Antigenic variation in *Plasmodium chabaudi chabaudi* AS

by

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For Andrew,
without whom this thesis may never have been completed.
For Ewan, Seona and again Andrew,
without whom this thesis would have been completed three years ago.
I would not be without any of them.

CONTENTS

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DECLARATION

Other than the mosquito transmission of *Plasmodium chabaudi*, which was performed by Professor David Walliker (University of Edinburgh), I declare the work described in this thesis to be my own.

Some of the work presented herein has been published previously elsewhere:

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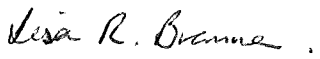

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ABBREVIATIONS

Ab Antibody; ADCC Antibody-dependent cellular cytotoxicity; ADCI Ab-dependent cellular inhibition; Ag Antigen; B cell B lymphocyte; BCIP/NPT Bromochloroindolyl phosphate/nitro blue tetrazolium; BP Blood passage; BSA Bovine serum albumin; °C Degrees celsius; CHEF Contour-clamped homogeneous electric fields; CMI Cell-mediated immunity; CO_2 Carbon dioxide; CSP Circumsporozoite protein; CTL Cytotoxic T lymphocyte; d Day(s); DMSO Dimethyl sulfoxide; DNA Deoxyribonucleic acid; *E. coli Escherichia coli*; EDTA Ethylenediamine tetraacetic acid; FCS Foetal calf serum; FITC Fluorescein isothiocyanate; g Acceleration in the earth's gravitational field; g Gramme(s); G Gauge; GUP Glasgow University Protozoology (prefix used to describe numbered batches of stabilate); H-2 Mouse major histocompatability complex; HAT Hypoxanthine, aminopterin, thymidine; Hb Haemoglobin; HEPES *N*-2-hydroxyethylpiperazine-*N'*-2 ethane sulfonic acid; HLA Human histocompatability leucocyte antigen; H_2O_2 Hydrogen peroxide; hr Hour(s); HRP Histidine-rich protein; HT Hypoxanthine, thymidine; HUVEC Human umbilical vein endothelial cells; ^{125}I Iodine 125; ICAM-1 Intercellular adhesion molecule-1; IFA Indirect fluorescent antibody; IFAT Indirect fluorescent antibody test; IFN Interferon; Ig Immunoglobulin; IGSS Immunogold silver staining; IL Interleukin; i.p. Intraperitoneally; ITS Insulin, transferrin, selenium; i.u. International unit(s); i.v. Intravenously; KAHRP (HRP1) Knob-associated histadine-rich protein; Kb Kilobase; kD Kilodalton(s); l Litre(s); LIFAT Indirect fluorescent antibody test performed on live parasites; M Molar; mAb Monoclonal antibody; Mb Megabase; MHC Major histocompatability complex; mg Milligramme(s); µg Microgramme(s); min Minute(s); ml Millilitre(s); ml Microlitre(s); mm Millimetre(s); MSP Merozoite surface protein; MT Mosquito transmission; MTRC Mosquito-transmitted recrudescent clone; MW Molecular weight; N_2 Nitrogen; NANP Asparagine-alanine-asparagine-proline amino acid repeat; NK cell Natural killer cell; ng Nanogramme(s); nm Nanometre(s); NL Normal light; NMS Normal mouse serum; NO Nitric oxide; NOS nitric oxide synthase; nRBC Normal/uninfected red blood cell; O_2 Oxygen; O_2^- Superoxide anion; OH· Hydroxyl radical; o/n Overnight; OPI Oxaloacetate, pyruvate, insulin; PBS Phosphate buffered saline; PEG Polyethylene glycol; PfEMP *P. falciparum* erythrocyte membrane protein; PFGE Pulsed field gel electrophoresis; p.i. Post infection; PMN Phagocytic mononuclear cell; PMSF phenylmethylsulfonyl fluoride; PWC Peritoneal wash cell; pRBC Parasitised/infected red blood cell; RBC Red blood cell; RC Recrudescent clone; RESA (Pf155) Ring-infected erythrocyte surface antigen; RL Reverse light; RNI Reactive nitrogen intermediate; ROI Reactive oxygen intermediate; RT Room temperature; SCID severe combined immune deficiency; s Second(s); S.D. Standard deviation; SDS-PAGE Sodium dodecyl

sulphate polyacrylamide gel electrophoresis; SICA Schizont-infected cell agglutination; S/N Supernatant; TBE Tris-borate/EDTA electrophoresis buffer; T cell T lymphocyte; Th T helper lymphocyte; TNF Tumour necrosis factor; TNS Tumour necrosis serum; TSP Thrombospondin; UV Ultra violet; VAT Variant antigen type; VCAM-1 Vascular cell adhesion molecule-1; v/v Volume per volume; WEP Wellcome Experimental Parasitology (prefix used to describe numbered batches of stabilate); WLEP Wellcome Laboratories for Experimental Parasitology; WHO World Health Organisation; w/v Weight per volume; > Greater than; < Less than; -ve Negative; +ve Positive; % Percentage point(s).

SUMMARY

Plasmodium chabaudi has been shown to undergo antigenic variation during the course of infection in mice. The importance of this model is the similarity and applicability of its features to infection of humans with *P. falciparum*. This thesis presents work performed using *P. chabaudi* to study various aspects of antigenic variation in asexual erythrocytic malaria parasites.

The course of infection of *P. chabaudi* in NIH mice shows an initial acute parasitaemia which clears to subpatency. This is usually followed, after a period of days, by a second, and occasionally a third, recrudescent parasitaemia of lesser magnitude and duration. A cloned parent parasite population and cloned parasite populations derived from a recrudescence of the parent were tested in an indirect fluorescent antibody test on live, schizont-infected RBC (live IFAT) using a panel of hyperimmune sera raised against these populations and against one of the recrudescent clones after mosquito transmission. This test can detect antigens on the surface of parasitised RBC. The results of this analysis indicated that all the recrudescent clones were antigenically different from the parent and some were different from each other. In total, including the parent, six variant antigen types (VATs) were identified. Some of these also appeared to vary in immunogenicity.

The effects of mosquito transmission on expression of variant antibodies was also examined using the panel of hyperimmune sera in the live IFAT. Mosquito transmission of two antigenically distinct recrudescent clone populations resulted in a change in antigenicity of both types to an apparently similar VAT, which had the same apparent identity as that of the original, post mosquito transmission but pre-cloning, parent population.

Comparison of the courses of infection of the parent and four of the recrudescent clone populations showed some differences in terms of the levels of peak primary parasitaemia, the preference for invasion of reticulocytes early in infection, and the timing of recrudescences. Analysis by live IFAT of recrudescences from these infections indicated further antigenic variation of these variant populations.

The rates of switching on of three minor VATs was measured during the exponential growth phase of the ascending primary parasitaemia, when immune-mediated killing is essentially absent. This showed that switching rates for individual VATs *in vivo* could be high, with rates varying depending on the VAT being switched on. By summation of rates, an overall minimum estimate of antigenic variation of 1.6% per asexual parasite generation was obtained.

The parent parasite population and four variant recrudescent clone populations were all found to sequester *in vivo*. Cytoadherence *in vitro* was also examined, by binding to 3T3 and B10D2 mouse fibroblast-like cell lines. Although overall binding

levels were low, specific binding of parasitised RBC was observed for all of the parasite populations tested, with the specificity of binding greater for some populations than for others.

Molecular karyotyping by pulsed field gel electrophoresis showed all the antigenically variant populations to have the same number of chromosomes and to have individual chromosomes of an identical size. This therefore demonstrated that they all of the VATs examined were originally derived from the same parasite isolate, and confirmed that the observed phenomenon referred to as antigenic variation is true phenotypic variation.

Production of monoclonal antibodies against parasitised RBC surface variant antigens was problematic, but did yield one monoclonal antibody of IgG_1 isotype. This monoclonal antibody reacted specifically by live IFAT with the VAT against which it was raised. It did not, however, detect any variant-specific bands by Western blotting.

The value of *P. chabaudi* in mice as a model system in which to study antigenic variation is confirmed herein by its application to a variety of studies involving the use of antigenically variant cloned parasite populations. The complementary aspects of antigenic variation examined include the dynamics of infection, sequestration *in vivo*, cytoadherence *in vitro*, modulation of antigenic phenotype by mosquito transmission, and the rate of switching of antigenic phenotype. The work presented in this thesis thus provides novel information on, and thereby extends our knowledge of, antigenic variation in malaria parasites.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Historical perspective and nature of the disease

The causative agent of malaria was discovered little more than a century ago, but references to the disease can be found in Egyptian hieroglyphics and in the Hindi vedic literature. The disease has long been associated with marshes, the breeding ground for the mosquito vector, and certain names for the disease reflect this, such as marsh fever or paludism (from the French for marsh). The term malaria is taken from the Italian 'mal aria' meaning 'bad air', and reflects a traditional view that the noxious gases emanating from marshlands contained the agents responsible for the disease.

Malaria transmission once occurred throughout most of the inhabited world, affecting most civilisations, causing incalculable morbidity and mortality. The disease has repeatedly affected the course of world history, especially during times of war (documented for Europe by Bruce-Chwatt & de Zulueta 1980). In World War II, the U.S. Army suffered more losses from malaria on the Pacific front than from battle injuries. This was to be repeated in Vietnam.

Large-scale spraying with the insecticides dichloro diphenyl trichloroethane (DDT) and hexachlorocyclohexane during the 1950s contributed to an estimated 400 million people no longer exposed to malaria, and eradication of the disease from most temperate regions (Nogeur *et al.* 1978). Despite this early success, malaria is still widespread throughout South and Central America, Africa and much of Asia. The rapid spread of resistance amongst both mosquito vectors and parasites to control measures has led to a resurgence of malaria, with the incidence of the disease increasing in many countries. Today, malaria is still the most important infectious disease in the world, endemic in 102 countries, with over half the population of the world at risk (Tropical Diseases Report 1995). Estimates suggest that over 400 million cases of malaria occur each year, with ≥ 2.5 million people dying from the disease, the majority being children < 5 years of age (Sturchler 1989).

In 1847, the first step towards identifying the causative agent of malaria was made by Heinrich Meckel, who described black pigment (now known to be haemozoin, a waste product of malarial metabolism) in the blood, spleen and liver of people who had died of malaria (Harrison 1978). In 1880, Laveran observed malaria parasites in the blood of infected individuals, describing crescent-shaped bodies now known to be gametocytes of *P. falciparum* . This was subsequently confirmed by Marchiafava & Celli (1883). Mosquito transmission (MT) of bird malaria was first demonstrated by Ross (Manson 1898), and confirmed for human malaria by Grassi in the same year (reviewed by Harrison 1978).

Malaria parasites are protozoa of the genus *Plasmodium,* which are classified in the phylum Apicomplexa. More than a hundred species of *Plasmodium* have been described, infecting reptiles, birds and mammals. Four species are commonly infective to humans: *P. falciparum, P. vivax, P. malariae* and *P. ovale.* Of these, *P. falciparum*

is the major cause of mortality.

The clinical disease associated with malaria infection covers a broad range of symptoms and pathology. Paroxysmal fever is the classical symptom, but other symptoms such as headaches, drowsiness, anaemia, hypoglycaemia, splenomegaly and hepatomegaly may occur (Ellis 1989; Molyneux 1989). The majority of severe disease is due to acute infections with *P. falciparum*, the most important manifestation being cerebral malaria, but other defining criteria include severe anaemia, renal failure, pulmonary oedema, circulatory collapse, spontaneous bleeding, repeated generalised convulsions, acidosis and haemoglobinuria (WHO 1990).

1.2 Life cycle

Figure 1.1 illustrates the life cycle of a mammalian malaria parasite.

1.2.1 Exoerythrocytic cycle

Malaria parasites enter the vertebrate host via the bite of an infected female mosquito. Sporozoites are injected via the proboscis from the salivary glands as the mosquito takes a blood meal. The inoculum is small, with one study showing an average of 15 sporozoites (Rosenberg *et al.* 1990). Other studies showed that > 98% of naturally infected mosquitoes transmitted < 25 sporozoites (Beier *et al.* 1991a) and > 80% of experimentally infective mosquitoes transmitted from 1-10 sporozoites (Beier *et al.* 1991b). The sporozoites circulate in the bloodstream for 15-60 min (Fairley 1947; Sinden & Smith 1982) before either invading liver hepatocytes directly (Shortt 1948; Shin *et al.* 1982) or indirectly after uptake by Kupffer cells (Smith *et al.* 1981). Within the hepatocyte, the parasites develop into exoerythrocytic schizonts (Garnham *et al.* 1966) by asexual multiplication. Mammalian malaria parasites are thought to undergo only one cycle of exoerythrocytic multiplication, this taking between 5.5-15 d for human malarias, depending on the species. With *P. falciparum* and *P. malariae* infections, this tissue schizogony follows sporozoite invasion directly, whereas for *P. vivax* and *P. ovale* infections, a proportion of the sporozoites first develop into latent hypnozoite forms which are responsible for producing relapses (Krotoski *et al.* 1982 a & b). The mature schizont contains around 30000 merozoites in the case of *P. falciparum*. These are released into the bloodstream upon rupture of the schizont and the host hepatocyte and invade RBC, where they commence a cycle of asexual multiplication responsible for the characteristic pathology of malaria.

1.2.2 Erythrocytic cycle

Invasion of RBC by merozoites is a complex process, commencing by attachment and orientation of the merozoite so that the apical complex is in contact with the RBC membrane, probably via a species-specific receptor. For *P. vivax* and *P. falciparum*,

these receptors are associated with the Duffy blood group Ags (Miller *et al.* 1975b) and glycophorin (Miller *et al.* 1977; Perkins 1984), respectively. A junction is formed between the RBC membrane and the merozoite plasma membrane (Aikawa *et al.* 1978). The parasite releases material from the rhoptries and micronemes, causing invagination of the RBC membrane, and the junction moves over the parasite which enters the invagination until it lies completely enclosed within the parasitophorous vacuole (Dvorak *et al.* 1975; Aikawa *et al.* 1978). During this entry process (reviewed by Mitchell & Bannister 1988; Bannister & Dluzewski 1990), the merozoite surface coat is sloughed off (Bannister *et al.* 1975; Miller *et al.* 1975a).

Upon entering a RBC, the parasite develops a vacuole and becomes a ring stage. It is called this due to the signet ring-like appearance upon examination of Giemsa's stained bloodsmears. This ring stage grows, feeding mostly on haemoglobin in the host cell, and producing malarious pigment, the vacuole disappears, and the ring stage becomes a trophozoite. Asexual multiplication (schizogony) ensues by repeated division of the parasite nucleus, the parasite segments to form a schizont containing merozoites, the number of which varies depending on the species, which regain their surface coat (Bannister *et al.* 1977). The erythrocytic schizont ruptures to release merozoites which can then invade further RBC. The timing of the erythrocytic cycle depends on the species of malaria parasite. This is 24 h for *P. chabaudi*, 48 h for *P. falciparum, P. vivax* and *P. ovale*, and 72 h for *P. malariae.* It is relatively synchronous, and it is the synchronous release of merozoites from the RBC, with destruction of the RBC membrane, which is responsible for the clinical manifestations of periodic fever and chills characteristic of malaria.

Following invasion of RBC, some merozoites develop into the sexual stages, gametocytes, within the RBC. Gametocytogenesis (reviewed by Mons 1985; Alano & Carter 1990) is poorly understood, but both micro and macrogametocytes can be found in an infection initiated with a single parasite (Carter & Walliker 1975). It is these gametocytes which are infective to the mosquito vector when ingested during a blood meal.

1.2.3 Development in the mosquito vector

When mature gametocytes are taken into the midgut of the mosquito vector, the RBC membrane is lost and gametogenesis occurs, to form micro and macrogametes. The microgametocyte divides mitotically 3x (Sinden 1981) and exflagellates, releasing 8 flagellated microgametes. These fertilise the macrogametes, forming diploid zygotes. These transform into motile ookinetes which cross the gut wall within 24 h, undergoing meiosis to form haploid oocysts, situated between the the gut epithelium and the basal lamina of the mosquito mid gut wall (Sinden & Strong 1978). The oocyst divides many time over a period of 10-16 d, depending on external environmental conditions, to form

sporozoites, which are released into the haemocoel when the oocyst ruptures. The number of sporozoites produced is estimated to vary from 1000-10000 (Garnham 1966). Pringle (1965) counted 9555 sporozoites in an individual *P. falciparum* oocyst, and Rosenberg & Rungsiwongse (1991) counted a mean of 3688 sporozoites per *P. vivax* oocyst, a mean of 3386 sporozoites per *P. falciparum* oocyst, and 7521 sporozoites in an individual *P. cynomolgi* oocyst. These motile sporozoites migrate and penetrate into the lumen of the mosquito salivary glands, becoming infective to the vertebrate host (Vanderberg 1975).

1.2.4 Longevity of infections in humans

Infections in humans can be of considerable longevity (Phillips 1983). The lifespan of *P. malariae* can be decades, the parasites seeming able to evade complete elimination by the host's immune system. *P. vivax* is estimated to have a lifespan of 3-4 years. *P. falciparum* is considered to have a lifespan of about 12 months, but the duration of infections tends to become shorter as the host's immunity increases. The longevity of infection is therefore a balance between the protective responses of the host and the ability of the parasites to evade such responses.

1.3 Laboratory models

The development of a method for *in vitro* culture of *P. falciparum* (Trager & Jensen 1976) has provided material for the biochemical and molecular analysis of this parasite, and certain species of *Aotus* and *Saimiri* monkeys have been examined as hosts for *P. falciparum* and *P. vivax* for vaccine and other studies (Collins *et al.* 1983; Gysin & Fandeur 1983). A *Plasmodium* parasite able to infect the common marmoset, *Callithrix jacchus* may represent the successful adaptation of a human malaria parasite to a commonly available primate. This was initially thought to be *P. vivax* (Mitchell *et al.* 1988), but is now believed to be *P. malariae* (Mons & Sinden 1990). However, human malarias in monkeys can give unpredictable results, and for practical and ethical reasons the use of such models can be hard to justify. Various species of rodent, avian and non-human primate plasmodia are therefore used for laboratory study of the biology of malaria parasites.

Of the non-human primate malarias, *P. knowlesi* and *P. cynomolgi* have been widely used in the rhesus monkey *Macaca mulatta*. *P. brasilianum* and *P. simium* have also been used in the New World monkeys *Aotus trivirgatus* and *Saimiri sciureus*, respectively (reviewed by WHO 1987). The laboratory use of primates is, however, severely restricted.

Much early laboratory work was performed using avian species of malaria parasites. *P. cathemerium* and *P. relictum* were used in canaries, and *P. gallinaceum* and *P. lophurae* in chickens and ducks. The discovery of malaria parasites in rodents

by Vincke & Lips (1948) in Katanga provided a major breakthrough in the laboratory study of malaria. *P. berghei* was transmitted successfully from naturally infected tree rats (*Thamnomys surdaster*) to laboratory mice and rats by blood inoculation. Several other rodent species have subsequently been isolated, and the availability of all these rodent species has enabled genetic, chemotherapy and immunity studies to be performed. Four rodent species are recognised: *P. berghei, P. chabaudi, P. vinckei* and *P. yoelii.* All except *P. berghei* contain two or more subspecies. Precise identification of particular parasite strains has been achieved by the behaviour and structure of blood stage parasites, serology, isoenzyme types and patterns of cross protection (Carter & Diggs 1977). These studies have provided defined rodent models, but care must be taken in extrapolating results from these models to human malarias.

