate of production of new red blood cells to replace the lost cells (Jarra & Brown, 1989). This is another reason why only animals with parasitaemias of < 25 % were used for further experiments.

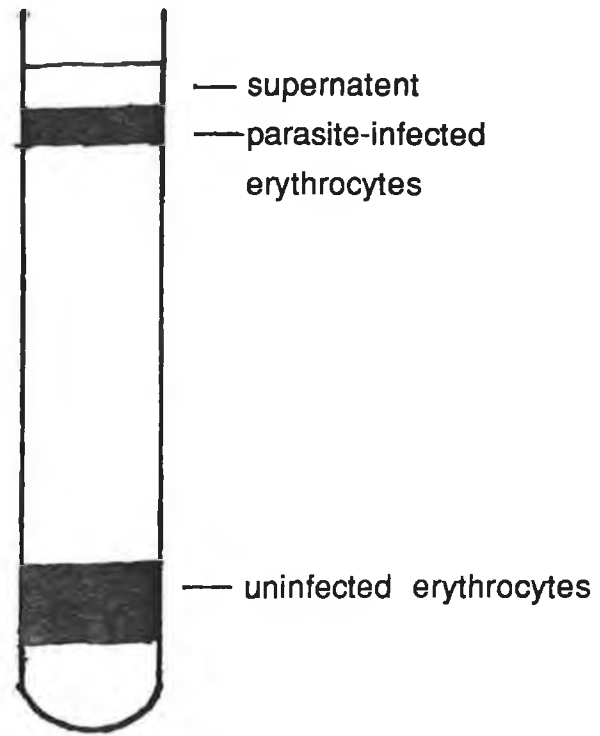


Fig. 3.2 Centrifuged Percoll gradient, showing the top band containing > 95 % parasite-infected cells and the bottom band containing uninfected cells.

3.4.2 Erythrocyte invasion assay

Plasmodium c. chabaudi AS-infected erythrocytes purified on Percoll were incubated with fresh (target) erythrocytes in 1 ml of complete RPMI - 1640 containing 10 % FCS, in a 24-well plate. The cells were incubated for 26 hours, under low oxygen tension (approximately 5 % or less) and at 37 ^{0}C. After the incubation period, the cells were washed with FCS and smears made. Analysis of the giemsa stained slides showed that using 4 x 10 ^{6} cells / ml parasitised erythrocytes and 4 x 10^7 / ml rat erythrocytes (in a 1 : 10 ratio) resulted in the successful re-invasion of target erythrocytes. The invasion level achieved was 1.2 +/- 0.2 %. Human cells were used as a negative control as they are refractory to invasion *in vivo*. No invasion was observed into human erythrocytes by *P. c. chabaudi* parasites *in vitro.*

Levels of invasion of 1.2 +/- 0.2 %, while adequate to provide proof of invasion were not considered suitable for assays investigating the effects of various treatments on invasion. Therefore attempts were made to obtain higher levels of invasion. Initially, the number of parasite-infected erythrocytes added to invasion assays was doubled (2 x 10^6 - 4 x 10^6), thereby decreasing the ratio of parasitised cells to target cells to 1 : 5. This resulted in an invasion level almost double that of the level in the original assay (Table 3.2a). When the total concentration of cells in culture was doubled (target cells, 2 x 10^7-> 4 x 10^7 / ml and parasitised cells, 2 x 10^6 -> 4 x 10^6 / ml), the levels of invasion more than doubled. The ratio was then changed to 1 : 5 (parasitised cells, 8 x 10^6; target cells, 4 x 10^7) and a four-fold increase in the levels of invasion was achieved when compared to the results of the initial invasion assay.

Other researchers routinely remove leucocytes from parasite-infected erythrocyte preparations. The simplest method involves the use of the ion-exchange column CF11 (Richards & Williams, 1973) though other methods, such as Wunderlich's (Wunderlich *et al*, 1987) use of sea sand overlaid with Sephadex G 25 and SE - cellulose, are also used. In order to study the effect of a CF11 column on invasion levels, infected blood was passed over a column equilibrated with RPMI - 1640 prior to purification of the infected cells. Both CF11 column-

Table 3.2a **Estimation of cell numbers and ratios necesary for successful *in vitro* invasion**

Target cells	Cell number target / parasitised	Ratio	Relative invasion levels
rat	$2 \times 10^7 / 2 \times 10^6$	10 : 1	100.0
	$2 \times 10^7 / 4 \times 10^6$	5 : 1	192.9
	$4 \times 10^7 / 4 \times 10^6$	10 : 1	242.9
	$4 \times 10^7 / 8 \times 10^6$	5 : 1	400.0

Invasion levels were between 1.2 and 5.6 %, in all experiments.

Table 3.2b **The effect of a CF11 column on levels of invasion**

Target cells	Treatment	Cell number target / parasitised	Relative invasion levels
rat	none	$4 \times 10^7 / 8 \times 10^6$	100.0
	CF11	"	175.5
mouse	none	"	100.0
	CF11	"	119.3

Invasion levels were between 1.2 and 5.6 %, in all experiments.

treated cells and non-treated cells were tested in invasion assays using either mouse or rat cells as the target cells (4×10^7 / 8×10^6 cells). The results are shown in Table 3.2b. The use of the CF11 column improved the invasion levels into mouse and rat cells, though the effect was more pronounced with rat erythrocytes. It was also noted that there was a higher level of invasion into mouse erythrocytes compared to rat erythrocytes. The invasion rate into rat cells was 1.85 %, whereas that into mouse cells was 2.85 %.
In the experiments carried out above 10 % FCS was used. Different concentrations of FCS and rat serum (RS) were tested within the invasion assay (the parasite-infected erythrocyte preparation had been passed over a CF11 column) and it was discovered that 50 % FCS improved the rate of invasion achieved when compared to 10 % FCS ($p \leqslant 0.05$). RS, however significantly increased the invasion rate compared to 10 and 50 % FCS (Table 3.2c). The RS was heat-inactivated for the invasion assays as non-treated serum caused the clumping of mouse cells and the destruction of human, rabbit and guinea pig erythrocytes.
While 10 % RS improved the invasion rate it was observed that 50 % RS caused the lysis of all erythrocytes in the assay.

Table 3.2c The effect of different serums on levels of erythrocyte invasion

Target cells	Treatment	Cell number target / parasitised	Relative invasion levels
mouse	10% FCS	$4 \times 10^7 / 8 \times 10^6$	100.0
	50% FCS	"	211.8
	10% RS	"	329.4
	50 % RS	"	Lysis

Invasion levels were between 1.2 and 5.6 %, in all experiments.

As the project developed a further modification was made to the invasion assay. Instead of adding *P. c. chabaudi* AS-infected cells to the target cells directly after purification, the parasites were cultured for four hours *in vitro* in a 5 ml culture flask to allow development of the parasites to late trophozoites / segmentors prior to their addition to target erythrocytes. This modification led to easier handling of the assay as the parasitised erythrocytes were prepared at a different time to the preparation of the target erythrocytes. In summary, the erythrocyte invasion assay developed included the following steps;

a) Infected mice were bled at between 13.00 - 13.30 (Fig. 3.3a)

b) The infected blood was passed over a CF11 column, washed and the cells were purified on a Percoll gradient to a level of > 95 % (Fig. 3.3b).

c) The purified parasites were then cultured for four hours to allow for parasite development (Fig. 3.3c).

d) The cultured parasites were added to target cells and cultured for 19 hours (Fig. 3.3d).

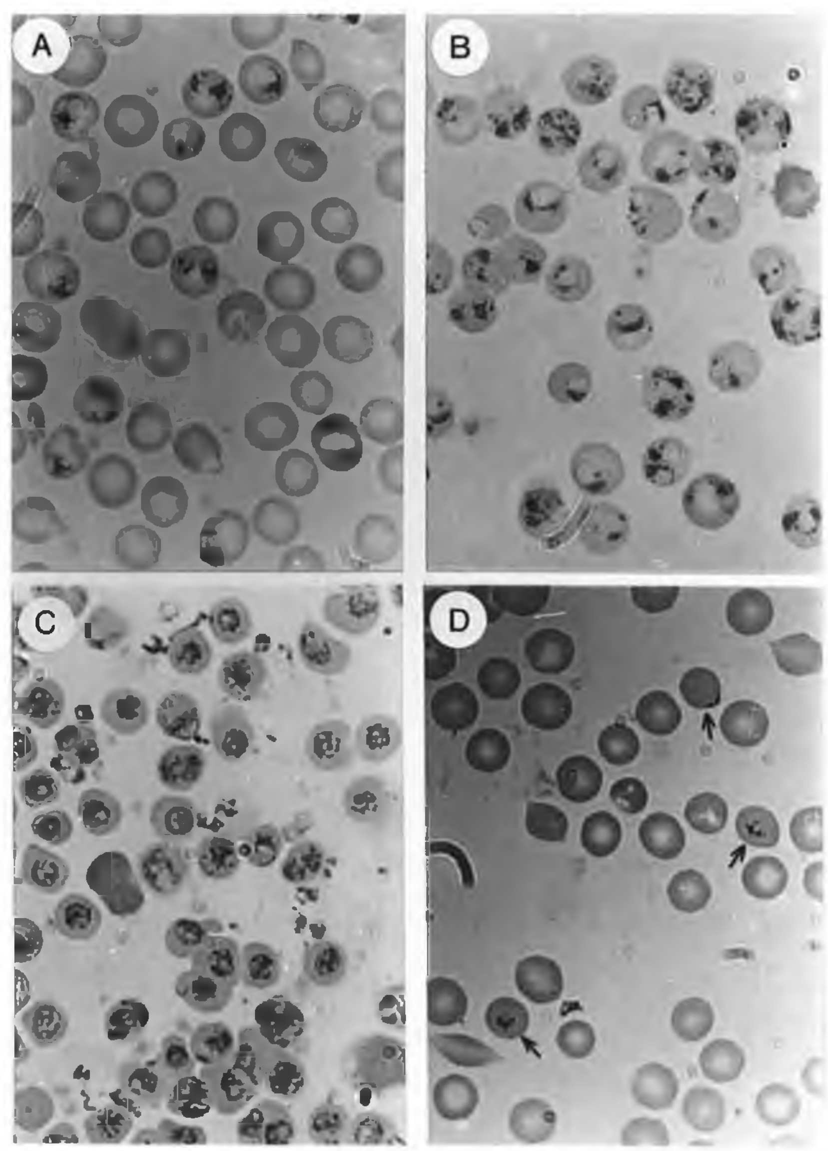


Fig. 3.3 Purification of *Plasmodium c. chabaudi* -infected erythrocytes, *in vitro* culturing and reinvasion. Blood obtained from infected CD - 1 mice (A) was layered onto a 74 % Percoll gradient. After centrifugation the parasite-infected erythrocytes banded at the top of the Percoll (B) and were removed, washed and maintained in culture for 4 hr (C) before adding to target erythrocytes. Reinvasion was assessed by estimating the number of ring-stage parasites in erythrocytes 19 hr later (D, arrowed). (D) Shows reinvasion into erythrocytes obtained from 8 week old CD - 1 mice.

3.5 The mechanism of erythrocyte invasion by malaria parasites.

The malarial merozoite has the unique ability to distinguish between erythrocytes of host and non-host erythrocytes. The basis of this selectivity is the recognition, at a molecular level, by the parasite of specific ligands on the erythrocyte surface. Only for three species of *Plasmodium* (*P. falciparum, P. vivax* and *P. knowlesi*) have the ligands been outlined in any detail (Table 1.3). The ligand system for rodent malarias remains largely unexplored and there is no information available on the mechanism by which *P. chabaudi* invades erythrocytes due to the absence of an *in vitro* erythrocyte invasion assay. With the development of this assay, the molecular mechanism of invasion can now be studied.

To help understand the mechanisms of invasion, a four step study was carried out. These steps were to investigate the susceptibility of :

a) erythrocytes from various species to invasion by *P. c.chabaudi* AS

b) erythrocytes from various strains of mice to invasion

c) erythrocytes (rodent) of varying ages to invasion

d) enzyme-treated erythrocytes

3.5.1 Invasion of erythrocytes obtained from various species

Plasmodium c. chabaudi is a rodent malaria which *in vivo* infects mice and rats but whose infection is refractory in other rodent species, including guinea pigs and rabbits (Wery, 1968) as well as higher mammals e.g. humans. Erythrocytes from mice, rats, guinea pigs, rabbits and humans were obtained and added to parasitised erythrocytes (8×10^6 parasitised cells / 4×10^7 target erythrocytes) in a 24-well tissue culture plate containing complete RPMI - 1640 supplimented with 10 % RS and incubated for 19 hours, under standard conditions (Section 2.13).

On analysis of the results of the assay (Fig. 3.4) invasion levels of approximately 6 % were achieved with mouse erythrocytes. Levels of invasion in the region of 3.7 % were achieved with rat cells. No newly invaded erythrocytes were detected with the assays carried out on guinea pig, rabbit and human erythrocytes. These cells therefore proved to be resistant to invasion by *P. c. chabaudi* AS *in vitro*, as was expected.

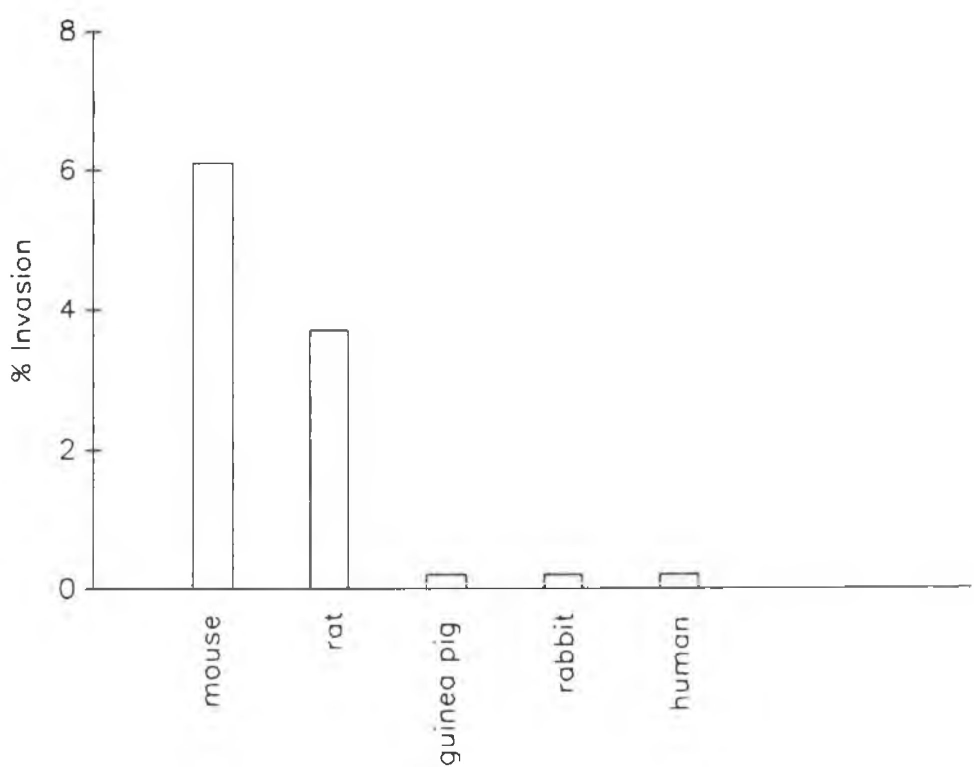


Fig. 3.4 Invasion into erythrocytes obtained from various species

3.5.2 Invasion of erythrocytes obtained from different strains of mice

Wery (1968) and Bafort (1971) noted that some mice were "susceptible" to *P. chabaudi* infection, with the parasitaemia increasing to 50 %, becoming chronic and then latent within 20 days, while some mice were "resistant", where the parasitaemia reached about 20 % before becoming became latent. In some strains of mice infection with *P. c. chabaudi* is fatal (Carter & Walliker, 1975) while in others it is non-fatal and follows the characteristic course of infection of a peak in parasitaemia followed by recrudescence (Jarra & Brown, 1989). *Plasmodium c. chabaudi* AS invasion assays were carried out using target erythrocytes obtained from three commonly used strains of laboratory mouse; Balb / c, Schofield and CD - 1. In our laboratory *P. c. chabaudi* proved to be fatal in Balb / c mice, while CD - 1 mice were susceptible and Schofield mice were resistant. The target cells were added to parasitised erythrocytes as before (Section 2.13). On analyis of invasion after the 19 hour assay period, successful invasion was observed with erythrocytes from the three strains, with levels of invasion in the region of 6 % being achieved (Fig. 3.5). This value was similar for all the strains, indicating that there was no significant difference between the susceptibility of erythrocytes from these different strains *in vitro*.

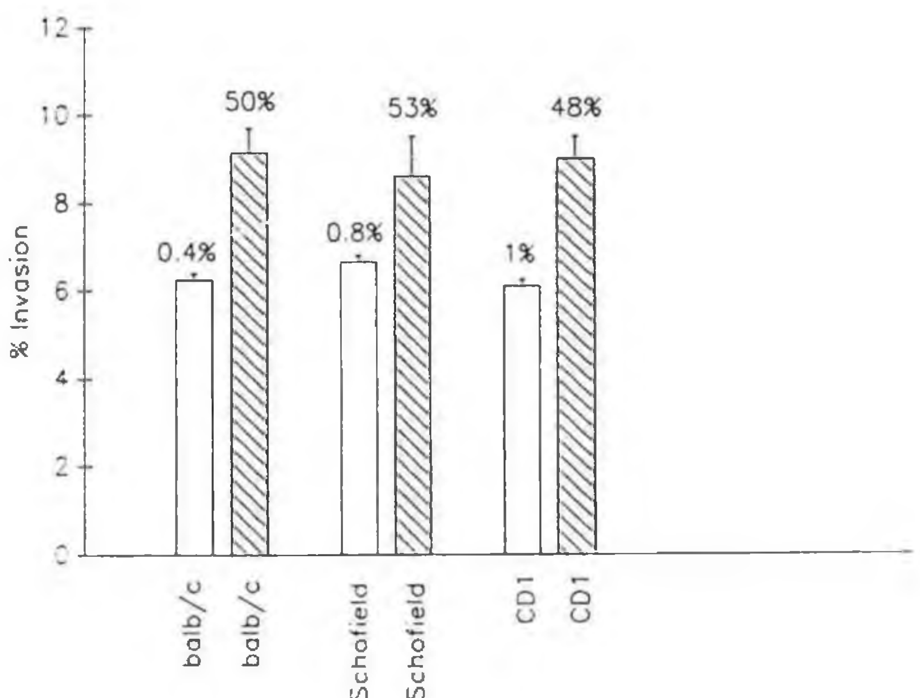


Fig. 3.5 Invasion of erythrocytes obtained from different strains of mice. [hatched box] erythrocytes from PHZ - treated mice. [open box] erythrocytes from untreated mice. (The percentage of reticulocytes is shown above their respective columns).

3.5.3 Invasion into erythrocytes from different age groups

Plasmodium chabaudi has in the past been characterised by it's lack of a preference for reticulocytes (Landau, 1965). Carter & Walliker (1975) reported that this species of the malaria parasite has a strong preference for mature erythrocytes (normocytes), though where infection with this parasite resulted in considerable anaemia and reticulocyte production, the reticulocytes were often invaded (Carter & Walliker, 1975; McDonald, 1977). Jarra & Brown (1989) reported that no preference was exhibited by *P. c. chabaudi* for either normocytes or reticulocytes.

Adult Schofield, Balb / c and CD - 1 mice (8 - 10 weeks old) were treated with phenylhydrazine to induce reticulocytosis (stimulation of red blood cell production due to the destruction of circulating red blood cells). The treatment of animals with phenylhydrazine resulted in an increased proportion of reticulocytes in the blood preparations used for invasion assays. Levels of > 50 % reticulocytes, as detected by methylene blue staining, were routinely achieved. Untreated mice contained approximately 1 % reticulocytes. Erythrocyte invasion assays were carried out according to Section 2.13, using blood cells from the three strains of mice, both treated and untreated. It is shown in Fig. 3.5 that invasion levels into phenylhydrazine-treated mice were increased significantly when compared to untreated mice ($p \leqslant 0.01$), with an increase in invasion levels of approximately 40 % being obtained in the three strains of mice. While there was a significant increase in the invasion levels between treated and untreated mice, there was no significant difference between invasion levels into erythrocytes in blood preparations from the three strains of mice.

Red blood cells from adult mice (8 - 10 weeks old) and immature mice (2 weeks old) were also used in erythrocyte invasion assays. Young mice in general have slightly elevated numbers of reticulocytes in their blood. The invasion assays were carried out as before (Section 2.13). Examination of the stained slides of these cells showed that increased levels of invasion were achieved with the blood cells obtained from young mice (Fig. 3.6). While this increase was not as great as that observed with phenylhydrazine-treated mice, it was still significant ($p \leqslant 0.05$). A possible explanation for the smaller increase

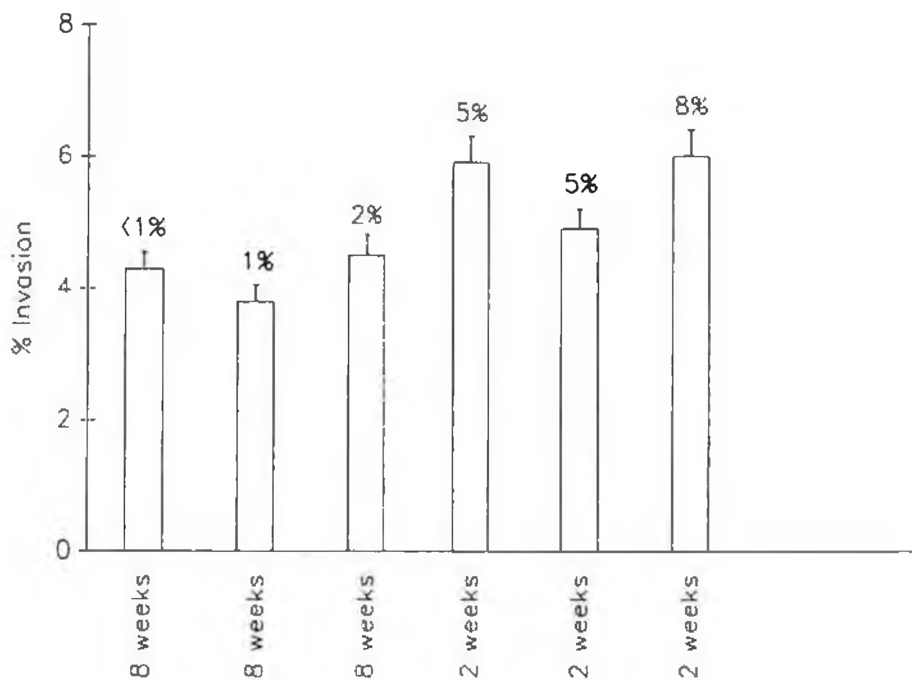


Fig. 3.6 Invasion assay into erythrocytes from two different age groups. Cells from adult (3) and immature (3) mice were used. (The percentage reticulocytes is shown above their respective columns).

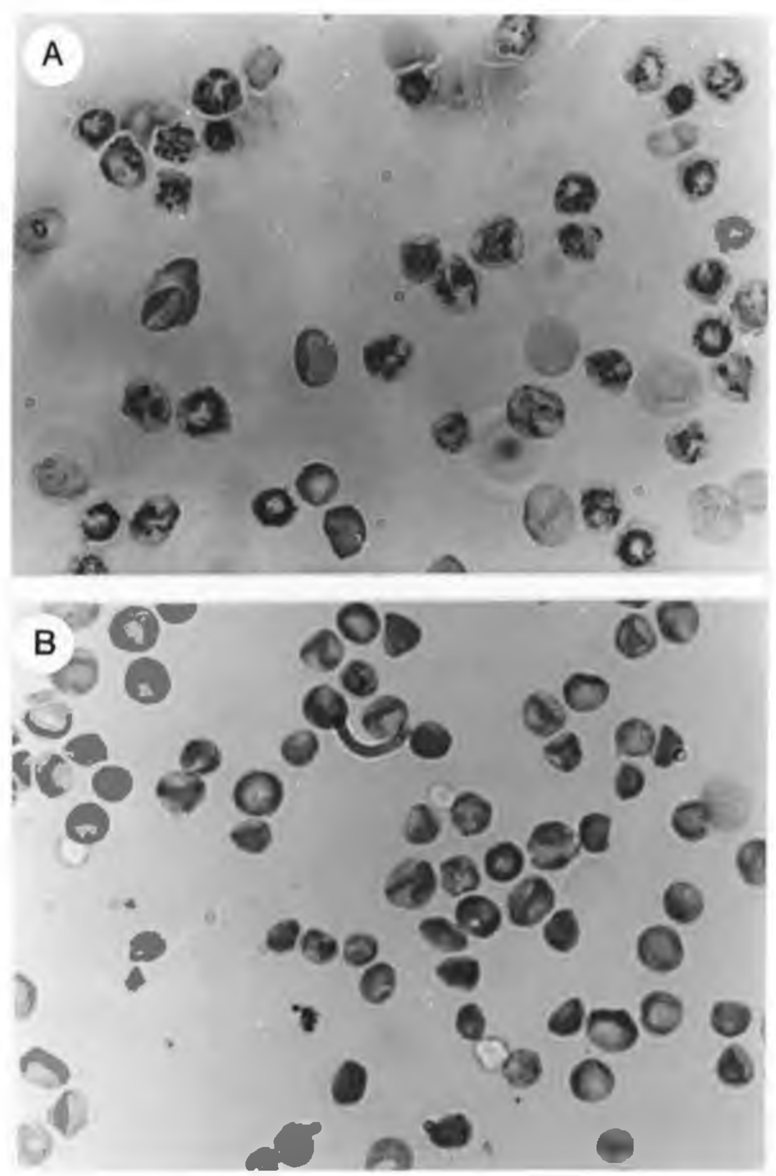


Fig. 3.7 Maturation of reticulocytes *in vitro*. Blood obtained from phenylhydrazine-treated, 8 week old CD - 1 mice was stained with methylene blue and the percentage reticulocytes estimated before (Panel A, reticulocytes = 56.6 %) and after (Panel B, reticulocytes = 1.2 %) maintainance *in vitro* for 19 hours under the same conditions as the invasion assays.

is the fact that the reticulocyte numbers in the two week old mice (5 - 8 %) were considerably lower than those of phenylhydrazine-treated mice.

Development of reticulocytes *in vitro*.

To investigate whether the increased level of invasion that was observed in invasion assays using blood from phenylhydrazine-treated mice was due to preferential invasion of reticulocytes, the cells were examined using methylene blue staining. By studying the relative numbers of reticulocytes and normocytes in the assays after the 19 hour incubation period, more information could be gained about the cell type that was invaded by *P. c. chabaudi* AS. Unfortunately, on analysis of the blood cells after incubation under the same conditions as an invasion assay, the reticulocyte count had decreased from 56.6 % to 1 %, as detected by methylene blue staining (Fig. 3.7). Because of this reticulocyte maturation, it was not possible to compare the relative invasion of *P. c. chabaudi* AS into reticulocytes and normocytes. Therefore it was concluded that there was increased invasion into cell preparations enriched with reticulocytes, although not necessarily into the reticulocytes themselves.

3.5.4 Invasion of P. c. chabaudi AS into enzyme treated or chemically modified cells

The process of invasion depends on the capability of merozoites to recognise ligands on the erythrocyte membrane. The study of the effects of enzyme treatment or chemical modification of murine erythrocytes on the invasive process, using *in vitro* invasion assays, can be used to define the chemical nature of these ligands on the cell surface essential to successful invasion.

a) proteinase digestion

Several proteinases were used to digest target cells by cleaving specific peptide links in membrane polypeptides before the cells were used in invasion assays to ascertain the effect of the enzyme on invasion levels. Four proteinases were used to digest the proteins on target cell membranes (trypsin, chymotrypsin, papain and protease XXV [Pronase E]). Both rat and mouse cells were treated with proteases.The target cells were digested for two hours at 37 ^{0}C before being washed in complete RPMI - 1640 and protease inhibitors. The cells were then washed again in medium. These cells were added to the invasion assay and incubated for 19 hours in an invasion assay (Fig. 3.8).

When cells were digested with trypsin, there was a significant reduction of 25 % in invasion levels obtained with a 1 mg / ml solution of the enzyme ($p < 0.05$), while there was no effect with lower concentrations. A reduction in invasion of 66 % was observed in cells treated with 1 mg / ml of chymotrypsin ($p < 0.05$) while the reduction of 39 % with 10 µg / ml of enzyme was not statistically significant. While there was no effect on invasion observed with enzyme digestion with papain, a reduction was observed with Pronase. A reduction of 57 % was detected upon digestion with a 1 mg / ml solution of Pronase ($p < 0.05$) while the reduction of 35 % observed with a 10 µg / ml solution of the enzyme was not significant.

Upon proteinase digestion of rat cells (Fig. 3.9), there was no significant effect on the levels of invasion achieved into the treated

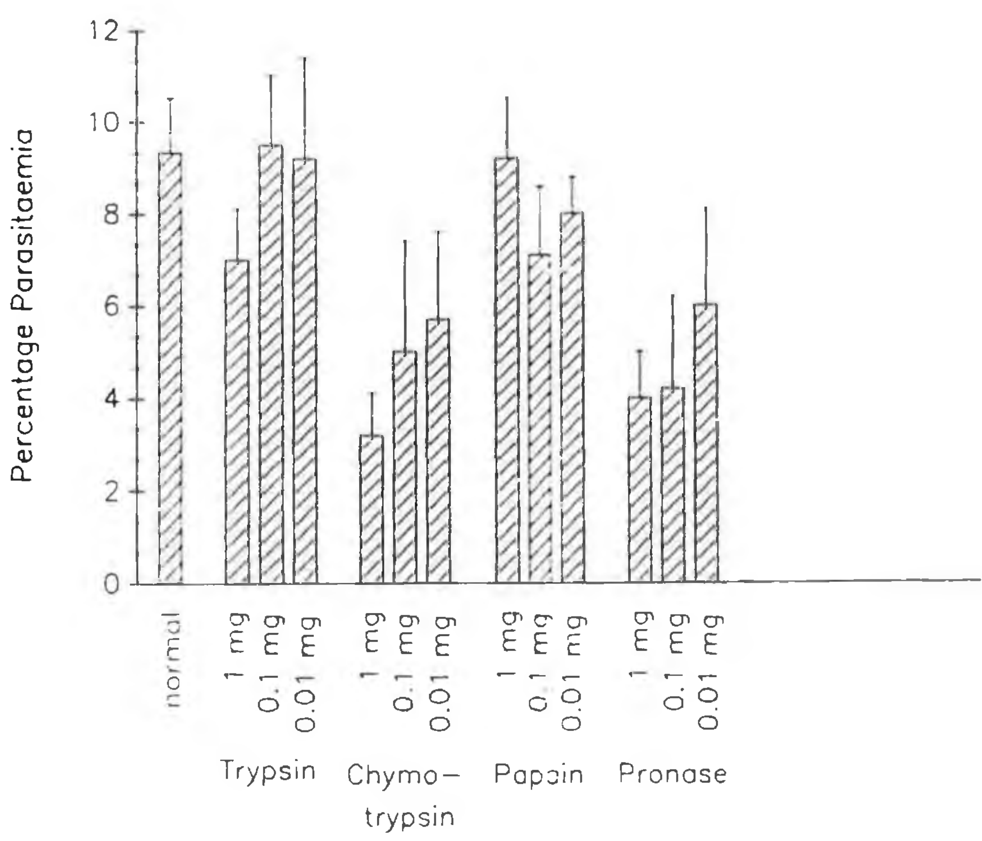


Fig. 3.8 Invasion of murine erythrocytes after proteinase digestion. Target cells were digested at different concentrations of enzyme for two hours, at 37 ^{0}C. The cells were then washed in protease inhibitors and medium, and added to the invasion assay. The level of invasion (expressed as Percentage Parasitaemia) into treated cells was then analysed and compared to invasion into untreated (normal) cells.

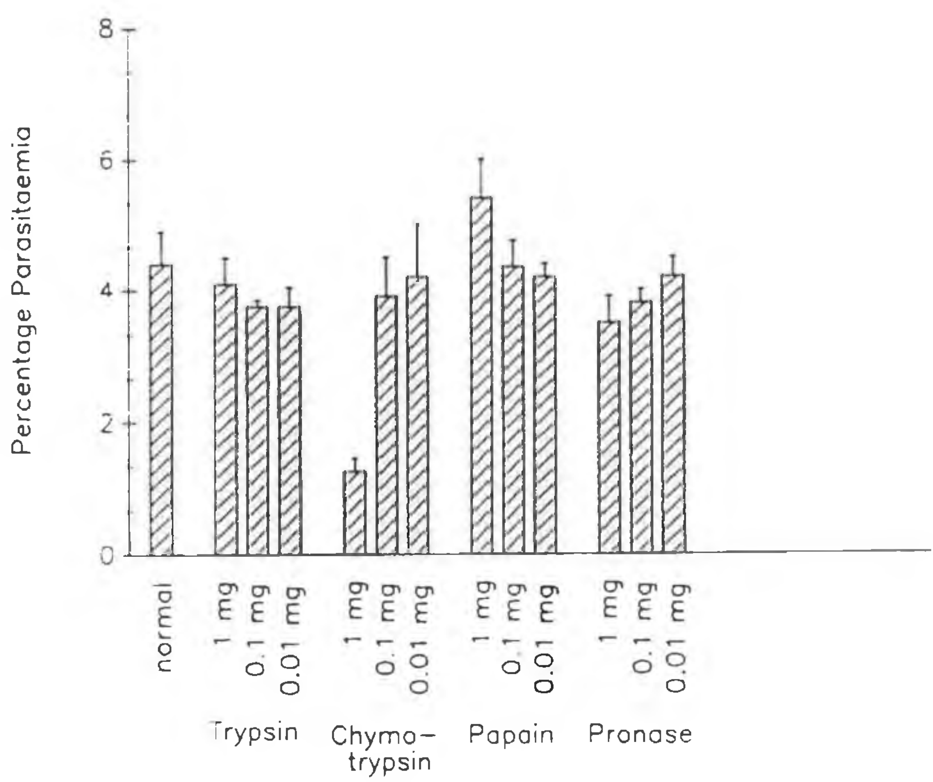


Fig. 3.9 Invasion of rat erythrocytes after proteinase digestion. Target cells were digested at different concentrations of enzyme for two hours, at 37 ^{0}C. The cells were then washed in protease inhibitors and medium, and added to the invasion assay. The level of invasion (expressed as Percentage Parasitaemia) into treated cells was then analysed and compared to invasion into untreated (normal) cells.

target erythrocytes with the exception of chymotrypsin and Pronase. There was a reduction of 71.6 % with a 1 mg / ml solution of chymotrypsin and a reduction of 20.3 % with a 1 mg / ml solution of Pronase ($p < 0.05$).

During the maturation process from reticulocyte to normocyte the cell membrane, it's skeleton and the internal cytoskeleton undergo various changes. At least two processes are involved: (1) the appearance and assembly of red cell-specific components and (2) the loss of constituents which are specific for the non-differentiated precursors. For example the expression of cell surface glycophorins during differentiation were found to reach maximal antigenic expression in mature erythrocytes (Fukuda *et al*, 1980). Based on the differences in cell membrane between normocytes and reticulocytes, cells were obtained from phenylhydrazine-treated mice and were digested with proteinases. The enzyme digestion was carried out on blood cells enriched with 42 % reticulocytes to monitor if an increased level of inhibition was linked to higher numbers of reticulocytes.
No significant effect was detected when the cells were digested with trypsin, pronase and papain (Fig. 3.10). A significant reduction of 52 % was observed with blood cells that had been digested with a 1 mg / ml solution of chymotrypsin ($p \leq 0.05$). Similar results were obtained when erythrocyte preparations with high and low reticulocyte counts were used.

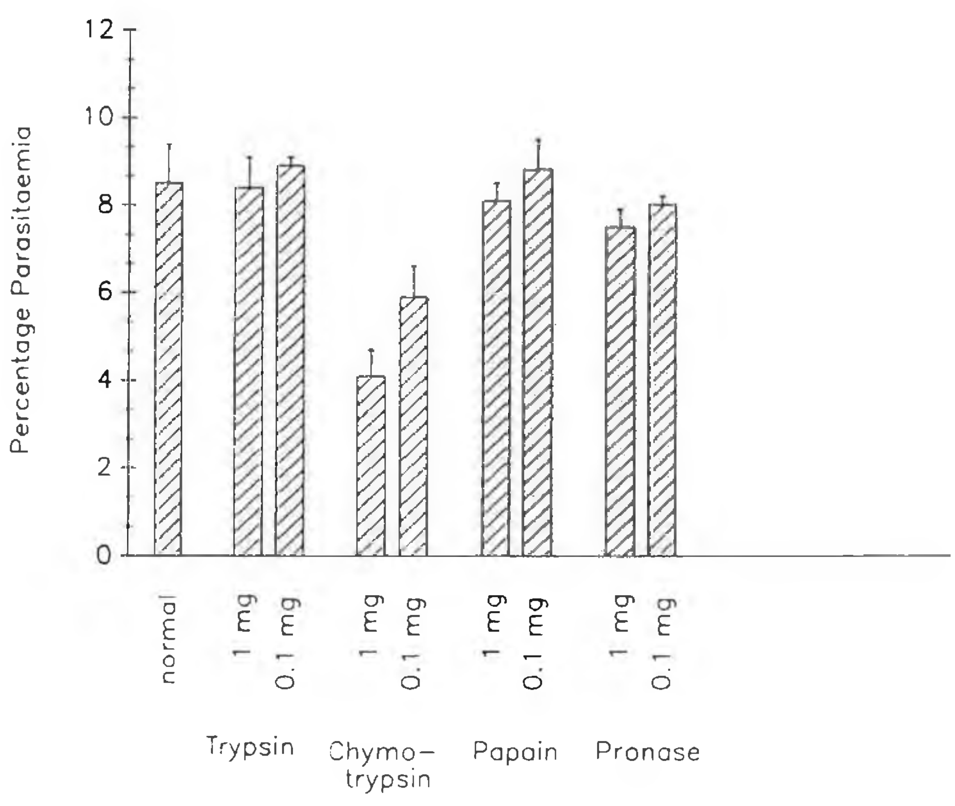


Fig. 3.10 Invasion of PHZ - treated murine erythrocytes after proteinase digestion. Target cells were digested at different concentrations of enzyme for two hours, at 37 ^{0}C. The cells were then washed in protease inhibitors and medium, and added to the invasion assay. The level of invasion (expressed as Percentage Parasitaemia) into treated cells was then analysed and compared to invasion into untreated (normal) cells.

b) neuraminidase digestion

N-Acetylneuraminic acid (NeuNAc) is the terminal sugar residue of the O-linked tetrasaccharide linked to erythrocyte sialoglycoproteins, glycophorins. Neuraminidase is a sialase which cleaves off these terminal NeuNAc residues, which are implicated in *P. falciparum* invasion (Perkins & Holt, 1988).

Mouse cells were treated for two hours at 37 ^{0}C in varying concentrations of neuraminidase. Analysis of stained slides after the assay incubation period was completed showed that there was no significant effect on the levels of invasion achieved when compared to the untreated target cells (Table 3.3).

Blood cells from phenylhydrazine-treated mice (reticulocytes > 50 %) were also digested with neuraminidase to investigate whether this enzyme had an effect on invasion into target cells containing a high proportion of reticulocytes; however no effect on invasion was observed compared to untreated cells (Table 3.3).

Table 3.3 Neuraminidase digestion of mouse erythrocytes

Neuraminidase (1 Unit / ml)	Relative Invasion
no treatment	100
100 mU	98.9
50 mU	107.5
25 mU	96.8
10 mU	104.3
5 µl	91.3
reticulocytes / 50 mU	101.5
reticulocytes / 25 mU	96.3

c) Sodium periodate treatment of target erythrocytes

Sodium periodate treatment results in the cleavage of the exocyclic hydroxyl groups of the terminal NeuNAc but leaves it's COOH group unaltered (Perkins & Holt, 1988). Initially treatment of target cells was carried out by treating the cells with periodate prepared in PBS. The cells were then washed a number of times to remove excess periodate and the cells added to parasitised erythrocytes in an invasion assay. However, with this method there was no effect on invasion. If the cells were treated with 1 % glycine after periodate treatment of the cells to prevent the reformation of the exocyclic hydroxyl groups cleaved by the chemical, there was a significant inhibitory effect on the levels of invasion obtained (Fig. 3.11). Invasion was inhibited by greater than 50 % with 20 mM sodium periodate and the effect gradually decreased to 28 % with 0.01 mM periodate. The inhibition of invasion was significant at ail concentrations of sodium periodate used with the exception of 0.01 mM ($p < 0.05$). There was no affect on invasion levels when the target erythrocytes were treated with 1 % glycine alone.

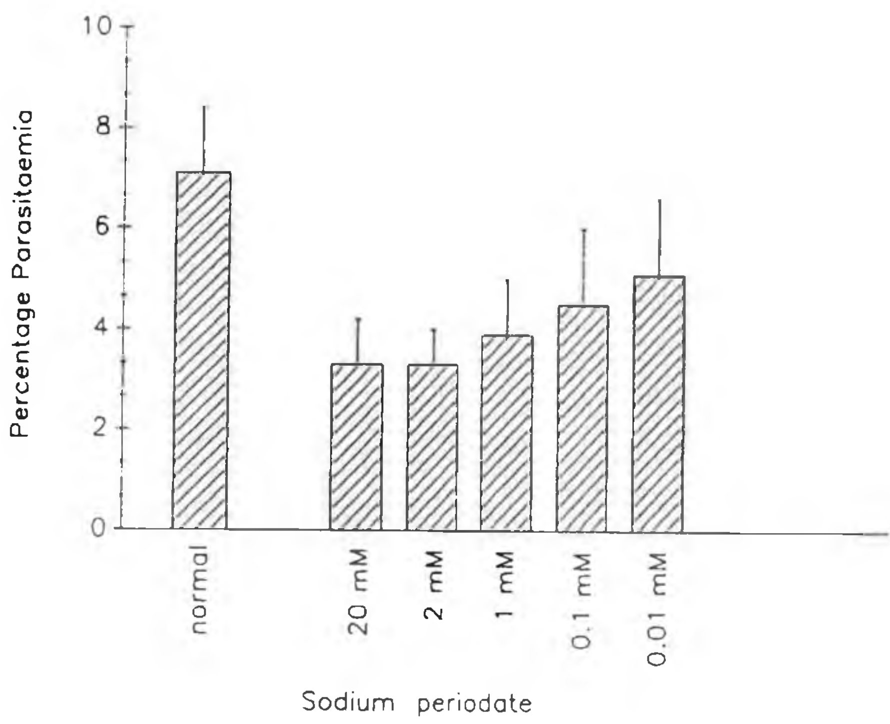


Fig. 3.11 Invasion of murine erythrocytes after sodium periodate treatment. Target cells were incubated in sodium periodate for five minutes, washed and incubated for a further 30 minutes in 1 % glycine. The erythrocytes were washed and then added to the invasion assay. The level of invasion (expressed as Percentage Parasitaemia) into treated cells was then analysed and compared to invasion into untreated (normal) cells.

3.6 Investigations to improve erythrocyte invasion assays with new commercially available media

RPMI - 1640 is a nutritionally complex medium that is routinely used as the basal medium for the culture of many malaria species including *P. c. chabaudi* (McDonald & Sherman, 1980; Miller *et al*, 1983, Mons *et al*, 1983; Trager & Jensen, 1976). It was for this reason that RPMI - 1640 was used for the development of a *P. c. chabaudi* AS invasion assay. Since no report was available indicating this medium as the most suitable for rodent malaria maintenance plus the fact that there are now a large number of commercial media available, it was decided to test eight nutritionally complex media as a basal medium and to compare them to RPMI - 1640 using the erythrocyte invasion assay.

3.6.1 Study of a range of commercially available media for use in P. c. chabaudi *AS invasion assays*

In the first set of experiments, parasites were purified and cultured for four hours in RPMI -1640 to allow parasite development. The schizont-infected erythrocytes were then added to microtitre wells containing erythrocytes suspended in the various commercially available media being tested and incubated for 19 hours. The cells were removed from culture and the percentage of newly invaded cells estimated by giemsa staining. It was clear from the levels of merozoite invasion that there is considerable variation in the suitability of the different media for the *in vitro* culturing of *P. c. chabaudi* AS. Based on this difference the media were divided into three groups (Table 3.4).
Invasion levels in the Group I media were 50 % below those observed with RPMI - 1640, while the Group II media had invasion levels that were similar to RPMI -1640 (Fig. 3.12). However the media in Group III consistently and significantly improved merozoite invasion as compared with RPMI - 1640 ($p < 0.05$). It was noted that the newly invaded parasites also showed a variation in their rates of maturation (Table 3.4), which was indicated by a variation in the number of ring-stage parasites that had matured to trophozoites. An improved rate of parasite development was particularly evident in William's E medium,

Table 3.4 Rate of maturation of *P. c. chabaudi* AS parasites during invasion assays.

Medium	% Invasion[a]	% ring - stage	% trophozoites
RPMI - 1640	4.2 +/- 0.3	92.6 +/- 2.0	7.4 +/- 2.0
Group I			
BGJb	1.9 +/- 0.7	89.9 +/- 1.2	10.1 +/- 1.2
CMRL	1.5 +/- 0.5	95.4 +/- 3.5	4.6 +/- 3.5
NCTC	1.3 +/- 0.5	96.4 +/- 2.6	3.6 +/- 2.6
Group II			
McCoy's	4.0 +/- 0.4	84.1 +/- 6.9	15.9 +/- 6.9
MEM	3.5 +/- 0.2	92.3 +/- 1.3	7.7 +/- 1.3
Group III			
BME	6.0 +/- 1.5	81.2 +/- 10.2	18.8 +/- 10.2
DMEM	6.0 +/- 0.35	79.8 +/- 3.5	20.0 +/- 3.5
William's E	5.1 +/- 0.7	54.3 +/- 9.8	45.7 +/- 9.8

a = average of a minimum of three experiments

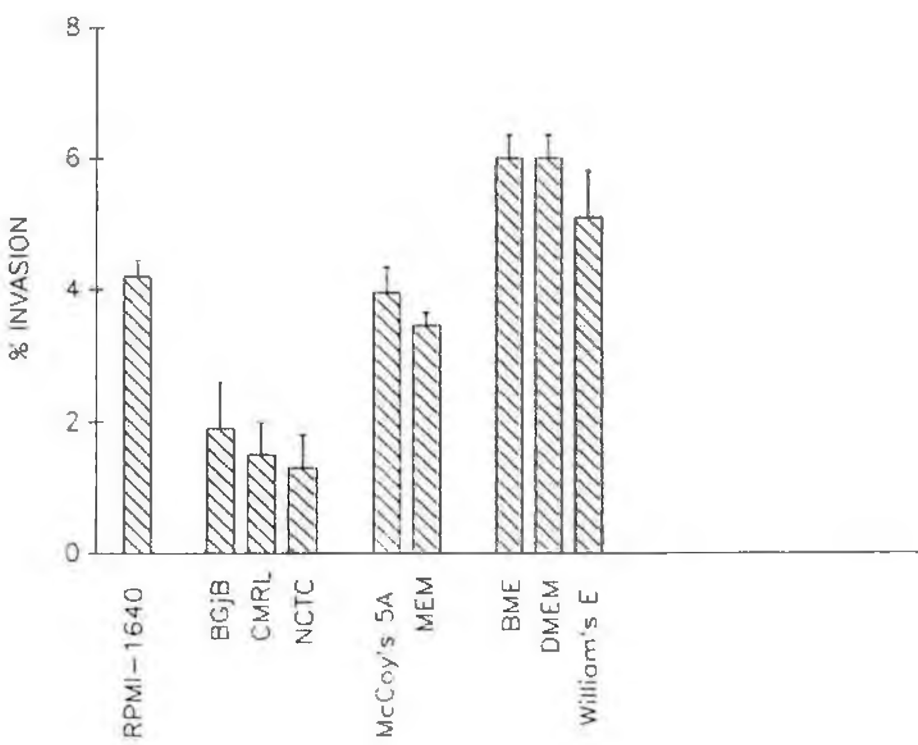


Fig. 3.12 Investigation into the suitability of a range of commercially available media for *P. c. chabaudi* invasion assays. Comparison of invasion where the parasites were cultured for four hours in RPMI - 1640 before being assayed

in which 45.7 % of the newly invaded parasites had developed to trophozoites, whereas in RPMI - 1640 only 7.4 % had reached this stage ($p < 0.05$).

In the second set of experiments the initial four hour culture was carried out in either RPMI - 1640, BME, DMEM or William's E before incubation with target erythrocytes in their respective medium (Fig. 3.13). The observed merozoite invasion levels were greater with BME, DMEM and William's E when compared to RPMI - 1640 ($p < 0.05$).

On analysis of the parasite-infected erythrocytes after the initial four hour incubation and prior to their addition to the invasion assay, trophozoite development into schizonts appeared to be more rapid than in RPMI - 1640. The parasites themselves also appeared to be healthier.

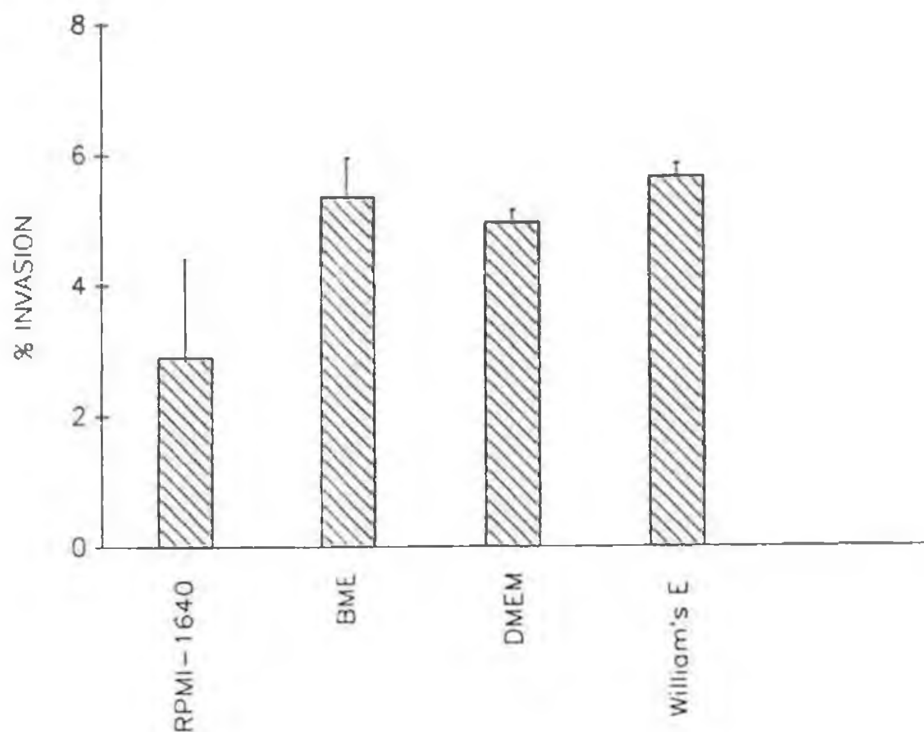


Fig. 3.13 Investigation into the suitability of a range of commercially available media for *P. c. chabaudi* invasion assays. Comparison of invasion where the parasites were cultured for four hours in their respective medium before being assayed.