1.4 Host resistance to malaria infection

The dynamic interactions of the host-parasite relationship plays a major part in the eventual outcome of infection. Host resistance to the parasite is a major element in this interaction. The ability of the host to control malaria infection may take two forms, innate and acquired resistance. Innate resistance is expressed regardless of previous exposure, and has no immunological specificity, but can be parasite-specific. Acquired resistance requires previous exposure and is immunological in nature. Between these two is non-specific resistance, which is immunologically mediated, but requiring exposure to an organism or substance unrelated to malaria parasites which stimulates the host to kill parasites.

1.4.1 Innate resistance

Certain innate characteristics of the host can either protect completely or lessen the severity of malaria in individuals. In populations exposed to high rates of malaria infection, genetic alterations resulting in such characteristics would increase an individual's chance of survival and reproduction, and would therefore spread through a population (Haldane 1949). A number of conditions have been associated with protection from malaria, mostly associated with host RBC and affecting the asexual erythrocytic stages of the parasite.

There are certain RBC phenotypes which affect the ability of parasites to invade RBC. The Duffy Ag has been shown to be necessary for invasion of human RBC by *P. knowlesi in vitro* (Miller *et al.* 1975 a & b) and is involved in RBC invasion by *P. vivax* , as individuals who are -ve for the Duffy blood group Ag are resistant to infection with *P. vivax* (Miller *et al.* 1976, 1977; Spencer *et al.* 1978). This explains the long-standing observation of an association between the high frequency of the Duffy -ve genotype and resistance to *P. vivax* in Africa (Boyd & Stratman-Thomas 1933; Bray 1958). RBC lacking glycophorin A show reduced invasion by *P. falciparum*

merozoites (reviewed by Pasvol & Jungery 1983), though very low numbers of individuals carrying this phenotype have been described worldwide. It is therefore unlikely that there is a selective advantage of this trait for malaria resistance. RBC deficient for glycophorin B also show reduced invasion by *P. falciparum*, and individuals carrying this phenotype reach frequencies in malarious areas characteristic of a balanced polymorphism. Therefore, there may be a selective advantage of this trait for malaria. Ovalocytosis, a morphological RBC variant phenotype, occurs in up to 20% of Melanesians in malarious areas of Papua New Guinea. Such individuals have lower parasitaemias than normal when infected with *P. falciparum, P. vivax,* and *P. malariae* (Serjeantson *et al.* 1977). These RBC are resistant to invasion by parasites due to an altered cytoskeletal structure (Kidson *et al.* 1981). A recent study has compared the prevalence of the deletion in the band 3 (AE1) gene that causes ovalocytosis in populations with different clinical status of malaria in Papua New Guinea (Genton *et al.* 1995). There was a clear decrease in prevalence of band 3 deletion with increasing disease severity, with no heterozygous individuals among the cerebral malaria cases.

RBC age may also affect their susceptibility to invasion by malaria parasites (reviewed by Bray & Garnham 1982). *P. vivax* and *P. ovale* predominantly invade reticulocytes or slightly older normocytes. *P. falciparum* seems to show a preference for metabolically young RBC (Phillips 1983). Preferences for RBC of different ages are also observed in rodent malarias (Cox 1988).

Other RBC abnormalities may affect the intraerythrocytic development of malaria parasites (reviewed by Nagel 1990), thus resulting in less severe disease. Sickle cell anaemia has long been associated with resistance to falciparum malaria in areas of hyperendemicity (Allison 1954). Sickle haemoglobin results from a single amino acid substitution, valine for glutamic acid at position 6 in the β-globin chain. A similar incidence of infection is observed in individuals with the sickle cell trait, both homozygous (HbSS) and heterozygous (HbAS), as in individuals with normal haemoglobin (HbAA), but less severe disease tends to occur in those with sickle cell trait (Gilles *et al.* 1967). One mechanism by which HbS is thought to protect is through the accelerated destruction of pRBC, as a rapid sickling of infected RBC is observed at low O_2 tensions (Friedman 1979). Parasites are also unable to develop normally in sickled cells at low O_2 tensions (Friedman 1978), possibly due to low intracellular potassium levels (Friedman *et al.* 1979a), and invasion of HbS-containing cells at low O_2 is inhibited (Pasvol *et al.* 1978). Thus, both invasion and development are inhibited under low O_2 conditions to which pRBC are exposed, particularly in the spleen (Friedman & Trager 1981) and also during deep vascular sequestration.

Other haemoglobinopathies which are associated with malaria include HbC and HbE, and β-thalassaemia, the frequencies of which are increased in areas of malaria

endemicity and which protect against severe, non-severe and mild infections (Siniscalo *et al.* 1966; Bodmer & Cavalli-Sforza 1976; Flatz 1967). It has long been assumed that the high gene frequency of α-thalassaemia, the most common of known human genetic disorders (affecting up to 80% of some malaria-endemic populations), likewise reflects selection by, and protection from, malaria; indeed a detailed study in Melanesia (Flint *et al.* 1986) which showed α^+-thalassaemia gene frequencies of 68% and 10% in areas of intense transmission and no transmission, respectively, corroborates this view. A recent study in Vanuatu, however, surprisingly found an increased incidence of malaria in α^+-thalassaemic children, the effect being most marked for those < 4 years of age and for *P. vivax* (Williams *et al.* 1996). Paradoxically, this has been interpreted as evidence for a protective effect of α-thalassaemia against *P. falciparum*, early infection by the non-lethal *P. vivax* acting as a natural vaccine through induction of limited cross-species protection to prevent or attenuate subsequent severe *P. falciparum* infections.

Glucose-6-phosphate dehydrogenase deficiency is another genetically-determined RBC abnormality associated with protection against malaria, the distribution of which occurs frequently in malarious areas of Africa (Allison 1960; Luzzatto 1979). The mechanisms of protection may be via reduced parasite growth (Pasvol *et al.* 1977; Friedman *et al.* 1979b; Nagel *et al.* 1981; Roth *et al.* 1983), or an increased susceptibility to mononuclear phagocytes and oxidative damage (reviewed by Yuthavong *et al.* 1990).

The genetic background of the host and environmental factors may also affect the susceptibility to malaria and the severity of disease. Inbred strains of mice may differ in their susceptibility to malaria infections (reviewed by Stevenson 1990). Genetic factors have been implicated in the pathogenesis of human cerebral malaria and hyperreactive malarial splenomegaly, and there is evidence of genetic control of immune responses to synthetic *P. falciparum* sporozoite vaccines (reviewed by Stevenson 1990). Hill *et al.* (1991) have observed an association between certain HLA class I and class II haplotypes and protection from severe malaria in West Africa.

The nutritional status of the host may also influence malaria infections. In rodent malaria models, diet changes have been shown to be a variable in the host-parasite system (Gilks *et al.* 1989), and rodents maintained solely on milk suffer less severe infection (Maegraith *et al.* 1952). The inhibitory effects of a milk-only diet may explain the lower than expected malaria infection rate in infants < 1 year in endemic areas (Phillips 1983). Feeding malnourished children during famine relief can result in outbreaks of malaria soon after, so called 'feeding malaria' (Murray *et al.* 1981).

1.4.2 Acquired resistance

Immune responses to malaria can be complex, involving different mechanisms and directed against different parasite stages. Acquired immunity is a general feature of the

host immune response, which can be manifest both as anti-parasite and anti-disease immunity, and has been studied extensively. It is generally species-specific and parasite stage-specific, with immunity largely directed against the asexual erythrocytic stages which are responsible for the symptoms of disease.

1.4.2 a The immune responses to pre-erythrocytic stages

Natural immune responses to sporozoites can be detected in humans, though sporozoites persist in the bloodstream for only a very short time. There is conflicting evidence regarding the role of Ab in anti-sporozoite immunity; however, it does appear that Ab must play some part (reviewed by Taylor 1990). Abs against sporozoites have been identified in sera from populations living in endemic areas (Nardin *et al.* 1979; Tapchaisri *et al.* 1983; Hoffman *et al.* 1986; Del Giudice *et al.* 1987 a & b). Sterile immunity can be obtained against challenge with rodent malaria viable sporozoites after vaccination of mice with irradiated sporozoites (Nussenzweig *et al.* 1967; Beaudoin *et al.* 1976), and a correlation between protection and prechallenge Ab titres has been reported (Hansen *et al.* 1979). Antisporozoite Ab in humans, however, appears to be poorly developed under natural conditions, does not appear to be boosted by reinfection, and does not correlate with protection against malaria infection (Webster *et al.* 1988). Passive transfer of Ab at the time of sporozoite challenge in mice leads to an increase in the rate of sporozoite clearance and a reduction in the number of exoerythrocytic stages in the liver (Nussenzweig *et al.* 1972), but unlike vaccination studies, rarely results in complete protection against sporozoite challenge (Verhave *et al.* 1978). Chen *et al.* (1977) found that immunisation of B cell-deficient mice with irradiated *P. berghei* sporozoites protected most animals against challenge with homologous viable sporozoites, therefore suggesting that resistance to this stage could be mediated by Ab-independent mechanisms. There is now compelling evidence that cell-mediated immune responses play an essential role in immunity to sporozoites (Chen *et al.* 1977; Spitalny *et al.* 1977; Egan *et al.* 1987; Schofield *et al.* 1987a; Weiss *et al.* 1988; reviewed by Schofield 1989).

As outlined, it is likely that Ab and T cells play a role in controlling the survival of sporozoites, but once the parasites are within hepatocytes, it appears that Ab-independent mechanisms alone are relevant in controlling liver stage infection (Schofield *et al.* 1987a). IFN-γ inhibits the development of liver stage parasites *in vitro* (Ferreira *et al.* 1986; Maheshwari *et al.* 1986; Schofield *et al.* 1987b), and *in vivo* it appears that $CD8^+$ cells are involved in IFN-γ-mediated protection, as immunised mice depleted of these cells lose their immunity (Schofield *et al.* 1987a). $CD8^+$ cells may also be directly cytotoxic to liver stage parasites (Schofield 1989). However, it now appears probable that the main mechanism for intrahepatic killing of parasites is the production of NO by hepatocytes, stimulated by IFN-γ or TNF-α (Green *et al.* 1990;

Nüssler *et al.* 1991).

1.4.2 b Immune responses to sexual stages

Abs against gametes suppress infectivity of malaria parasites to mosquitoes, and Abs against zygotes and ookinetes can also suppress development of parasites in the mosquito. It is clear that such Abs present in sera mediate transmission-blocking immunity within mosquitoes (Gwadz 1976; Kaushal *et al.* 1983; Rener *et al.* 1983; Vermeulen *et al.* 1985, 1986; Munesinghe *et al.* 1986). The effects of these Abs in the mosquito appear to be mediated by agglutination, preventing fertilisation, by complement-mediated lysis, and possibly by preventing penetration of the midgut wall (Kaushal *et al.* 1983; Rener *et al.* 1983; Grotendorst *et al.* 1984; Vermeulen *et al.* 1985; reviewed by Carter 1988). Transmission-blocking Abs have been shown to occur naturally during *P. vivax* infection (Mendis *et al.* 1987), though such Ab is known to both inhibit and enhance infectivity to mosquitoes at different concentrations (Peiris *et al.* 1988).

Abs do not appear to be effective against gametocytes in the vertebrate host (Cohen *et al.* 1961), though immunity has been demonstrated against circulating intracellular gametocytes (Harte *et al.* 1985). This immunity thus appears to be T cell-dependent, Ab-independent and mediated by cytokines (Naotunne *et al.* 1990). Crisis serum inhibits the ability of gametocytes of *P. cynomolgi* to infect mosquitoes, and this inhibitory effect of crisis serum is blocked by Abs against IFN-γ and TNF-α (Naotunne *et al.* 1991). However, gametocyte killing appears to require additional and as yet undefined complementary factors in crisis serum (Naotunne *et al.* 1991; Karunaweera *et al.* 1992).

1.4.2 c Immune responses to asexual erythrocytic stages

1. The antibody response

Malaria infection stimulates a rapid increase in both malaria-specific and non-specific Ig synthesis (McGregor *et al.* 1956; Cohen *et al.* 1961). Specific Ab production may contribute to the clearance of some species of malaria parasites from the infected host (Freeman *et al.* 1980), but most of the Abs formed appear to have no protective effect, and in general, there is little correlation between total anti-malarial Ab and protective immunity, though specific Ab levels do appear to correlate positively with exposure to *P. falciparum* (Thelu *et al.* 1991). Most of the total Ig synthesised has no apparent reactivity with plasmodial Ags (Abele *et al.* 1965; Targett & Voller 1965; Cohen & Butcher 1969). Such Abs have been shown to react with a variety of host Ags (Deans & Cohen 1983; Ternynck *et al.* 1991), probably contributing to the immunopathology of malaria, though some may also have a protective effect (Schetters *et al.* 1989).

Evidence supporting a protective role of Ab against asexual erythrocytic stage

malaria parasites includes results of the passive transfer of immune sera and mAbs. IgG from protected adults has been shown to reduce parasitaemia in children (Cohen *et al.* 1961; McGregor 1964; Sabchareon *et al.* 1991), and IgG-mediated protection has also been demonstrated in various animal models (Diggs & Osler 1969; Diggs *et al.* 1972; Phillips & Jones 1972; Green & Kreier 1978; Reese & Motyl 1979). Passive transfer experiments show considerable variation, especially in rodents. This variation appears to be due to the timing of serum collection and the amounts of serum transferred. The protective activity of transferred sera was shown to increase with time during a primary infection (McDonald & Phillips 1980), with highest activity at the time of parasite elimination (Phillips & Jones 1972; Murphy 1979), and protective activity diminishing rapidly after parasite clearance (Hamburger & Kreier 1976; Murphy 1979). Transfer of sera will include other serum components, but a mAb has been shown to be protective against *P. yoelii* (Majarian *et al.* 1984), indicating that Ab alone can be sufficient for conferring protection.

A role for specific Ab in immunity is also indicated from adoptive transfer experiments, where transfer of B cell-enriched populations of immune spleen cells gives protection (Gravely & Kreier 1976; McDonald & Phillips 1978; Ferraroni & Speer 1982) and transfer of B cells with T cells gives increased protection compared to T cells alone (Brown *et al.* 1976 a & b). B cells have also been shown to be necessary for the transfer of protective immunity to *P. chabaudi* in SCID mice and lethally irradiated mice, and clearance of parasites correlated with specific Ab in the serum (Meding & Langhorne 1991; Taylor-Robinson & Phillips 1993a).

There are several possible roles for anti-malarial Ab in protection (reviewed by Cohen 1979; Taylor & Siddiqui 1982; Taylor 1990), the relative importance of which is unclear. Abs have been shown to interfere with invasion of RBC by merozoites *in vivo* (Quinn & Wyler 1979a) and *in vitro* (Cohen *et al.* 1969; Cohen & Butcher 1970), but there is little evidence that Abs have any effect on the intraerythrocytic development of parasites (Cohen *et al.* 1969; Cohen & Butcher 1970, 1971; Mitchell *et al.* 1976). Abs may be important in preventing sequestration (David *et al.* 1983; Udeinya *et al.* 1983), and Ab titres to neo-Ags on the surface of schizont-infected RBC of *P. falciparum*, which are linked to cytoadherence and sequestration of parasites (see 1.5.3), have been shown to correlate with protection (Marsh *et al.* 1989). Abs also mediate phagocytosis of parasites (Hunter *et al.* 1979; Langreth & Reese 1979; Shear *et al.* 1979; Jain & Vianyak 1986). The appearance of Abs mediating phagocytosis of merozoites is thought to correlate with protective immunity (Druilhe & Khusmith 1987). Studies have also implicated Ab-dependent cellular cytotoxicity (ADCC) (Brown & Smalley 1980; Lunel & Druilhe 1989). and Ab-dependent cellular inhibition (ADCI) (Bouharoun-Tayoun *et al.* 1990, 1995) in limiting parasite growth and invasion *in vitro*, lending support for an anti-malarial role of Ab.

2. The cell-mediated response

Whilst Ab-mediated mechanisms clearly play a part in immunity to blood stage malarial parasites, cell-mediated responses are also necessary. Ag-specific T cells appear to play an essential role, providing both help for specific Ab production and initiating and modifying non-Ab cell-mediated effector mechanisms.

The role of CMI has been examined most closely in murine models. Evidence for the involvement of T cells in immunity to blood stage malarial parasites includes studies of B cell-deficient and T cell-deficient animals and adoptive transfer experiments.

Mice rendered B cell-deficient by anti-μ treatment suffer increased severity of acute *P. yoelii* infection (Weinbaum *et al.* 1976a), but when infected and drug-cured, they subsequently develop a prolonged low-level parasitaemia and are resistant to homologous parasite challenge (Roberts & Weidanz 1979). However, B cell-deficient mice infected with *P. chabaudi adami* spontaneously resolve acute infections (Grun & Weidanz 1981). These results indicate that non-Ab, T cell-dependent mechanisms can function both in resistance to reinfection and in suppressing acute disease, but that different mechanisms of immunity may operate, depending on the species of malaria parasites studied.

When infected with a variety of murine malarias, animals rendered T cell-deficient by thymectomy suffer more severe and prolonged parasitaemia and increased mortality (Brown *et al.* 1968a; Stechschulte 1969; Chapman & Hanson 1971; Jayawardena *et al.* 1977; Cottrell *et al.* 1978; McDonald & Phillips 1978; Cavacini *et al.* 1986). Likewise, nude (nu/nu) mice, which are congenically athymic and therefore T cell-deficient, suffer exacerbated and often fatal malarial infections (Clark & Allison 1974; Weinbaum *et al.* 1976b; Roberts *et al.* 1977; Eugui & Allison 1980; Grun & Weidanz 1981; Brinkmann *et al.* 1985; Brake *et al.* 1986, 1988; Cavacini *et al.* 1986, 1990; Mogil *et al.* 1987; Vinetz *et al.* 1990; Meding & Langhorne 1991; Watier *et al.* 1992). Such results demonstrate the role of an intact thymus, and therefore T cells, in immunity to malaria, but give no indication of the mechanisms involved.

Adoptive transfer experiments using rodent models show that immune T cell-enriched cell populations can confer some protection against malarial infection (Brown *et al.* 1976 a & b; Gravely & Kreier 1976; McDonald & Phillips 1978, 1980; Jayawardena *et al.* 1982; Brinkmann *et al.* 1985; Cavacini *et al.* 1986; Fahey & Spitalny 1986). In addition, a synergistic effect of enhanced protection can be obtained with the transfer of T and B cells together (Brown *et al.* 1976b; Gravely & Kreier 1976; Jayawardena *et al.* 1982), indicating a role for T cells in immunity to malaria by functioning as T helper (Th) cells for the production of specific Ab.

It is apparent that the mechanisms by which T cells mediate protection are multifaceted and may vary in importance in different rodent models. However, the

consensus from adoptive transfer experiments is that in rodents, T cells mediating protection against asexual erythrocytic malaria parasites are of the helper/inducer phenotype (L3T4$^+$; Ly-4$^+$; CD4$^+$) and possess αβ T cell receptors (Jayawardena *et al.* 1982; Brinkmann *et al.* 1985; Brake *et al.* 1986, 1988; Cavacini *et al.* 1986; Taylor-Robinson & Phillips 1993a, 1994a; Taylor-Robinson *et al.* 1993). *In vivo* depletion studies also implicate CD4$^+$ T cells in protective immunity (Süss *et al.* 1988; Kumar *et al.* 1989; Langhorne *et al.* 1990; Vinetz *et al.* 1990; Taylor-Robinson *et al.* 1993; Taylor-Robinson & Phillips 1994a). CD4$^+$ Th cells in mice, and probably in humans, can be further subdivided into Th1 and Th2 subsets, defined according to the pattern of cytokines produced (Mosmann *et al.* 1986; Mosmann & Coffman 1987). In its simplest form, this paradigm indicates that Th1 cells secrete IL-2 and IFN-γ and Th2 cells secrete IL-4 and provide help for specific Ab production. In *P. chabaudi* infections, these two subsets appear to be important at different times, with Th1 cells predominating early in infection, and Th2 cells predominating later (Langhorne 1989; Langhorne *et al.* 1989 a & b, 1990; Taylor-Robinson & Phillips 1992). Ag-specific T cell lines and clones of either subset can confer protection upon adoptive transfer to immunocompromised mice (Taylor-Robinson & Phillips 1993a, 1994a; Taylor-Robinson *et al.* 1993). Serum cytokine profiles of patients with *P. falciparum* and *in vitro* stimulation of peripheral blood lymphocytes from malarious individuals have indicated that both Th1 and Th2 cells are also activated during human infection (Troye-Blomberg & Perlmann 1988; Troye-Blomberg *et al.* 1990; Mshana *et al.* 1991).

The possible mechanisms by which CD4$^+$ T cells mediate protection against asexual erythrocytic malaria parasites appear to be by providing help for specific Ab production, direct killing by T cells, or by activation of other effector cells by the secretion of cytokines. It appears likely that all three mechanisms play some part, depending on the time during infection and the model being studied. The CD4$^+$ T cells involved in providing help for specific Ab production are likely to be exclusively of the Th2 subset, and adoptive transfer of Th2 cells against *P. chabaudi* has been shown to induce high levels of IgG_1 (Taylor-Robinson *et al.* 1993). Direct killing of parasites by CD4$^+$ T cells could possibly occur by production of toxic factors. Th1 cells have recently been shown to produce NO (Taylor-Robinson *et al.* 1994), which has been shown to be toxic to malaria parasites *in vitro* (Rockett *et al.* 1991). It is likely that this production of NO by Th1 cells contributes to the peak of NO shown to occur at peak parasitaemia in mice infected with *P. chabaudi* and protected by adoptive transfer of malaria-specific Th1 cells (Taylor-Robinson 1995). As well as possible direct killing mechanisms, Th1 cells, by the production of cytokines, mediate other non-Ab effector mechanisms due to other activated effector cells. Such mechanisms may include phagocytosis by macrophages, Ab-independent cellular cytotoxicity, and the production of RNI and ROI.

T cells that express γδ T cell receptors constitute only a small minority of peripheral T cells in mice and humans but have become associated with a variety of infectious and parasitic diseases, including malaria (Haas *et al.* 1993). An increase in the number and proportion of peripheral blood γδ T cells has been observed during acute *P. falciparum* infections (Ho *et al.* 1990; Roussilhon *et al.* 1990) and during fever paroxysm associated with *P. vivax* infection (Perera *et al.* 1994). An expansion of γδ T cells was also reported for peripheral blood from non-immune individuals in response to *P. falciparum* pRBC *in vitro*, with significant production of IFN-γ and TNF-α (Behr & Dubois 1992; Goodier *et al.* 1992), leading to the consensus that γδ T cells may be involved in malaria pathogenesis (Langhorne *et al.* 1992). Experiments in murine models to determine a possible protector function of γδ T cells in blood stage malaria indicate a minor role, as γδ T cell-deficient mice clear infections with *P. yoelii* (Tsuji *et al.* 1994) and *P. chabaudi* AS (Langhorne *et al.* 1995; Taylor-Robinson 1995), while in each case αβ T cell-deficient mice fail to control parasitaemia. It appears that γδ T cells are not effective alone in providing help for generation of malaria-specific Abs, but they may influence the quality and quantity of Ig secreted (Langhorne *et al.* 1995). As γδ T cells can be cytolytic (Haas *et al.* 1993), it is possible that any anti-parasitic effects they may exhibit is through acting as non-MHC-restricted cytotoxic cells (Ho *et al.* 1990). In this regard, it has been shown that human γδ T cells can inhibit the growth of *P. falciparum in vitro*, with activity directed primarily against the extracellular merozoite (Elloso *et al.* 1994).

3. The reticulo-endothelial system

Macrophages are thought to be important in controlling blood stage malaria infections through phagocytosis and/or the release of extracellular mediators. For many years, phagocytosis (Taliaferro 1929) was considered the principle mechanism by which macrophages effected immunity. A sharp increase in blood monocytes and an accumulation of macrophages in the spleen and liver has since been identified in experimental malaria infections (Jayawardena *et al.* 1977, Lee *et al.* 1986), as has increased phagocytosis (Lucia & Nussenzweig 1969; Sheagren *et al.* 1970; Criswell *et al.* 1971; Loose & DiLuzio 1976). The ingestion process is thought to be mediated by disease-associated Igs which bind to the surface of pRBC (Lustig *et al.* 1977).

Activated macrophages may also mediate pRBC destruction by the release of factors toxic to the intracellular parasite (Clark *et al.* 1981; Allison & Eugui 1982). The mechanisms by which macrophage secretion products destroy blood stage parasites are discussed in 'cytokines' (see 1.4.2.c 4, below). The recruitment and activation of macrophages and monocytes is mediated by such cytokines as IFN-γ, IL-2, IL-6 and macrophage chemotactic factor (Liew & Cox 1991), secreted by T cells, which are

themselves activated by exposure to plasmodial mitogens as well as specific parasite Ags (Wyler & Gallin 1977; Ockenhouse & Shear 1983).

In human malaria infections, both pRBC and nRBC have been observed within splenic macrophages *in vivo* (Pongponratn *et al.* 1987). The part played by immune phagocytosis in the clearance of *P. falciparum* is controversial. In Thai patients with falciparum malaria, the activity of monocytes from cases of uncomplicated malaria was significantly increased compared to healthy controls (Ward *et al.* 1984). In contrast, the activity of monocytes from cerebral malaria sufferers was within normal limits. In another study, the clearance *in vivo* of IgG-coated RBC was accelerated in some but not all patients (Ho & Webster 1990) with a significant +ve correlation between the half-time for clearance of sensitised RBC from the circulation and the level of parasitaemia. The apparently normal rate of parasite clearance seen in patients with high parasitaemias suggests a failure to augment splenic Fc receptor function and consequent phagocytic activity in the face of a considerable antigenic challenge. Together, these studies indicate that immune clearance through phagocytosis is important in reducing parasitaemia to subpatency, thereby controlling the acute phase of infection. The failure of immune clearance in some instances may be related to the development of severe clinical illness, including cerebral manifestations.

4. Cytokines

The first direct support for cytokine production in response to malarial Ags was provided by Wyler & Gallin (1977), who identified a mononuclear cell chemotactic factor in spleen cell extracts from malarious mice and monkeys. Since spleen extracts of *P. berghei* -infected nude mice lacked significant activity, it was concluded that the chemotactic activity was secreted by, or dependent upon, T cells and their precursors. Lelchuk *et al.* (1984) showed that the ability of spleen cells from mice infected with *P. berghei* or *P. yoelii* to produce IL-2 following concanavalin A stimulation was greater early in both infections, a finding also shown with *P. chabaudi* (Langhorne *et al.* 1989 a & b; Taylor-Robinson & Phillips 1994 b). Langhorne (1989) attributed IL-2 secretion to the Th1 subset of $CD4^+$ cells which predominate during the clearance of the primary parasitaemia to subpatent levels.

Interferons are increasingly being considered important in acquired immunity to asexual erythrocytic malaria parasites. Administration of exogenous IFN inducers or IFN-containing serum delayed the progress of *P. berghei* infection in mice (Jahiel *et al.* 1968, 1970), while treatment with anti-mouse IFN globulin accelerated infection (Sauvager & Fauconnier 1978). The presence of IFN-γ in the sera of infected humans and mice has been reported (Eugui & Allison 1982, Rhodes-Feuillette *et al.* 1985). T cells from malarious patients and immune individuals in endemic areas can secrete IFN-γ and IL-2 upon stimulation with homologous Ag (Sinigaglia & Pink 1985, Troye-

Blomberg *et al.* 1985, 1987; Riley *et al.* 1988).

IFN-γ has by itself no effect on erythrocytic malaria parasites (Ferreira *et al.* 1986). However, as IFN-γ is capable of activating macrophages with enhanced microbicidal activity, its production is considered central to CMI to intracellular microorganisms (Murray 1988). Experimental evidence from *in vitro* and *in vivo* studies implicates IFN-γ in acquired immunity to blood stage malaria. Ockenhouse & Shear (1984) demonstrated that macrophages recovered from normal mice could be activated *in vitro* to destroy *P. yoelii* pRBC after incubation in IFN-containing S/N obtained from Ag-stimulated spleen cells from *P. yoelii*-immune mice. In further studies, these investigators showed that the addition of anti-IFN-γ Ab to crude lymphokine S/N blocked macrophage-mediated parasite destruction, and demonstrated that recombinant IFN-γ activated human macrophages to induce the appearance of crisis forms of *P. falciparum* in cultures of human pRBC (Ockenhouse *et al.* 1984).

Treatment of mice with exogenous IFN-γ has a protective effect during blood stage infection with various rodent malarias (Clark *et al.* 1987; Bienzle *et al.* 1988; Shear *et al.* 1989), and can also enhance antimalarial chemotherapy to *P. vinckei* (Kremsner *et al.* 1991). In *P. chabaudi* AS-infected mice, the peak of endogenous IFN-γ production occurred just before peak parasitaemia, and correlated directly with a relatively high frequency of IFN-γ-secreting T cells in the spleen (Slade & Langhorne 1989, Stevenson *et al.* 1990; Taylor-Robinson & Phillips 1994b). *In vivo* depletion of IFN-γ by treatment with mAbs exacerbated infection (Slade & Langhorne 1989, Stevenson *et al.* 1990). Furthermore, in mice depleted of $CD4^+$ T cells, and thus unable to produce IFN-γ, treatment impaired host resistance to *P. chabaudi* AS infection (Meding *et al.* 1990). Administration of IFN-γ in combination with chloroquine during the late stage of *P. vinckei* malaria, however, did not prevent a lethal outcome, despite effective parasite clearance (Kremsner *et al.* 1992). This suggests that IFN-γ has a pivotal role in host immunity to malaria, but that factors in addition to this pluripotent cytokine may be important in parasite clearance.

Inflammatory mediators such as TNF can be induced in macrophages activated by IFN-γ (Mosmann & Coffman 1987) in response to malarial parasite stimulation (Bate *et al.* 1988). TNF may contribute to protective CMI but is also linked to the pathology of cerebral malaria (Grau *et al.* 1987). The direct parasiticidal effect of TNF is controversial, as the toxicity of recombinant TNF-α towards pRBC has yet to be demonstrated *in vitro*. However, TNF is present in very high amounts in human serum taken from malaria-infected individuals (Scuderi *et al.* 1986). Furthermore, TNF-containing serum and partially purified TNF can kill murine (Taverne *et al.* 1981) and human (Haidaris *et al.* 1983, Carlin *et al.* 1985) blood stage parasites *in vitro*. There is good evidence that serum-extracted TNF inhibits the *in vivo* growth of *P. vinckei* (Clark *et al.* 1981) and *P. yoelii* (Taverne *et al.* 1982), and that administration of recombinant

TNF-α *in vivo* reduces parasitaemia in mice infected with *P. chabaudi* (Clark *et al.* 1987) and both lethal and non-lethal strains of *P. yoelii* (Taverne *et al.* 1987). The mechanism by which TNF exerts its deleterious effects on pRBC remains to be elucidated, but as it is toxic to the host animal, whether or not it exerts a beneficial effect or is pathogenic may depend on the sensitivity of the individual to TNF and its level in the serum.

Kumaratilake *et al.* (1991) have demonstrated an enhanced neutrophil-mediated killing of *P. falciparum* by IFN-γ and TNF-β (lymphotoxin). This supports a role for both Th1 and Th2 $CD4^+$ T cells in immunity to malaria, as IFN-γ and TNF-β are Th1-derived cytokines and killing of *P. falciparum* and *P. berghei* parasites by neutrophils is Ab-dependent (Kumaratilake *et al.* 1991, 1992; Waki 1994).

IL-4 can depress the macrophage-mediated killing of *P. falciparum* (Kumaratilake & Ferrante 1992). This finding may be explained by results from studies of other parasitic diseases in which the ability of IL-4 to inhibit the microbicidal functions of IFN-γ-activated macrophages *in vitro* has been demonstrated (Liew *et al.* 1991; Oswald *et al.* 1992). However, *P. chabaudi* infection of mice in which the IL-4 gene has been inactivated by gene targetting is cleared with kinetics similar to wild-type littermates (von der Weid *et al.* 1994). At present, therefore, the role of IL-4 in host protection against malaria is unresolved.

Another cytokine attracting attention as a determinant of development of acquired immunity is IL-12, originally identified as NK cell stimulating factor. Produced most notably by monocyte-macrophages and B cells, in response to infectious agents, IL-12 induces NK and T cells to produce IFN-γ and TNF-α, thereby enhancing their cytotoxic activity and stimulating their proliferation in combination with other activators, such as IL-2 (Trinchieri 1993). With regard to malaria, IL-12 has been shown to regulate the development *in vivo* of protective CMI to *P. chabaudi* via a Th1 $CD4^+$ T cell response, which involves IFN-γ and TNF-α (Stevenson *et al.* 1995), and is in part NO-dependent (Taylor-Robinson *et al.* 1993; Stevenson *et al.* 1995).

5. Reactive oxygen intermediates

The release of IFN-γ and other cytokines from $CD4^+$ T cells stimulates cells of the mononuclear phagocytic cell lineage to exert anti-parasitic effects, either directly by phagocytosis, or more often through the release of ROI which, in turn, may generate more stable parasiticidal components (Allison & Eugui 1983; Clark *et al.* 1987; Golenser *et al.* 1992).

Injection of agents known to generate ROI, including t-butylhydroperoxide (Wood & Clark 1982; Clark *et al.* 1983) and alloxan (Clark & Hunt 1983) suppressed parasitaemias in *P. vinckei*-infected mice. The chemical generation of ROI, such as H_2O_2, superoxide anions (O_2-) and hydroxyl radicals (OH·) may mimic this mechanism

of CMI against blood stage malaria. Indeed, not only have ROI been shown to be toxic to asexual stages of a variety of different *Plasmodium* species, both *in vitro* and *in vivo* (Dockrell & Playfair 1983), free radical scavengers have exacerbated *P. c. adami* infections (Clark *et al.* 1987). Moreover, in strains of mice susceptible to *P. chabaudi*, the oxidative capacity of macrophages was shown to be significantly reduced compared to that of macrophages from resistant mouse strains (Stevenson *et al.* 1992).

Since ROI are extremely short-lived molecules, it is assumed that they exert their activity locally within the liver and spleen, through lipid peroxidation leading to the generation of toxic aldehydes (Allison & Eugui 1983; Clark *et al.* 1987; Rockett *et al.* 1988). These may then circulate in the blood and effect parasite (and tissue) damage at more distant sites.

6. Reactive nitrogen intermediates

Nitric oxide (NO), a highly diffusible cellular mediator involved in a wide range of biological effects, has been indicated as a cytotoxic agent released by leucocytes in response to malaria infection. The first suggestion that an oxygen-independent mechanism for parasite killing existed came from cases of chronic granulamatous disease, in which oxidative metabolism is impaired, macrophages (Ockenhouse *et al.* 1984) and PMN cells (Kharazmi *et al.* 1984) were capable of inhibiting the growth of pRBC. Cavacini *et al.* (1989) also reported proficiency of killing in hosts possessing cells deficient in the respiratory burst. This mechanism was shown to involve the cytokine-induced synthesis of RNI from L-arginine by macrophages, neutrophils, hepatocytes and endothelial cells (Green *et al.* 1990). NO inhibits iron sulphur-dependent enzymes involved in cellular respiration and energy production and may react with a ROI to yield the highly reactive OH· and the more stable NO· (James & Hibbs 1990; Liew & Cox 1991).

Serum levels of cytokines known to induce NO synthesis, such as TNF and IL-1, are increased in acute *P. falciparum* infections (Clark *et al.* 1992) and killing of asexual *P. falciparum* parasites *in vitro* correlates with detection of increased levels of RNI derivatives, following incubation with high concentrations of RNI generators (Rockett *et al.* 1991) or human monocytes (Gyan *et al.* 1994). Concentrations of NO known to be physiologically relevant, such as those produced by activated macrophages, are usually cytostatic rather than cytotoxic to *P. falciparum in vitro* (Balmer *et al.* 1995; Taylor-Robinson 1997). The role of NO in protection against *P. chabaudi* AS has been demonstrated *in vivo* (Taylor-Robinson *et al.* 1993, 1996). A sharp peak of NO production, measured as serum nitrate, consistently paralleled peak parasitaemia. Treatment with an inhibitor of NOS abolished completely NO production and mice suffered extended primary parasitaemias. However, blockade of NO production later in infection had no observable effect on the level or duration of recrudescent

parasitaemias. Treatment of infected CD4-depleted mice, protected by the adoptive transfer of a Th1 clone, with the NOS inhibitor resulted in severe infection with significantly increased parasitaemia and 90% mortality within 20 d p.i.. This suggests that NO plays a crucial role in protection against blood stage malaria but at present its exact involvement is not clear. On the one hand, Th1 cell secretion of IFN-γ may activate macrophages to produce large amounts of NO (Marletta *et al.* 1988; Stuehr & Nathan 1989) to kill the parasites directly. Alternatively, NO may have an indirect effect by causing blood vessel vasodilation (Knowles & Moncada 1992), leading to less efficient parasite sequestration in deep tissue capillaries, allowing removal of parasites by macrophages (Taylor-Robinson *et al.* 1993).

A link between NO production and cerebral malaria has also been suggested (Clark *et al.* 1991; Clark & Rockett 1994). During infection, NO produced in excess by TNF-stimulated vascular cells, or directly by *P. falciparum* pRBC (Ghigo *et al.* 1995), could diffuse to local neurons, causing a disruption of the regulation of glutamate-induced neural NO, thereby interfering with neurotransmission and causing coma (Clark *et al.* 1991, 1992). Several studies have, however, demonstrated an inability of NO inhibitors to influence the development of cerebral malaria in the mouse model, *P. berghei* ANKA, even upon intracranial administration (Senaldi *et al.* 1992; Asensio *et al.* 1993; Kremsner *et al.* 1993), implying that NO blockade *in vivo* is not able to protect against pathology. While these reports may appear to conflict, Grau & de Kossodo (1994) proposed that NO may mediate early changes in cerebral malaria, such as neurotransmission disturbances, when the neurological syndrome is still reversible, but that NO is unlikely to be involved in the actual processes causing neurovascular damage at the advanced stages of the condition.