3.6.2 Radio-isotope incorporation

Rate of maturation of parasites in the various media was examined using radiolabelled amino acid uptake. Purified parasite-infected erythrocytes were cultured in radioisotope-containing medium over the four hour primary incubation period. The uptake of [^{3}H]isoleucine and [^{35}S]methionine was tested in BME, DMEM, William's E and RPMI - 1640. Uptake of both isotopes by developing parasites was greater in BME, DMEM and William's E when compared to RPMI - 1640, with BME resulting in the highest rate of uptake (Fig. 3.14).

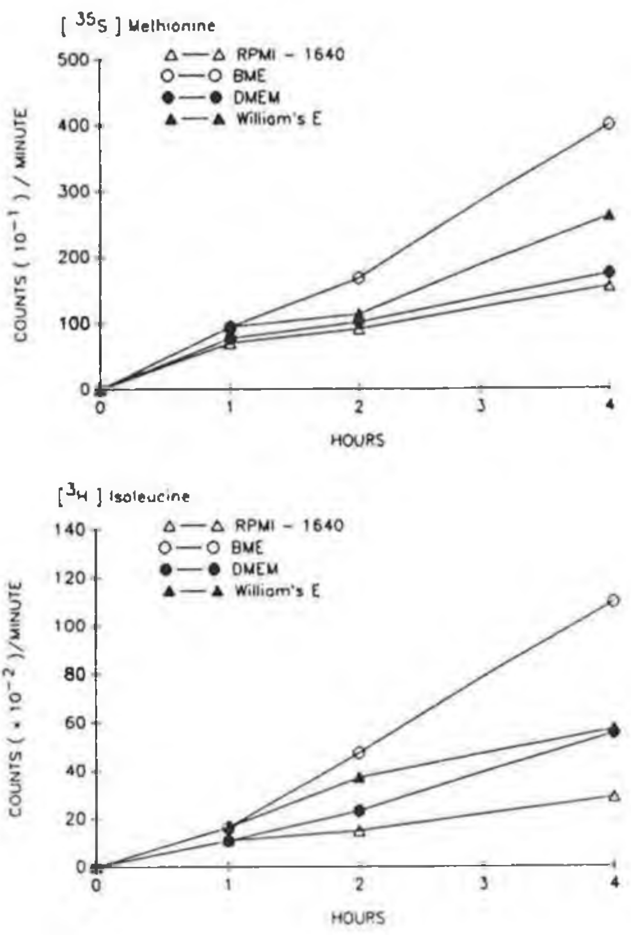


Fig. 3.14 Radioisotope incorporation by *P. c. chabaudi* parasites. Upper panel shows the incorporation of [^{35}S]methionine by parasite-infected erythrocytes cultured in a range of selected media, while the lower panel shows the incorporation of [^{3}H]isoleucine by parasites.

3.6.3 *Formulation of a new medium for* P. c. chabaudi *AS* in vitro *invasion assays*

Since BME resulted in the best rate of trophozoite to schizont maturation (Section 3.6.2) and William's E showed superior ring-stage to trophozoite development (Section 3.6.1), combinations of the two media were examined in the invasion assay. It was found that the two media complimented each other with increased levels of invasion consistently recorded with three parts BME and one part William's E, compared to RPMI - 1640 and William's E alone ($p \leqslant 0.05$). The invasion level was higher than with BME alone though not significantly so (Fig. 3.15).

When the level of maturation of parasites into young trophozoites was examined, it was found that the combination of three parts BME and one part William's E did not significantly improve the rate of maturation when compared to BME alone (Table 3. 5) although the difference was highly significant when compared to RPMI - 1640 ($p \leqslant 0.05$).

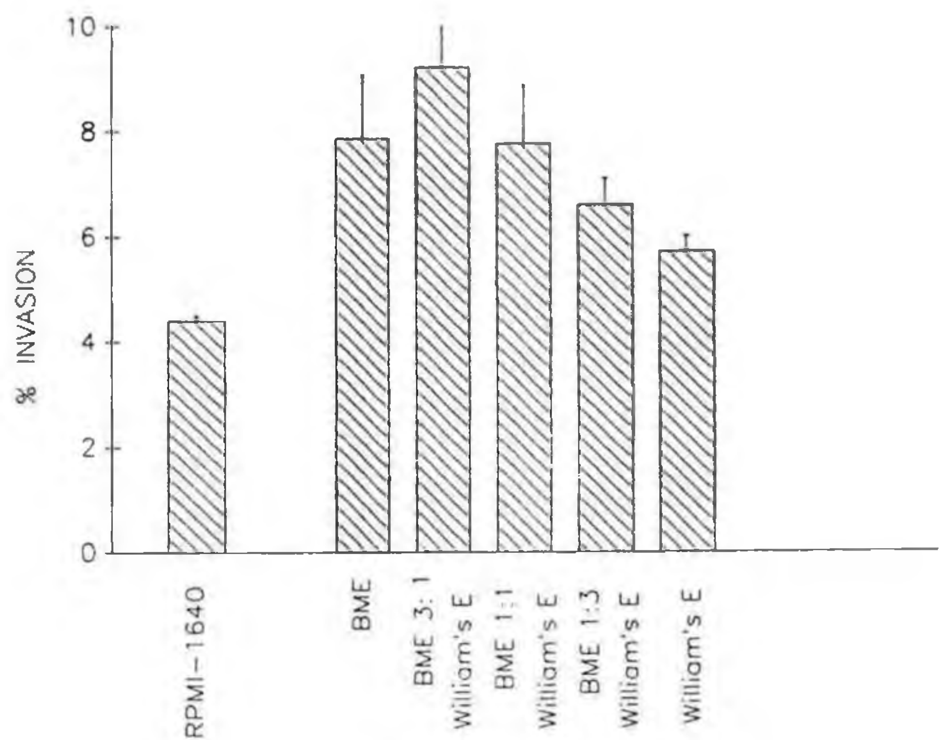


Fig. 3.15 Formulation of a new medium for *P. c. chabaudi* invasion assays. Comparison of BME and William's E medium ratios and their effect on the level of invasion achieved.

Table 3.5 **Rate of maturation of *P. c. chabaudi* AS parasites during invasion assays.**

Medium	% Invasion	% ring - stage	% trophozoite
RPMI - 1640	4.5 +/- 0.1	98.12 +/- 0.4	1.88 +/- 0.4
BME	6.7 +/- 1.2	84.9 +/- 0.6	15.1 +/- 0.6
BME 3 : 1 W-E *	8.0 +/- 1.2	84.1 +/- 5.8	15.9 +/- 5.8
BME 1 : 1 W-E	6.6 +/- 1.1	83.4 +/- 2.9	16.5 +/- 2.9
BME 1 : 3 W-E	6.1 +/- 0.5	71.5 +/- 3.5	18.5 +/- 3.5
William's E	5.4 +/- 0.3	66.2 +/- 6.5	23.8 +/- 6.5

* W-E , William's E

3.7 Identification of *P.c.chabaudi* AS proteins involved in erythrocyte re-invasion

3.7.1 Comparison of protein profiles of in vitro *cultured* P. c. chabaudi AS *parasites*

During parasite development after invasion, parasites are metabolically very active, synthesising nucleic acids, proteins, lipids, mitochondria and ribosomes and assembling these components into new merozoites. The bulk of the protein production takes place in the period leading up to schizogony when the merozoites are produced via segmentation. *Plasmodium c. chabaudi* trophozoite-infected erythrocytes were cultured for six hours to allow development to schizonts. The parasite protein profiles were analysed by extracting soluble proteins from the parasites and electrophoretically separating them.

The protein profile was studied at time 0 (13.00 hrs, Fig. 3.1, Lane 1) and after three (Fig. 3.16, Lane 2) and six hours (Fig. 3.16, Lane 3) of development in culture. A large number of proteins were detected by coomassie staining of the profiles. Most of the proteins were common to the three stages, however a protein of 162 kDa (Fig. 3.16, arrowed) was not visualised in the profile of *P. c. chabaudi* parasites at time zero but was present at three and six hours. This protein appeared to be present in a greater concentration at six hours. Two proteins of molecular weights 87 kDa and130 kDa (Fig 3.16, arrowed) which were present at time zero, appeared to increase in concentration with culturing.

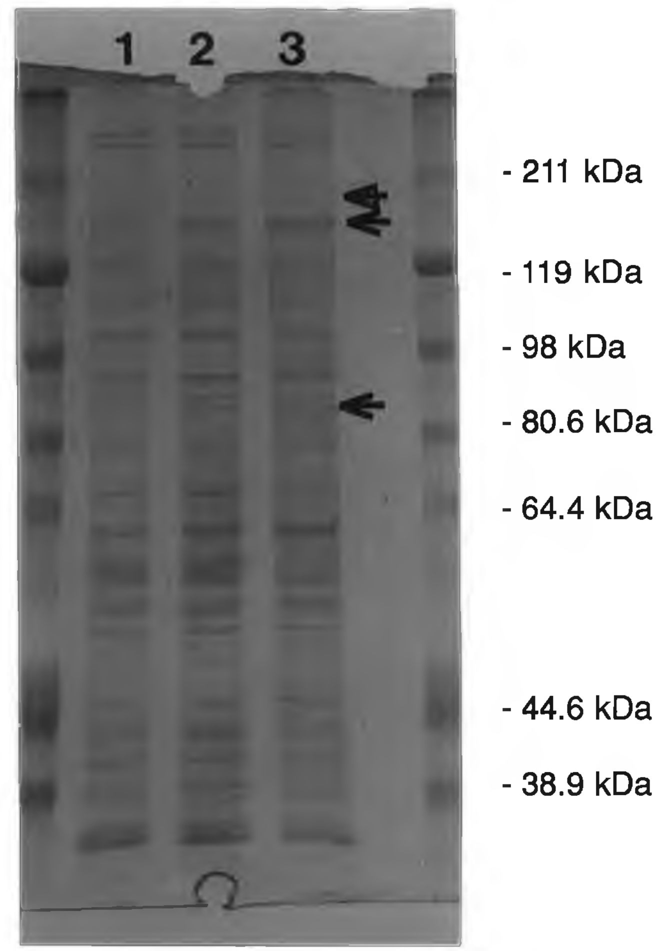


Fig. 3.16 Protein profiles of *P. c. chabaudi* parasite extracts. Parasite-infected erythrocytes were obtained from CD - 1 mice at 13.00 hrs (Lane 1). The purified parasite-infected erythrocytes were then cultured for periods of 3 hrs (Lane 2) and 6 hrs (Lane 3). A band of 162 kDa (arrowed) appeared with parasite development over the 6 hour period, while bands of 87 and 130 kDa increased in concentration with development (arrowed).

3.7.2 Binding assays using [^{35}S]methionine-labelled culture supernatent

Successful invasion of target erythrocytes by merozoites requires the attachment of the merozoite to the cell after properly orientated contact. The attachment occurs through an interaction between the ligands on the erythrocyte and receptors on the merozoite surface after which invasion may proceed. The presence of ligand molecules on the erythrocyte surface is essential for successful merozoite attachment and in their absence invasion can not occur.

Plasmodium c. chabaudi trophozoite-infected erythrocytes were cultured overnight, past the rupture stage in culture medium containing [^{35}S]methionine. The culture was centrifuged to remove cell debris and merozoites and the supernatant retained. Receptor binding assays were carried out by incubating erythrocytes with the metabolically labelled proteins in the culture supernatant. The mixture was centrifuged through silicone oil and the bound proteins eluted with 1.5 M NaCl. The proteins were examined by SDS gel electrophoresis and fluorography.

A large number of *P. c. chabaudi* proteins were radioactively labelled with [^{35}S]methionine (Fig. 3.17, Lane 1). A protein of > 200 kDa and another of 130 kDa from cultured *P. c. chabaudi* bound to and were eluted from mouse erythrocytes (Fig. 3.17, Lane 3) using 1.5 M NaCl. These proteins were the most selectively removed, though there were faint traces of other proteins. When receptor binding assays were carried out using human erythrocytes, no proteins were eluted from the cells (Fig. 3.17, Lane 2).

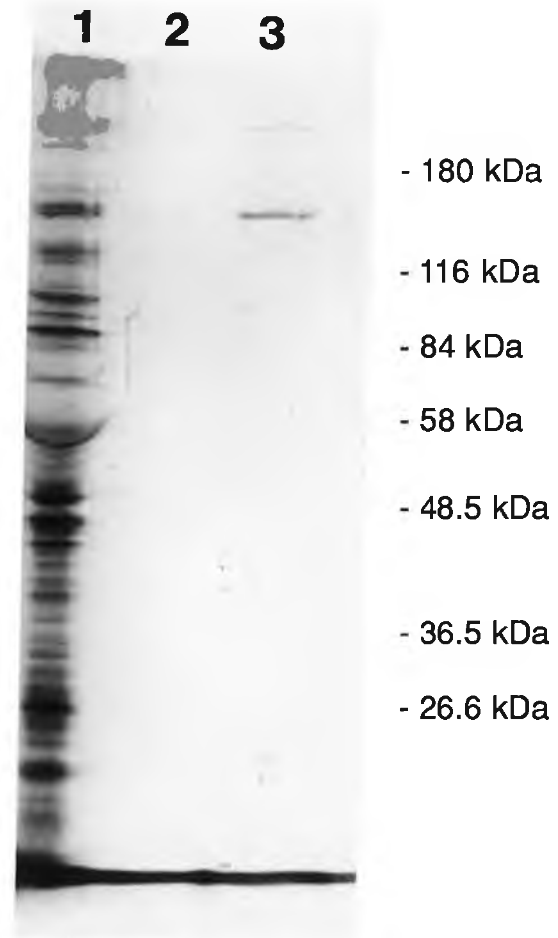


Fig. 3.17 Identification of *P. c. chabaudi* 130 kDa putative erythrocyte receptor. Receptor binding assays were carried out by incubating target erythrocytes with metabolically labelled parasite proteins in culture supernatent. The cells were washed and the bound proteins were removed by elution with 1.5 M NaCl. Receptor binding assays were carried out with human erythrocytes (Lane 2) and with erythrocytes obtained from CD - 1 mice (Lane 3). Total [^{35}S]-metabolically labelled protein from *P. c. chabaudi* culture is shown in Lane 1.

3.7.3 Heparin-Sepharose CL-6B chromatography

The selective binding of a 135 kDa polypeptide produced by *P. knowlesi* merozoites to heparin was demonstrated by passing [^{35}S]-methionine-labelled *P. knowlesi* culture supernatent over Heparin-Sepharose (Dalton *et al*, 1988). This polypeptide has been shown to bind to the Duffy antigen on human blood cells (Dalton *et al*, 1991) and it is believed that it is involved in a direct receptor-like interaction between *P. knowlesi* merozoites and the Duffy blood group antigen during the invasion process.

An extract of *P. c. chabaudi* parasites which had been matured *in vitro* for six hours was passed over a Heparin-Sepharose column and the bound proteins were eluted with a salt solutions of increasing molarity. Two bands of 130 kDa and 62 kDa bound to the column and were selectively eluted with increasing salt concentrations (Fig. 3.18, Lane 4, arrowed), as visualised by coomassie staining. The 62 kDa protein was a major stained protein in the parasite extract whereas the 130 kDa was not.

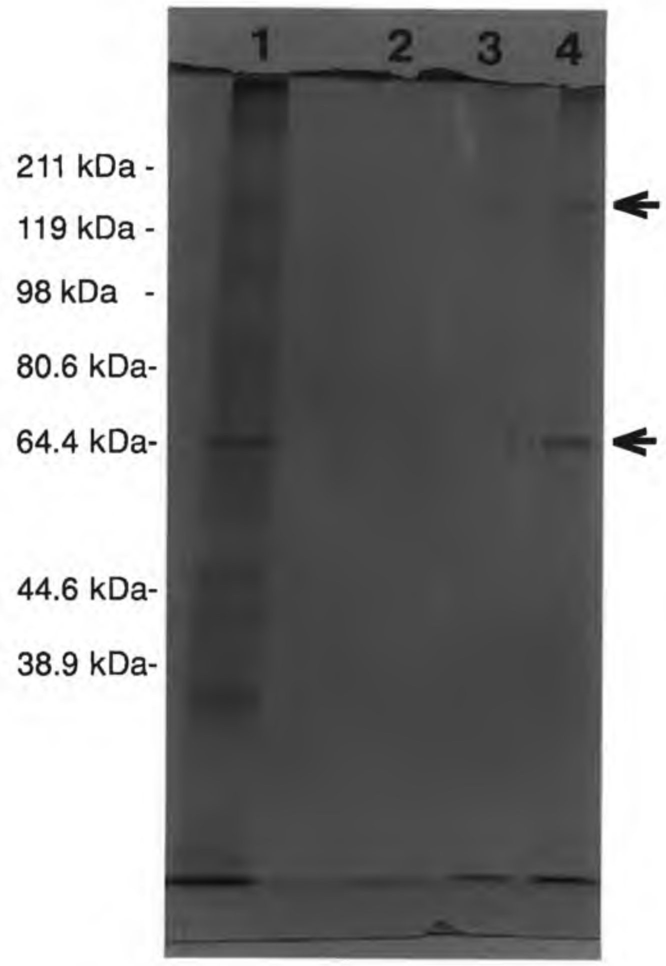


Fig. 3.18 Binding of protein extracts from *P. c. chabaudi* cultured for 6 hrs to Heparin-Sepharose CL-6B. Total protein in extract (Lane 1) was applied to the Heparin-Sepharose column and the bound proteins were eluted with 400 mM NaCl (Lane 2), 800 mM NaCl (Lane 3) and 1.5 M NaCl (Lane 4).

3.8 Polyclonal anyisera to *P. c. chabaudi* AS parasite proteins

3.8.1 Preparation of antiserum against various P. c. chabaudi antigen preparations

The identification of antigens that may be involved in invasion is essential to understanding the invasion process. These antigens may be accessible to antibodies that could block this process and thus prevent disease.

Various antigen preparations were prepared from *P. c. chabaudi* parasites and used to raise polyclonal antibodies in rats. An antigen preparation consisting of culture supernatant was obtained by culturing parasites to rupture and allowing merozoite release. The merozoites were removed from the culture by centrifugation and the supernatant retained (TCS). The merozoite pellet was Triton-X extracted and the soluble proteins that were released were used as a source of antigen (TXP). A third antigen preparation consisted of proteins from the culture supernatant that bound to and were salt eluted from mouse erythrocytes (EBP). A fourth antigen preparation was obtained by culturing *P. c. chabaudi* parasites to schizogony and extracting the parasite proteins by homogenisation in PBS (TSP). The final antigen preparation was made from the salt elute from a Heparin-Sepharose column over which had been passed extracted schizont parasites (HEP).

Antibodies were raised to each of these antigen extracts in laboratory rats. The antibody titres in the serum of the immunised rats was estimated using ELISA techniques. The rats were bled and their serum was adsorbed by incubating the serum with mouse cells to remove any antibodies in the serum that were specific to mouse erythrocytes. The various antibody titres were as follows;

a) Total Culture Supernatent (TCS) > 1 / 12800
b) Triton - X extracted Pellet (TXP) > 1 / 12800
c) Erythrocyte binding Protein (EBP) > 1 / 6400
d) Total Schizont Protein (TSP) > 1 / 6400
e) Heparin column elute (HEP) > 1 / 6400

3.8.2 Study of antiserum in erythrocyte invasion assays for blocking activities

The various adsorbed antisera were tested at several dilutions in erythrocyte invasion assays to observe their effect on levels of invasion (Figs. 3.19 and 3.20). Each antiserum dilution was added to invasion assay wells and the assay incubated for 19 hours. No effect was detected with any dilution of anti - TXP (1 / 10 -> 1 / 100). Anti - EBP resulted in an inhibition of invasion of 29.1 +/- 0.9 % at 1 / 25, which was not significant. No effect was observed at 1 / 100 dilution (Fig. 3.19A). There was a slight inhibitory effect (25.7 %) observed with a 1 / 25 dilution of anti - TSP, but it was not significant (Fig. 3.19 B).

Anti - TCS and anti - HEP had the greatest effect on merozoite invasion levels. Antibodies to TCS resulted in an inhibition of 54 +/- 9.1 % at a dilution of 1 / 10 and an inhibition of 32 +/- 6.3 % at a dilution of 1 / 200 (Fig. 3.20A). These levels of blocking were all significant ($p < 0.05$). Anti - HEP inhibited invasion by 45.1 +/- 6.8 % at 1 / 10 while the inhibition exhibited at 1 / 100 dilution was not significant (Fig. 3.20B).

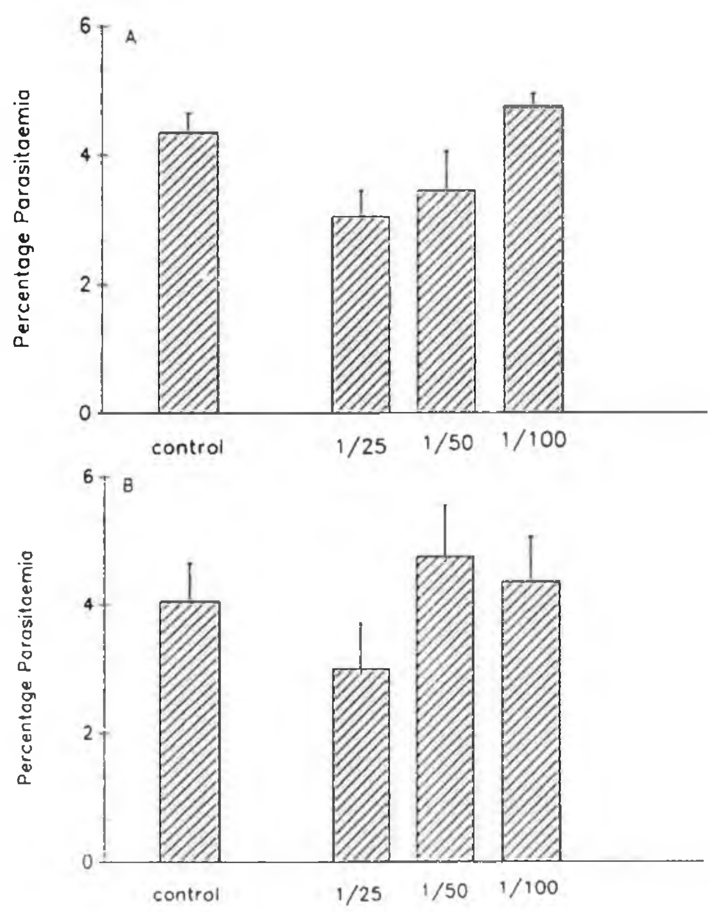


Fig. 3.19 The effects of antisera on levels of invasion achieved in invasion assays. A, the effect of anti - EBP on invasion. b, the effect of anti - TSP on invasion levels.

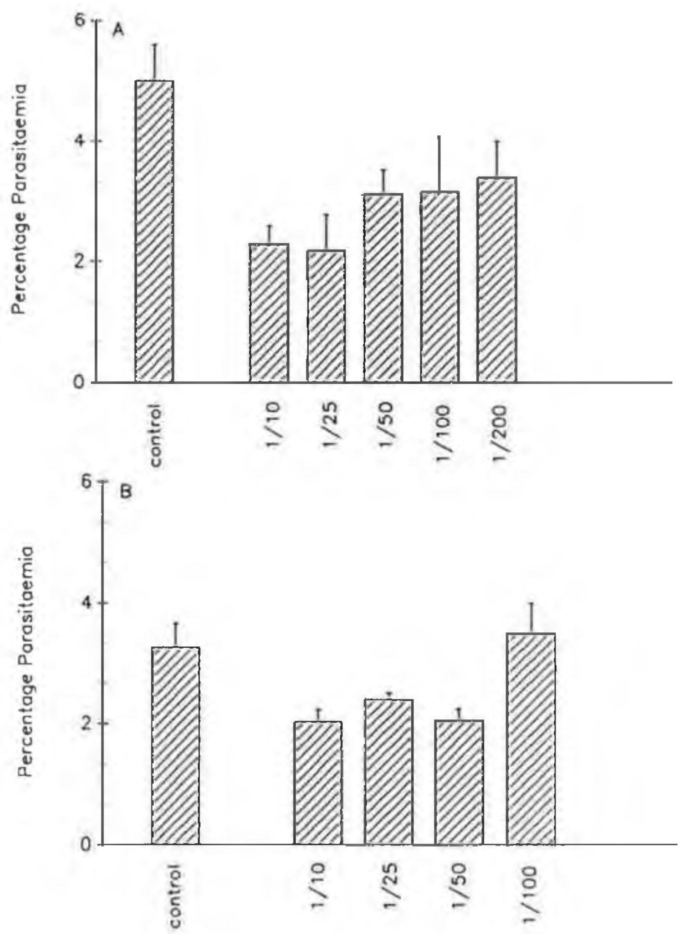


Fig. 3.20 The effects of antisera on levels of invasion achieved in invasion assays. A, the effect of anti - TCS on invasion. b, the effect of anti - HEP on invasion levels.

3.9 Characterisation of polyclonal antibodies

The reactivity of antibodies with *P. c. chabaudi* antigen in each of the antisera prepared was investigated using the following techniques;

a) immunoblotting
b) radio-immunoprecipitation
c) immunofluorescence

3.9.1 Immunoblots

The panel of antisera raised against parasite preparations were analysed using immunoblotting techniques. The antibodies were used to probe nitrocellulose filters bearing electrophoretically separated extracts of *P. c. chabaudi* parasites that had been cultured for six hours to reach schizogony (Fig. 3.21). When these extracts were probed with serum from a control rat (un-immunised), no proteins were visualised (Fig. 3.21, Lane 1).

i) *TXP.*

Antibodies in the anti - TXP serum were reactive with the following polypeptides; 110 kDa (doublet), 90 kDa, 44 and 41 kDa - weakly stained (Fig. 3.21, Lane 2).

ii) *TCS*

A number of polypeptides reactive with this anti-serum were detected (Fig. 3.21, Lane 3). These included proteins of molecular weights 130 kDa, 110 kDa, 107 kDa, 97 kDa, 94 kDa, 87 kDa,62 kDa, 43 kDa and 41 kDa.

iii) *EBP*

When anti - EBP was used to probe nitrocellulose filters bearing protein extracts, no reactive proteins were visualised (Fig. 3.21, Lane 4).

iv) *TSP*

Antibodies in the anti - TSP serum were reactive with a number of proteins (Fig. 3.21, Lane 5). These proteins were of molecular weights

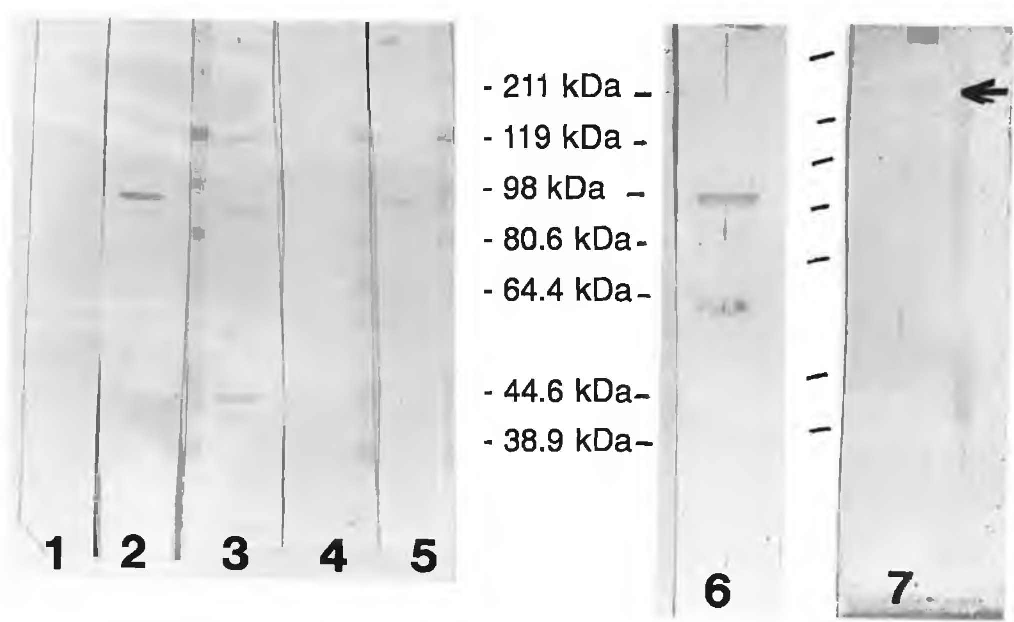
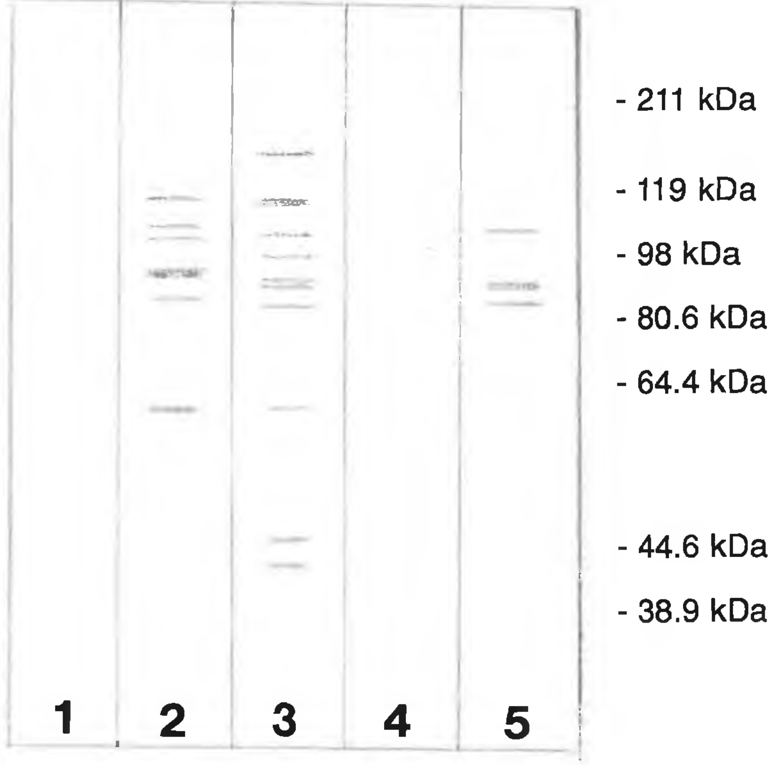


Fig. 3.21 Immunoblotting with polyclonal antisera. Nitrocellulose filters bearing electrophoretically separated parasite proteins were probed with control antiserum (Lane 1), anti - TXP (Lane 2), anti - TCS (Lane 3), anti - EBP (Lane 4), anti - TSP (Lane 5), anti - HEP (Lane 6) and anti - 130 kDa (Lane 7). (The bands did not come up clearly in the photograph of the immunoblot, therefore a schematic diagram of the immunoblot is shown below).



of approximately 107, 94 and 87 kDa.

v) *HEP*

When anti - HEP serum was used to visualise proteins on nitrocellulose filters, two major proteins were detected (Fig. 3.21,Lane 6). They consisted of a doublet of 94 kDa and a single protein of 62 kDa.

vi) *Eluted antibody (anti - 130 kDa)*

Plasmodium c. chabaudi extracts were electrophoretically separated and transferred to nitrocellulose. The filters were probed with anti - TCS and the antibody that bound to the 130 kDa molecule was eluted from the blot. This antibody was then used to probe a blot of the *P. c. chabaudi* extract. A band corresponding to the 130 kDa protein was visualised, indicating that a monospecific antibody was present in the elute (Fig. 3.21, Lane 7, arrowed)

Plasmodium c. chabaudi parasite-infected erythrocytes were cultured from time 0 (13.00) for a period of three and six hours. The parasites were extracted, separated electrophoretically and the proteins transferred to nitrocellulose filters. The filters were probed with control serum and anti - TCS serum, the most reactive antiserum (Fig. 3.22). No proteins were visualised when control serum was used to probe the extracts (Fig. 3.22, Lanes 1 - 3). When time zero extract was probed with anti - TCS, three proteins were detected (Fig. 3.22, Lane 4), of molecular weights 107 kDa, 104 kDa and 94 kDa. After three hours of culturing a further protein band of 87 kDa was visualised using anti - TCS (Fig. 3.22, Lane 5). When six hour parasite extracts were probed with anti - TCS, protein bands of 130 kDa, 116 kDa and 97 kDa and a very faint band of 62 kDa were visualised in addition to the proteins already visualised (Fig. 3.22, Lane 6). A number of low molecular weight bands were also present; 41 kDa and 39 kDa.

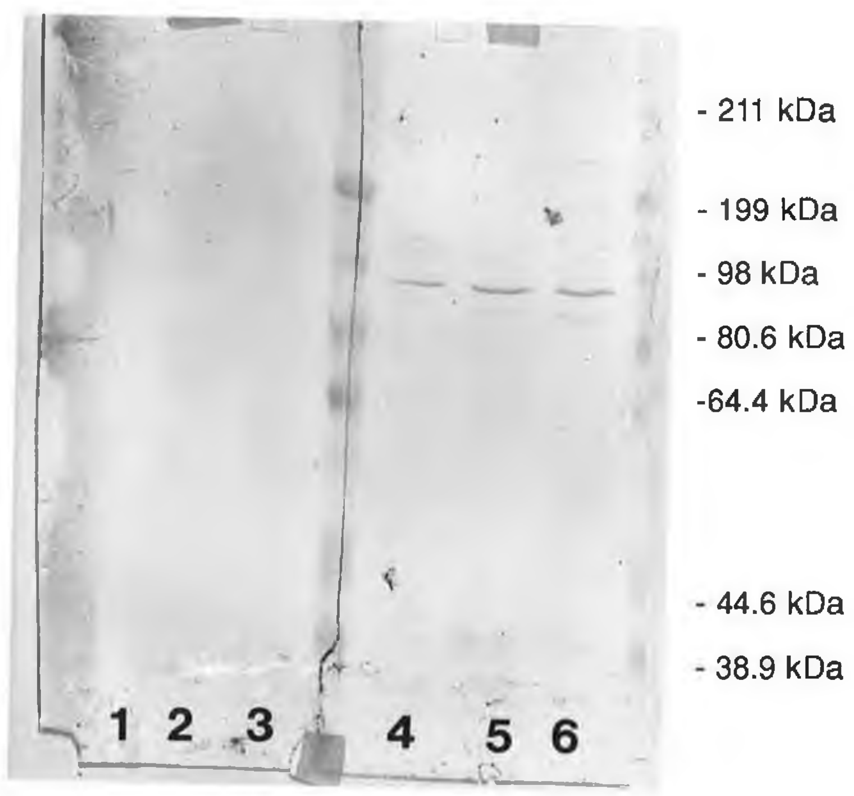


Fig. 3.22 Immunoblotting of parasite proteins from different developmental stages. Extracts of parasites at the middle-trophozoite stage (time 0), late-trophozoite stage (three hour culture) and schizont-stage (six hour culture) were probed with control antiserum (Lanes 1, 2 and 3) and with anti - TCS (Lanes 4, 5, and 6).

3.9.2 Immunoprecipitation of parasite proteins

The chief function of red blood cells is to transport oxygen and carbon dioxide around the body and while their metabolism is geared to carry out this task, they do not have the capacity to produce proteins (Beuther, 1975). Therefore any proteins that incorporate radiolabelled amino acids e. g. [^{35}S]methionine, during the *in vitro* culture of malaria parasites in medium containing radiolabelled amino acids, can only be of parasite origin. The polyclonal antibodies were used in radio-immunoprecipitation experiments. For these experiments *P. c. chabaudi* parasites were radio-labelled *in vitro* for six hours using [^{35}S]methionine and PBS extracts prepared. The pattern of proteins precipitated by the antisera is shown in Fig. 3.23. No proteins were precipitated with control serum. A greater number of immunoreactants were detected by the antibodies using immuno-precipitation than with immunoblotting. Different profiles were obtained with the various antibody preparations.

Over 15 proteins were precipitated with anti - TXP, including a band of approximately 130 kDa (Fig. 3.23, Lane 3). The strongest reaction was to two bands of approximately 116 and 112 kDa. Fewer proteins were precipitated with anti - TSP (eight bands clearly detected, though some faint bands were detected). Of these, three bands of molecular weights 94, 38 and 33 kDa were prominent. The 38 kDa band appears to be unique to anti - TSP (Fig. 3.23, Lane 4) .

Seventeen major bands plus a number of faint bands were detected with anti -TCS (Fig. 3.23, Lane 5). These bands appeared to include all the bands detected by the other anti-sera in the panel, with the exception of the 38 kDa band detected by anti - TSP. Among the proteins precipitated by anti - TCS is a protein of molecular weight approximately 116 kDa which was detected by immunoblotting, as well as a band of approximately 130 kDa.

Only one major band was detected with anti - EBP (Fig. 3.23, Lane 6). A band of approximately 41 kDa was also detected with anti - TXP and anti - TCS. Other bands show up faintly with a longer exposure to X - ray film. When anti - HEP was used to precipitate proteins, five major and a number of faint bands were detected (Fig. 3.23, Lane 7). The bands of 116, 94 and 62 kDa gave the strongest precipitation

reaction.

No bands were detected when the anti - 130 kDa serum was used to precipitate proteins (Fig. 3.23, Lane 2). There was definitely no evidence for the 130 kDa band that was detected using immunobloting.

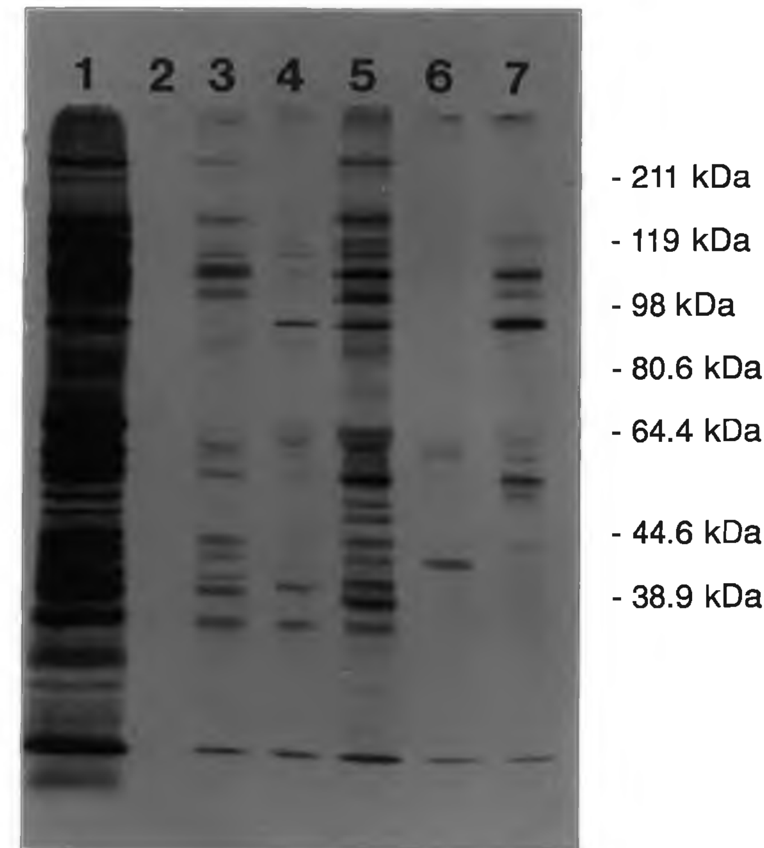


Fig. 3.23 Immunoprecipitation of radiolabelled proteins extracted from *P. c. chabaudi* parasites cultured for 6 hrs. The total [^{35}S]-metabolically labelled proteins in extracts of *P. c. chabaudi* parasites (Lane 1) were then immunoprecipitated with anti - 130 kDa (Lane 2), anti - TXP (Lane 3), anti - TSP (Lane 4), anti - TCS (Lane 5), anti - EBP (Lane 6) and anti - HEP (Lane 7). No bands were detected with the control serum.

3.9.3 Indirect immunofluorescence of parasitised cells

Immunofluorescence analysis is a method of localising antigens and antibodies on or in cells and tissues. It is a sensitive and specific method but one that is fraught with the same problems as any immunological method e.g. potential non-specific artifacts. Therefore all experiments using this technique required strict controls for non-specific staining. These controls were as follows;

a) the use of diluent (PBS - Tween) instead of primary antibody
b) anti-rat FITC-conjugated antibody with no primary antibody
c) anti-mouse FITC-conjugated antibody with no primary antibody
d) rat serum from an non-immunised rat (negative control)

These controls were carried out to ensure that any reactivity observed was due to antibodies in the test antisera and was not due to non-specific binding. Antiserum that had been incubated with mouse erythrocytes to remove any anti-erythrocyte antibodies (absorbed antisera) was used for all tests. Mouse serum from a mouse that was immune to *P. c. chabaudi* infection (immune serum) was used as the positive control.

A brief summary of the results obtained is shown in Table 3.6. In all the tests carried out no reactivity was observed with diluent, FITC - conjugated mouse antibody or FITC-conjugated rat antibody.

In the first set of experiments immunofluoresence was carried out on purified *P. c. chabaudi-* infected erythrocytes that had been cultured for six hours, resulting in very mature parasites, including schizonts, being present on the antigen slides. When the negative control serum was tested, a minute amount of immunofluorescence was detected. However, on closer examination, the areas of immunofluorescence were not localised to any particular cell type or extra-cellular structure and the fluorescence quenched rapidly. Positive control immune serum was tested at various dilutions ranging from neat to a 1 in 1250 dilution. A very strong positive reaction was visible even at the lowest dilution. Fluorescence was detected on the rim of infected erythrocytes as well

Table 3.6 Reactivity of polyclonal antisera in immuno-fluorescence studies

Anti - sera	*P. c. chabaudi*		*P . falciparum*
	rings	schizonts	
negative control	-	-	ND
positive control	+++	+++	ND
anti - TCS 91:1250)	+++	+++	-
anti - TSP (1:1250)	+++	+++	+
anti - EBP (1:250)	+	++	+
anti - T - XP(1:1250)	+++	++	++
anti - HEP (1:1250)	++	++	-
Elute(anti-130 kDa) (neat)	-	+	-

- background fluoresence
+ detectable activity, but very low levels (< 1 : 50)
++ good reactivity (1 : 250 - 1 : 1250)
+++ very strong reaction (> 1 : 1250)
ND Not Determined

as on the parasites themselves. This fluorescence appeared to be connected to the presence of parasite proteins in the host erythrocyte membrane, as desribed by Coppel *et al,* (1984) and Haldar *et al,* (1986). No fluorescence was associated with the membrane of uninfected cells.

There was a strong positive reaction to parasitised blood smears when anti - TXP was tested (> 1 : 1250). Extra-cellular parasites gave the strongest reaction (Fig 3.24, Panel A), while the amount of fluorescence shown by intra-cellular parasites appeared to increase with the maturity of the parasite contained in the cell. Parasites that had begun to segment or that contained merozoites fluoresced the strongest.

Anti - TCS resulted in strong reactivity (> 1 : 1250), (Fig 3.24, Panel B). Fluorescence was also observed on parasites that were extra-cellular. Fluorescence intensity was greater with increasing maturity of the parasites. A classic " bunch of grapes " fluorescence was observed with the anti - TCS. This formation results from fluorescence of a mature schizont just prior to rupture, where each merozoite is a "grape". The erythrocyte membrane around the schizont is also stained with antibody.

When anti - TSP was tested it resulted in a strong level of reactivity, with a pattern of fluorescence that was very similar to that obtained with anti - TCS, i.e. stronger fluorescence on extra-cellular parasites. There was also very faint fluorescence associated with the erythrocyte membrane (data not shown).

Anti - EBP gave a positive result at a dilution of 1 /10 -> 1 / 250; however no fluorescence was detectable at 1 : 1250. Again extra-cellular parasites showed greater intensity of fluorescence by comparison with intra-cellular parasites. Fluorescence was localised in the parasite cytoplasm of infected cells and no reactivity was observed with the erythrocyte membranes (data not shown).

Smears of infected blood probed with anti - HEP exhibited barely detectable levels of fluorescence at the lower antibody dilutions. Extra-cellular parasites showed a stronger fluorescence when compared to intra-cellular parasites. There was a general fluorescent staining of the parasite cytoplasm of all the intra-cellular parasites (data not shown).

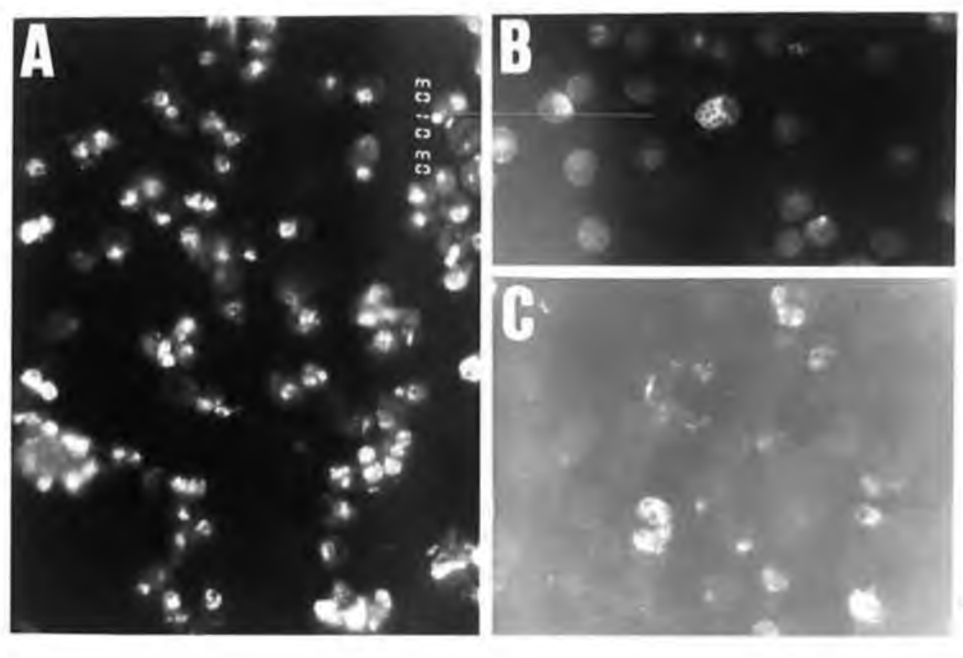


Fig. 3.24 Immunofluorescence of parasite-infected erythrocytes probed with polyclonal antisera against *P. c. chabaudi* parasite proteins. Slides of murine erythrocytes containing *P. c. chabaudi* schizonts were probed with anti - TXP (Panel A) and anti - TCS (Panel B). Slides of human erythrocytes containing *P. falciparum* schizonts were also probed with anti - TCS (Panel C).

The eluted antibody (anti - 130 kDa) was used to probe smears of infected blood. Fluorescence was detected though it was quite faint and tended to fade rapidly despite the presence of the anti-quenching agent " DABCO ". Most of the fluorescence observed tended to be associated with parasitised erythrocytes that were usually in quite poor condition, probably from mechanical shearing during smearing. When the fluorescence observed with anti - 130 kDa was compared to the fluorescent staining observed with the negative control serum, there was an apparent increase in the level of fluorescence. It was not possible however, to determine if the fluorescent pattern was the different (both seemed to associate with cellular material whose nature was not always easy to elucidate).

Smears of erythrocytes infected with the human malaria *P. falciparum* were probed with the panel of anti-sera for cross-reactivity studies. From examination of the slides under Differential Interference Contrast (DIC) microscopy, the parasites were well developed (late trophozoites, schizonts). No fluorescence was observed with anti - TCS, anti - HEP or anti - 130 kDa. The levels of fluorescence detected with the remaining three anti-sera were very low. Anti - TXP (Fig. 3.24, Panel C) gave the strongest response with staining of all intra-cellular parasites, as detected by DIC. Both anti - EBP and anti - TSP were positive but levels of reactivity were weak even at the 1 : 50 dilution.

The panel of antisera was used to probe smears of blood from infected animals at a time when the parasites were at the ring-stage of development. No fluorescence was detected with the negative control serum while the immune serum (positive control) resulted in a fluorescent rim around infected cells and fluorescence of the ring-stage of the parasite.

When smears prepared with ring-stage parasites were probed with anti - TCS, anti - TSP or anti - TXP, similar patterns of fluorescence were observed (Fig 3.25, Panel B). Individual ring-stage parasites fluoresced in such a way that the rim of the parasite was clearly visible (Fig 3. 25, Panel B - arrowed). Very young rings were detected by fluorescence that were not detected by DIC. Any extra-cellular parasite material again fluoresced more strongly than intra-cellular parasites.

When the parasite-infected erythrocytes were probed with anti - TSP, strong levels of fluorescence were associated with the erythrocyte membrane (Fig. 3.34, Panel B - arrowed).

Anti - HEP also showed fluorescence with ring-stage parasites, however whereas with the three anti-sera described above the fluorescence was continuous around the parasite, with anti - HEP, the fluorescence was discontinuous and fragmented (Fig 3.25, Panel A - arrowed).

No fluorescence was observed with neat anti - 130 kDa and only very faint fluorescence detected with neat anti - EBP.

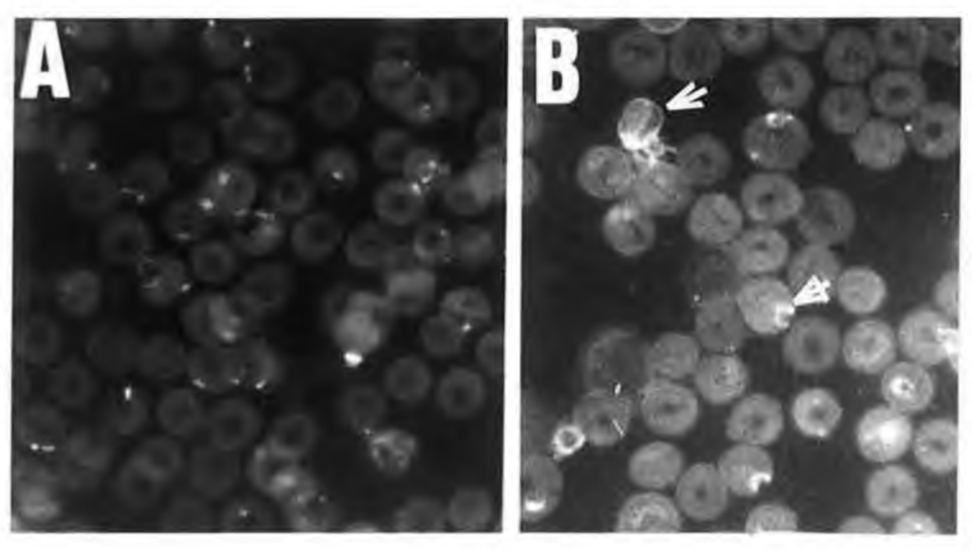


Fig. 3.25 Immunofluorescence of *P. c. chabaudi* ring-infected erythrocytes probed with polyclonal antisera against *P. c. chabaudi* parasite proteins. Panel A, ring-infected erythrocytes probed with anti - HEP, showing the discrete nature of the fluorescence on the rings-stages of the parasite. Panel B, ring-infected erythrocytes probed with anti - TSP. Note the continuous nature of the fluorescence on the parasite and the fluorescence on the erythrocyte membrane (arrowed).