7. The involvement of the spleen

As an organ of prime importance to host defence against blood pathogens and that responsible for removing damaged and effete RBC from the circulation, the spleen is thought necessary for resolution of malaria infection. Taliaferro & Cannon (1936) first reported that during a primary infection, the spleen becomes massively enlarged, splenomegaly, which is a hallmark of malaria, and observed increased numbers of differentiated macrophages phagocytosing parasites in the spleens of *P. brasilianum*-infected Panamanian monkeys. More recently, the total number of splenic macrophages has been shown to increase greatly during *P. berghei* and *P. yoelii* infections (Wyler & Gallin 1977; Lelchuk *et al.* 1979).

Non-lethal challenges may become lethal and latent infections may relapse following splenectomy (Taliaferro 1929; reviewed by Wyler *et al.* 1979). However, splenectomy does not always worsen infection. One study reported that splenectomy did not affect the outcome of infection with *P. yoelii* (Dockrell *et al.* 1980). These

contradictory reports may be explained by the finding that the spleen is beneficial for the host early in infection, but later promotes chronicity of infection with some plasmodia (Wyler *et al.* 1979). Splenectomy removes a large population of effector cells (Brown *et al.* 1976a). However, this is probably not as important as the loss of the normal splenic architecture and filtering ability of the spleen. Phillips (1970) and Oster *et al.* (1980) showed, using several rodent malarias, that mice reconstituted with spleen cell suspensions after splenectomy exhibited infections similar to those in splenectomised controls.

The reason for the spleen being so vital in malaria infections appears to be a physical role in trapping pRBC, enabling localised elimination of parasites (Conrad & Dennis 1968; Schnitzer *et al.* 1972; Wyler *et al.* 1981). *P. berghei*-infected RBC are removed more rapidly than are nRBC from the circulation into the spleen (Quinn & Wyler 1979b; Wyler *et al.* 1981). The site where filtration occurs is thought to be the red pulp (Weiss 1979), a unique structure of the spleen not present in other lymphoid organs. The intermediate circulation in the red pulp consists of arterioles opening into cords that are connected to sinuses. This structure brings pRBC in close apposition to macrophages, which appear to be selectively held in the filtration beds of the reticular meshwork (Weiss 1983 a & b). pRBC can then be eliminated by direct phagocytosis or by the cytotoxic effects of monokines and other macrophage-derived factors. Phagocytosis of *P. knowlesi*-infected RBC by cordal macrophages has been observed in rhesus monkeys (Schnitzer *et al.* 1972).

Another filter system of the red pulp exists where blood leaves the cord and enters the lumen of the vascular sinus by passing between endothelial cells (Weiss 1979). RBC passing through must be pliant. When RBC deformability is reduced, as has been shown for pRBC (Miller *et al.* 1971b), passage is delayed. Such a concentration of pRBC was first reported for *P. brasilianum* infection (Taliaferro & Cannon 1936).

The capacity of the spleen to clear parasites from the blood varies considerably during the course of infection. After a brief initial phase of activity, splenic clearance falls to subnormal levels until crisis, when active clearance is restored (Quinn & Wyler 1979b; Wyler *et al.* 1981). There is also a switch in *P. berghei* infection from an open blood flow through the locules of filtration beds, during normal or heightened clearance, to a closed blood flow, away from the locules, during depressed clearance (Quinn & Wyler 1979b; Wyler *et al.* 1981). Evidence for such a change in the flow of pRBC through the spleen during the transition to an immune state during infection with another murine model, *P. c. adami*, is, however, lacking (Yadava *et al.* 1996), suggesting that some alteration in immune effector function, rather than microcirculatory changes, may be crucial to parasite killing.

The phenomenon of crisis is perhaps the most striking instance of splenic control of malaria, when pRBC spontaneously and rapidly disappear from the blood (Taliaferro

& Cannon 1936; Taliaferro & Mulligan 1937; Taliaferro & Taliaferro 1944; Quinn & Wyler 1979b, 1980; Wyler *et al.* 1979, 1981; Wyler 1983). Crisis fails to occur in the absence of the spleen. The disappearance of circulating pRBC in crisis is due to their removal on the filtration beds of the red pulp and their destruction by macrophages held there (Taliaferro & Cannon 1936; Taliaferro & Mulligan 1937; Yadava *et al.* 1996).

Early in *P. yoelii* infections, a rapid activation of reticular cells provides a competent blood-spleen barrier (Weiss *et al.* 1986; Weiss 1989, 1990). This appears to protect proliferating and differentiating populations of erythroblasts, lymphocytes and macrophages by ecluding pRBC from filtration beds. This barrier permits the development of a rising parasitaemia and anaemia (McGhee 1960; Zuckerman 1960). At crisis, the barrier relaxes, resulting in pRBC entering the fitration beds of the spleen, where they are destroyed, and reticulocyte stores being released into the circulation (Weiss *et al.* 1986). Filtration capacities of the spleen, blood flow alterations and control of malaria seem to be intrinsically related. These depend on the formation of the reticular cell blood-spleen barrier, and indeed, it has been speculated that the very nature of the spleen may have been driven by malaria (Weiss 1990).

In addition to the role of the spleen in host resistance, there is another spleen-parasite interaction which may affect the outcome of infection. Expression of surface variant Ags is dependent on the presence of the spleen in some species of malaria parasites, including *P. falciparum* (Hommel *et al.* 1983), *P. knowlesi* (Barnwell *et al.* 1983 a & b), *P. fragile* (Handunnetti *et al.* 1987) and *P. chabaudi* (Gilks *et al.* 1990). Sequestration, whereby pRBC cease circulating and remain in the blood vessels of various organs, and which is linked to the expression of such surface variant Ags (see 1.5.2), has also been shown to be dependent on the presence of the spleen (David *et al.* 1983; Gilks *et al.* 1990). These observations suggest that the expression of such Ags on pRBC and sequestration by parasites may be adaptations for survival in the presence of a potentially destructive spleen.

1.5 Immune evasion

The persistence of malaria blood stage infections has been well documented (Cohen 1980; Terry 1988). Such observations imply that either there is an incomplete immune response by the host, or that immune evasion strategies are being successfully employed by the parasites. The balance between the immune response mounted by the host and evasion of this response by the parasites will ultimately determine the survival of parasites both in the infected host and in the population.

1.5.1 Antigenic diversity

Malaria parasites present a diverse array of Ags to the host immune system. This diversity is multifaceted, with different Ags occurring at different life cycle stages of

the parasite, different forms of a particular Ag in different parasite strains or isolates and within a strain or parasite clone. Such antigenic diversity may account for the slow development of immunity in natural malaria infections in humans, and the survival of parasites despite specific immune responses (see 1.4.2).

Many apparently stage-specific Ags have been described (reviewed by Newbold 1985; Kemp *et al.* 1990), some of which are considered of importance in eliciting immune responses against the parasites. These stage-specific Ags are therefore targets for the development of vaccines against malaria (see 1.6).

The expression of different forms of a particular Ag by different strains/isolates of a malaria parasite is well-documented (reviewed by Newbold 1985; Kemp *et al.* 1990; Anders 1991) and is the usual definition of antigenic diversity. The means by which isolates have been defined and antigenic diversity recognised include isoenzyme typing (Sanderson *et al.* 1981), *in vitro* drug sensitivity (reviewed by Peters 1985), two-dimensional electrophoresis (Tait 1981; Fenton *et al.* 1985), serotyping of S-Ags (Wilson 1980) and studies using mAbs (McBride *et al.* 1982). Such methods have indicated that there is a considerable degree of antigenic diversity in malaria parasites. Isolates exhibiting antigenic diversity may be derived from different geographical locations, different individuals at the same location and different malaria bouts from the same individual. Antigenic diversity may also be seen in pRBC taken at various times from an isolate *in vitro* or during an infection *in vivo*.

Mechanisms by which antigenic diversity arises (reviewed by Kemp *et al.* 1990; Anders 1991) include failure to express Ags, probably more common *in vitro* than *in vivo*, simple mutational events and major polymorphisms such as expression of different repeat sequences and intragenic recombination. Using PFGE, considerable variation in chromosome sizes between different parasite cloned isolates has been observed, which is associated with antigenic diversity (reviewed by Kemp *et al.* 1990).

Antigenic diversity within an infection or *in vitro* may also arise due to antigenic variation (see 1.5.2), which may be considered a subset of antigenic diversity. Moreover, the variant Ags are themselves highly diverse between different parasite isolates and strains (Hommel *et al.* 1983; Aley *et al.* 1984; Leech *et al.* 1984; Marsh & Howard 1986; Magowan *et al.* 1988; Forsyth *et al.* 1989; Newbold *et al.* 1992; Iqbal *et al.* 1993).

1.5.2 Antigenic variation

Antigenic variation is defined as variation within a clone of a particular organism, as opposed to antigenic diversity, which denotes variation between clones, strains, lines etc. Antigenic variation is now recognised to be an immune evasion strategy utilised by many parasitic organisms, including malaria parasites. By periodically changing their antigenic profile to avoid elimination by the host's immune system, infectious

organisms undergoing antigenic variation thus gain a selective advantage. Parasitic organisms shown to utilise antigenic variation as an immune evasion strategy include bacteria, for example, *Mycoplasma hyorhinis* (Rosengarten & Wise 1990, 1991), *Neisseria gonorrhoeae* (Hagblom *et al.* 1985) and *Borrelia* (reviewed by Barbour 1990; Wilske *et al.* 1992), and protozoa (reviewed by Turner 1992). Among the protozoa, antigenic variation has been the most extensively studied and is best understood in African trypanosomes (reviewed by Vickerman 1978, 1989; Borst & Cross 1982; Cross 1990; Barry & Turner 1991; Turner 1992). Other parasitic protozoa which undergo antigenic variation include *Trichomonas vaginalis* (Alderete *et al.* 1985, 1986 a & b, 1987; Alderete 1987), *Giardia lamblia* (reviewed by Nash 1989), *Babesia* (Phillips 1971; Allred *et al.* 1994), and malaria parasites (reviewed by Howard 1984; Hommel 1985).

The first indication of antigenic variation occurring in malaria parasites came from studies of relapsing infections of *P. berghei* in mice (Cox 1959, 1962). These studies showed that acute infections drug treated subcuratively produced a latent infection with periodic recrudescences. Mice infected with a parent population and given a latency-inducing treatment were shown to be more susceptible to heterologous challenge with recrudescent parasites than to homologous challenge with the parent parasites, indicating that the populations were antigenically distinct (Cox 1959). Upon infection of naive mice, it was also suggested that there were differences in virulence and development of immunity between these recrudescences and the parent population (Cox 1962). *P. berghei* parasites surviving in mice following passive immunisation with immune serum from *P. berghei*-infected rats (Briggs *et al.* 1968) or mice (Wellde & Diggs 1978) were resistant to the same serum in subsequent experiments, again indicative of antigenic variation having occurred. Whether the immune serum (Ab) played an inductive or selective role in the emergence of an antigenically variant population cannot be ascertained from these experiments. The work described above, though strong evidence for the occurrence of antigenic variation in *P. berghei*, was all performed using uncloned parasite lines. Wery *et al.* (1979), however, using cloned lines of *P. berghei* ANKA strain, isolated several parasite populations from successive recrudescences of chronic infections induced by multiple infection and drug cure. Cross challenge experiments with these recrudescent populations showed that mice immunised with one recrudescent population were more resistant to homologous challenge than to challenge with recrudescent populations taken from the same mouse at different times (Wery & Timperman 1979). This, therefore, was strongly suggestive of antigenic variation occurring during the course of *P. berghei* infection, confirming the results of earlier work, but with cloned parasites.

Another murine malaria parasite shown to undergo antigenic variation is *P. chabaudi*. The first indication of this came from passive transfer studies using cloned

lines of *P. c. chabaudi* AS in NIH mice (McLean *et al.* 1982b). Immune serum was collected from mice following resolution of the acute infection before any recrudescence had occurred. This immune serum significantly delayed the homologous parasite population reaching 2% parasitaemia compared to NMS upon passive transfer. Six out of 7 recrudescent populations were found to be less sensitive than the original infecting population to this immune serum, therefore indicating these recrudescences were antigenically different from the infecting population from which they were derived; antigenic variation had occurred. Similar experiments in CBA/Ca mice also demonstrated antigenic variation in breakthrough populations from passively protected mice (Jarra *et al.* 1986). Heterogeneity in sensitivity/resistance to immune serum of clones from a recrudescence in the NIH system have also been observed (McLean *et al.* 1986a), indicating a mix of antigenic types. This same passive transfer system also indicated a reversion of an antigenic variant to a basic 'parental' type after transmission through mosquitoes (McLean *et al.* 1987) and that antigenic variants could be detected as early as d 13 p.i., a time when the primary parasitaemia is still patent but in remission (McLean *et al.* 1990).

An indirect fluorescent antibody test (IFAT) which detects Ags on the surface of live, schizont-infected RBC (Hommel & David 1981, Hommel *et al.* 1982) has been adapted to *P. chabaudi* and used to recognise antigenic variants of this parasite (McLean *et al.* 1986b; Gilks *et al.* 1990). This has shown cloned recrudescent populations to be both different from the initial infecting parental cloned population and from each other, using both immune sera, collected upon resolution of the acute parasitaemia, and hyperimmune sera (Brannan *et al.* 1993; see chapter 3). These results and others presented in this thesis further demonstrate the occurrence of antigenic variation in *P. chabaudi.*

Until recently, the parasite most studied in investigations of antigenic variation and variant Ags in malaria parasites was *P. knowlesi.* Eaton (1938) showed that schizont-infected RBC can be agglutinated by immune serum. Using this schizont-infected cell agglutination (SICA) test, antigenic variation during chronic infections of *P. knowlesi* in rhesus monkeys was first described (Brown & Brown 1965, 1966; Brown *et al.* 1968b). Chronic infections were induced by subcurative drug treatment, resulting in a series of distinct recrudecent parasitaemia peaks. Parasites collected from different recrudescences were shown to be antigenically different using the SICA test. Serum from monkeys immunised with different populations reacted only with the homologous population and serum collected during chronic infections reacted only with parasite populations collected before the serum sample and not with parasite populations collected afterwards. These results indicated that each wave of parasitaemia expressed different SICA Ags on the surface of RBC. Serum reactivity to variant parasites in the SICA test was also shown to be species- and strain-specific (Brown *et al.* 1968b).

Voller & Rossan (1969b) also showed that parasites isolated from different recrudescences of chronic *P. knowlesi* infection were antigenically distinct. The SICA test has also indicated that *P. knowlesi* appears to undergo antigenic variation upon transmission through mosquitoes (Draper & Voller 1972).

Expression of the SICA Ag(s) is dependent on the presence of an intact spleen (Barnwell *et al.* 1982) and the ability of *P. knowlesi* to vary the SICA Ag during infection is apparently dependent on the presence of appropriate variant-specific Ab. A study by Brown (1973) strongly implies that this variation is Ab-induced rather than immunoselective. Variant-specific Ab levels determined by the SICA test do not correlate with protective immunity (Brown *et al.* 1970 a & b; Butcher & Cohen 1972). However, either variant-specific opsonising Ab (Brown *et al.* 1970b; Brown 1971) or specific inhibitory Ab assayed by *in vitro* culture (Butcher & Cohen 1972) consistently correlated with immune status and such Ab was predominantly variant-specific. Brown & Hills (1974) proposed that SICA Abs induce antigenic variation and opsonising Abs are parasiticidal. Both types of variant-specific Abs can be detected during *P. knowlesi* infection in rhesus monkeys, SICA Abs appearing much earlier than opsonising Abs (Brown & Hills 1974). As the host develops immunity during chronic infection, both Ab types appear much more quickly and simultaneously.

The early *P. knowlesi* studies described above were all performed using uncloned parasite lines but subsequent studies with cloned parasites have confirmed many of the earlier results and the occurrence of antigenic variation in *P. knowlesi* (Barnwell *et al.* 1983 a & b) The SICA Ag(s) has been identified from parasite clones as a high MW protein of between 180-225 kD by immunoprecipitation only with the homologous anti-variant Ab (Howard *et al.* 1983). These Ags are soluble in SDS but not Triton X-100 (Howard & Barnwell 1984), are malarial proteins, quantitatively minor, present at the cell surface and susceptible to trypsin (Howard *et al.* 1983, 1984). Howard & Barnwell (1985) detected at least 10 different variant Ag phenotypes by immunochemical analysis and showed that in SICA-negative pRBC obtained by passage in splenectomised monkeys, there is a lack of expression of the variant Ag rather than expression of different non-functioning variants.

Other simian malaria parasites have been shown to undergo antigenic variation but have not been studied as extensively as *P. knowlesi.* Voller & Rossan (1969a) found evidence of antigenic variation occurring in *P. cynomolgi bastianellii* and Handunnetti *et al.* (1987) showed antigenic variation in *P. fragile*. In the latter study, the parasites were studied in their natural host, *Macaca sinica*, the toque monkey. Antigenic variation in this natural host-parasite combination was shown to occur during the spontaneous evolution of infection and there was a sequential order of appearance of variant antigenic types.

Evidence of antigenic variation occurring in human malaria parasites is confined

to *P. falciparum*. The first indications came from studies on *P. falciparum* infections in the squirrel monkey, *Saimiri sciureus* (Hommel *et al.* 1983). Immune monkey serum was used in IFATs on live, schizont-infected RBC to detect Ags on the surface of the RBC, and using this method, parasites isolated during recrudescent peaks were shown to be antigenically different from the original parasite population. In total, 7 variants were derived from the Indochina-1 strain of *P. falciparum* in this study. This work was performed with an uncloned isolate of *P. falciparum* and therefore cannot be taken as conclusive of antigenic variation occurring in *P. falciparum*. However, cloned isolates were also studied and shown to undergo modulation of surface Ags upon transfer from splenectomised to intact monkeys, indicating the occurrence of antigenic variation in clonal *P. falciparum*. DNA fingerprinting studies with variant populations of the Indochina-1 strain of *P. falciparum* showed that phenotypic variation, detected by variant-specific sera and IGSS, was not accompanied by major genomic reorganisation (Hommel *et al.* 1991). In another study, resistant parasites emerged from a Palo Alto strain *P. falciparum* infection in *Saimiri* monkeys after passive transfer of specific Abs. Monkeys primed against the original parasites were susceptible to challenge with the resistant ones, and vice versa (Fandeur *et al.* 1995). The resistant parasites were found to be antigenically distinct from the original infecting parasites but molecular typing indicated them to be isogenic.

Cloned *P. falciparum* has also been shown to undergo antigenic variation *in vitro* , using agglutination, cytoadherence inhibition and immunoprecipitation (Biggs *et al.* 1991), or a 'mixed agglutination assay' (Roberts *et al.* 1992). The latter study demonstrated that antigenic variation may occur *in vitro* at a rate as high as 2% per generation in the absence of immune pressure.

The parasite protein involved in antigenic variation in *P. falciparum* is known as PfEMP1 (*P. falciparum* erythrocyte membrane protein 1) (Biggs *et al.* 1991; Roberts *et al.* 1992). As with the SICA Ag of *P. knowlesi*, this molecule was identified as a strain-specific malarial Ag exposed on the surface of infected RBC by immunoprecipitation using strain-specific sera, with different parasite strains possessing proteins of varying MW (Leech *et al.* 1984; Howard *et al.* 1988). PfEMP1 is a high MW protein of between 200000-350000 D, quantitatively minor, soluble in SDS but not in Triton X-100, suggestive of a close association with the RBC cytoskeleton (Howard *et al.* 1988), and susceptible to trypsin (Leech *et al.* 1984). It therefore shares several properties with the SICA Ag of *P. knowlesi* (reviewed by Howard & Barnwell 1983; Howard 1984). Generally, sera which react positively by live IFAT with, or which agglutinate with, *P. falciparum*-infected RBC are the only sera to immunoprecipitate ^{125}I-labelled PfEMP1 from any particular strain (Howard *et al.* 1988; van Schravendijk *et al.* 1991; Biggs *et al.* 1992).

PfEMP1 exhibits a high degree of antigenic diversity between different parasite

isolates (Hommel *et al.* 1983; Aley *et al.* 1984; Leech *et al.* 1984; Marsh & Howard 1986; Magowan *et al.* 1988; Forsyth *et al.* 1989; Newbold *et al.* 1992; Iqbal *et al.* 1993). Such studies also show that individuals respond to *P. falciparum* infection by producing isolate-specific Abs against PfEMP1. Levels of these Abs have been shown to correlate with protection against disease (Marsh *et al.* 1989), indicating that variant Ags may be important targets for protective immune responses against *P. falciparum* (Mendis *et al.* 1991).

The molecular basis of antigenic variation in *Plasmodium* remains to be fully elucidated. However, a family of 50-150 genes shown to encode PfEMP1 has recently been identified (Baruch *et al.* 1995; Su *et al.* 1995), which should open the way to a fuller understanding of the genetic mechanisms underlying antigenic variation. Members of the *var* gene family are expressed differentially in different parasite lines (Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995), with transcription of distinct *var* genes corresponding to expression of distinct variant Ags on the surface of pRBC (Smith *et al.* 1995). These genes are scattered over multiple malaria chromosomes (Su *et al.* 1995; Peterson *et al.* 1995), with some in clusters (Su *et al.* 1995) and are located in the subtelomeric regions (Rubio *et al.* 1996). It is estimated that they constitute 6% of the malaria genome, and, as they appear to be evolving at a very high rate, a substantial proportion may be non-functional (Borst *et al.* 1995).

The strain-specific sera initially used to identify PfEMP1 (Leech *et al.* 1984) were shown to be strain-specific by their ability to inhibit cytoadherence of pRBC *in vitro* (Udeinya *et al.* 1983). This was in itself suggestive of a link between expression of variant Ags and cytoadherence properties of *P. falciparum* pRBC. These cytoadherence properties are described in 1.5.3. Both characteristics arise at a similar time, during the later stages of the erythrocytic cycle and a link between the adherent and antigenic components of the surface of pRBC was proposed as early as 1981, by Udeinya *et al.*, on the basis of both occurring at knobs. Antigenic variation in *P. falciparum* is modulated by the spleen (Hommel *et al.* 1983), as is sequestration and the ability of pRBC to cytoadhere *in vitro* (David *et al.* 1983). Expression of *P. falciparum* variant Ags detected by live IFAT is sensitive to trypsin (Hommel *et al.* 1983), as is PfEMP1 (Leech *et al.* 1984), and cytoadherence (David *et al.* 1983). Such observations reinforced the concept of a linkage between variant Ags and cytoadherence (David *et al.* 1983; Hommel 1985), whilst at the time evidence for this was only circumstantial. Further evidence arose when expression of variant Ags was shown to correlate with different cytoadherence phenotypes *in vitro* (Magowan *et al.* 1988). A link between antigenic variation and sequestration in *P. chabaudi* was demonstrated (Gilks *et al.* 1990), and antigenic variation of *P. falciparum in vitro* is associated with size changes in PfEMP1 and changes in adhesive phenotype (Biggs *et al.* 1992; Roberts *et al.* 1992). In the published work describing the cloning of the gene for PfEMP1, Abs generated

against recombinant fusion proteins recognised PfEMP1, reacted by live IFAT with the surface of pRBC in a strain-specific manner and blocked adherence to CD36 (Baruch *et al.* 1995). Switches in expression of *var* genes also result in changes in antigenic and cytoadherent phenotypes (Smith *et al.* 1995). Finally, PfEMP1 has recently been shown directly to bind to CD36, ICAM-1 and TSP (Baruch *et al.* 1996). These two immune evasion mechanisms employed by pRBC, antigenic variation and cytoadherence, are thus due to the same parasite molecule, and therefore inextricably linked. The identification of the *var* gene family will hopefully lead to an increased understanding of both the molecular basis of antigenic variation and cytoadherence, which may guide vaccine development and therapeutic approaches to decreasing the pathology of malaria due to sequestration *in vivo*.

1.5.3 Sequestration and Cytoadherence

Some species of malaria parasites show withdrawal from the peripheral circulation (sequestration) of late trophozoite- and schizont-containing pRBC (Garnham 1966). In *P. falciparum* infections in humans, sequestration of schizonts is almost complete, and occurs in post-capillary venules of a variety of organs including the placenta (Jilly 1969; McGregor 1978; Bray & Sinden 1979) and heart (Merkel 1946), but most notably the brain (Rigdon 1942; Spitz 1946; Clark & Tomlinson 1949; MacPherson *et al.* 1985; Oo *et al.* 1987). This appears to be the major contributing factor in the development of cerebral malaria (MacPherson *et al.* 1985; Oo *et al.* 1987; Warrell 1987; Aikawa 1988). Sequestration is also seen to a similar extent in *P. falciparum* infections in *Aotus* monkeys, though the major sites are the heart, adipose tissue and spleen (Miller 1969; Voller *et al.* 1969; Gutierrez *et al.* 1976), without major cerebral involvement. In *Saimiri* monkeys, *P. falciparum* also sequesters, but this is not as marked as in humans (David *et al.* 1983). It is apparent, therefore, that host factors contribute to the extent and site of sequestration, at least in *P. falciparum* infections. Sequestration does not occur in any of the other human malarias (Howard 1988).

Some other primate malarias exhibit sequestration to a degree. This is most marked with *P. coatneyi* and *P. fragile* in both natural and unnatural hosts (Desowitz *et al.* 1969; Fremount & Miller 1975), with parasites localising mostly to cardiac muscle, but also to adipose tissue and small bowel mucosa. *P. knowlesi* shows only slight sequestration at low parasitaemias in rhesus monkeys, with parasites localising to the liver and small intestine (Miller *et al.* 1971a). At higher parasitaemias, schizonts are seen in peripheral blood (Miller *et al.* 1971a).

Sequestration also occurs in some murine malarias. In *P. berghei*, the major sites are bone marrow, liver and spleen (Alger 1963; Miller & Fremount 1969). Cerebral involvement has also been observed in the ANKA strain of *P. berghei*, with accumulation of pRBC, nRBC and macrophages in cerebral blood vessels in mice

(Mackey *et al.* 1980; Rest 1982). This has been used as a model for human cerebral malaria (e.g. Grau *et al.* 1987). In *P. chabaudi* infection, sequestration occurs (McDonald & Phillips 1978; Cox *et al.* 1987; Gilks *et al.* 1990), with the liver as the major site of pRBC accumulation (Cox *et al.* 1987) but with no cerebral sequestration. However, in mixed experimental infections of *P. berghei* and *P. chabaudi*, cerebral sequestration of *P. chabaudi* can be observed (Dennison & Hommel 1993; Hommel 1993).

Sequestration is clearly a parasite-induced process (Chulay & Ockenhouse 1990). Hypotheses proposed to account for this (reviewed by Howard 1988) include the requirement for a relatively anoxic environment, and avoidance of splenic filtration. *P. falciparum* asexual stage parasites, particularly the mature forms, grow best *in vitro* under conditions of relatively low oxygen tension (Scheibel *et al.* 1979), conditions similar to those encountered in the sites of sequestration *in vivo*. Mature *P. falciparum*-infected RBC contain a large parasite inclusion and have greatly impaired deformability compared to nRBC and to RBC containing early asexual stage parasites (Cranston *et al.* 1984). By sequestering and thereby not passing through the spleen, the splenic mechanisms for removal of such 'damaged' RBC (Quinn & Wyler 1979b; Wyler *et al.* 1981) are avoided. There are also parasite-derived neo-Ags expressed on the surface of pRBC containing mature asexual stage parasites (see 1.5.2). Sequestration allows immune recognition and clearance in the spleen (reviewed by Kreier & Green 1980; see 1.4.2 c, section 7) to be avoided. Further evidence for the hypothesis of splenic avoidance comes from work showing that sequestration ceases in splenectomised animals (David *et al.* 1983). Such hypotheses may account for the greater virulence of *P. falciparum* over the other human malarias, which do not sequester.

Sequestration is due to cytoadherence of mature pRBC to endothelial cells lining post-capillary blood vessels (Miller 1969; Luse & Miller 1971; MacPherson *et al.* 1985; Oo *et al.* 1987; Aikawa 1988). Mature *P. falciparum* pRBC also cytoadhere to human platelets (Ockenhouse *et al.* 1989), monocytes (Barnwell *et al.* 1985; Goldring *et al.* 1992), lymphocytes, neutrophils, plasma cells (Ruangjirachuporn *et al.* 1992), uninfected RBC (known as 'rosetting') (David *et al.* 1988; Handunnetti *et al.* 1989; Udomsangpetch *et al.* 1989b; Wahlgren *et al.* 1989), and also other pRBC (Roberts *et al.* 1992). Mostly, these observations have been made *in vitro*, although rosetting has been demonstrated *in vivo* (David *et al.* 1988). It is likely that all of these cell-cell interactions occur *in vivo*, but to what extent is uncertain (Berendt *et al.* 1990, 1994; Howard *et al.* 1990).

In vitro cytoadherence of *P. falciparum* pRBC to a variety of cell lines or transfected cells expressing human endothelial cell surface proteins has also been observed (reviewed by Hommel 1990; Pasloske & Howard 1994a). Reports of this phenomenon include binding to human umbilical vein endothelial cells (HUVEC)

(Udeinya *et al.* 1981), C32 amelanotic melanoma cells (Schmidt *et al.* 1982), SK-MEL-23 melanoma cells (Panton *et al.* 1987), CD36-deficient C32 cells (Ockenhouse *et al.* 1991a), human dermal microvasculature endothelial cells (Johnson *et al.* 1993), myelomonocytic U937 cells (Goldring *et al.* 1992), human brain capillary endothelial cells (Smith *et al.* 1992), and Chinese hamster ovary cells stably transfected with genes for human CD36 or intercellular adhesion molecule 1 (ICAM-1) (Hasler *et al.* 1993). Binding studies using many of these cell lines have been critical to identifying the human molecules likely to act as receptors on endothelial cells for pRBC. The molecules shown to mediate binding *in vitro* include TSP (Roberts *et al.* 1985), CD36 (Barnwell *et al.* 1985), ICAM-1 (Berendt *et al.* 1989), vascular cell adhesion molecule 1 (VCAM-1), E-selectin (Ockenhouse *et al.* 1992b) and chondroitin-4-sulphate (Rogerson *et al.* 1995). *In vitro* cytoadherence of *P. chabaudi* pRBC to some mouse cell lines has also been observed (Cox *et al.* 1987), but the host molecules acting as receptors have not been identified.

TSP is a secreted glycoprotein expressed in a number of cell types including endothelial cells, epithelial cells, smooth muscle, fibroblasts and macrophages (Lawler 1986). It is present *in vivo* at low levels throughout the microvasculature (Turner *et al.* 1994). TSP is a multifunctional, multidomain protein which can bind to many different ligands, and is thought to be involved in a number of pathogenic events requiring immobilisation in blood vessels, including adhesion of sickled reticulocytes (Sugihara *et al.* 1992) and of *Babesia bovis*-infected RBC (Parrodi *et al.* 1989) to endothelium. *P. falciparum*-pRBC were found to bind to purified TSP immobilised to plastic (Roberts *et al.* 1985); this binding is calcium-dependent and is inhibited by both anti-TSP Ab and soluble TSP (Roberts *et al.* 1985; Barnwell *et al.* 1989). Anti-TSP Ab and soluble TSP also inhibited pRBC binding to rat microvessels in an *ex vivo* model (Rock *et al.* 1988) and were initially reported to inhibit pRBC binding to C32 amelanotic melanoma cells (Roberts *et al.* 1985); this finding has since been challenged (Barnwell *et al.* 1989; Sherwood *et al.* 1990), implying that TSP is unnecessary for binding to these cells. Nearly all wild isolates of *P. falciparum* examined bind to immobilised TSP (Sherwood *et al.* 1987; Hasler *et al.* 1990). This property of pRBC seems to be invariant, with no alterations in levels of binding to TSP observed with antigenic switching *in vitro* and concomitant changes in binding to CD36 and ICAM-1 (Gardner *et al.* 1996).

CD36 is an integral membrane glycoprotein found on a variety of cell types including endothelial cells, platelets, monocytes, macrophages, erythroid precursors and melanoma cells (Talle *et al.* 1983; Knowles *et al.* 1984; Barnwell *et al.* 1985; Edelman *et al.* 1986; Greenwalt *et al.* 1992). The biological function(s) of CD36 are unclear, but it has been reported to bind to TSP (Asch *et al.* 1987) and collagen (Tandon *et al.* 1989) and to act in signal transduction (Greenwalt *et al.* 1992). The first evidence of CD36 acting as an adhesive receptor for *P. falciparum* pRBC came from studies using mAb

OKM5, which was found to block binding of pRBC to C32 melanoma cells, monocytes and endothelial cells (Barnwell *et al.* 1985). The Ag recognised by this mAb has subsequently been identified as CD36 (Asch *et al.* 1987). pRBC also bind to purified CD36 immobilised on plastic (Barnwell *et al.* 1989; Ockenhouse *et al.* 1989; Hasler *et al.* 1990) and to COS cells and CHO cells transfected with genes encoding CD36 (Oquendo *et al.* 1989; Hasler *et al.* 1993). Anti-CD36 mAbs have been shown to block binding of pRBC to purified CD36, HUVEC, C32 melanoma cells and CD36-transfected cells (Barnwell *et al.* 1989; Berendt *et al.* 1989; Ockenhouse *et al.* 1989; Oquendo *et al.* 1989). CD36 in solution binds directly to pRBC and has been shown also to inhibit pRBC binding to the purified receptor, to C32 melanoma cells and to HUVEC (Barnwell *et al.* 1989; Ockenhouse *et al.* 1989). Studies examining wild isolates binding to purified CD36 either found little variation in binding ability (Hasler *et al.* 1990) or a wide degree of variation (Ockenhouse *et al.* 1991a). Binding studies using C32 melanoma cells, binding to which is predominantly CD36-dependent (Barnwell *et al.* 1989; Ockenhouse *et al.* 1991a), also found a wide variation in binding of wild isolates (Marsh *et al.* 1988; Ho *et al.* 1991). Binding to CD36 also changes with antigenic switching *in vitro*, indicating that adherence to CD36 is a variable property of pRBC (Gardner *et al.* 1996).

CD36 may also be involved in rosette formation, as it is found at low densities on RBC (van Schravendijk *et al.* 1992) and anti-CD36 mAbs and soluble CD36 can reverse rosetting (Handunnetti *et al.* 1992), although Wahlgren *et al.* (1994) claim that rosetting is dependent on CD36 in only a relatively small number of parasite lines.

ICAM-1 is an integral membrane glycoprotein expressed on the surface of lymphocytes, monocytes, macrophages, fibroblasts, epithelial cells and endothelial cells (Dustin *et al.* 1986). ICAM-1 is the ligand for the leucocyte function associated molecule 1 (LFA-1) (Marlin & Springer 1987) and is critically involved in leucocyte-leucocyte adhesion and leucocyte-endothelial adhesion (reviewed by Carlos & Harlan 1994). It is also the receptor for human rhinoviruses (Staunton *et al.* 1989). Expression of ICAM-1 can be induced on endothelial cells by inflammatory cytokines such as TNF, IL-1 and IFN-γ (Pober *et al.* 1986). ICAM-1 was identified as an adhesive receptor for *P. falciparum* when pRBC of a parasite line that was repeatedly selected for high levels of binding to HUVEC were found to bind to ICAM-1-transfected COS cells (Berendt *et al.* 1989). Anti-ICAM-1 mAbs inhibit binding to both HUVEC- and ICAM-1-transfected COS cells, while pRBC bind to purified ICAM-1 immobilised on plastic, which can be blocked by anti-ICAM-1 mAbs (Berendt *et al.* 1992; Ockenhouse *et al.* 1992a). Binding to ICAM-1 is highly variable between parasite isolates (Ockenhouse *et al.* 1991a) and changes with antigenic switching *in vitro* (Gardner *et al.* 1996).

VCAM-1 and E-selectin are two leucocyte adhesion molecules expressed on

activated but not on unactivated endothelial cells. Expression of these molecules can be induced by a number of stimuli, including TNF, IL-1, lipopolysaccharide (reviewed by Pigott & Power 1993) and pRBC (Udeinya & Akogyeram 1993). VCAM-1 and E-selectin immobilised on plastic were both found to bind pRBC of a particular wild isolate at low levels and anti-E-selectin Abs blocked adhesion of this wild isolate to TNF-activated HUVEC. A cloned parasite line derived from this isolate, which was obtained after selection for binding, showed increased binding to E-selectin and VCAM-1. Binding of this parasite clone to E-selectin was inhibited by anti-E-selectin Abs and to VCAM-1 by an anti-VCAM-1 mAb (Ockenhouse *et al.* 1992b).

Chondroitin-4-sulphate is a glycosaminoglycan expressed by various cell types and can be detected on resting human cerebral endothelium (Aikawa *et al.* 1990). *P. falciparum* pRBC selected for high binding to CHO cells were found to adhere to CHO cells expressing chondroitin sulphate but not to CHO cell mutants not expressing chondroitin sulphate (Rogerson *et al.* 1995). This binding was inhibited by pre-treating the CHO cells with chondroitinase. pRBC also bound to immobilised chondroitin-4-sulphate, which, as well as the binding to CHO cells, was inhibited by soluble chondroitin-4-sulphate. This adhesive phenotype may occur fairly frequently, and although binding is at low densities, it may be clinically relevant for some wild isolates of *P. falciparum* (Chaiyaroj *et al.* 1996).