3.10 *Plasmodium chabaudi* IP - IPC1 (clone F)

3.10.1 *Maintenance of P. chabaudi IP. - IPC1 in vivo*

Plasmodium chabaudi IP - IPC1 (clone F) parasites were received as a frozen stabilate, which was injected I. P. into CD - 1 mice at the same stage in the reverse cycle as the stabilate had been made. The infection was successful in this strain of mouse, achieving parasitaemias of 85 % and greater. This level of infection, however was often fatal. Frozen stabilates were made from infected blood when the parasitaemia was < 30 %, to ensure the good condition of the parasites (Fig. 3.26).

The daily cycle of parasite was also monitored, and it was found that the parasite was not as synchronous as *P. c. chabaudi* AS strain and did not sequester to the same high level. Despite these differences, the parasite was treated in the same manner as *P. c. chabaudi* AS strain, to allow the two strains of parasite to be compared more directly.

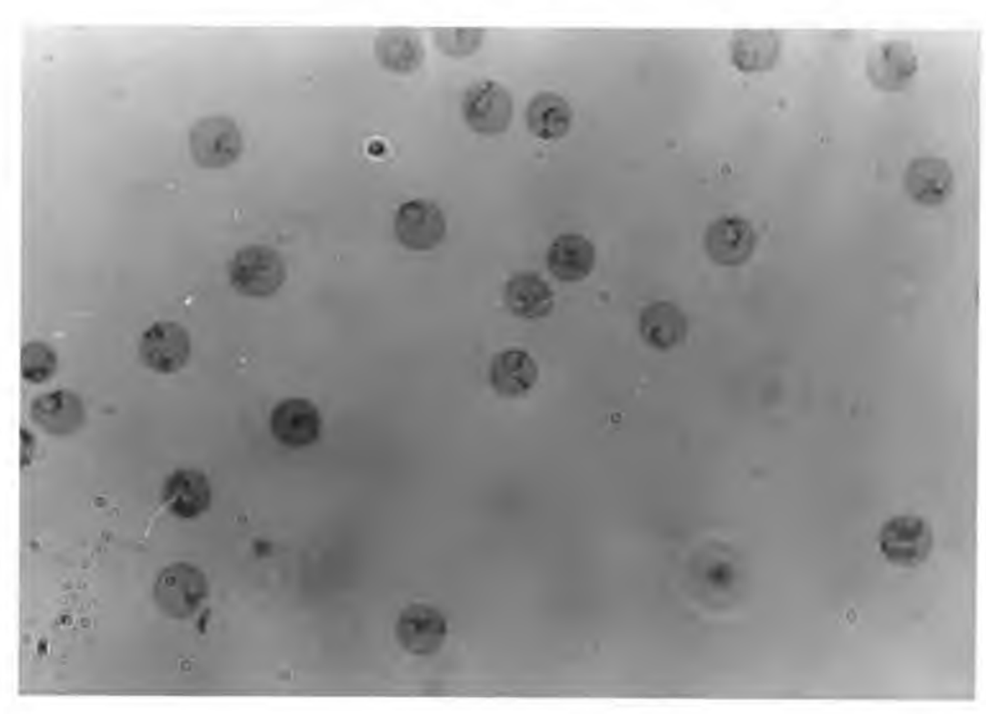


Fig. 3.26 *P. chabaudi* IP - IPC1 parasite-infected erythrocytes (> 80 % parasitised erythrocytes).

3.10.2 Parasite purification by Percoll

Mice infected with *P. chabaudi* IP - IPC1 parasites were bled at 13.00 hrs (the bleeding time of *P. c. chabaudi* AS strain) when their parasitaemia was approximately 25 %. The parasite-infected erythrocytes were passed over a CF11 column and the cells washed in RPMI - 1640. The pelleted cells were then layered onto a 74 % Percoll gradient and centrifuged as per *P. c. chabaudi* AS strain. The same cell banding pattern was observed and when the top band was analysed using giemsa staining, a purification in the region of 98 % had been achieved.

3.10.3 Erythrocyte invasion assay using P. chabaudi *IP - IP.C1*

Parasitised cells were obtained from animals with a parasitaemia of approximately 25 % and purified by Percoll density gradient centrifugation. These cells were cultured for four hours in BME - WE medium supplemented with 10 % RS (as developed for *P. c. chabaudi* AS). These parasitised erythrocytes (8 x 10^6 cells / well) were added to target mouse cells (4 x 10^7 cells / well) as detailed for *P. c. chabaudi* AS (Section 2.14). The assays were carried out with 5 and 10 % RS and 10 % FCS. Upon examination of giemsa stained smears of the cells after completion of the invasion assay, it was clear that not only did *P. chabaudi* IP - IPC1 survive when cultured in BME - WE and 10 % RS as opposed to RPMI - 1640 and 10 % FCS, but successful reinvasion was achieved. Ten percent rat serum also resulted in significantly elevated levels of invasion when compared to 10 % FCS and 5 % RS, where $p < 0.05$ (Fig. 3.27).

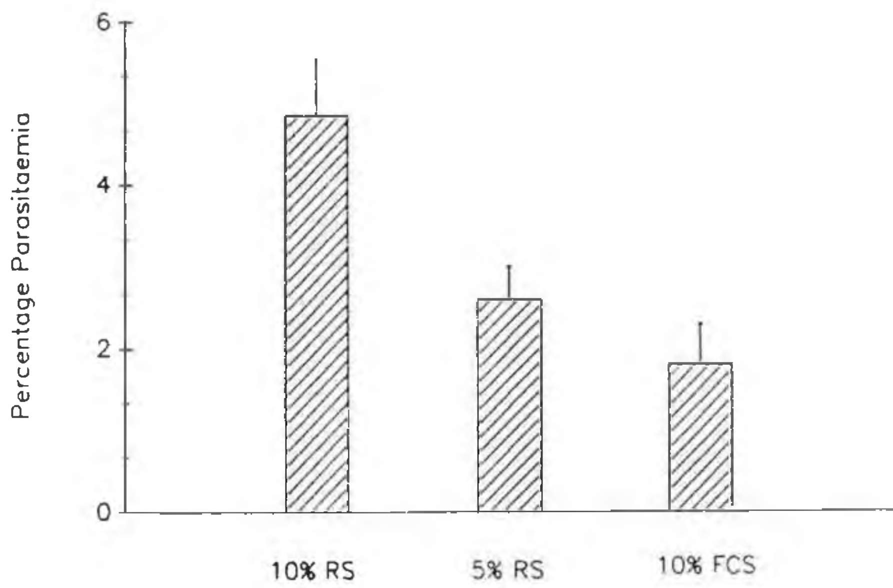


Fig. 3.27 The effects of serum on erythrocyte invasion levels of invasion achieved in invasion with *P. chabaudi* IP -IPC1 clone F. (Invasion levels are represented by Percentage Parasitaemia).

3.10.4 Enzyme digestion of erythrocytes for invasion assays using P. chabaudi IP - IPC1 -infected cells

Enzyme digestion of target cells using trypsin, chymotrypsin, Pronase and papain was carried out as described in Section 2.18. These cells were used as target cells in invasion assays with *P. chabaudi* IP - IPC1 parasites. On analysis of the giemsa stained smears, chymotrypsin digestion with 0.1 mg / ml of enzyme resulted in an inhibition in invasion levels of 31.6 +/- 3.1 % while 0.1 mg / ml of Pronase resulted in a inhibition of 12.4 +/- 1.1 % (Fig 3.28). These levels were significant ($p \leqslant 0.05$). Trypsin and papain digestion of erythrocytes had no effect on invasion levels into the cells.

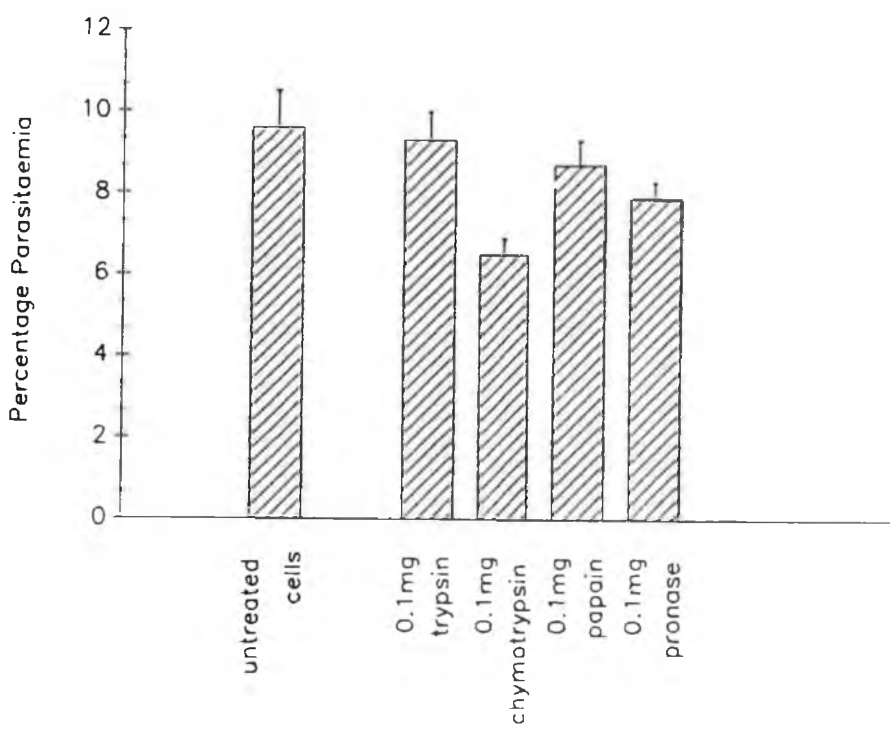


Fig. 3.28 Invasion of murine erythrocytes by *P. chabaudi* IP - IPC1 clone F after proteinase digestion. Target cells were digested at different concentrations of enzyme for two hours, at 37 ^{0}C. The cells were then washed in protease inhibitors and medium, and added to the invasion assay. The level of invasion (expressed as Percentage Parasitaemia) into treated cells was then analysed and compared to invasion into untreated (normal) cells.

Chapter Four

Discussion

4 General discussion

Malaria, one of the world's leading killers, is presently undergoing a resurgence in incidence, primarily due to the rapid development of single- and multi-drug resistance in strains of the parasite in Africa (SPRTTD, 1985). The human parasite *Plasmodium falciparum* is the species responsible for 80 % of all malaria cases worldwide and the majority of malaria-related deaths (90 - 99 %) are attributed to this species (SPRTTD, 1985). The development of resistance in strains of *P. falciparum* is of particular concern. There is now an increase in the amount of research being carried out into the development of new drugs and possible vaccines against the parasite.

The asexual erythrocytic cycle in the parasite's life cycle is the stage that is associated with the clinical manifestations of malaria. 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 might prevent disease; hence identification and characterisation of merozoite molecules involved in all of these events is important, as each represents a target against which a protective humoral immunity response could be directed.

Plasmodium falciparum has been studied quite extensively as the intraerythrocytic stages of the parasite can be maintained in continuous *in vitro* culture in the laboratory and can also be used to infect *Aotus* monkeys, thereby enabling the *in vivo* study of the parasite. The monkey model enables studies to be carried out into aspects of the parasite's life cycle that contribute to the severity of the disease and the parasite's evasion of the host's immune response e.g. antigenic variation and sequestration. However, monkeys are difficult to handle and are very expensive to maintain. In the past the rodent malarias have been used to study general aspects of the malaria life cycle *in vivo* because these models are very easy to maintain and to handle, and are considerably less expensive to work with.

The work presented in this thesis was carried out on the rodent malaria model, *Plasmodium chabaudi chabaudi*. This species infects laboratory mice and rats and was chosen for research purposes as it has a number of important characteristics in common with *P.*

falciparum. Both species undergo sequestration and recrudescence of infection. The recrudescent populations contain parasites that are antigenically different from the parasites that caused the original infection. *Plasmodium chabaudi* has been used as an *in vivo* model for the study of sequestration, antigenic variation and chronic parasitism and the relationship between all three (Cox *et al*, 1987; Gilks *et al*, 1990). Both species also invade mature erythrocytes and immature erythrocytes (reticulocytes). However there is evidence to suggest that *P. falciparum* preferentially invades reticulocytes and young red blood cells (Pasvol *et al*, 1980).

Elucidation of the molecular intricacies of erythrocyte invasion necessitates the development of *in vitro* methods that allow examination of each event in the invasion process. However, in the absence of such an assay for *P. chabaudi*, very little is known about the mechanism of invasion of host erythrocytes by the parasite. The development of a *P. chabaudi in vitro* erythrocyte invasion assay, would enable these processes to be studied in detail.

An *in vitro* invasion assay would allow the screening of antibodies for their ability to block invasion. The molecules which elicited the response could then be identified, characterised and used for initial vaccine trials. A number of merozoite putative erythrocyte receptors have been identified for *P. falciparum, P. vivax* and *P. knowlesi* (Mitchell *et al*, 1986; Barnwell et *al*, 1989). Therefore more than one receptor may be involved in host cell recognition and invasion by individual species of malaria. The existence of more than one invasion pathway has also been suggested (Hadley *et al*, 1987; Perkins & Holt, 1988). A number of other proteins have been identified and localised in specific organelles e.g. rhoptry proteins, which have been linked to processes involved in invasion such as red cell deformation and invagination (Perkins, 1989).

Analysis of this evidence implies that antibodies to a number of different parasite proteins would have to be tested, individually and in combination with other proteins, for their ability to block invasion and their possible use as vaccines.

4.1 Development of a simple *in vitro* erythrocyte invasion assay

Invasion assays that use schizont-infected cells are the simplest to perform and have been developed for a number of *Plasmodium* species (Miller *et al*, 1983; Mitchell *et al*, 1986; Clark *et al*, 1989; Barnwell *et al*, 1989). These assays involve the incubation of schizont-infected erythrocytes with target erythrocytes to allow the parasites to develop and rupture, and for the target erythrocytes to be subsequently invaded by these released merozoites. These types of erythrocyte invasion assays have been used to investigate inhibitors of invasion and antibody blocking of invasion. They are simple to perform and allow a number of assays to be carried out simultaneously. These are the requirements for a *P. c. chabaudi* erythrocyte invasion assay.

In order to obtain parasites for a *P. c. chabaudi in vitro* erythrocyte invasion assay, *P. c. chabaudi* AS strain was successfully established in male CD - 1 mice in an adjusted light cycle. Necessary requirements for the development of useful invasion assays include a) highly purified parasite-infected erythrocytes and b) erythrocytes containing parasites that are at the same stage of development (synchronous). Purification is necessary as scored invasions must have occurred into the target cells only and not into uninfected cells introduced with parasite-infected erythrocytes. Synchronisation of the parasites ensures that the parasites rupture at the same time and that invasion takes place during the course of the assay. Since *P. c. chabaudi* is a synchronous parasite *in vivo*, no methods were required to synchronise the parasite-infected cells.

The developmental stage of the parasites used in the assay is crucial; parasites just prior to their rupture are required. Mice were bled at 13.00 hours when the parasites were middle-stage trophozoites and before the parasite-infected erythrocytes started to withdraw from the peripheral circulation to the endothelial cells (sequestration). The animals were bled when they had a parasitaemia of between 20 - 25 %. If animals were bled at higher parasitaemias it was found that the health of the parasites deteriorated. Therefore 75 % of the erythrocytes were uninfected and had to be removed to obtain purified parasites. In the past other researchers dealing with the rodent malaria

P. berghei obtained low yields of purified parasites by centrifugation of infected blood when a high proportion of the parasites were schizonts. The top 10 - 20 % of the pelleted cells, which contained schizonts, was then removed and used as a source of parasites (Mons *et al*, 1985). Since this and other methods resulted in low purity and did not give consistent results, density centrifugation gradients were investigated and Percoll and Nycodenz gradients were developed for the purification of various species of malaria (Aley *et al*, 1984; Mons *et al*, 1988). Wunderlich *et al*, (1985) used a single step Percoll gradient containing FCS, to obtain approximately 90 % purified *P. chabaudi* parasites. The method of Wunderlich *et al*, (1985) was not reproducible in our hands and we developed a modified Percoll gradient to separate infected from uninfected cells. As *P. chabaudi* parasites are synchronous, a single-step gradient is sufficient to separate the two cell populations. The gradient as developed in our laboratory is a 12.17 ml 74 % Percoll gradient which is simple to perform and results in a purification of parasites of between 95 and 99 %. Once purified parasites were obtained, the successful culturing of the parasites through schizogony and onto rupture and merozoite release was achieved by incubating the parasitised cells in RPMI - 1640, the routinely used medium for the cultivation of all species of malaria parasite. The medium also contained 10 % FCS (heat-inactivated).

We initially found that by culturing blood from mice (unpurified parasites) with a parasitaemia of 25 % overnight, invasion occurred into uninfected cells. Therefore parasite invasion of host red blood cells was possible in RPMI - 1640. We used this medium to develop the first *in vitro* erythrocyte invasion assay for *P. c. chabaudi*.

The invasion assay that we developed includes an initial culturing of the parasite-infected erythrocytes at a density of 2.5×10^7 cells / ml for four hours prior to transferring 8×10^6 of these cells to fresh medium containing the target erythrocytes. During this period, the parasites develop and subsequently rupture within several hours of the transfer. This transfer step serves the following purposes. It allows for the replacement of the culture medium with fresh medium just prior to rupture and reinvasion, and as the cells are taken directly from the

the 5 ml culture and added to the assay wells, it reduces the amount of manipulation of the parasites to a minimum. As mouse erythrocytes are fragile, the minimising of cell manipulation is considered vital. The initial four hour culture period makes the setting up of the assay easier as while the parasites are maturing *in vitro* the target cells can be prepared.

Both the initial four hour culture and the assay incubation period are carried out under low oxygen tension (5 %) to allow for the development of schizonts, merozoite formation and release, and the reinvasion of new cells. While 1 % O_2 was found to result in higher levels of invasion with *P. falciparum* (Trager & Jensen, 1976), an oxygen tension of 5 % was conveniently obtained using the candle jar method.

Leucocyte removal by a number of different methods is routine in the cultivation of malaria parasites as the presence of the leucocytes may adversely affect the culture. The leucocytes may damage the parasites through their phagocytic activities or through the high levels of proteases and other degradative enzymes they contain (Howard *et al*, 1978). The leucocytes could also be a source of contaminating molecules that could interfere with the characterisation of intact parasite antigens and enzymes and therefore should also be removed from parasitised erythrocytes when the parasites are obtained for the purpose of protein purification or characterisation (Richards & Williams, 1973). A CF11 column was decided upon for leucocyte removal due to the ease with which it is set up, in comparison to other methods such as a 1 : 3 ratio of Sulphoethyl cellulose and Sephadex G - 25 (Howard *et al*, 1978) or seasand overlaid with Sephadex and cellulose (Wunderlich *et al*, 1985) all of which resulted in 80 to 90 % removal of leucocytes. Upon investigation of the effect of leucocyte removal on levels of invasion, it was found that while successful development and reinvasion of *P. c. chabaudi* parasites was not critically dependent on the removal of leucocytes, the levels of reinvasion were considerably improved. This is contrary to the findings of Richards & Williams (1972), which stated that the short-term culture of the rodent malarias was dependent of the removal of the leucocytes.

McLean *et al*, (1986) also cultured *P. chabaudi*-parasitised erythrocytes for 5 - 6 hours, in RPMI - 1640 in a candle jar, to obtain trophozoites / schizonts for immunofluorescence assays. The medium was supplemented with 5 - 10 % FCS (heat-inactivated). After testing various serum types and concentrations, it was found that 10 % heat-inactivated rat serum was the optimum for *P. c. chabaudi* AS. Rat serum is not the most suitable serum for all rodent malarias. McNally *et al*, (1992) found that 50 % FCS (heat-inactivated) resulted in the highest levels of invasion in a *P. berghei* erythrocyte invasion assay. The lysis of erythrocytes that was observed with higher levels of rat serum could be attributed to an excess of harmful components that were released into the serum upon clotting of the erythrocytes when the serum was being prepared.

Braun-Breton *et al*, (1992) developed a *P. chabaudi* erythrocyte invasion assay using merozoites obtained from mechanically disrupted, unpurified schizonts containing a DNA-binding bisbenzimidazole dye. These merozoites were then added to target erythrocytes and after approximately two hours, the number of bound merozoites was estimated by measurement of fluorescent patterns (small fluorescent cells, merozoites; large non-fluorescent cells, uninfected cells), followed by analysis using FACstar plus research software. After six to seven hours the number of invaded erythrocytes was counted and the level of invasion calculated. While attachment to target cells was in the region of 10 - 20 %, invasion levels of only 0.7 - 2 % were achieved. The assay itself as described by Braun-Breton *et al*, (1992) is complicated and requires elaborate and specialised, expensive equipment and the levels of invasion achieved were very low, to the point that the results are not statistically acceptable. The invasion assay developed here is simple to use and requires no elaborate equipment, while invasion levels of 5 - 6 % were routinely achieved. Similar invasion assays carried out with other *Plasmodium* species into their host erythrocytes resulted in invasion levels of 6 - 15 % with *P. knowlesi* (Miller *et al*, 1975; Haynes *et al*, 1988) and greater than 10 % with *P. falciparum* (Mitchell *et al*, 1986); therefore the levels of invasion obtained using our invasion assay are comparable to levels in other invasion assays.

4.2 The mechanism of erythrocyte invasion by *P. c. chabaudi*

Since no methods were available to study the intricacies of rodent invasion of host cells *in vitro*, very little is known at the molecular level about the recognition, attachment and invasion of erythrocytes by the rodent malarias. With the development of an *in vitro* invasion assay for *P. c. chabaudi*, we could now begin to study these mechanisms.

The *P. c. chabaudi in vitro* erythrocyte invasion assays were carried out with target cells obtained from adult mice, rat, rabbit, guinea pig and human cells. Merozoite invasion was observed into mouse and rat cells (6 and 3.7 % respectively), but no newly invaded parasites were visible in rabbit, guinea pig or human cells. This *in vitro* finding supports the *in vivo* observation that *P. c. chabaudi* infects mice and rats, while rabbits, guinea pigs and humans are not susceptible to *P. c. chabaudi* infection (Wrey, 1968). This implies the existence of an erythrocyte ligand on the surface of mouse and rat cells, that is necessary for invasion, and that is not present on erythrocytes from the other species examined.

Among the various species of malaria there exists a difference in parasite preference for erythrocytes of different ages. The human malaria *P. vivax* (Hingst, 1938), is characterised by a preference for invading immature red blood cells (reticulocytes) while *P. falciparum* invades mature and immature erythrocytes, without preference (Howard & Miller, 1981). There is some evidence to suggest that *P. falciparum*, however preferentially invades reticulocytes and young erythrocytes (Pasvol *et al*, 1980). A detailed study of the course of *P. c. chabaudi* (AS strain) parasitaemias in CBA / Ca mice showed that this parasite had no preference for reticulocytes (Jarra & Brown, 1989). When the *in vivo* reticulocyte level was enhanced to 50 % by phenylhydrazine treatment of mice prior to infection, it found that *P. c. chabaudi* merozoite invasion into mature erythrocytes (normocytes) and reticulocytes was similar. In contrast, Ott (1968), carried out a similar experiment studying *P. chabaudi* infection in phenylhydrazine-treated mice and concluded that this species preferentially invaded normocytes. Carter & Walliker (1975), also reported a *P. c.chabaudi*

preference for normocytes, based on the *in vivo* observation of parasitised cells during the course of a normal infection in laboratory mice.

Using our *P. c. chabaudi* invasion assay we could directly determine if this malaria species prefers reticulocytes and thus clarify the target cell requirements of the parasite. The results presented in this study are interesting to compare to the previous *in vivo* studies (Ott, 1968; Jarra & Brown, 1989). Initially it was found that there was a slight but significant increase in the levels of merozoite invasion into target cell preparations from immature mice compared to invasion into cells obtained from mature mice. The immature mice routinely had a reticulocyte count of between 5 and 8 %, as determined by methylene blue staining, while blood from mature mice normally contains approximately 1 % reticulocytes. We then showed that when *P. c. chabaudi* (AS strain) parasites are presented *in vitro* with target erythrocyte preparations containing almost equal numbers of reticulocytes and normocytes, the levels of merozoite invasion are consistently better than invasion levels into target cell preparations from untreated mice (approximately 1 % reticulocytes). This increase in invasion was considerably greater than the increase between target cell preparations from immature and mature mice. It can be concluded therefore that *P. c. chabaudi* preferentially invades target cell preparations containing elevated numbers of reticulocytes. Wrey (1968) observed that the course of *P. chabaudi* infection varied between different strains of mice. In some strains the peak of infection reached 50 % while in others only reached 20 %. Furthermore, Carter & Walliker (1975) found that *P. chabaudi* infection was fatal only in some strains of mice. In the course of our work we obtained target cell preparations, from three strains of laboratory mice which were either treated with phenylhydrazine or left untreated. A similar increase in levels of invasion was observed into reticulocyte preparations from the three strains *in vitro* over the preparations from untreated mice, indicating that the availability of erythrocyte ligands does not influence the course of parasitaemias in different mouse strains. However, the ability of different strains of mouse to produce reticulocytes could affect the course of infection (Wrey, 1968).

The rodent malaria *P. berghei,* is a species which is known to have a strong preference for invasion into reticulocytes, *in vivo* (Ramakrishnan & Prakash, 1950) and *in vitro* (McNally *et al*, 1992). *Plasmodium berghei* parasites, however, do invade normocytes in the absence of available reticulocytes. The increased levels of invasion by *P. c. chabaudi* parasites into target cell preparations containing an increased proportion of reticulocytes, may indicate that this species spontaneously arose from a line that had a preference for reticulocytes such as *P. berghei.* An example of such a divergence was the spontaneous emergence in the laboratory of a strain of *P. yoelii* which invaded both normocytes and reticulocytes (Yoeli *et al*, 1975) from a strain of *P. yoelii* which normally exhibits a preference for invading reticulocytes.