Several studies have investigated the relationship between cytoadherence and disease in *P. falciparum* infections. No correlation between disease severity and binding to TSP was noted in two separate studies (Sherwood *et al.* 1987; Hasler *et al.* 1990), with TSP binding being high in all isolates examined. Cytoadherence either to purified CD36 or to C32 melanoma cells does not differ significantly in isolates from cerebral malaria compared to isolates from non-severe cases (Marsh *et al.* 1988; Ho *et al.* 1991; Ockenhouse *et al.* 1991a; Treutiger *et al.* 1992). Also, no correlation between disease severity and binding to ICAM-1 or HUVEC was observed in three studies (Ockenhouse *et al.* 1991a; Cooke *et al.* 1993; Ringwald *et al.* 1993), although in cases of fatal malaria expression of ICAM-1 is markedly raised in vascular endothelium (Turner *et al.* 1994). Parasite isolates may also show high levels of cytoadherence in one assay but not in another (Goldring *et al.* 1992). All these binding studies, whilst indicating cytoadherence phenotypes of parasite isolates, do not reflect the actual receptor profiles of the original hosts, which may vary both quantitatively and qualitively between hosts, between different sites in individual hosts and at different times during infection, due to differential stimulation by various factors including cytokines. One study which examined both together, using the patient's own peripheral blood monocytes taken during acute infection and during convalescence, showed a correlation between binding of pRBC to 'acute' monocytes and disease severity (Goldring & Hommel 1992). A correlation between disease severity and rosetting has

also been observed for *P. falciparum* in some studies (Carlson *et al.* 1990; Ho *et al.* 1991; Treutiger *et al.* 1992; Ringwald *et al.* 1993; Rowe *et al.* 1995) Rosette formation may augment sequestration (Kaul *et al.* 1991; Nash *et al.* 1992), suggesting a role in the onset of severe disease. Another study, however, did not corroborate these findings (Al-Yaman *et al.* 1995), while rosetting has been observed among several isolates of *P. vivax* (Udomsangpetch *et al.* 1995), a species which does not cause cerebral malaria.

Uninfected RBC and ring-stage pRBC show none of the adherence characteristics displayed by late stage pRBC (Udeinya 1990). The acquisition of these adherence properties must reflect changes in the pRBC membrane, and occurs concurrently with the development of knobs and the expression of variant Ags on the surface of pRBC. Knobs can be seen by both scanning and transmission electron microscopy to be 100nm submembranous protusions on the pRBC surface, each underlaid by electron dense material thought to be the structural component of the knob (Trager *et al.* 1966; Luse & Miller 1971). This includes the knob-associated histidine-rich protein (KAHRP or PfHRP1) (Kilejian 1979) which is associated with the cytoskeleton. The number of knobs increases with parasite maturation, whilst the size of each knob decreases (Gruenberg *et al.* 1983). Knobs are usually the points of contact for cytoadherence (Luse & Miller 1971; Udeinya *et al.* 1981; MacPherson *et al.* 1985) and are the location of parasite adhesins (Nakamura *et al.* 1992; Baruch *et al.* 1995), but their function is unknown.

For many years, knobs were thought necessary for *P. falciparum* cytoadherence, but this is now known not to be the case. It is well established that knob formation is insufficient for cytoadherence (David *et al.* 1983; Udeinya *et al.* 1983) and cytoadherence of knobless strains has been observed upon repeated selection for binding to melanoma cells *in vitro* (Biggs *et al.* 1989; Udomsangpetch *et al.* 1989a). Other malaria species, including *P. knowlesi* (Miller *et al.* 1971a), *P. berghei* (Alger 1963) and *P. chabaudi* (Cox *et al.* 1987), as well as immature gametocytes of *P. falciparum*, sequester but do not possess knobs. However, it is likely that knobs are advantageous *in vivo*, as they have been present on all wild isolates examined (Sherwood *et al.* 1987, 1989; Marsh *et al.* 1988; Ruangjirachuporn *et al.* 1992). Knobs may play a role in aiding pRBC cytoadherence by either projecting adherence molecules out from the pRBC surface, in a similar, if less marked manner, to microvilli on leucocytes, thought to potentiate adhesion to endothelium (Picker *et al.* 1991; Berlin *et al.* 1995; Scholander *et al.* 1996), or by clustering adherence molecules (Nakamura *et al.* 1992), thereby increasing binding avidity by ensuring multiple bonds have to be broken at any one time in order to prevent or suspend pRBC cytoadherence.

Several molecules have been proposed as candidate adherence ligands on the surface of pRBC (reviewed by Hommel & Semoff 1988; Howard 1988). These include: HRP 1 (Kilejian 1979); PfEMP1 (Leech *et al.* 1984); PfEMP2, also called

mature parasite-infected erythrocyte surface Ag (MESA) (Coppel *et al.* 1986; Howard *et al.* 1987); an Ag called sequestrin (Ockenhouse *et al.* 1991b), which is probably PfEMP1 (Pasloske & Howard 1994a); Ag 332 (Mattei & Scherf 1992); and modified (Crandall *et al.* 1993) or truncated (Sherman *et al.* 1995) band 3. Molecules thought to be involved in rosetting, including blood group Ags, have also been identified (Carlson & Wahlgren 1992; Udomsangpetch *et al.* 1993; Rowe *et al.* 1994).

PfEMP1 has been identified as the parasite molecule involved in antigenic variation and involved in cytoadherence (see 1.5.2) and has now been shown directly to bind to CD36, ICAM-1 and TSP (Baruch *et al.* 1996). The general consensus is that PfEMP1 is the major parasite molecule involved in cytoadherence, although this does not exclude the possibility of other molecules being involved.

HRP1 is an 80-120kDa parasite protein exported to the RBC membrane during the later stages of the erythrocytic cycle (Kilejian 1979). It is associated with knobs but is not surface-exposed (Taylor *et al.* 1987) and is therefore unlikely to act as an adherence ligand. However, HRP1 may promote cytoadherence, possibly by aiding knob formation, a notion consistent with the observation that deletion of the HRP1 gene results in loss of knobs (Pologe & Ravetch 1986; Biggs *et al.* 1989).

PfEMP2 shows variation in MW and is associated with knobs, but is not exposed on the surface of pRBC (Coppel *et al.* 1986; Howard *et al.* 1987) and expression of this molecule is not required for cytoadherence (Petersen *et al.* 1989). It now seems unlikely that PfEMP2 is involved in cytoadherence.

Ag332, also called Pf332, is a giant protein of 2.5 MDa, identified by a human mAb, 33G2. This mAb inhibits cytoadherence of some parasite lines to melanoma cells (Udomsangpetch *et al.* 1989a), although not completely. Abs affinity purified on a Pf332 repeat peptide do not inhibit cytoadherence and it is likely that mAb 33G2 cross-reacts with another, as yet unidentified, molecule (Iqbal *et al.* 1993).

Band 3 is a transmembrane protein of 95kDa and is the major anion transporter in RBC. Two proteins which could not be labelled metabolically and were identified as cleavage products of band 3 were immunoprecipitated by mAbs which reacted with the surface of pRBC and blocked *in vitro* cytoadherence to C32 melanoma cells (Winograd & Sherman 1989; Crandall & Sherman 1991). Peptides representing regions of band 3 also block cytadherence *in vitro* and prevent sequestration *in vivo* in *P. falciparum*-infected monkeys (Crandall *et al.* 1993). Initially, it was thought that a modification of a host sequence resulted in the adherence properties of band 3. It is now suggested, however, that the conformation and topography of band 3 peptides is of importance, with extensive deformation of the protein structure in truncated forms on mature *P. falciparum* pRBC exposing a previously cryptic adhesin, pfalhesin (Guthrie *et al.* 1995; Sherman *et al.* 1995). The host receptor identified for this adherence is CD36 (Crandall *et al.* 1994). The role of truncated forms of band 3 in cytoadherence and sequestration

is as yet unclear, but it is possible that interaction with PfEMP1 is needed for efficient CD36-mediated binding. Pfalhesin may participate in rosetting as well as in cytoadherence, and in the absence of pRBC surface knobs, it is thought that rosetting may be the favoured of the two cell-cell interactions (Crandall *et al.* 1994; Sherman *et al.* 1995).

1.6 Vaccine development

The need for effective vaccines against malaria has become increasingly apparent due to the limited effectiveness of currently available control measures. The life cycle of *Plasmodium* offers several possible vaccine strategies. Many Ags are presented to the immune system, but most are not suitable as vaccine candidates as they show considerable antigenic diversity or are poorly immunogenic, or elicit an inappropriate immune response (Miller *et al.* 1986). Immunity to malaria parasites appears to be largely stage-specific. Therefore, an effective vaccine may need to be multicomponent, providing protective immunity by generating the appropriate immune response (Ab, $CD4^+$ or $CD8^+$ T cell) against more than one, and perhaps all, stages of the malaria life cycle (Nussenzweig & Long 1994). In theory, this could be achieved with synthetic peptide constructs, DNA vaccines, purified recombinant proteins, or through live viral, fungal or bacterial expression, and each of these approaches is being actively investigated.

1.6.1 Pre-erythrocytic stage targets

Sporozoites attenuated by irradiation have long been known to give excellent protection against subsequent viable challenge in animals, including humans (reviewed by Jones & Hoffman 1994), though many infective bites from irradiated mosquitoes are needed to confer resistance. Most attempts to reproduce this immunity have focussed on recombinant or synthetic expression of part of the circumsporozoite protein (CSP), and in particular, the region of the molecule that comprises tandem repeats of short sequences of amino acids. In *P. falciparum*, a four amino acid sequence, asparagine-alanine-asparagine-proline (NANP), is repeated, but perhaps because it is immunodominant during natural infections, the many small-scale clinical trials with candidate vaccines based on this structure have disappointed in terms of protection achieved (reviewed by Phillips 1992; Jones & Hoffman 1994). It may not be possible to reproduce the strong immunity induced by attenuated sporozoites in this way, as some of the long-lived protection elicited is thought to be a consequence of their ability to invade hepatocytes and thereby induce a variety of immune effector mechanisms targetting Ags other than CSP (Good *et al.* 1993). Nevertheless, further experimental and early clinical studies designed to enhance sporozoite-directed immunisation are continuing: these include use of multiple Ag peptides containing the NANP repeat as a

B cell epitope and T cell epitopes from tetanus toxin (Wang *et al.* 1995); immunisation with vaccinia and influenza virus constructs expressing B cell or $CD8^+$ T cell epitopes of CSP (Hoffman *et al.* 1994; Rodrigues *et al.* 1994); NANP repeats plus the C terminus of CSP co-expressed in yeast with hepatitis B surface Ag (Gordon *et al.* 1995); and oral immunisation with *P. falciparum* CSP expressed in *Salmonella typhi* (Gonzalez *et al.* 1994). A novel recent approach has been the injection of naked DNA encoding the CSP, which induced CTL and Ab responses and gave good protection against *P. yoelii* challenge in mice (Sedegah *et al.* 1994).

A different approach to induction of protective cytotoxic T lymphocyte (CTL) responses by vaccination has been pursued by Hill and colleagues. They showed that possession of the Bw53 class I HLA conferred protection against cerebral malaria and severe malarial anaemia (Hill *et al.* 1991). Reverse immunogenetics was then used in a search of pre-erythrocytic stage Ags for potential HLA-Bw53 epitopes. One epitope within the liver stage Ag (LSA-1), when expressed with HLA-Bw53, was recognised by CTLs from Gambians with the same class I Ag (Hill *et al.* 1992). Following extension of this study to include six HLA class I haplotypes common among both African and Caucasian populations, epitopes were found in four pre-erythrocytic stage Ags - CSP, LSA-1, TRAP (thrombospondin-related anonymous protein) and STARP (sporozoite threonine and asparagine rich protein). Screening cells from children and adults revealed CTLs in some individuals (Aidoo *et al.* 1995). The protective effect is presumed to be CTL destruction of infected hepatocytes, and the aim is that a subunit or recombinant vaccine based on the identified epitopes would induce significant CTL activity.

1.6.2 Transmission-blocking targets

The purpose of a vaccine against the sexual stages of the parasite would not be to protect the vaccinee from becoming infected, but instead the mosquito, thereby reducing the rate of transmission. In addition to a direct effect on the parasite inoculation rate, it would serve also to reduce the spread of genes responsible for drug or vaccine resistance, preserving the efficacy of other control measures. Effective, predominantly Ab-mediated, transmission-blocking immunity has been achieved experimentally and target Ags identified (reviewed by Kaslow 1993; Carter 1994). Immune responses directed against gamete Ags, such as Pfs230 and Pfs48/45 of *P. falciparum*, may be boosted further by infection as they are also expressed in circulating gametocytes. Although both have been sequenced and expressed in recombinant form, expression products that induce transmission-blocking Abs have not yet been made, probably due to difficulty in creating the tertiary structural conformation essential to the B cell epitopes (Carter *et al.* 1995). A second approach is to induce immune responses to ookinete surface Ags, such as Pfs25, expressed only in the mosquito. A yeast

recombinant form of Pfs25 has induced strong transmission-blocking immunity in rodents and monkeys (Kaslow *et al.* 1994) and has been approved for phase I clinical trials.

1.6.3 Asexual erythrocytic stage targets

The last decade has seen the identification, gene sequencing and expression, and experimental testing of several blood stage proteins as putative vaccine candidates (reviewed by Howard & Pasloske 1993; Jones & Hoffman 1994; Pasloske & Howard 1994b). As yet, none of these Ags appears to be especially potent in inducing protection alone, and it is therefore likely that an effective vaccine will combine a number of Ags. The most interesting approaches have been those that have tried to identify and then block functions vital to parasite development. Thus, the C-terminal 19 kDa portion of the merozoite surface Ag MSA-1 remains on the surface of merozoites while the rest of the molecule is cleaved and released at RBC invasion (Ling *et al.* 1994). Natural Ab responses to this fragment correlate with resistance, and vaccination with a recombinant form is highly effective in mice (Ling *et al.* 1994), although this success has yet to be repeated in primates. To optimise the outcome of vaccinations, highly conserved regions of immunogenic molecules rather than those that are variable are best selected. When this was done with MSA-2, immunity effective against heterologous as well as homologous challenge was achieved (Saul *et al.* 1992). The ectodomain of apical membrane Ag 1 (AMA-1) (Crewther *et al.* 1990) expressed in *E. coli* and refolded *in vitro* gave good but strain-specific protection against *P. chabaudi adami* in mice and a highly antigenic form of *P. falciparum* AMA-1 from *E. coli* has been prepared for clinical trials (Targett 1995).

1.6.4 SPf66

The vaccine developed by Patarroyo and colleagues is a synthetic peptide polymer. The monomeric form consists of the N terminal sequences from three asexual blood stage Ags, Pf83 (part of MSA-1), Pf35 and Pf55, hybridised with two NANP repeat sequences from the CSP of *P. falciparum*. The early trials of efficacy in South America involved many thousands of people and established its acute safety and immunogenicity but attracted criticism of the methodology employed (reviewed by Tanner *et al.* 1995). Subsequent trials reported from Colombia (Valero *et al.* 1993), Tanzania (Alonso *et al.* 1994), The Gambia (D'Alessandro *et al.* 1995; Leach *et al.* 1995) and Thailand (Nosten *et al.* 1996), and, when completed, the current further trials in The Gambia, Tanzania and Colombia, have been the subject of careful consideration. In Colombia, an overall protective efficacy of 33.6% against clinical malaria was achieved in an area of low transmission. In Tanzania, where malaria transmission is perennial and more intense, children 1-4 years of age were vaccinated and a level of protection of 31% was

reported. By contrast, in the Gambian trial, in which infants 6-11 months old were recruited, SPf66 did not protect against a first clinical bout of malaria, overall incidence of malaria attacks, or infection. It is thought that the young age of the Gambian children may have precluded acquisition of a level of immunological competence to be able to respond adequately to vaccination, probably linked to a prior lack of clinical malaria, whereas the Tanzanian children would have experienced one or more attacks, and perhaps had developing immunity as a consequence (D'Alessandro *et al.* 1995; Targett 1995). A further two year surveillance in The Gambia is in progress and it is possible that some protection may be seen in subsequent transmission seasons. The disappointing results of the recently published Thai trial, which showed an efficacy of -9% over a 15 month period among children aged 2-15 years living in an area of low and seasonal *P. falciparum* and *P. vivax* transmission (Nosten *et al.* 1996), suggests, however, that the initial optimism with which SPf66 was received may not be warranted.

The results of the SPf66 trials conducted in South America give an apparently reproducible, if relatively modest, level of protection. The true efficacy in trials elsewhere, with their much greater parasite challenge, may fall somewhere between the published results from Tanzania and those from The Gambia and Thailand, or the reported differences may be real, reflecting differences in age, exposure and parasite diversity. In order to establish precisely how the vaccine works, and whether it can lessen debilitation and severe morbidity as well as impacting on mortality, particularly in Africa, further field studies are required. However, the borderline efficacy reported against clinical malaria in the last two published trials, in The Gambia (D'Alessandro *et al.* 1995) and Thailand (Nosten *et al.* 1996), questions the justification for further evaluation of the vaccine potential of SPf66.

1.7 History and biology of *Plasmodium chabaudi chabaudi*

Plasmodium chabaudi chabaudi (hereafter referred to as *P. chabaudi*) was first isolated from the blood of thicket rats, *Thamnomys rutilans*, caught in the Central African Republic by Landau in 1965. The parasites infect mainly mature RBC (Landau 1965), although they can invade reticulocytes later in infection (Carter & Walliker 1975; Jarra & Brown 1989). Multiple infection of RBC with *P. chabaudi* can also occur (Carter & Walliker 1975).

P. chabaudi provides a good and accessible model for many aspects of malaria research. It has some important similarities to *P. falciparum* and is recognised as an animal model for the human parasite (Long 1988; Mons & Sinden 1990; Gilks *et al.* 1990). It forms a chronic, recrudescing, bloodstream infection which, in the natural host, can last for at least 2-3 years (Landau & Boulard 1978). Bloodstream infections are synchronous, although the asexual erythrocytic cycle is completed in only 24 h.

Peripheral withdrawal of schizonts to deep tissue capillaries also occurs (McDonald 1977; McDonald & Phillips 1978; Gilks *et al.* 1990) and antigenic variation of pRBC surface Ags is a feature of infection (McLean *et al.* 1982b).

Cloned, well-characterised lines of *P. chabaudi* were established by Carter & Walliker (1975) in laboratory mice from wild-caught isolates without any need for adaptation. These clones have been passaged cyclically in *Anopheles stephensi* and are free from contamination with other rodent malaria species and from pathogens such as *Eperythrozoon coccoides* and *Haemobartonella muris* (Cox 1978, 1988). Isoenzyme patterns have been established, which have well-defined provenances and remain close to those of the original isolate (Beale *et al.* 1978; Walliker, personal communication).

The AS strain of *P. chabaudi* in inbred NIH mice has a low rate of mortality and had been extensively used previously for various biological and immunological studies and in work examining antigenic variation in malaria parasites. This was therefore the parasite-host combination of choice in this study. NIH mice show a genetically-determined resistance to *P. chabaudi* AS (Stevenson *et al.* 1982), with infections lasting up to two months. The course of infection typically shows an acute primary parasitaemia followed by a period of subpatency and one or sometimes two short lasting recrudescences of low parasitaemia (McLean *et al.* 1982a).

1.8 Experimental Rationale

Antigenic variation is now an accepted feature of most, if not all, malaria parasites. It is a phenomenon that may be of importance in the severity and duration of malarial infection and disease (e.g. reviewed by Miller *et al.* 1994), but which is, however, still not fully understood.

The host-parasite relationship of *P. chabaudi* in NIH mice has been studied for several years in Professor Phillips' laboratory, in terms of both host immunity to infection and immune evasion by parasites. *P. chabaudi* has been shown to undergo antigenic variation during the course of infection using a passive transfer system (McLean *et al.* 1982b). By this method, analysis of recrudescent populations indicated a mix of antigenic types (McLean *et al.* 1986a), and changes in antigenic type after MT (McLean *et al.* 1987). Antigenic variants could also be detected as early as d 13 p.i. (McLean *et al.* 1990). An indirect fluorescent antibody test which detect Ags on the surface of live, *P. chabaudi* schizont-infected RBC (live IFAT) has been developed (McLean *et al.* 1986b). A cloned parent parasite population and parasite clones derived from a recrudecence of the parent infection were examined in this test using a panel of immune sera collected on d 16 & 17 p.i.. From the recrudescent parasites, a mix of antigenically variant populations were detected, different from the parent population. However, the immune sera reacted homologously at low titres with only some of the parasite populations (Brannan *et al.* 1993).

These observations represented the starting point for the work presented in this thesis. These same cloned *P. chabaudi* populations were studied in a series of experiments, with the aim of increasing our knowledge and understanding of antigenic variation in asexual erythrocytic malaria parasites.

As a result of the low or absent reactivity with the immune sera, a panel of hyperimmune sera was raised against the parasite populations, which was then used in the live IFAT to detect the antigenically variant populations. This confirmed the results attained with the immune sera, and demonstrated that some, but not all, the hyperimmune sera could react homologously to a high titre with the surface of pRBC. These sera were then also used to examine the effect of MT on the expression of variant Ags.

The live IFAT analysis showed possible differences in the immunogenicity between different variant parasite populations. Therefore, the behaviour of some populations was studied *in vivo*, in terms of the overall pattern of infection, reticulocyte invasion, and whether recrudescences were again antigenically variant from the infecting population.

Antigenic variation has been shown to occur at very high rates, up to 2% per generation, in *P. falciparum in vitro* (Roberts *et al.* 1992). Determination of the rate of antigenic variation is important as it pertains directly to the nature of the host-parasite relationship. Such a determination for *P. chabaudi in vivo* was deemed feasible with the availability of sera specific for some antigenic variants, and the use of a detection method similar to the live IFAT, IGSS (Hommel *et al.* 1991), which results in permanent preparations of pRBC detected by sera. Analysis of very large numbers of pRBC of individual variant Ag types (VATs) was possible, enabling the measurement of switching rates of individual VATs, and thereby providing estimates of overall rates of variation of *P. chabaudi in vivo.*

Expression of surface variant Ags has been correlated with cytoadherence of *P. falciparum in vitro* (Magowan *et al.* 1988), while antigenic variation in *P. falciparum* is associated with changes in cytoadherence phenotypes (Biggs *et al.* 1992; Roberts *et al.* 1992). A link has also been reported between sequestration and expression of variant Ags in *P. chabaudi* (Gilks *et al.* 1990). It was therefore considered of interest to examine the *P. chabaudi* variant populations in terms of both sequestration *in vivo* and cytoadherence *in vitro.* Given the link between loss of cytadherence and subtelomeric deletions in *P. falciparum* (Biggs *et al.* 1989), the chromosomes of the *P. chabaudi* variant populations were also examined by PFGE.

MAbs are considered powerful tools for applying to immunochemical and molecular studies. In the context of the project, mAbs specific for surface variant Ags may be used to examine the relationship between antigenic variation, sequestration and cytoadherence of malaria-infected RBC. To this end, mAb production against variant

parasite populations of *P. chabaudi* was undertaken.

The implications of the results presented, possibilities for future research and the role of murine models in the study of antigenic variation in malaria parasites are discussed.

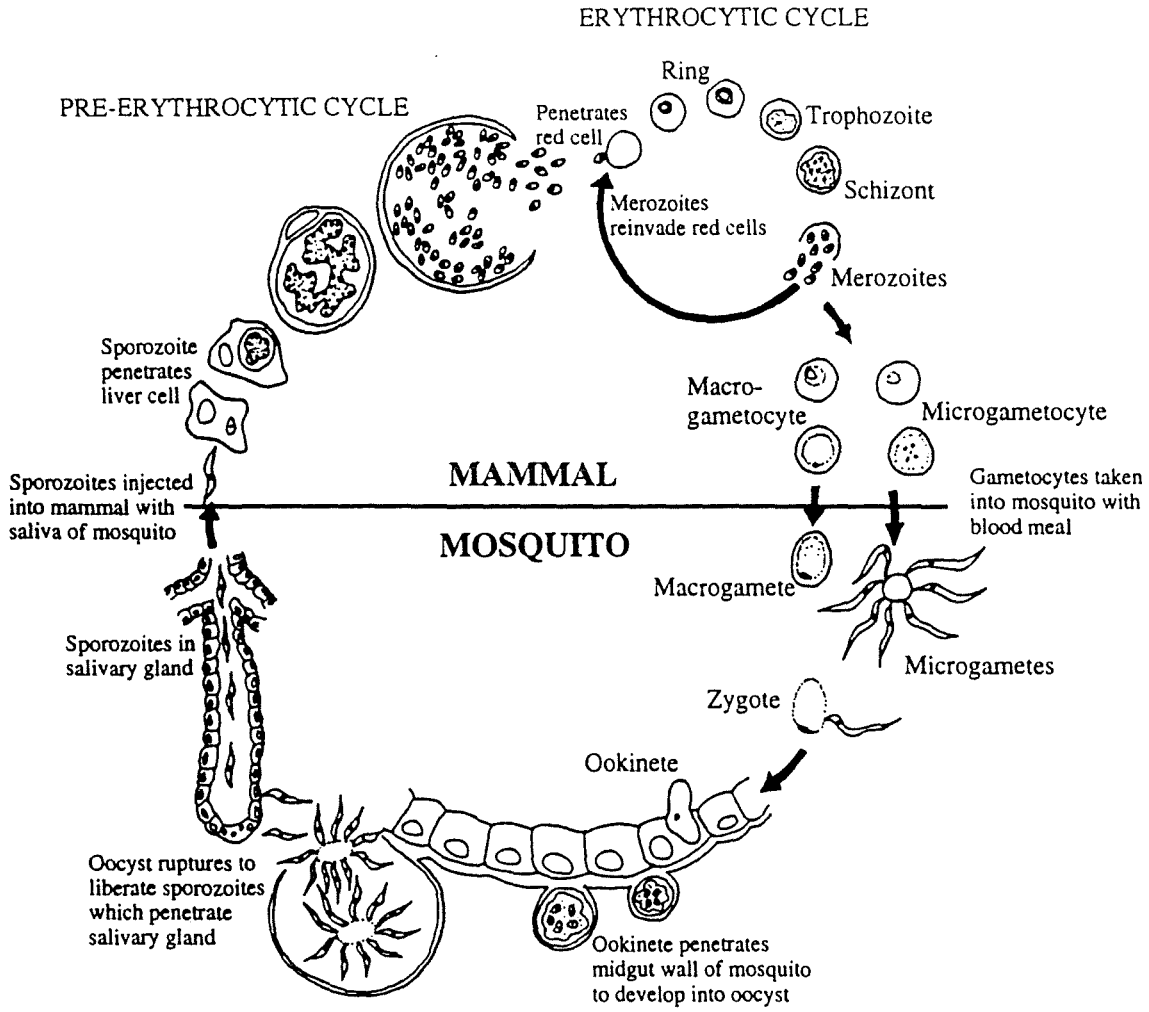


Figure 1.1 The life cycle of *Plasmodium* spp. in mammals (adapted from Vickerman & Cox 1967)

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

Inbred male NIH mice were used for most animal experiments. These were either bred in the WLEP animal house breeding facility or supplied by Interfauna (Huntingdon). Inbred BALB/c mice were also bred inhouse. All mice were kept at 22°C ± 2°C with 12 h normal light (NL) from either 0800 to 2000 h or a reverse light (RL) cycle from 2000 to 0800 h. They were fed on pelleted Labsure CRM breeder diet (Special Diet Services) and given both food and water *ad libitum*. For all experimental procedures, mice aged 8-16 weeks were used. Mice in RL were kept in this light cycle for a minimum of one week before use.

2.2 Parasites

The AS strain of *P. chabaudi chabaudi* had been isolated originally from thicket rats (*Thamnomys rutilans*) for Professor David Walliker (University of Edinburgh) in March 1969. The parasites were provided as a cloned mosquito-transmitted line by Professor Walliker to the University of Glasgow in 1973. The line has since been cloned twice by limiting dilution (Walliker *et al.* 1971). This cloned line is referred to as the parent population and all parasites have been derived from this. The history of the parent population is detailed in Fig. 2.1.

A recrudescence was collected from a mouse initially infected with the parent population when the parasitaemia was 1.54%. This and the infecting parent population were then cloned by limiting dilution as above. Cloning of the recrudescence yielded 10 clones. The derivation of these clones is described by McLean *et al.* (1986a). Herein, these recrudescent clones are referred to as recrudescent clone (RC) 1-10. The derivation and history of these recrudescent populations is detailed in Fig. 2.2.

All parasites were maintained in the laboratory by cryopreservation and serial subpassage of infected blood in mice (see 2.3 and 2.4).

2.3 Maintenance of parasites

For longterm preservation, parasite stabilates were stored in liquid N_2 (-196°C) (BOC). When required, infected blood was recovered from stabilate by the method of Mutetwa & James (1984 a & b). Each stabilate was defrosted by immersion of the cryotube (Nunc, Gibco) in water at 37°C, and then diluted with an equal volume of 15% w/v glucose in PBS (pH 7.2) (see Appendix A). This was then immediately injected i.v., via the lateral tail vein, into one or two naive mice.

Parasites were maintained by blood passage in mice every 3-4 d. Mice were bled by cardiac puncture, under ether anaethesia, into sodium heparin (1000 i.u./ml, Evans Medical Ltd.) in PBS at 10 i.u./ml blood. The infected blood was injected i.v. either immediately into recipient mice, or diluted to the required concentration of parasites in RPMI 1640 (Gibco) (see Appendix B) containing 5% FCS (Gibco), the parasites being

stored on ice until inoculation.

All parasite populations used experimentally were no more than 2-3 blood passages out of stabilate.

2.4 Cryopreservation of parasites

Parasites were cryopreserved as stabilates using the method of Phillips & Wilson (1978). Infected mice were bled by cardiac puncture when the majority of parasites were early ring stages (before 1000 h NL mice; after 1600 h RL mice) and the heparinised blood diluted 1:1 with sorbitol-glycerol (see Appendix C) added dropwise with frequent mixing (Gray & Phillips 1981). This was aliquoted into cryotubes (0.2-0.3 ml/tube) and snap frozen in liquid N_2. Each batch of stabilate was allocated a Wellcome Experimental Parasitology (WEP) number for reference.

2.5 Determination of parasitaemia

Parasitaemias were evaluated by examination of thin blood smears made from tail blood of infected mice. For NL mice, bloodsmears monitoring the course of infection were taken daily before 1200 h, and for RL mice, before 0900 h, in both cases before any peripheral withdrawal had occurred. In some experiments, on one day of infection only, hourly bloodsmears were taken either throughout the night (NL) or throughout the day (RL), over the period of time when peripheral withdrawal during schizogony occurs.

The blood smears were air-dried, fixed in 100% methanol (Analar, BDH Ltd.) for 1-2 min and stained in 10% Giemsa's stain (Gurr, BDH Ltd.) in phosphate buffer (pH 7.4) (see Appendix A) for 30 min. They were then rinsed in tap water and air-dried before examination under oil immersion using x100 objective and x10 eyepiece lenses on a Leitz S.M. Lux binocular optical microscope.

Parasitaemias were obtained by counting the % of RBC that were parasitised (pRBC). Parasitaemias were considered to be subpatent when no parasites were observed in 50 fields of view (approximately 10000 RBC). If the parasitaemia was ≥ 2-3% (> 3-4 parasites in a field of view), counts were made of 1-3 fields (at least 500 RBC). Lower parasitaemias were evaluated by counting numbers of parasites in 30 fields of view.

2.6 Presentation of parasitaemic data

For each course of infection, the day of infection was termed d 0. The course of infection in a group of mice is represented graphically by plotting the geometric mean of the parasitaemia (mean $\log_{10}$ of the number of pRBC/10^5 RBC) against time (expressed in days). Where parasitaemias were followed by hourly bloodsmears over one day of infection, these are presented graphically as mean % parasitaemia.

Peak parasitaemia data are presented graphically as median values ± interquartile

ranges, and where parasitaemic data are presented graphically in conjunction with reticulocyte data, median values are used.

2.7 Cloning of parasites

Parasites were cloned by limiting dilution in mice following the method of Walliker *et al.* (1971). Parasitised blood was collected from a *P. chabaudi*-infected mouse early in infection when the parasitaemia was < 2%, in order to minimise the risk of there being multiply-infected RBC. The parasitaemia was determined (see 2.5) (at least 2000 RBC were counted to ensured accuracy), and an accurate RBC count performed using a haemocytometer (improved Neubauer) in order to calculate the concentration of parasites in the blood. The blood was diluted accordingly in RPMI 1640 with 5% FCS and 1% normal mouse blood. Mice were infected i.v. with 0.2 ml of a suspension of infected blood containing 1 pRBC/ml medium. The mice were checked for parasites 8-15 d later, and where present, were preserved as stabilate.

2.8 Culture of parasites

Withdrawal from the peripheral circulation and sequestration in deep vascular tissue has been shown to occur in some strains of *P. chabaudi* (Shungu & Arnold 1972). Therefore, in order to obtain schizont/late trophozoite stage parasites of *P. chabaudi* , it is necessary to collect earlier stages by cardiac puncture from mice before sequestration occurs and to grow the parasites in short term *in vitro* culture.

Infected blood was collected before 0900 h by cardiac puncture into sodium heparin (see 2.3) from mice kept in RL. The blood was washed once in RPMI 1640 and the RBC resuspended to a 10% haematocrit in medium with 5% FCS. This was dispensed into 33 mm diameter plastic Petri dishes (Cel-Cult, Sterilin) (1.5 ml/dish) and cultured in a candle jar at 37°C by the method of Trager & Jensen (1976). Development was monitored by examination of Giemsa's stained bloodsmears from the cultures and the RBC collected from culture when schizonts were beginning to appear (usually after approximately 2 h), or later when most parasites observed were schizonts (3-4 h).

2.9 Raising hyperimmune sera

Hyperimmune sera were raised as described by Brannan *et al.* (1993) by infecting mice repeatedly with cloned populations of *P. chabaudi*, according to a method suggested by Gilks & Newbold (personal communication), thereby maximising the immune response to a particular variant type.

Mice were infected initially with 5 x 10^4 pRBC of a particular cloned population. This primary infection was allowed to clear completely before subsequent challenge. The secondary boost was of 1 x 10^7 pRBC given 81 d p.i., with subsequent boosts of 2.5 x 10^8, 1.5 x 10^8, 3 x 10^8 and 2.5 x 10^9 pRBC given at monthly intervals following the

secondary boost. After the final boost, mice were killed and bled for serum by cardiac puncture 7-9 d later.

2.10 Collection of serum

Larger volumes of serum were collected from immunised mice by exsanguination by cardiac puncture under ether anaethesia. The blood was collected into hard glass 2 ml tubes (BDH) and allowed to clot at 37°C for 30 min. The clot was then loosened from the edge of the container with a glass Pasteur pipette (Bilbate), and incubated o/n at 4°C for the clot to contract fully. The serum was then pipetted off into a microcentrifuge tube (Treff, Scotlab), any contaminating RBC removed by centrifugation (300 g for 5 min) (MSE Microcentaur, Fisons), and the serum pooled, where appropriate, then aliquoted and stored frozen at -20°C.

Smaller volumes (up to 100 µl) were collected by bleeding mice from the tail into hard glass 2 ml tubes. Mice were prewarmed under a heat lamp and then 1-2 mm was snipped off the end of the tail using clean, sharp scissors. Tubes were filled to approximately 1 cm depth with blood, and the blood allowed to clot and the serum collected and stored as described above.

2.11 Mosquito transmission of parasites

This was performed as described by McLean *et al.* (1987) and Brannan *et al.* (1993) in collaboration with Professor David Walliker (University of Edinburgh). Mice were infected with 10^6 pRBC of a particular parasite population. When gametocytes could be observed in the blood, previously starved *Anopheles stephensi* mosquitoes were allowed to feed on the infected mice. The day on which this was performed varied with different experiments, but was between 5-9 d p.i.. If mature oocysts were observed in the mosquitoes, they were then fed on an uninfected mouse 15 d after the infecting blood meal. Recipient mice were monitored for patent parasitaemia by daily bloodsmears, and stabilate made as appropriate.

2.12 Statistical analysis

Peak parasitaemia data were compared using the non-parametric Kruskal-Wallis one-way analysis of variance by ranks. Using this method of analysis, it is possible to perform multiple comparisons between different groups. This analysis was performed using the Minitab software program, followed by manual calculations to determine exactly if and where any significant differences were occurring.

Infected reticulocyte data were analysed using the Wilcoxin signed rank test, again using Minitab.

2.13 Indirect fluorescent antibody test on live, schizont-infected RBC

The 'live IFAT' method used was essentially that described by McLean *et al.* (1986b). Parasites collected at a parasitaemia of 20-30% from mice kept in RL were harvested from short term culture when just entering schizogony. After one wash in PBS/5% FCS (250 g for 5 min), 6-8 µl of packed RBC were added with mixing to 100 µl of appropriate dilutions of test antisera in microcentrifuge tubes and incubated at 37°C for 30 min. RBC were pelleted and washed x2 in 1 ml PBS/5% FCS by microcentrifugation at 300 g for 1 min, before addition of 100 µl biotinylated anti-mouse IgG (Sigma) (1:50 in PBS/5% FCS) to each tube with mixing. After a further 30 min incubation, washing was repeated as above, and 100 µl of phycoerythrin-streptavidin (Sera-Lab) (1:100 in PBS/5% FCS) added to each tube with mixing. The tubes were then incubated for 30 min at 37°C, washed as above and the RBC resuspended in 30 µl PBS/5% FCS. These were kept at 4°C until examined under a Leitz Ortholux optical microscope with UV light source using a rhodamine filter.

2.14 Preparation of monoclonal antibodies

The methodologies followed for growing hybridomas and screening for mAb production were adapted largely from those described by Harlow & Lane (1988).

2.14.1 Immunisation of mice

Parasites of each variant type were recovered from stabilate (see 2.3) and subsequently passaged into BALB/c mice, from which stabilates were prepared. All immunisations were performed using this BALB/c parasite material. Groups of 2-3 BALB/c mice were infected initially with 5 x 10^4 pRBC of a particular parasite population. The infection was allowed to clear completely before further challenge. Mice were then inoculated another 3-4 times with increasing numbers of pRBC (1 x 10^7 - 3 x 10^8 pRBC i.v.), 1-3 months apart.

2.14.2 Growing myeloma cells

Myeloma cell line X63Ag8.653 was used. Approximately 5-7 d before fusion, cells were recovered from stabilate in liquid N_2 by defrosting an ampoule in a waterbath at 37°C. The cells were resuspended using a Pasteur pipette and transferred to a sterile universal, the ampoule being washed out with prewarmed RPMI 1640. After washing by centrifugation (250 g for 5 min) x2 in 10 ml medium at RT, the cells were resuspended in 5 ml complete medium (15% FCS; Flow) (screened for growth of myeloma cell line X63Ag8.653), and incubated in a 25 ml tissue culture flask (Greiner) at 37°C, 5% CO_2. The flask was examined for cell growth using an optical microscope with inverted light source (Leitz). Usually, 1 day after initiating the culture, 5 ml fresh, prewarmed complete medium was added. On d 2, 5 ml was removed and replaced with

5 ml fresh complete medium. By d 3, the cells were usually confluent. If so, they were resuspended using a Pasteur pipette and transferred to 30-40 ml complete medium in a 75 ml flask, retaining a small amount of cells in the original flask in fresh complete medium. Cells were thus kept in log phase growth by subculture as necessary until used for fusions or frozen as stabilate in liquid N_2.