A clearer understanding of *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* develop into young erythrocytes within 24 - 36 hours after their release from the bone-marrow into the circulation (Gronowicz *et al*, 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 the reticulocytes had matured, losing their ribonucleic acid and could not be visualised with methylene blue or other stains. It was not possible, therefore, to determine what proportion of parasites had invaded reticulocytes.

Malaria parasites use ligands present on the surface of erythrocytes to recognise their host cells. Through enzyme treatment or chemical modification of host erythrocytes, information about the nature of the erythrocyte ligands can be learned. Our *in vitro* erythrocyte invasion assay allowed such experiments to be carried out.

Murine erythrocytes were treated with various enzymes or with sodium periodate. Papain is a cysteine proteinase, while trypsin and chymotrypsin are both serine proteinases. These three enzymes are endopeptidases, which can act upon their specific cleavage sites at any location in the amino acid chain on the protein. The specific cleavage sites are shown in Fig. 4.1. Trypsin is the more specific of the three (Lehninger, 1975). Pronase is a general proteinase and

contains some chymotrypsin and trypsin activity. Neuraminidase removes sialic acid residues from glycophorins on the erythrocyte membrane while sodium periodate treatment results in the cleavage of the exocyclic hydroxyl groups of the terminal NeuNAc of the glycophorin but leaves it's COOH group unaltered (Perkins & Holt, 1988).

Plasmodium c. chabaudi merozoite invasion into trypsin-, chymotrypsin- or Pronase-treated murine erythrocytes was less when compared to invasion into normal erythrocytes. Chemical modification of erythrocyte ligands with sodium periodate also leads to a reduction in levels of merozoite invasion. In contrast, papain or neuraminidase treatment of murine erythrocytes had no effect on merozoite invasion. Chymotrypsin resulted in the greatest amount of reduction of invasion (66 %), while Pronase treatment reduced invasion levels by 57 %. Trypsin treatment of murine erythrocytes decreased invasion levels by 25 %.

Invasion by *P. knowlesi* into human erythrocytes treated with chymotrypsin or Pronase was reduced by almost 100 %, while trypsin or neuraminidase treatment had no effect on *P. knowlesi* invasion into human erythrocytes, indicating the protein or lipoprotein nature of the erythrocyte ligand for this parasite (Miller *et al*, 1973). In contrast *P. falciparum* merozoite invasion into chymotrypsin- or Pronase-treated erythrocytes is not effected but invasion is inhibited by > 95 % when the cells are treated with neuraminidase or trypsin. Pronase treatment of the target erythrocytes results in a slight inhibitory effect on the ability of *P. falciparum* to invade human erythrocytes (Breuer *et al*, 1983). It is generally accepted that the *P. falciparum* erythrocyte ligands are the glycoproteins, glycophorin A and B; however, Hadley *et al*, (1987) and Perkins & Holt (1989) have suggested that there is a second invasion pathway available to *P. falciparum* merozoites, which is independent of glycophorin A and B (neuraminidase-independent). It was found that *P. falciparum* invasion occurred into M^kM^k cells. These cells lack glycophorin A and B and hence the merozoites must recognise another ligand on the erythrocyte membrane of these cells (Hadley *et al*, 1987).This ligand is trypsin-sensitive and is located on another molecule.

Sodium periodate treatment of murine erythrocytes reduced levels of

```
 H   H        H   R2
 |   |        |   |
-N - C - C - | - N - C - C -
     |   ||       |   ||
     R1  O        H   O
```

amino acid 1   amino acid 2

Trypsin	amino acid 1 = Lys or Arg
	amino acid 2 = non-specific
Chymotrypsin	amino acid 1 = Phe, Trp or Try
	amino acid 2 = non-specific
Papain	amino acid 1 = non-specific but Arg or Lys preferred
	amino acid 2 = non-specific

Fig. 4.1 Site of enzyme cleavage of peptide bonds

P. c. chabaudi merozoite invasion, implying that the exocyclic hydroxyl group is the important group in the interaction between the merozoite and a terminal sugar group. However, neuraminidase which removes the terminal NeuNAc group from glycophorins, does not affect invasion. Trypsin, chymotrypsin and Pronase also cleave sialoglycoproteins from human erythrocyte membranes (Bender *et al*, 1971; Seaman & Uhlenbruck, 1963; Hubbard & Cohen, 1972). Therefore, one possible pathway for invasion of *P. c. chabaudi* may involve sialoglycoproteins as erythrocyte ligands.
Results obtained using enzyme-treated rat erythrocytes were similar to those obtained using enzyme-treated mouse erythrocytes. Hence the ligand required for *P. c. chabaudi* invasion of mouse and rat cells is sensitive to the same treatment . This implies that the ligand required for invasion by the parasite is the same or related.
Erythrocyte preparations from normal and phenylhydrazine-treated mice were treated with trypsin, chymotrypsin, Pronase, papain, neuraminidase or sodium periodate. The results obtained with cells from the phenylhydrazine-treated mice were the same as those obtained when erythrocytes from normal mice were used. The erythrocyte ligand required for *P. c. chabaudi* invasion is therefore present on both normocyte and reticulocyte membrane surfaces.

4.3 Improvement to the *in vitro* erythrocyte invasion assay

When attempting the *in vitro* cultivation of parasitised cells, two inter-related problems exist.
a) The erythrocyte surface must be maintained in a condition that allows attachment and invasion by malaria parasites.
b) The erythrocyte must provide the optimum environment for the development to maturity of the intra-cellular parasite, it's rupture and entry into new host cells and the development therein.
Initial research led to the development of the Harvard growth medium (Ball *et al*, 1945) for the cultivation of *P. knowlesi* -parasitised erythrocytes. This medium was used to cultivate a number of different species of malaria including the human malarias *P. falciparum* and *P. vivax* as well as the rodent malarias *P. berghei* and *P. vinckei* (Geiman *et al*, 1966). Parasite growth was supported in all cases. The

Harvard medium was gradually modified until Trager (1976) assayed *P. falciparum* growth in a number of commercially available media by comparison with Ball - Geiman modified Harvard medium (Geiman *et al*, 1966). He found that RPMI - 1640 resulted in better multiplication rates and allowed the parasites to be cultured for a longer period. Trager & Jensen (1976) used RPMI - 1640 supplemented with glucose, hypoxanthine, L - glutamine, gentamicin, bicarbonate and serum for their breakthrough in malaria cultivation, when they achieved the first *in vitro* continuous cultivation of *P. falciparum*. This medium is the routinely used medium for the cultivation of all *Plasmodium* species. RPMI - 1640 is a nutritionally complex medium that was originally developed for the cultivation of human leucocytes (Moore *et al*, 1967), however there is no evidence to suggest that it is the most suitable medium for the culture of rodent malaria parasites such as *P. chabaudi*. In the last 20 years a large number of nutritionally complex media have been developed and become available commercially. Eight of these nutritionally complex media, were selected for study. All the media chosen contain inorganic salts, glucose, amino acids and vitamins and are used for the culture and maintenance of mammalian cells (Table 4.1).

On analysis of *P. c. chabaudi* merozoite invasion levels into murine erythrocytes in the presence of the various media, there was a significant variation in the suitability of the various media for the support of *P. chabaudi* growth and re-invasion. In all of the media tested the parasites developed to cell rupture. Target erythrocytes were maintained in a healthy condition, in all media, as judged by cell morphology under the microscope. However, *P. c. chabaudi* parasites cultured in the Group I media failed to re-invade target erythrocytes to the same degree as those cultured in RPMI - 1640. The parasites cultured in Group II media supported merozoite invasion levels that were approximately the same as those achieved using RPMI - 1640. However, when the parasites were cultured in the Group III media, invasion levels that were significantly above those obtained with parasites cultured in RPMI - 1640, were achieved. The newly invaded parasites also showed an improved rate of maturation in the Group II media as indicated by an increase in the number of newly invaded

Table 4.1 ***In vitro*** **application of selected culture medium**

Culture medium	Application
BGJb	designed for growth of foetal long bones
BME	used for diploid or primary mammalian cells
CMRL	used for Earle's "L" cells
DMEM	broad spectrum of mammalian cell lines
McCoy's 5A	designed for human lymphocyte propagation
MEM	broad spectrum of mammalian cell lines
NCTC	growth of hybridoma cell lines
RPMI - 1640	designed for human leucocyte cultivation
William's E	designed for long-term cell cultures of rat liver epithelial cells

parasites that had developed past the ring-stage and into trophozoites. This increased rate of ring to trophozoite development was especially noticeable with William's E medium.
The rate of *P. c. chabaudi* development from trophozoite to schizont-stage in the presence of the Group III media was also studied using radio-labelled amino acid incorporation into parasite proteins as an indicator of parasite growth. Parasites cultivated in BME resulted in the highest rate of amino acid incorporation, implying that it supported the greatest rate of development at this stage. We developed a medium for the cultivation of *P. c. chabaudi* based on the improved rate of schizont-stage development observed with parasites cultured in BME and the increased rate of development of newly invaded parasites observed with parasites suspended in William's E medium. This medium combined BME and William's E in a ratio of 3 : 1 (BME - WE) and this supported merozoite invasion levels 35 - 45 % greater than those achieved in invasion assays carried out with RPMI - 1640. There was also an improvement in the proportion of newly invaded parasites that had developed to trophozoites; approximately 15 % of parasites had developed to the trophozoite stage with BME - WE when compared to 2 % with RPMI - 1640.
Previous work carried out on *P. chabaudi* (McLean *et al*, 1986; Gilks *et al*, 1990) used RPMI - 1640 although Coombs & Gutteridge (1975) used Modified Eagles MEM supplimented with glycylglycine, HEPES, TES and sodium phosphate buffer. Both media have similar compositions and in our laboratory MEM resulted in invasion levels comparable to those obtained with RPMI - 1640.
Divo *et al*, (1985) working with *P. falciparum* analysed the parasite's requirements for the individual constituents of RPMI - 1640 in continuous culture. A basic medium containing inorganic salts plus HEPES, glucose and hypoxanthine was prepared, to which the amino acids and vitamins contained in RPMI - 1640 were added. Each amino acid and vitamin was then omitted individually and the effect on invasion levels monitored. With this technique, it was found that only calcium pantothenate, cystine, glutamate, isoleucine, methionine, proline and tyrosine were required for successful reinvasion of cells by *P. falciparum* merozoites. The complete absence of either amino acids or

vitamins resulted in drastically reduced rates of parasite growth and invasion. The finding that the absence of either folate or *p*-aminobenzoic acid (PABA) had no effect on invasion is contrary to previous *in vivo* evidence (Jacobs, 1964; Ferone, 1977).
On analysis of the components of the different media tested in our study, including RPMI - 1640, a number of observations can be made (Appendix 2). There is no single component present or absent in Group III media which accounts for the improved invasion levels observed. It must be noted however that amino acids and amino acids can be obtained by parasites from serum and from the enzymatic breakdown of the host cell's haemoglobin (Goldberg & Slater, 1992). Upon analysis of the components of Group III media, we found that the components that Divo *et al*, (1985) found to be essential for the successful reinvasion of host erythrocytes by malaria parasites, were not necessarily present. BME lacks tyrosine, proline and glutamine and DMEM lacks tyrosine and cystine. The essential components of Divo *et al*, (1985) were present in William's E. All three media contained either PABA or folate, vitamins which were not required for reinvasion according to Divo *et al*, (1985).
A metalloaminopeptidase has been identified in our laboratory which is possibly involved in haemoglobin digestion (Curley *et al,* 1993).The presence of metal ions (Appendix 2) in the Group III media could improve the rate of parasite development and reinvasion into erythrocytes by enhancing or stabilising this aminopeptidase activity.
In conclusion, a medium which supports higher levels of invasion and faster rates of *in vitro* development of the malaria parasite *P. chabaudi*, has been developed. The specific constituents of this medium which are responsible for the improvements cannot be determined however.

4.4 *Plasmodium c. chabaudi* parasite receptor molecules

Successful invasion of erythrocytes depends upon the recognition of the host erythrocytes by specific parasite receptors. These parasite receptors bind to the erythrocyte ligands on the cell membrane (Section 4.2). Putative receptor molecules have been suggested for *P. falciparum, P. knowlesi* and *P. vivax* (Brown & Coppel, 1991; Camus &

Hadley, 1985; Epstein *et al*, 1981; Haynes *et al*, 1988; Miller *et al*, 1988; Perkins, 1989; Perkins & Rocco, 1988; Wertheimer & Barnwell, 1989). These proteins, such as the *P. falciparum* protein EBA - 175, are synthesised during schizogony and are found on the merozoite. When *P. falciparum* is cultured *in vitro,* it is also found in the culture medium (Orlandi *et al*, 1990).

Plasmodium c. chabaudi parasites were cultured from middle-stage trophozoites to schizogony (schizonts). At various times during the culturing period, parasites were removed from the culture and their proteins PBS - extracted. Upon comparison of the protein profiles of middle-stage trophozoites, late-stage trophozoites and segmentors it was seen that a protein of 162 kDa was present in the late-stage trophozoites and segmentors but was not observed in the middle-stage trophozoites. Two proteins (87 kDa and 130 kDa) were present in all three developmental stages but their synthesis increased with maturation of the parasite.

Haynes *et al*, (1988) identified a *P. knowlesi* 135 kDa protein as a putative receptor. Radiolabelled proteins released by *P. knowlesi in vitro* were used in receptor binding studies and a protein of 135 kDa was found to bind selectively to human erythrocytes. This protein is a minor component of the *P. knowlesi* malaria radiolabelled proteins released into culture supernatant at the time of merozoite release and reinvasion. A similar approach was employed to identify a *P. c. chabaudi* receptor. *Plasmodium c. chabaudi* parasites were labelled by culturing the parasites until erythrocyte rupture in medium containing [^{35}S]methionine. A large number of soluble parasite proteins were labelled. These labelled parasite proteins were then incubated with host erythrocytes to determine if any of the proteins bound specifically to the erythrocytes. Two proteins were identified as binding to murine erythrocytes. A protein of > 200 kDa was occasionally detected, while a protein of 130 kDa consistently bound to murine cells. The > 200 and 130 kDa proteins did not bind to human erythrocytes, cells that are refractory to invasion. These 130 and 200 kDa proteins can therefore be considered a putative receptors for *P. c. chabaudi.* It is interesting to note the similarity in size between the 130 kDa *P. chabaudi* protein and the 135 kDa protein of Haynes *et al*, (1988), the receptor binding

Adams *et al*, 1990) have been cloned. In spite of the differences in molecular size and the ligands they bind to, the structure of these genes is very similar. The open reading frame consists of 5 exons (Adams *et al*, 1992).

1. signal peptide
2. erythrocyte binding domain
3. transmembrane domain

4. & 5. cytoplasmic domains

Within the erythrocyte binding domain there are 5' and 3' cysteine rich regions that show high levels of homology (approximately 70 %). In every case the cysteine residues are positionally conserved, as are most of the aromatic residues (Adams *et al*, 1992). In the case of *P. falciparum*, there are two copies of the 5' cysteine region. This accounts for the increased size of the erythrocyte binding protein of this species; 175 kDa as opposed to 135 kDa (Adams *et al*, 1992). On the basis of this homology, it has been proposed that the Duffy binding proteins and the *P. falciparum* EBA-175 protein are members of the same gene family.

We have shown the existence of a *P. chabaudi* erythrocyte binding protein which is in the same size region as the 135 kDa Duffy receptor molecule. The presence of a 130 kDa erythrocyte binding protein of *P. berghei* has also been discovered (McNally, personal communication).

Preliminary work has demonstrated the presence of a gene in the genome of *P. chabaudi* that is homologous to the erythrocyte receptor genes of *P. knowlesi, P. vivax* and *P. falciparum* (Curley, personal communication). This implies that the rodent malarias may synthesise receptors homologous to the primate malaria receptors, and which could belong to the same gene family.

Future work is needed to determine if the product of the *P. c. chabaudi* gene is the *P. c. chabaudi* 130 kDa erythrocyte binding molecule identified in this study.

4.5 Analysis of the polyclonal antisera

The basis for the development of a malaria vaccine is the identification of parasite proteins involved in the invasion process against which individuals can be immunised in order to produce antibodies that will block the invasion process and thus prevent the disease. A simple *in vitro* invasion assay would allow the screening of such antibodies for blocking abilities.

Polyclonal antisera to various parasite protein preparations (each preparation contained a number of different proteins) were raised. The antisera were developed to identify parasite antigens against which antibodies capable of blocking invasion could be produced. The antibodies were characterised by immunoblotting, immunoprecipitation and immunofluorescence. Each antiserum was tested in our invasion assay to study its effect on the invasion process.

Each antiserum was used to probe nitrocellulose filters bearing electrophoretically separated *P. c.chabaudi* parasite protein extracts and a characteristic pattern of reactivity was detected with each. A number of the parasite antigens were found in several of the antisera, while others were unique to a particular antiserum. Proteins that had been identified as having possible roles in invasion (the 130 kDa as an erythrocyte binding protein; 87 and 162 kDa proteins which were produced during schizogony) were compared to the antigens recognised by antibodies in the antisera. A number of observations could be made. The 162 kDa protein appears to be non-immunogenic in rats as it was not detected by any of the antisera produced. Antibodies to a 87 kDa protein were detected in the antisera raised to the proteins contained in culture supernatent (TCS) and the proteins extracted from schizonts (TSP), while antibodies to a 130 kDa protein were only detected in anti - TCS. The fact that this antigen was detected with anti - TCS, implies that the protein is secreted. However, antibodies to the 130 kDa protein would also have been expected in anti - TSP and anti - TXP since this protein must be synthesised by schizonts and merozoites. However none were detected. When the antiserum raised to parasite molecules eluted from murine erythrocytes (anti - EBP) was tested in immunoblotting experiments, no antigens were detected. However a 41 kDa antigen was detected in

immunoprecipitation experiments (see below), implying that the antibody to the 41 kDa protein only recognises the native form. Examination of the antiserum raised against the two proteins eluted from the Heparin-Sepharose column (130 and 62 kDa), showed that the antiserum reacted with two antigens of 94 and 62 kDa. The 130 kDa protein was not detected, suggesting that it was not immunogenic in this preparation. However an antigen of 94 kDa was detected with anti - HEP, an antigen that was not seen on electrophoresis gels of the proteins eluted from a heparin column. The 94 kDa parasite protein is a major parasite protein and is strongly reactive in anti - TCS and anti - TSP.

When the panel of antisera were used in immunoprecipitation experiments, a greater number of immunoreactive antigens were detected compared to the immunoblotting experiment, indicating that this method is more sensitive. All the proteins precipitated with the antisera, with the exception of a protein of molecular weight 38 kDa with anti - TSP, were contained in the precipitation pattern obtained with anti - TCS. A 130 kDa protein was precipitated with anti - TCS and anti - TXP, and may correspond to the 130 kDa protein, previously identified as a putative erythrocyte receptor of *P. c. chabaudi.*

Anti - EBP precipitated a protein of 41 kDa, not detected with immunoblotting. The specific precipitation of this antigen is surprising as a protein of that molecular size was not found to bind to murine erythrocytes in receptor binding studies. Anti - HEP was raised to proteins eluted from a Heparin-Sepharose column (130 and 62 kDa), and precipitated a small number of proteins (116, 94 and 60 kDa). However, it did not precipitate a 130 kDa protein. Both anti - TCS and anti - HEP blocked invasion *in vitro* (see below).

When the panel of antisera was used for immunofluorescence studies, all gave positive reactions. When sera from an immune mouse was tested, a strongly positive reaction resulted. Fluorescent staining of the parasites themselves was observed, as well as immunofluorescence located on the erythrocyte membrane of the parasite-infected cells. This erythrocyte membrane fluorescence was also detected with two of the polyclonal antisera, anti - TSP and anti - TCS. Parasite proteins have been shown to be inserted into the erythrocyte membranes of

parasitised cells. Brown *et al*, (1984) cloned an antigen (RESA) found on the surface of erythrocytes infected with ring-stage *P. falciparum* parasites. It is thought that RESA (m.w. 155 kDa) is first synthesised in the maturing trophozoite and is particularly abundant in merozoites. RESA is released from the rhoptries of the apical pore, around the time of merozoite invasion and is then transferred to the erythrocyte membrane of the recently invaded cell.

The parasites themselves showed a general fluorescent staining with all of the antisera, with no difference in the fluorescence patterns. This was expected due to the large number of antigens recognised by each of the antisera. The greater intensity of fluorescence observed with extra-cellular parasites may be a result of the parasites being more accessible to the antibodies.

The panel of antisera was used to stain erythrocytes infected with ring-stage parasites and gave quite striking results. All of the antisera resulted in fluorescent staining of the parasites. Fluorescence of the erythrocyte membrane was detected again with anti - TSP. The intensity of the fluorescence was much greater in these ring-infected erythrocytes. Perlmann *et al*, (1984) observed with *P. falciparum,* that when parasites were in the early stages of their developmental cycle (rings / trophozoites), the fluorescent staining of RESA proteins in infected host erythrocyte membranes by immune serum is stronger. This finding supports our observations.

There was intense fluorescent staining of the ring-stage parasites themselves with the panel of antisera. Anti - EBP which immunoprecipitates only the 41 kDa antigen, gives fluorescent staining over the whole of the parasite (both ring-stage and schizont). This immunogenic protein must be a general parasite protein, however it's function is unknown.

Anti - HEP was different to the other antisera as the fluorescence was not continuous around the ring, but was particulate. This implied that the antibodies were against proteins that were intermittently spaced on the parasite surface. In both immunoblotting and radio-immunoprecipitation experiments anti - HEP sera reacted with a small number of antigens. Therefore this difference in pattern is not surprising.

A protein of 105 / 96 kDa , has been identified, which is present in the erythrocyte membrane of *P. chabaudi*-infected erythrocytes and which cross-reacts with RESA (Gabriel *et al*, 1986; Perlmann *et al*, 1984; Wandiworanun *et al*, 1987). Weiser *et al*, (1988) reported on the appearance of phosphoproteins on the host erythrocyte surface of *P. chabaudi* infected parasites (73, 76 and 90 kDa). The structure of an acidic phosphoprotein of *P. chabaudi* has recently been characterised by Deleersnijider *et al*, (1992) which is associated with the erythrocyte membrane. It is possible that the fluorescence in the membrane of infected erythrocytes observed in this study is due to the presence of antibodies reactive with some or all of these antigens described by Gabriel *et al*, (1986),Weiss *et al*, (1988) and Deleersnijider *et al*, (1992).

Slides of *P. falciparum*-infected erythrocytes were also probed with our antisera and very low levels of cross-reactivity were detected with anti - TSP, anti - EBP and anti - TXP. The pattern of fluorescence was similar to the pattern when *P. c. chabaudi*-infected erythrocytes were used. Since anti - TCS contains antibody against a 130 kDa molecule, we attempted to isolate sera specific to this molecule from immunoblots and use it in immunoprecipitation and immunofluorescent studies. A monospecific anti - 130 kDa sera was isolated, but whilst the antisera was reactive on immunoblots, it did not immunoprecipitate a 130 kDa antigen, nor was it reactive in immunofluorescence experiments. This lack of reactivity may be due to the fact that epitopes to antibodies eluted from immunoblots are accessible only when the antigen is denatured (in immunoblots, the samples are boiled in the presence of mercaptoethanol) and hence would not immunoprecipitate the native protein.

The antisera were screened for their ability to block re-invasion of target cells *in vitro*. Anti - TCS and anti - HEP blocked invasion at a rate of approximately 50 % at a 1 / 10 dilution of antiserum, while anti - TSP resulted in a 25 % blocking of invasion at a 1 / 25 dilution. Anti - EBP appeared to block invasion but the amount of blocking was not significant, while anti - TXP failed to have any effect on invasion. From analysis of the antigens reactive with antibody in each antiserum, anti - TCS reacted with the greatest range of antigens. Anti - HEP contains

antibodies to a few proteins only (116, 94 and 62 kDa mainly), antibodies reactive with these antigens are also present in anti - TCS. These immunogenic proteins are implicated in the invasion process. Previously, no assay was available to study the blocking abilities of antibodies against *P. c. chabaudi* parasite proteins. We have shown that our *in vitro* erythrocyte invasion assay is suitable for studying the blocking abilities of antibodies to parasite proteins. Using this assay we have achieved significant blocking of invasion using polyclonal antibodies developed against a number of parasite proteins. Future work in this area should involve the identification of a specific antigen or antigens against which antibodies could be raised that would block invasion. These antigens could then be used to immunise mice against *P. c. chabaudi* infection.

4.6 *Plasmodium c. chabaudi* IP - IPC1 strain

A second strain of *P. chabaudi*, *P. chabaudi* IP - IPC1 (Hommel *et al*, 1982; Falanga *et al*, 1984) was obtained as a frozen stabilate from Balb / c mice. We found that in CD - 1 mice the strain was more virulent and parasitaemias of greater than 80 % were obtained. These infections were normally fatal to the host mouse. We wished to investigate whether the methods we developed could be applied to other strains of *P. chabaudi*, therefore we treated this strain in the same manner as we did *P. c. chabaudi* AS strain. The animals were bled at 13.00 hours and after CF11 column treatment and washing, the cells were layered onto the Percoll gradient and centrifuged. The top band of cells that was formed contained greater than 98 % purified parasite-infected cells, indicating that the gradient was suitable for the purification of more than one strain of *P. chabaudi*. When these parasites were used in our erythrocyte invasion assay, invasion levels of 5 to 10 % were routinely achieved in BME - WE containing 10 % rat serum. This indicates the suitability of our erythrocyte invasion assay for application to other strains of *P. chabaudi*, and possibly other rodent malarias.
Erythrocytes were treated with trypsin, chymotrypsin, papain and Pronase and were used as target cells for invasion assays using *P. chabaudi* IP - IPC1. The pattern of inhibition of invasion into cells

treated with the different enzymes was similar to that described for *P. c. chabaudi* AS strain. It is suggested therefore that the erythrocyte ligands for the two strains of parasite are the same.

4.6 Conclusions

We have developed a method for the purification of *P. c. chabaudi* parasites which results in greater than 95 % purified parasites. This purification procedure can also be applied to other strains of *P. chabaudi*. The purified parasites were then used to carry out *in vitro* erythrocyte invasion assays. These assays, as developed during this research, are simple to perform, require no elaborate equipment and allow a number of assays to be carried out simultaneously. This assay was used to investigate a number of parameters that were set during the development of *in vitro* cultivation techniques for other species of malaria parasites. We found that 5 % O_2 allowed the parasites to develop to maturity, merozoite release to occur and reinvade new host cells. The removal of leucocytes improved invasion levels as did the replacement of FCS with rat serum. Since the routinely used medium for the cultivation of malaria parasites was originally developed for human leucocytes, we investigated the possibility that the medium was not the most suitable medium for murine malaria. A number of commercially available media were tested in our invasion assay. Based on the results of the survey, in which several media supported improved levels of merozoite invasion, a medium combining BME and William's E medium was adopted as the medium for the cultivation of *P. c. chabaudi*.

A second strain of *P. chabaudi* was tested in our invasion assay and we found that the assay can be used for other strains of the parasite. It is also possible that similar invasion assays can be developed for other species of malaria.

The invasion assay was also used to study the parasites requirements for ligands on murine erythrocytes. We found that *P. c. chabaudi* invades only mouse and rat cells due to the presence of a specific ligand on these cells. The parasite also has a preference for target cell preparations which contain high levels of reticulocytes. The erythrocyte ligand was found to be a glycoprotein. However, the

existence of a second pathway has been suggested by the fact that enzyme treatment only partially inhibited invasion.
A putative merozoite receptor of 130 kDa was identified by erythrocyte binding studies. Antibodies to a 130 kDa protein were present in a polyclonal antiserum raised against culture medium in which parasites were maintained until rupture (anti - TCS). It is possible that this receptor binds to glycoproteins on murine erythrocyte membranes. A monospecific anti - 130 kDa antibody was isolated but in insufficient quantities for further characterisation. A protein of 41 kDa was also identified by immunoprecipitation with anti - EBP, the function of which not understood.
Two antisera prepared during this project, including anti - TCS which contained an anti - 130 kDa antibody, blocked invasion of erythrocytes in our *P. c. chabaudi* merozoite *in vitro* invasion assay. These findings lead us to be optimistic about the further characterisation of proteins involved in erythrocyte invasion. These proteins could then be used in future vaccine trials in mice to determine if protection against malaria can be induced.

Chapter Five

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Appendices

Appendix 1

Statistical Tables

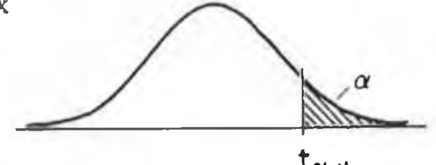
Table 7

PERCENTAGE POINTS OF THE t DISTRIBUTION

The table gives the value of $t_{\alpha;\nu}$ — the 100α percentage point of the t distribution for ν degrees of freedom.

The values of t are obtained by solution of the equation:-

$$\alpha = \Gamma\{\tfrac{1}{2}(\nu+1)\}\{\Gamma(\tfrac{1}{2}\nu)\}^{-1}(\nu\pi)^{-1/2}\int_t^{\infty}(1+x^2/\nu)^{-(\nu+1)/2}dx$$



Note. The tabulation is for one tail only i.e. for positive values of t. For |t| the column headings for α must be doubled.

$\alpha =$	0.10	0.05	0.025	0.01	0.005	0.001	0.0005
ν = 1	3.078	6.314	12.706	31.821	63.657	318.31	636.62
2	1.886	2.920	4.303	6.965	9.925	22.326	31.598
3	1.638	2.353	3.182	4.541	5.841	10.213	12.924
4	1.533	2.132	2.776	3.747	4.604	7.173	8.610
5	1.476	2.015	2.571	3.365	4.032	5.893	6.869
6	1.440	1.943	2.447	3.143	3.707	5.208	5.959
7	1.415	1.895	2.365	2.998	3.499	4.785	5.408
8	1.397	1.860	2.306	2.896	3.355	4.501	5.041
9	1.383	1.833	2.262	2.821	3.250	4.297	4.781
10	1.372	1.812	2.228	2.764	3.169	4.144	4.587
11	1.363	1.796	2.201	2.718	3.106	4.025	4.437
12	1.356	1.782	2.179	2.681	3.055	3.930	4.318
13	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	1.333	1.740	2.110	2.567	2.898	3.646	3.965
18	1.330	1.734	2.101	2.552	2.878	3.610	3.922
19	1.328	1.729	2.093	2.539	2.861	3.579	3.883
20	1.325	1.725	2.086	2.528	2.845	3.552	3.850
21	1.323	1.721	2.080	2.518	2.831	3.527	3.819
22	1.321	1.717	2.074	2.508	2.819	3.505	3.792
23	1.319	1.714	2.069	2.500	2.807	3.485	3.767
24	1.318	1.711	2.064	2.492	2.797	3.467	3.745
25	1.316	1.708	2.060	2.485	2.787	3.450	3.725
26	1.315	1.706	2.056	2.479	2.779	3.435	3.707
27	1.314	1.703	2.052	2.473	2.771	3.421	3.690
28	1.313	1.701	2.048	2.467	2.763	3.408	3.674
29	1.311	1.699	2.045	2.462	2.756	3.396	3.659
30	1.310	1.697	2.042	2.457	2.750	3.385	3.646
40	1.303	1.684	2.021	2.423	2.704	3.307	3.551
60	1.296	1.671	2.000	2.390	2.660	3.232	3.460
120	1.289	1.658	1.980	2.358	2.617	3.160	3.373
∞	1.282	1.645	1.960	2.326	2.576	3.090	3.291

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Appendix 2

Composition of Media

BGJb Medium
(Original[1] BGJb Medium)

Component	041-02581 1X Liquid mg/L
INORGANIC SALTS:	
$NaH_2PO_4 \cdot H_2O$	–
$MgSO_4 \cdot 7H_2O$	200.00
KCl	530.00
KH_2PO_4	160.00
$NaHCO_3$	3500.00
NaCl	8000.00
OTHER COMPONENTS:	
Calcium Lactate	555.00
D-Glucose	5000.00
Phenol Red	20.00
Sodium Acetate	–
AMINO ACIDS:	
L-Alanine	–
L-Arginine	–
L-Arginine HCl	75.00
L-Aspartic Acid	–
$L\text{-Cysteine HCl} \cdot H_2O$	90.00
L-Glutamine	200.00
Glycine	–
L-Histidine	–
$L\text{-Histidine HCl} \cdot H_2O$	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine	–
L-Lysine · HCl	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	–
L-Serine	–
L-Threonine	75.00
L-Tryptophan	40.00
L-Tyrosine	40.00
DL-Valine	–
L-Valine	65.00
VITAMINS:	
α-tocopherol phosphate (disodium salt)	1.00
Ascorbic acid	–
Biotin	0.20
D-Ca pantothenate	0.20
Choline Chloride	50.00
Folic Acid	0.20
i-Inositol	0.20
Nicotinamide	20.00
Para-aminobenzoic acid	0.20
Pyridoxal Phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04

BME[1] Basal Medium (Eagle)

Component	41-02310 1X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	140.00
KCl	400.00
KH_2PO_4	60.00
$MgCl_2$ (anhyd.)	–
$MgCl_2 \cdot 6H_2O$	100.00
$MgSO_4$ (anhyd.)	–
$MgSO_4\ 7H_2O$	100.00
NaCl	7500.00
$NaHCO_3$	350.00
$NaH_2PO_4 \cdot H_2O$†	–
Na_2HPO_4 (anhyd.)	–
$Na_2HPO_4 \cdot 7H_2O$	90.00
OTHER COMPONENTS:	
D-Glucose	1000.00
HEPES	5958.00
Phenol Red	10.00
Sodium Succinate	–
Succinic Acid	–
AMINO ACIDS:	
L-Arginine	17.40
L-Arginine · HCl	–
L-Cystine	12.00
L-Cystine · 2HCl	–
L-Glutamine	–
L-Histidine	8.00
$L\text{-Histidine HCl} \cdot H_2O$	–
L-Isoleucine	26.00
L-Leucine	26.00
L-Lysine	29.20
L-Lysine · HCl	–
L-Methionine	7.50
L-Phenylalanine	16.50
L-Threonine	24.00
L-Tryptophan	4.00
L-Tyrosine	18.00
L-Tyrosine (disodium salt)	–
L-Valine	23.50
VITAMINS:	
Biotin	1.00
D-Ca pantothenate	1.00
Choline Bitartrate	–
Choline Chloride	1.00
Folic Acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	9.10
Thiamine HCl	1.00

CMRL 1066' Medium

Component	041-01530 1X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	200.00
KCl	400.00
$MgSO_4 \cdot 7H_2O$	200.00
NaCl	6799.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00
OTHER COMPONENTS:	
Cocarboxylase	1.00
Coenzyme A	2.50
Deoxyadenosine	10.00
Deoxycytidine	10.00
Deoxyguanosine	10.00
Diphosphopyridine Nucleotide	7.00
Ethanol for solubizing lipid components	16.00
Flavin Adenine Dinucleotide	1.00
D-Glucose	1000.00
Glutathione (reduced)	10.00
5-Methyl-deoxyctidine	0.10
Phenol Red[a]	20.00
Sodium Acetate · $3H_2O$	83.00
Sodium Glucuronate · H_2O	4.20
Thymidine	10.00
Triphosphopyridine Nucleotide	1.00
Tween 80®	5.00
Uridine Triphosphate	1.00
AMINO ACIDS:	
L-Alanine	25.00
L-Arginine HCl	70.00
L-Aspartic acid	30.00
L-Cysteine HCl · H_2O	260.00
L-Cystine	20.00
L-Glutamic acid	75.00
L-Glutamine	–
Glycine	50.00
L-Histidine HCl · H_2O	20.00
Hydroxy-L-proline	10.00
L-Isoleucine	20.00
L-Leucine	60.00
L-Lysine · HCl	70.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine	40.00
L-Valine	25.00

Component	041-01530 1X Liquid mg/L
VITAMINS:	
Ascorbic acid	50.00
Biotin	0.01
D-Ca pantothenate	0.01
Cholesterol	0.20
Choline Chloride	0.50
Folic Acid	0.01
i-Inositol	0.05
Niacin	0.025
Niacinamide	0.025
Para-aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine	0.025
Riboflavin	0.01
Thiamine HCl	0.01

DMEM/F12(1:1) Medium

Component	041-01330 1X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$	116.60
$CuSO_4 \cdot 5H_2O$	0.0013
$Fe(No_3)_3 \cdot 9H_2O$	0.05
$FeSO_4 \cdot 7H_2O$	0.417
KCl	311.80
$MgCl_2$	–
$MgCl_2 \cdot 6H_2O$	61.00
$MgSO_4$	–
$MgSO_4 \cdot 7H_2O$	100.00
NaCl	6995.95
$NaHCO_3$	1200.00
$NaH_2PO_4 \cdot H_2O$	62.50
Na_2HPO_4	–
$Na_2HPO_4 \cdot 7H_2O$	134.00
$ZnSO_4 \cdot 7H_2O$	0.432
OTHER COMPONENTS:	
D-Glucose	3151.00
HEPES	3574.50
Na Hypoxanthine	2.39
Linoleic Acid	0.042
Lipoic Acid	0.105
Phenol Red	8.10
Sodium Putrescine · 2HCl	0.081
Sodium Pyruvate	55.00
AMINO ACIDS:	
L-Alanine	4.45
L-Arginine · HCl	147.50
L-Aspargine · H_2O	7.50
L-Aspartic Acid	6.65
L-Cysteine · HCl · H_2O	17.56
L-Cystine · 2HCl	31.29
L-Glutamic Acid	7.35
L-Glutamine	365.00
Glycine	18.75
L-Histidine HCl · H_2O	31.48
L-Isoleucine	54.47
L-Leucine	59.05
L-Lysine · HCl	91.25
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	17.25
L-Serine	26.25
L-Threonine	53.45
L-Tryptophan	9.02
L-Tyrosine · 2Na · $2H_2O$	55.79
L-Valine	52.85
VITAMINS:	
Biotin	0.0035
D-Ca Pantothenate	2.24
Choline Chloride	8.98
Folic Acid	2.65
i-Inositol	12.60
Niacinamide	2.02
Pyridoxal HCl	2.00
Pyridoxine HCl	0.031
Riboflavin	0.219
Thiamine HCl	2.17
Thymidine	0.365
Vitamin B_{12}	0.68

McCoys 5A Medium (modified)[1,2,3]

Component	041-06600 1X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	100.00
KCl	400.00
KH_2PO_4	–
$MgCl_2 \cdot 6H_2O$	–
$MgSO_4$ (anhyd.)	–
$MgSO_4 \cdot 7H_2O$	200.00
NaCl	6460.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot H_2O$	580.00
$Na_2HPO_4 \cdot 7H_2O$	–
OTHER COMPONENTS:	
Bacto-peptone	600.00
HEPES	–
D-Glucose	3000.00
Glutathione (reduced)	0.50
Phenol Red	10.00
AMINO ACIDS:	
L-Alanine	13.90
L-Arginine · HCl	42.10
L-Asparagine[a]	45.00
L-Aspartic Acid	19.97
L-Cysteine[b]	31.50
L-Glutamic Acid	22.10
L-Glutamine	219.20
Glycine	7.50
L-Histidine HCl · H_2O	20.96
L-Hydroxyproline	19.70
L-Isoleucine	39.36
L-Leucine	39.36
L-Lysine · HCl	36.50
L-Methionine	14.90
L-Phenylalanine	16.50
L-Proline	17.30
L-Serine	26.30
L-Threonine	17.90
L-Tryptophan	3.10
L-Tyrosine	18.10
L-Tyrosine (disodium salt)	–
L-Valine	17.60
VITAMINS:	
Ascorbic Acid	0.50
Biotin	0.20
Choline Chloride	5.00
D-Ca Pantothenate	0.20
Folic Acid	10.00
i-Inositol	36.00
Nicotinamide	0.50
Nicotinic Acid	0.50
Para-aminobenzoic Acid	1.00
Pyridoxal HCl	0.50
Pyridoxine HCl	0.50
Riboflavin	0.20
Thiamine HCl	0.20
Vitamin B_{12}	2.00

Minimum Essential Medium' (MEM)

Component	042-01430 10X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	2000.00
KCl	4000.00
$MgSO_4$ (anhyd.)	–
$MgSO_4 \cdot 7H_2O$	2000.00
NaCl	68000.00
$NaHCO_3$	–
$NaH_2PO_4 \cdot H_2O$*	1400.00
OTHER COMPONENTS:	
D-Glucose	10000.00
Phenol Red	100.00
Sodium Succinate	–
Succinic Acid	–
AMINO ACIDS:	
L-Arginine · HCl	1260.00
L-Cystine	240.00
L-Cystine · 2HCl	–
L-Glutamine	–
L-Histidine HCl · H_2O	420.00
L-Isoleucine	520.00
L-Leucine	520.00
L-Lysine · HCl	725.00
L-Methionine	150.00
L-Phenylalanine	320.00
L-Threonine	480.00
L-Tryptophan	100.00
L-Tyrosine	360.00
D-Valine	–
L-Valine	460.00
VITAMINS:	
D-Ca Pantothenate	10.00
Choline Bitartrate	–
Choline Chloride	10.00
Folic Acid	10.00
i-Inositol	20.00
Nicotinamide	10.00
Pyridoxal HCl	10.00
Riboflavin	1.00
Thiamine HCl	10.00

RPMI 1640 Medium'

Component	042-02511 10X Liquid mg/L
INORGANIC SALTS:	
$Ca(NO_3)_2 \cdot 4H_2O$	1000.00
KCl	4000.00
$MgSO_4$ (anhyd.)	–
$MgSO_4 \cdot 7H_2O$	1000.00
$MnSO_4 \cdot H_2O$	–
NaCl	60000.00
$NaHCO_3$	–
Na_2HPO_4 (anhyd.)	–
$Na_2HPO_4 \cdot 7H_2O$	15120.00
OTHER COMPONENTS:	
D-Glucose	20000.00
Glutathione (reduced)	10.00
HEPES	–
Phenol Red	50.00
AMINO ACIDS:	
L-Arginine (free base)	2000.00
L-Asparagine	500.00
L-Aspartic Acid	200.00
L-Cystine	500.00
L-Cystine · 2HCl	–
L-Glutamic Acid	200.00
L-Glutamine	–
Glycine	100.00
L-Hisitdine (free base)	150.00
L-Hydroxproline	200.00
L-Isoleucine (allo free)	500.00
L-Leucine (methionine free)	500.00
L-Lysine · HCl	400.00
L-Methionine	150.00
L-Phenylalanine	150.00
L-Proline (hydroxy L-proline free)	200.00
L-Serine	300.00
L-Threonine (allo free)	200.00
L-Tryptophan	50.00
L-Tyrosine	200.00
L-Tyrosine (disodium salt)	–
L-Valine	200.00
VITAMINS:	
Biotin	2.00
D-Ca Pantothenate	2.50
Choline Chloride	30.00
Folic Acid	10.00
i-Inositol	350.00
Nicotinamide	10.00
Para-aminobenzoic Acid	10.00
Pyridoxine HCl	10.00
Riboflavin	2.00
Thiamine HCl	10.00
Vitamin B_{12}	0.05

NCTC–135[1] Medium

Component	041-01350 1X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	200.00
KCl	400.00
$MgSO_4 \cdot 7H_2O$	200.00
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00
OTHER COMPONENTS:	
Ethyl Alcohol	40.00
D-Glucose	1000.00
D-Glucuronolactone	1.80
Phenol Red (sodium salt)	20.00
Sodium Acetate · $3H_2O$	50.00
Sodium Glucuronate · H_2O	1.80
Tween 80®	12.50
COENZYMES:	
CoA (coenzyme A)	2.50
DPN (diphosphopyridine nucleotide)	7.00
FAD (flavin adenine dinucleotide)	1.00
TPN (triphosphopyridine nucleotide, monosodium)	1.00
TPP (cocarboxylase)	1.00
UTP (uridine triphosphate, sodium)	1.00
REDUCING AGENTS:	
Ascorbic Acid	50.00
Glutathione, monosodium	10.00
NUCLEIC ACID DERIVATIVES:	
Deoxyadenosine	10.00
Deoxyguanosine	10.00
Deoxycytidine HCl	10.00
5-Methylcytosine	0.10
Thymidine	10.00
AMINO ACIDS:	
L-Alanine	31.48
L-α-Amino-n-butyric Acid	5.51
L-Arginine HCl	31.16
L-Asparagine · H_2O	9.19
L-Aspartic Acid	9.91
L-Cystine	10.49
D-Glucosamine · HCl	3.85
L-Glutamic Acid	8.26
L-Glutamine	135.73
Glycine	13.51
L-Histidine · HCl · H_2O	26.65
Hydoxy-L-Proline	4.09
L-Isoleucine	18.04
L-Leucine	20.44
L-Lysine · HCl	38.43
L-Methionine	4.44
L-Ornithine · HCl	9.41
L-Phenylalanine	16.53
L-Proline	6.13
L-Serine	10.75
L-Taurine	4.18
L-Threonine	18.93
L-Tryptophan	17.50
L-Tyrosine	16.44
L-Valine	25.00

Component	041-01350 1X Liquid mg/L
VITAMINS:	
α-Tocopherol Phosphate (disodium salt)	0.025
d-Biotin	0.025
Calciferol (Vitamin D_2)	0.25
D-Ca Pantothenate	0.025
Choline Chloride	1.25
Folic Acid	0.025
i-Inositol	0.125
Menadione (Vitamin K)	0.025
Niacin	0.0625
Niacinamide	0.0625
Para-aminobenzoic Acid	0.125
Pyridoxal HCl	0.0625
Pyridoxine HCl	0.0625
Riboflavin	0.025
Thiamine HCl	0.025
Vitamin A (crystallized alcohol)	0.25
Vitamin B_{12}	10.00

Williams' Medium E[1]*

Component	041-02551 1X Liquid mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	200.00
$CuSO_4 \cdot 5H_2O$	0.0001
$Fe(NO_3)_3 \cdot 9H_2O$	0.0001
KCl	400.00
$MgSO_4$	400.00
$MgSO_4 \cdot 7H_2O$	200.00
$MnCl_2 \cdot 4H_2O$	0001
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00
$ZnSO_4 \cdot 7H_2O$	0.0002
OTHER COMPONENTS:	
D-Glucose	2000.00
Glutathione	0.05
Methvl Linoleate	0.03
Phenol Red	10.00
Sodium Pyruvate	25.00
AMINO ACIDS:	
L-Alanine	90.00
L-Arginine	50.00
$L\text{-Asparagine} \cdot H_2O$	20.00
L-Aspartic Acid	30.00
L-Cysteine	40.00
L-Cystine	20.00
L-Glutamic Acid	50.00
Glycine	50.00
L-Histidine	15.00
L-Isoleucine	50.00
L-Leucine	75.00
L-Lysine HCl'	87.50
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	30.00
L-Serine	10.00
L-Threonine	40.00
L-Trvptophan	10.00
L-Tyrosine	35.00
L-Valine	50.00

Component	041-02551 1X Liquid mg/L
VITAMINS:	
Ascorbic Acid	2.00
Biotin	0.50
D-Ca Pantothenate	1.00
Choline Chloride	1.50
Ergocalciferol	0.10
Folic Acid	1.00
i-Inositol	2.00
Menadione Sodium Bisulphate	0.01
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
α-Tocopherol Phosphate, Disodium	0.01
Thianine HCl	1.00
Vitamin A Acetate	0.10
Vitamin B_{12}	0.20

Appendix 3

Research communications :

O' Donovan. S. & Dalton, J. P. (September, 1989) Identification of a putative *Plasmodium berghei* (a rodent malaria parasite) reticulocyte receptor. Biochemical Society Meeting, Cork.

O' Donovan, S., McNally. J. & Dalton, J. P. (September, 1990) Identification of a putative erythrocyte receptor of *Plasmodium chabaudi*. Biochemical Society Meeting, Dublin.

O' Donovan, S., McNally, J. & Dalton, J. P. (September, 1991) Development of rodent malaria *in vitro* invasion asays. Irish Society of Immunology Meeting, Dublin.

O' Donovan, S., McNally, J. & Dalton, J. P. (November, 1991) *In vitro* erythrocyte invasion assays for the rodent malarias, *P. berghei* and *P. c. chabaudi*. Congressa Latino Americano De Parasitologia, Montevideo, Uraguay, S.A.

O' Donovan, S., McNally, J. & Dalton, J. P. (Feburary, 1992) Development of *in vitro* invasion assays for the rodent malarias, *P. berghei* and *P. c. chabaudi*. British Society of Parasitology, Malaria Meeting, London.

Appendix 4

Publications:

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

O' Donovan, S. M. & Dalton, J. P. (1992) *Plasmodium c. chabaudi*: improvements to an *in vitro* invasion assay. J. Eukaryotic Mirobiol. 40, 152 -154.

Dalton, J. P., McNally, J. & O' Donovan, S. M. 1993. *In vitro* assays for the study of erythrocyte invasion by malarial parasites. Parasitol. Today. 9, 109 - 111.

Curley, G. P., O' Donovan, S. M., McNally, J., Mullally, M., O' Hara, H., O' Callaghan, S.-A. & Dalton, J. P. (submitted) Aminopeptidases from *Plasmodium falciparum, Plasmodium chabaudi chabaudi* and *Plasmodium berghei.*

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 or candle jar at 37° C. For *P. vivax* invasion assays, the percentage of immature erythrocytes in the target cells is increased by processing cells over percoll or Nycodenz cushions[5-7]. The schizont : target cell ratio is generally 1 : 5 or 1 : 10 with the number of target cells typically 2–5 x 10^7. At these ratios, sufficient numbers of merozoites, that allow good statistical analysis of data, will invade. Alternatively, assays can be performed in Eppendorfs (cheaper microcentrifuge tubes may leach chemicals that are toxic to parasites) which are then gased (6% CO_2, 3% O_2, 91% N_2) and placed on a rotating wheel at 37° C. After allowing time for rupture, re-invasion and development of parasites to visible ring forms (usually six hours for *P. knowlesi* assays, overnight for *P. falciparum* assays, and six to eight hours for *P. vivax*), the erythrocytes are removed from the wells and washed once with foetal calf serum before preparing smears which are then Giemsa stained. Invasion of erythrocytes is estimated microscopically by calculating the percentage of erythrocytes containing one or more ring-form parasites. These percentages usually range from 5 to 12%. It is clear, therefore, considering the parasitaemia at which the assay is initiated, that many merozoites do not successfully re-invade. At which stage of invasion the difficulty for merozoites occurs is not known; unruptured cells are rarely seen.

The assays described above are useful for testing or searching for erythrocyte invasion-blocking antibodies. The mechanism by which an antibody blocks invasion can be either a specific inhibition of the activity of merozoite molecules, such as enzymes or molecules that interact with erythrocyte ligands, or by an agglutination of merozoites. These two means of blocking can be differentiated by preparing Fab fragments. Fab fragments of antibodies that block by agglutination will have no

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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[6].

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 × 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].

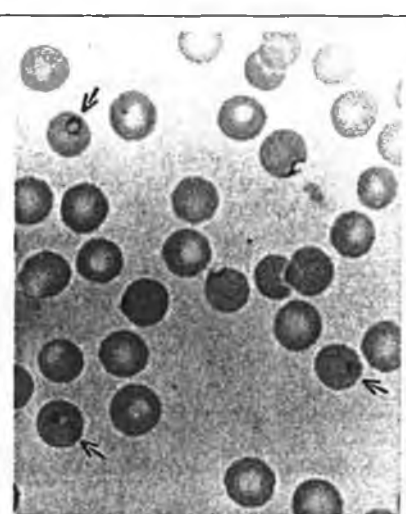

***Fig. 1.** Photograph of Giemsa-stained smears on a microscope slide, showing newly invaded ring-stage parasites (arrows) of* Plasmodium chabaudi chabaudi *in target (mouse) erythrocytes 19 h after initiation of the invasion assay,* in vitro.

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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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.

* Reprint requests to Dr J. P. Dalton.

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

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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 $2·5 \times 10^7$ 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.

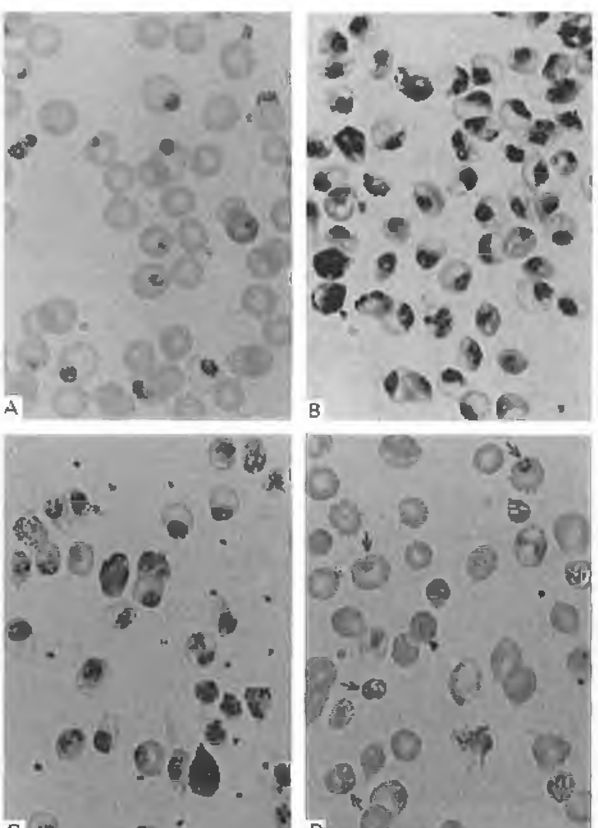


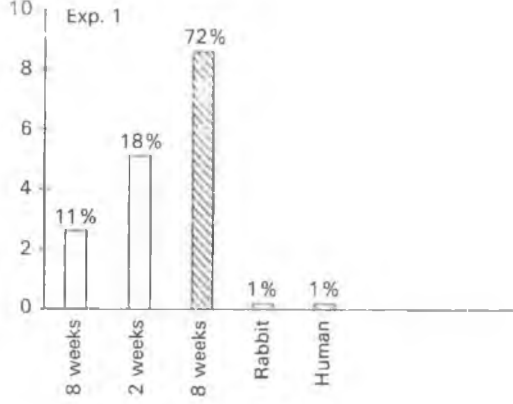
Fig. 1. Purification of *Plasmodium berghei* mature trophozoite-infected erythrocytes, *in vitro* culturing and reinvasion. Blood from an infected Balb/c mouse (A) was layered on a step-wise Percoll gradient as described in the Materials and Methods section. After centrifugation the erythrocytes infected 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.

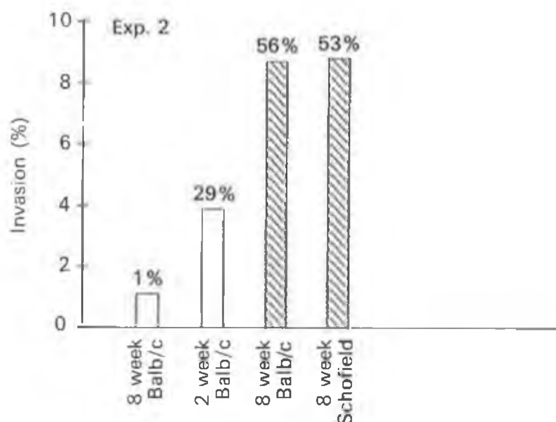
RESULTS

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





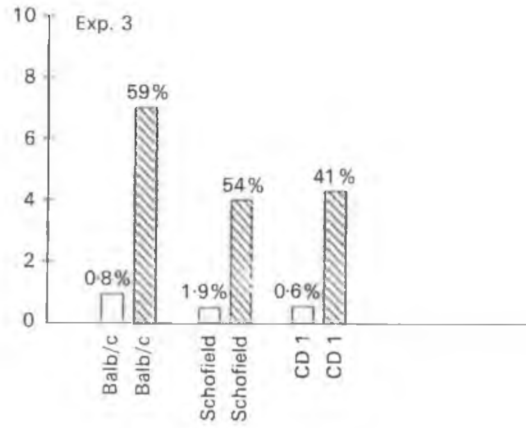


Fig. 2. Invasion of erythrocytes *in vitro* by *Plasmodium berghei*. Exp. 1, comparison of *P. berghei* invasion rates into erythrocytes obtained from 2-week-old mice, 8-week-old mice, phenylhydrazine-treated 8-week-old mice (all Balb/c), rabbit and human. Exp. 2, comparison of invasion rates into erythrocytes 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.

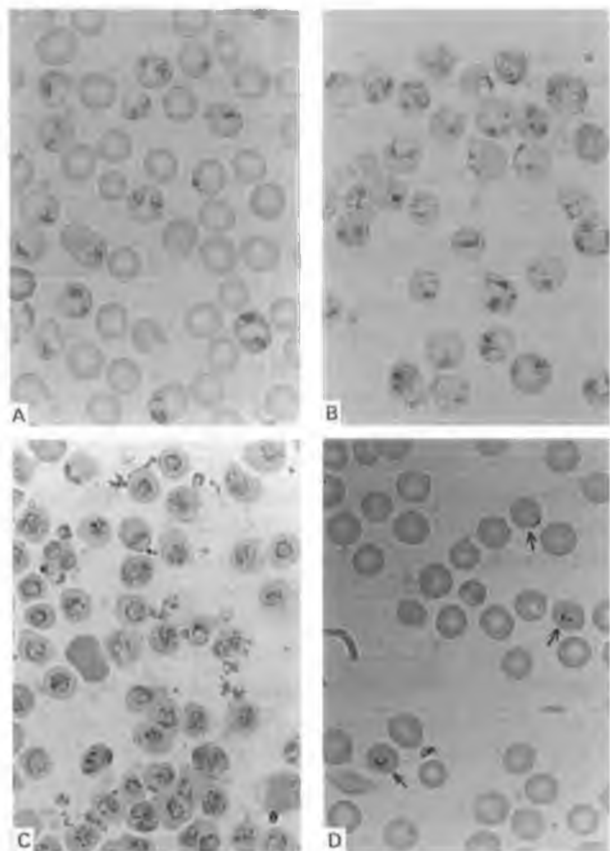
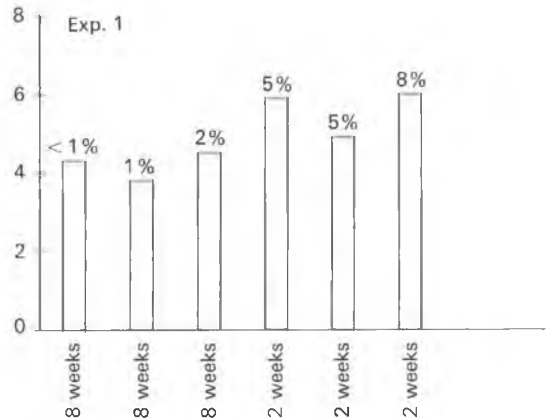
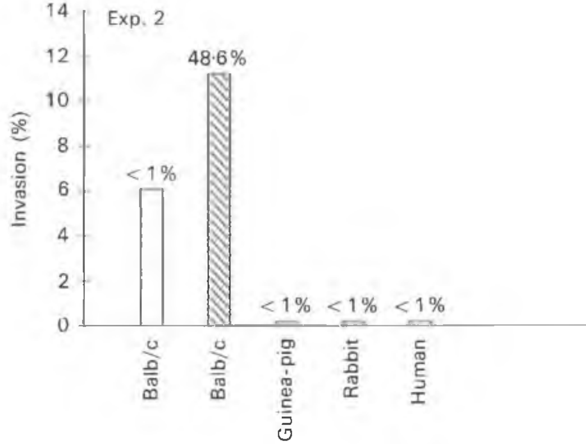


Fig. 3. Purification of *Plasmodium c. chabaudi*-infected erythrocytes, *in vitro* culturing and reinvasion. Blood obtained from infected CD-1 mice (A) was layered onto a 74 % Percoll gradient prepared as described the Materials and Methods section. After centrifugation the parasite-infected erythrocytes 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.





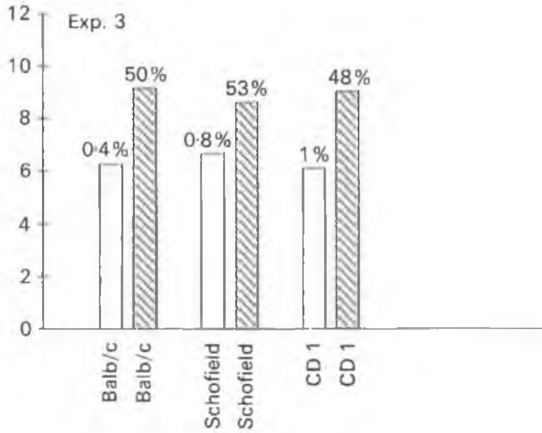


Fig. 4. Invasion of erythrocytes *in vitro* by *Plasmodium c. chabaudi*. Exp. 1, comparison of *P. c. chabaudi* invasion rates into erythrocytes obtained from 2-week-old and 8-week-old Balb/c mice. Exp 2, comparison of invasion rates into erythrocytes obtained from normal and phenylhydrazine-treated 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.

The authors would like to thank Caroline Wilson for maintaining the mice and for her technical assistance. We would also like to thank Dr K. N. Brown for the gift of the *P. c. chabaudi* AS strain. The help given by Dr L. H. Miller and Diana Hudson in initiating malaria research in Dublin is greatly appreciated. S.M.O'D. is supported by a grant kindly provided by the Health Research Board, Ireland. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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An Improved Medium for *Plasmodium chabaudi* In Vitro Erythrocyte Invasion Assays

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ABSTRACT. RPMI-1640 is routinely used as the basal medium for the in vitro maintenance of malaria parasites. In this study we tested several commercially available nutritional media in a *Plasmodium chabaudi chabaudi* erythrocyte invasion assay and showed that three media, BME Basal Medium—modified, Dulbecco's Modified Eagle Medium, and William's Medium E, improved the level of merozoite invasion when compared with RPMI-1640. These media improve the rate of maturation of newly invaded rings to young trophozoites. Radioisotope incorporation by trophozoites maintained in these three media was also improved when compared to trophozoites maintained in RPMI-1640. BME Basal Medium—modified, or a combination of three parts BME Basal Medium—modified with one part William's Medium E, supported higher levels of erythrocyte invasion by merozoites. We suggest that either of these media replace the currently used RPMI-1640 for in vitro studies on *P. c. chabaudi.*

Supplementary key words. Malaria, rodent.

RECENTLY we described a method for performing in vitro erythrocyte invasion assays using the rodent malaria *Plasmodium chabaudi chabaudi* [3]. In these assays trohozoite-infected erythrocytes were purified and maintained in vitro for 4 h to allow the parasites to mature to schizonts, before adding them to normal erythrocytes. Following a 19-h incubation, the cells were removed, smeared and stained. Successful merozoite invasions were then estimated by counting the number of ring or young trophozoite-infected erythrocytes. Since RPMI-1640 is the standard medium used in the in vitro culture of a number of species of malaria including *Plasmodium falciparum, Plasmodium knowlesi, Plasmodium berghei* and *P. c. chabaudi* [6, 1, 4, 2, respectively] we chose this medium as the basal medium for our assays. In this study, we report on work carried out to evaluate commercially available media for the in vitro culture of *P. c. chabaudi.* Our results show that BME Basal Medium—modified (BME) or a simple medium prepared by combining BME and William's Medium E (William's E) provides better support for growth of *P. c. chabaudi* and improves the level of merozoite invasion in our erythrocyte invasion assays.

MATERIALS AND METHODS

Parasites and animals. *Plasmodium chabaudi chabaudi* (AS strain) was maintained in CD-1 mice (Biological Laboratories, Ballina, Ireland) by passage, via intra-peritoneal injection of infected blood. Mice were kept in artificial daylight (24 h–12 h).

Preparation of target erythrocytes. Blood from Balb/c mice was obtained by cardiac puncture into heparinised syringes and was then washed with 10 ml of phosphate buffered saline (PBS) by centrifugation at 450 × *g* for 5 min. The pelleted cells were then resuspended in 0.5 ml PBS. Blood was obtained from New Zealand White rabbits in a similar fashion for control erythrocytes as these cells are refractory to *P. c. chabaudi* invasion [3]. Serum was obtained from adult Wistar rats. All animals were bred in Dublin City University.

***Plasmodium chabaudi chabaudi* erythrocyte invasion assay.** The invasion assay was carried out as detailed by McNally, O'Donovan and Dalton [3]. *Plasmodium chabaudi chabaudi* trophozoite-infected erythrocytes were obtained by bleeding mice with a parasitemia of 15–25% and purifying the parasites on a 74% percoll gradient. The infected cells, which formed a band on top of the gradient, were washed and cultured for 4 h at 37° C in a candle jar in RPMI-1640 medium containing 10% rat serum to achieve an oxygen tension of approximately 5%. Then 5×10^6 parasitised erythrocytes from this culture were added to 2×10^7 target erythrocytes in a final culture volume of 1 ml per well. After 19 h the cells were washed and the cells examined using Giemsa staining. The percentage of erythrocytes infected with ring or young trophozoite-stage parasites was determined using light microscopy.

Media preparation. The following media were obtained from Gibco Laboratories, Paisley, United Kingdom: BGJb Medium; BME Basal Medium—modified (BME); CMRL-1066 (CMRL); Dulbecco's Modified Eagle Medium (DMEM); McCoy's 5A Medium—modified (McCoy's); Modified Essential Medium (MEM); NCTC-135 (NCTC); RPMI-1640 and William's Medium E (William's E). Each medium was supplemented with 20 ml 1.0 M Hepes buffer, 5 mg hypoxanthine, 2 g glucose, 2 mg gentamicin, 10 ml 200 mM L-glutamine and 42 ml of 5% sodium bicarbonate solution per litre. Hepes, bicarbonate and L-glutamine were not added if they were already present in the medium. One millilitre rat serum was then added per 9 ml of basal medium to give the complete medium.

[^{3}H]Isoleucine and [^{35}S]methionine incorporation by in vitro cultured parasites. Ten microcuries of [^{3}H]isoleucine or 2.5 μCi [^{35}S]methionine were added to tissue culture wells containing 1

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Table 1. Rate of maturation of parasites during invasion assay.

Medium	% Invasion[a]	% Ring-stage	% Trophozoites
RPMI-1640	4.2 ± 0.27	92.6 ± 2.0	7.4 ± 2.0
BGJb	1.9 ± 0.70	89.9 ± 1.2	10.1 ± 1.2
CMRL	1.5 ± 0.50	95.4 ± 3.5	4.6 ± 3.5
NCTC	1.3 ± 0.50	96.4 ± 2.6	3.6 ± 2.6
McCoy's	4.0 ± 0.40	84.1 ± 6.9	15.9 ± 6.9
MEM	3.5 ± 0.20	92.3 ± 1.3	7.7 ± 1.3
BME	6.0 ± 1.50	81.2 ± 10.2	18.8 ± 10.2
DMEM	6.0 ± 0.35	79.8 ± 3.5	20.0 ± 3.5
William's E	5.1 ± 0.70	54.3 ± 9.8	45.7 ± 9.8

[a] Mean of three experiments ± standard deviation.

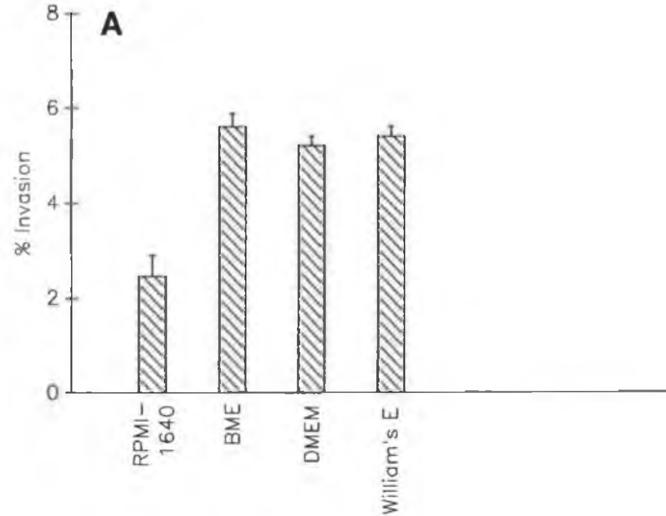
× 10^7 trophozoite-infected cells in either RPMI-1640, BME, DMEM or William's E medium. After 1, 2, and 4 h of culture, under the same conditions as before, the cells were removed from duplicate wells, washed three times in PBS and lysed in 1 ml PBS containing 1% Triton-X. Proteins were precipitated by the addition of trichloroacetic acid (TCA) to a final concentration of 10%, followed by centrifugation at 10,000 *g*. The pellets were washed with cold acetone, re-centrifuged and resuspended in 1 ml 0.5% Triton-X, sonicated and aliquots (50 μl) mixed with 4 ml Ecocint (Manville, NJ) for measurement of incorporated radioactivity using liquid scintillation counting.

RESULTS AND DISCUSSION

RPMI-1640 is a nutritionally complex medium that is routinely used as the basal medium for the culture of many malaria species including *P. c. chabaudi* [1, 2, 4, 6]. For this reason, when developing erythrocyte invasion assays for *P. c. chabaudi*, we chose RPMI-1640 as our basal medium [3]. Since no report was available indicating this medium as the most suitable for maintenance of this rodent malaria, we tested eight commercially available nutritionally complex media as basal media and compared them to RPMI-1640 in our erythrocyte invasion assays.

In the first set of experiments purified trophozoite-infected erythrocytes were initially incubated in RPMI-1640 to allow parasite development. These schizont-infected cells were added to microtitre wells containing target erythrocytes suspended in the various test media, and incubated for 19 h before the cells were removed and the number of newly invaded cells estimated. It was clear from the levels of merozoite invasion that there is considerable variation in media suitability for the in vitro culturing of *P. c. chabaudi*, and based on this we divided the media into three groups (Table 1). Merozoite levels in Group 1 media (BGJb, CMRL and NCTC) were 50% below those observed with RPMI-1640. In Group 2 media (McCoy's and MEM), invasion levels were similar to RPMI-1640. The media in Group 3 (BME, DMEM and William's E) consistently and significantly improved the invasion of merozoites as compared to RPMI-1640 ($P \leq 0.05$). The newly invaded parasites also showed an improved rate of maturation in the Group 3 media as indicated by the greater number of ring-stage parasites that had matured to trophozoites (Table 1). This improvement was particularly evident in the William's E medium, in which 45.7% of newly invaded parasites had developed to trophozoites, whereas in RPMI-1640 only 7.4% had reached this stage ($P \leq 0.05$).

In the second set of experiments, trophozoite-infected erythrocytes were allowed to mature to schizonts in RPMI-1640, BME, DMEM and William's E before incubation with target erythrocytes in their respective medium (Fig. 1A). Again, greater merozoite invasion levels were observed with BME, DMEM and William's E compared to RPMI-1640 ($P \leq 0.05$). It appeared from microscopic examination of the slides that trophozoite development to schizonts was more rapid within the 4-h primary incubation period in BME, DMEM and William's E, and the parasites themselves also appeared healthier. To determine if this was the case we examined the rate of radiolabelled amino acid uptake as a growth indicator over the 4-h primary incubation period. The incorporation of [^{3}H]isoleucine and [^{35}S]methionine by developing trophozoites was greater when the parasites were maintained in BME, DMEM and William's E compared to RPMI-1640; BME resulted in the best amino acid incorporation followed by William's E and DMEM (Fig. 2).



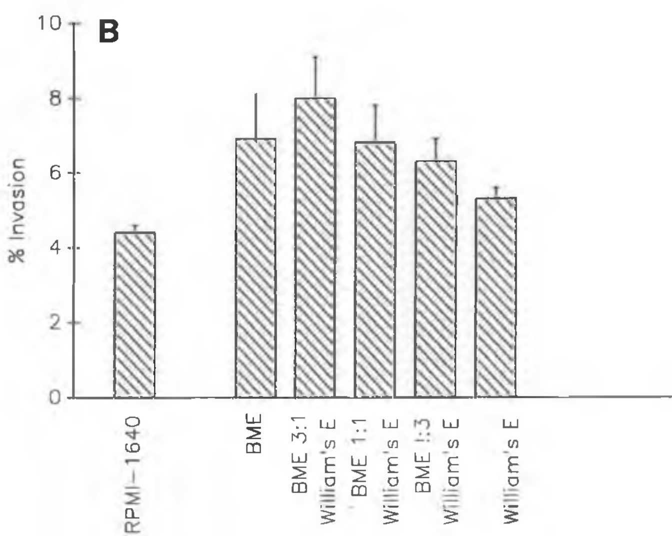
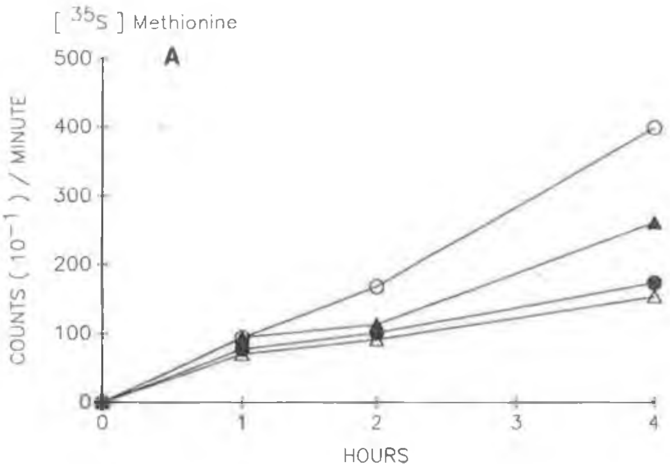


Fig. 1. Invasion of erythrocytes cultures in selected media. A. Comparison of invasion where the parasites were cultured for 4 h in their respective medium before being assayed. B. Comparison of BME and William's E medium ratios and their effects on the level of invasion achieved.

Since BME is the most suitable medium for *P. c. chabaudi* trophozoite to schizont maturation and William's E is superior for ring to trophozoite development, combinations of the two media were tested in our invasion assays. In these assays the two media complemented each other, with a higher level of



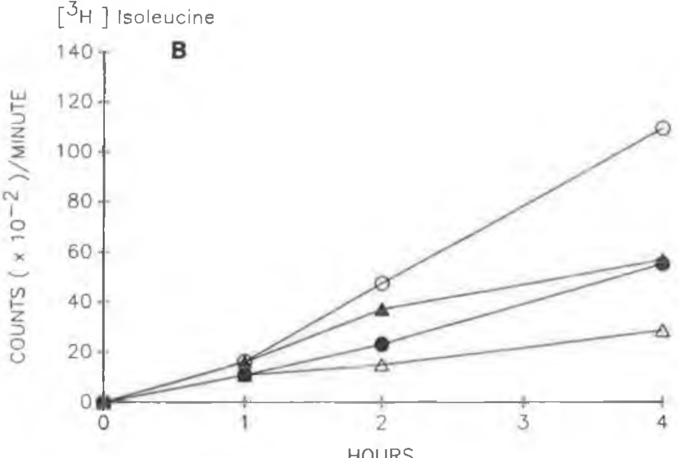


Fig. 2. Incorporation of radioisotope by *P. c. chabaudi* parasites. **A.** Incorporation of [^{35}S]methionine by *P. c. chabaudi* parasites cultured in a selected range of media. **B.** Incorporation of [^{3}H]isoleucine by parasites cultured in selected media.–△–, RPMI-1640; –○–, BME; –●–, DMEM; –▲–, William's E.

invasion being consistently observed with a mixture of three parts BME and one part William's E compared to RPMI-1640 or to William's E alone ($P \leq 0.05$); however, merozoite invasion with this combination of media was not significantly improved when compared to BME alone (Fig. 1B). In these experiments the percentages of newly invaded parasites maturing to young trophozoites were 1.88 ± 0.4 in RPMI-1640; 15.1 ± 0.7 in BME; 23.8 ± 6.5 in William's E; and 15.9 ± 5.8 in the combination of three parts BME and one part William's E. Therefore the combination of three parts BME and one part William's E medium did not significantly improve the maturation of the newly invaded parasites compared to BME alone.

In conclusion, we recommend that BME or the 3:1 combination of BME and William's E medium replace the traditionally used RPMI-1640 medium for in vitro studies using *P. c. chabaudi.* Although in the study of Trager [5], RPMI-1640 proved to be the most suitable of several media for the in vitro maintenance of *P. falciparum,* neither BME nor William's E was included in the test. Therefore, these media were worthy of examination, both for the cultivation of *P. falciparum* and the long-term culture of *P. c. chabaudi.*

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