2.14.3 Preparation of spleen cells for fusions

Mice were sacrificed 3-4 d after receiving the final boost of pRBC. The spleen was removed using aseptic techniques and dissociated in a sterile 9 cm diameter plastic Petri dish containing RPMI 1640 medium by pushing through a stainless steel sieve (mesh size 0.025 mm^2) using the plunger of a 5 ml sterile plastic syringe (Becton Dickinson). The cells were disaggregated further by passing up and down the 5 ml syringe and transferred to a sterile universal, leaving behind any connective tissue debris and large clumps of cells. The spleen cells were washed x2 in 25 ml of medium (250 g for 5 min), resuspended in 5 ml medium and cell count/viability determined by haemocytometry (see 2.14.5).

2.14.4 Preparation of myeloma cells for fusion

Myeloma cells growing in log phase were harvested from culture (at least one 75 ml flask) and washed x2 in RPMI 1640 medium (250 g for 5 min), pooling cells into one universal after the first spin. The myeloma cells were resuspended in 10 ml medium and the cell count/viability determined.

2.14.5 Determination of cell viability and cell counts

Viabilities of cells were determined using the trypan blue exclusion test (Naysmith & James 1968). An appropriate dilution of cells was made in RPMI 1640 or in PBS and then further diluted 1:1 in a solution of cold, 0.2% w/v trypan blue (Gurr, BDH Ltd.) in PBS. This suspension was then examined in a haemocytometer under phase contrast on an optical microscope (x40 objective, x10 eyepiece) to ascertain the cell concentration and viability. Dead cells were recognised by morphology and uptake of trypan blue.

2.14.6 Cell fusion

10^8 viable spleen cells were mixed with 10^7 viable myeloma cells in a sterile plastic universal and centrifuged (250 g for 5 min). All the medium was discarded and the cells resuspended gently by flicking the tube. 1 ml of the fusogen polyethylene glycol (PEG) 1500 solution in HEPES buffer (Boeringer-Mannheim) (prewarmed to 37°C) was added dropwise, rotating the universal gently, followed by the dropwise addition of 20 ml of complete RPMI 1640 (15% FCS) (prewarmed to 37°C). The cells were washed, resuspended gently in 5 ml of warm complete medium, and incubated at 37°C, 5% CO_2

for 2 h.

After incubation, the fused cells were centrifuged (250 g for 5 min). The S/N was discarded and the cells resuspended in 95 ml complete medium containing HAT (see Appendix B) and peritoneal wash cells (PWC) (see 2.14.7). The cells were then plated out into 5 sterile, flat-bottomed 96 well microtitre plates (Sterilin), 200 µl/well, with the first two rows of the first plate containing myeloma cells and PWC in HAT medium as a negative control. The plates were then transferred to a 5% CO_2, 37°C incubator. Wells were screened for hybridoma growth 8-10 d later.

2.14.7 Preparation of peritoneal wash cells (PWC)

PWC were added routinely to hybridoma cultures as feeder cells. These were collected from BALB/c mice by peritoneal lavage with 5 ml ice-cold RPMI 1640, followed by aseptic aspiration of the cells into a 5 ml syringe with a 21 G needle. The cells were washed in 20 ml ice-cold medium (250 g for 5 min at 4°C) and resuspended in ice-cold complete medium (15% FCS) at appropriate dilutions (see Appendix B).

2.14.8 Growing hybridoma cells

In wells where hybridomas were observed to be growing, medium was collected for screening for specific Ab production in the live IFAT. 50-100 µl/well of medium were replaced every 2-3 d with fresh HAT medium plus OPI (see Appendix B). When the hybridoma colonies were nearing confluency, they were transferred to 0.5 ml pre-conditioned medium (see Appendix B) in 24 well plates, with 200 µl being transferred back to the original well in the 96 well microtitre plate. The cultures in the 24 well plates were given fresh HT medium (see Appendix B) (0.2-0.4 ml) every 2-3 d, depending on growth. When confluent, the cells were transferred to 1-1.5 ml pre-conditioned medium in 6 well plates, by which time all fresh medium added was free of any HAT or HT. When confluent, the cultures were transferred to 25 ml culture flasks in 5 ml complete medium, which was replenished as necessary. Cells were frozen as stabilate from 24 well plates, 6 well plates and flasks, and on occasion, complete 96 well microtitre plates were frozen (see 2.14.10). S/N were collected from plates and flasks throughout.

2.14.9 Cloning of hybridomas

Hybridomas were cloned, usually from cultures at the 24 well stage, but on occasion, earlier or later than this, by limiting dilution in 96 well microtitre plates at a dilution of 1 cell/well or 0.5 cell/well. Complete medium plus OPI and PWC was used and 50 µl medium was replaced every 2-3 d. Plates were screened for growth of clones 8-9 d after cloning, and S/N from wells with hybridomas growing were screened for Ab by live IFAT. Positive clones were grown up as described above (see 2.14.8).

2.14.10 Cryopreservation of myelomas and hybridomas

Myeloma and hybridoma cells were cryopreserved as stabilate in liquid N_2. Cells were collected from culture and centrifuged (250 g for 5 min). S/N were removed and the cells resuspended in DMSO/10% FCS at 1-3 x 10^6 cells/ml, if possible (sometimes where hybridoma cultures did not contain this many cells, they were frozen at lower concentrations). 1 ml aliquots were dispensed into cryotubes and frozen by controlled cooling at a rate of approximately 1°C/min in the vapour phase of liquid N_2, using a freezing tray (Taylor-Wharton). After a minimum of 4 h, the cryotubes were transferred to the liquid phase for long term storage.

On occasion, 96 well microtitre plates were frozen at -70°C following the method of Wells & Price (1983). When growth in the wells could be seen macroscopically by eye, S/N were collected (100 µl/well) for testing and replaced with fresh medium. 24 h later, S/N were aspirated and 50 µl of DMSO/10 % FCS added to each well. Each plate was wrapped in clingfilm (Clingo-Rap), placed in an insulator bag (Jiffy Packaging Co.) and frozen by transfer to a -70°C freezer.

Hybridoma cells cryopreserved in liquid N_2 were recovered from frozen in the same manner as for myeloma cells (see 2.14.2) and cultured in an appropriate volume of medium. 96 well microtitre plates cryopreserved at -70°C were recovered from frozen by the addition of 150 µl prewarmed complete medium, followed by incubation at 37°C, 5% CO_2 for 5 min. The freezing medium was aspirated and 200 µl fresh complete medium plus OPI added to each well. These hybridoma cultures were then grown as described in 2.14.8.

2.14.11 Ascites production

Ascitic fluid was raised in BALB/c mice primed with pristane (Sigma) (0.5 ml, i.p.) 1 week prior to injection with hybridoma cells. Between 5 x 10^5-5 x 10^6 cells were injected i.p. and mice monitored for ascites after 1-2 weeks. Ascites were drained aseptically from the peritoneal cavity using a 19 G needle, clarified by centrifugation (300 g for 5 min), aliquoted and stored at -20°C, with repeated freeze-thawing avoided. Mice were drained of ascitic fluid as necessary up to 4 times. Mice were then exsanguinated by cardiac puncture under terminal ether anaethesia (see 2.10), and the serum pooled, aliquoted and stored at -20°C.

2.14.12 Antibody isotyping

MAbs were isotyped by Ouchterlony double diffusion. A solution of 2% agar in barbitone buffer (see Appendix A) was melted in a waterbath at 100°C, and poured onto pre-coated slides (see Appendix C) on a levelling table. When set, wells were formed using a 7-well gel punch, followed by extraction of the gel plugs with a Pasteur pipette connected to a vacuum pump. Appropriate dilutions of mAbs raised against mouse

isotypes IgG1, 2a, 2b, 3, IgA and IgM (Sigma) were placed in the peripheral wells, and of the test mAb (ascitic fluid) in the centre well. The plates were incubated at 4°C for 24 h, then washed in excess PBS for 24 h to remove free protein, covered with filter paper, and dried o/n at 37°C. Precipitation was visualised by staining in 0.1% Coomassie Brilliant Blue R-250 solution (Sigma) (see Appendix C) followed by immersion in 0.3% v/v glacial acetic acid destain solution until lines were clearly visible. A line of precipitate between the centre well and one of the outer wells indicated the isotype of the test mAb.

2.15 *In vitro* cytoadherence

2.15.1 Maintenance of adherent cell lines

(a) B10 D2 cell line

This cell line was kindly supplied by the Department of Cell Biology, University of Glasgow, as a growing culture. It is a mouse lung endothelial cell line which has been maintained in long term *in vitro* culture for > 20 years. Cells were incubated in 25 ml tissue culture flasks in 5 ml of Ham's F-10 medium (Gibco) (see Appendix B), with 5% FCS and a medium supplement of ITS (Sigma) at 37°C. Medium was changed every 3-4 d as required. When cultures were confluent, cells were trypsinised using 10% Trypsin (Sigma) in PBS to detach them from the bottom of the flask. The culture medium was removed and the cultures washed with 5 ml PBS. 1 ml of 10% Trypsin was added for 30 s with rocking. Excess trypsin was removed, leaving only residual amounts in the flask, and the flask placed at 37°C for 5-15 min, until the cells were observed to be detached from the flask. They were then resuspended in fresh medium and split as appropriate into 2-3 flasks, 5 ml/flask. The cells were maintained in culture until used in binding assays or frozen as stabilate using the same method as for myeloma cells (see 2.14.10).

(b) 3T3 and 3T3 A31 cell line

The 3T3 cell line and the 3T3 clone A31 line were acquired from the European Collection of Animal Cell Cultures, Porton Down, as frozen stabilates. Both these cell lines were cultured in DMEM medium (Gibco) (see Appendix B), containing 10% FCS. They were defrosted by immersion of the cryotube in a 37°C water bath and the cells washed in 10 ml of warm medium (100 g for 5 min), before being resuspended in 5 or 10 ml of complete medium and cultured in 25 ml tissue culture flasks at 37°C, 5% CO_2. When confluent the cultures were split as appropriate into 3-5 flasks as for the B10 D2 cell line, but using 10% Trypsin/EDTA (Sigma) in PBS. The cells were maintained in culture until used in binding assays or frozen as stabilate (as for 2.14.10).

For binding assays, cells were transferred to 33 mm 1.5 ml Petri dishes, 5 x 10^4 cells /ml, and cultured for 48 h before use in binding assays.

2.15.2 Binding assay

Binding assays were performed according to the method of Cox *et al.* (1987). Adherent cell lines were transferred to 33 mm 1.5 ml Petri dishes, 5 x 10^4 cells/ml for the B10 D2 line, and 1 x 10^4/ml for the 3T3 line, and cultured for 48 h prior to the binding assay. Parasitised blood was collected from infected mice kept in RL, diluted with normal blood to 7% parasitaemia, and cultured short term as described (see 2.8), but in RPMI 1640/10% FCS. The medium was removed from the adherent cell cultures and the cell monolayer washed x2 in RPMI 1640. The parasite cultures were then transferred to the adherent cell Petri dishes when the parasites were just entering schizogony. These were then incubated at 37°C for 1 h, with gentle rocking every 10 min. The RBC were then removed by washing several times with medium, and the cell monolayer fixed with 2% glutaraldehyde in PBS for 30 min. Excess glutaraldehyde was removed and the cells stained with Giemsa's (10% in phosphate buffer) for 10 min. Binding was assessed by counting the number of pRBC bound to 500 cells.

2.16 Immunogold silver staining (IGSS)

The technique of immunogold staining of pRBC followed by silver enhancement (Hommel & Semoff 1988; Chadwick *et al.* 1989) was used in order to evaluate the % of pRBC expressing variant Ags during the course of *P. chabaudi* infection. Infected blood was collected from mice kept in RL and cultured short term as described (see 2.8). Parasites were harvested from culture and washed in PBS/5% FCS. 6-8 µl of infected blood was incubated successively for 30 min at 37°C with 100 µl of appropriate dilutions of hyperimmune sera in PBS, then 100 µl rabbit anti-mouse IgG (1:20 in PBS) (Sera-Lab), and finally 100 µl Protein-A gold conjugate (5 nm particle size, 1:10 in PBS) (Auroprobe EM, Amersham). After each incubation, RBC were pelleted and washed x2 in 1 ml PBS/5% FCS. The RBC were then diluted to approximately 10^7/ml in PBS and thin blood films prepared using a cytospin centrifuge (10^6 RBC/well, 150 g for 10 min) in order to maintain the integrity of individual RBC. The slides were air-dried and the immunogold staining visualised by silver enhancement. The slides were fixed in 100% methanol for 2 min and air-dried, followed by washing x3 for 5 min in ddH_2O. Excess water was removed from each slide and 2-4 drops of silver stain solution (1:1 enhancer:initiator) (IntenSE^{TM} M, Amersham) applied to each blood smear preparation for 15-18 min. The slides were then washed x3 in excess ddH_2O and air-dried before staining in Giemsa's (10% in phosphate buffer) and examination by optical microscopy under oil-immersion.

2.17 Preparation of parasite lysates

'One-step ghosts' and total parasite lysates were made following the method of Newbold *et al.* (1982). Infected blood was collected by cardiac puncture from RL mice at

parasitaemias of ≥ 30%, and a RBC count performed by haemocytometry. The blood was passed through a sterile Whatman CF11 powdered cellulose column (3ml CF 11 to 1ml whole blood), prewetted with RPMI 1640 at 37°C, to remove leucocytes from the sample (Beutler *et al.* 1976), washing through with prewarmed RPMI 1640, and the filtrate washed. The pRBC were then cultured short term until schizonts developed (see 2.8). The pRBC were harvested from culture and washed x3 in PBS at 4°C. For one-step ghost preparations, approximately 5 x 10^8 RBC were mixed rapidly with 1 ml 5mM sodium phosphate (pH 8.0) containing 2mM PMSF (Sigma) and 20 µg/ml DNase (Type 1, Sigma). This was incubated for 5 min at 20°C and the membranes collected by microcentrifugation (300 g for 2 min). The S/N was discarded and the pellet stored at -70°C. For cell lysate preparations, the washed cells were resuspended to approximately 1-2 x 10^9 RBC/ml in PBS containing 2mM PMSF and 20 µg/ml DNase, kept on ice. Cells were lysed by 2 x 5 s pulses of an MSE sonicator (Fisons) on maximum power. The lysates were aliquoted and stored at -70°C.

2.18 Determination of total protein concentration

Protein concentrations of samples for analysis were determined by spectrophotometric measurement at 595 nm using the Coomassie blue G-250 Pierce protein assay reagent (Pierce Chemical Co.), based on the method of Bradford (1976). The microassay procedure was used, whereby protein concentrations in the range of 1-25 µg/ml can be determined. A known protein concentration series between 1-25 µg/ml was prepared by diluting a 2 mg/ml stock BSA standard (Pierce) in PBS. 1 ml of protein assay reagent was added with mixing to 1 ml of each of the dilute standards and the unknown protein sample (diluted as appropriate) in clean test tubes. PBS was used as a blank. Absorbance was read at 595 nm on a UV spectrophotometer (Pye Unicam PU 8600) against the blank, the value of which was then subtracted from each protein absorbance to give the net absorbance for each sample tested.

2.19 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.19.1 Electrophoresis

Separation of proteins was carried out by the method of Laemmli (1970), using the gel electrophoresis apparatus GE 2/4 LS (Pharmacia). 0.7 mm thick 5-25% gradient gels, consisting of 120 mm separating gel and 10 mm stacking gel, were prepared (see Appendix C) using a gradient former (Pharmacia) and peristaltic pump (LKB). Samples were mixed with SDS-PAGE sample buffer (see Appendix A) and boiled for 10 min prior to loading onto gel. Sample buffer contained 5% ME or 1 mg/ml iodoacetamide (Sigma) for reducing and non-reducing conditions, respectively. Usually, 20 µl sample buffer was added to 20 µl protein sample diluted in PBS for each well, loading 60 µg protein sample/well. The MW of parasite proteins were estimated by reference to MW

marker proteins (Pharmacia 17-0446-01) (MW range 14-94 kDa).

Electrophoresis was carried out for 4 h at a constant current of 40 mA/gel, or for 16 h o/n at 8 mA/gel, using SDS-PAGE running buffer (see Appendix A). Gel tanks were cooled by circulating water at 4°C to minimise any gel distortion due to heating during electrophoresis.

2.19.2 Staining polyacrylamide gels for protein

Proteins were visualised following electrophoresis by incubating gels for 2h in 0.1% Coomassie Brilliant Blue R-250 in a solvent solution of 25% methanol, 10% acetic acid and 1% glycerol, followed by destaining in the solvent until a clear background was obtained. Stained gels were dried onto filter paper (Whatman) using a gel slab drier (Bio-Rad, 1125B) at 80°C.

2.20 Western blotting

2.20.1 Transfer to nitrocellulose

Immunoblotting was performed by the method of Towbin *et al.* (1979). 5-25% SDS gels were run as described (see 2.19), using gels with a single large well (10x standard size) for samples and, on either side, standard wells for MW markers. Proteins were electrophoretically transferred onto nitrocellulose (Hybond C-extra, 0.45 μm) (Amersham), using a Tris-glycine/SDS transfer buffer, pH 7.0 (see Appendix A) in a Trans-blot cell (Bio-Rad) at a constant current of 100 mA for 16 h at 4°C.

2.20.2 Enzyme-linked antibody detection system

Following transfer, the nitrocellulose membrane was air-dried and cut into 1 cm wide strips. One sample strip and the strips for MW markers were stained in 0.1% amido black (BDH) in 45% methanol, 10% acetic acid for approximately 15 min to visualise proteins, then destained in the solvent. The remaining strips were incubated for 1 h in 4 ml 20% soya milk/0.5% Tween-20 (Sigma) in wash buffer (see Appendix A) to block non-specific binding of anti-serum to the nitrocellulose. After this, and for all subsequent steps, the membrane was washed x3 for 5 min in wash buffer. The nitrocellulose strips were then incubated for 90 min with primary Ab (immune mouse sera) (4 ml/strip), diluted 1:500 in wash buffer, followed by incubation for 1 h with a secondary layer of anti-mouse IgG conjugated to alkaline phosphatase (1:500 dilution in PBS, 4 ml/strip). All incubations and washes were carried out on a rocking table at RT.

Specific binding was visualised by incubating the strips in a solution of the substrate, NBT/BCIP (see Appendix C) at a final concentration of 0.1% AP buffer (see Appendix A), allowing 2 ml/strip. The reaction was terminated when bands could be seen clearly, before non-specific background staining occurred, by removing the substrate solution and adding EDTA (10 mM in PBS), 2 ml/strip. The strips were then

air-dried and stored away from direct light.

2.21 Pulsed field gel electrophoresis (PFGE)

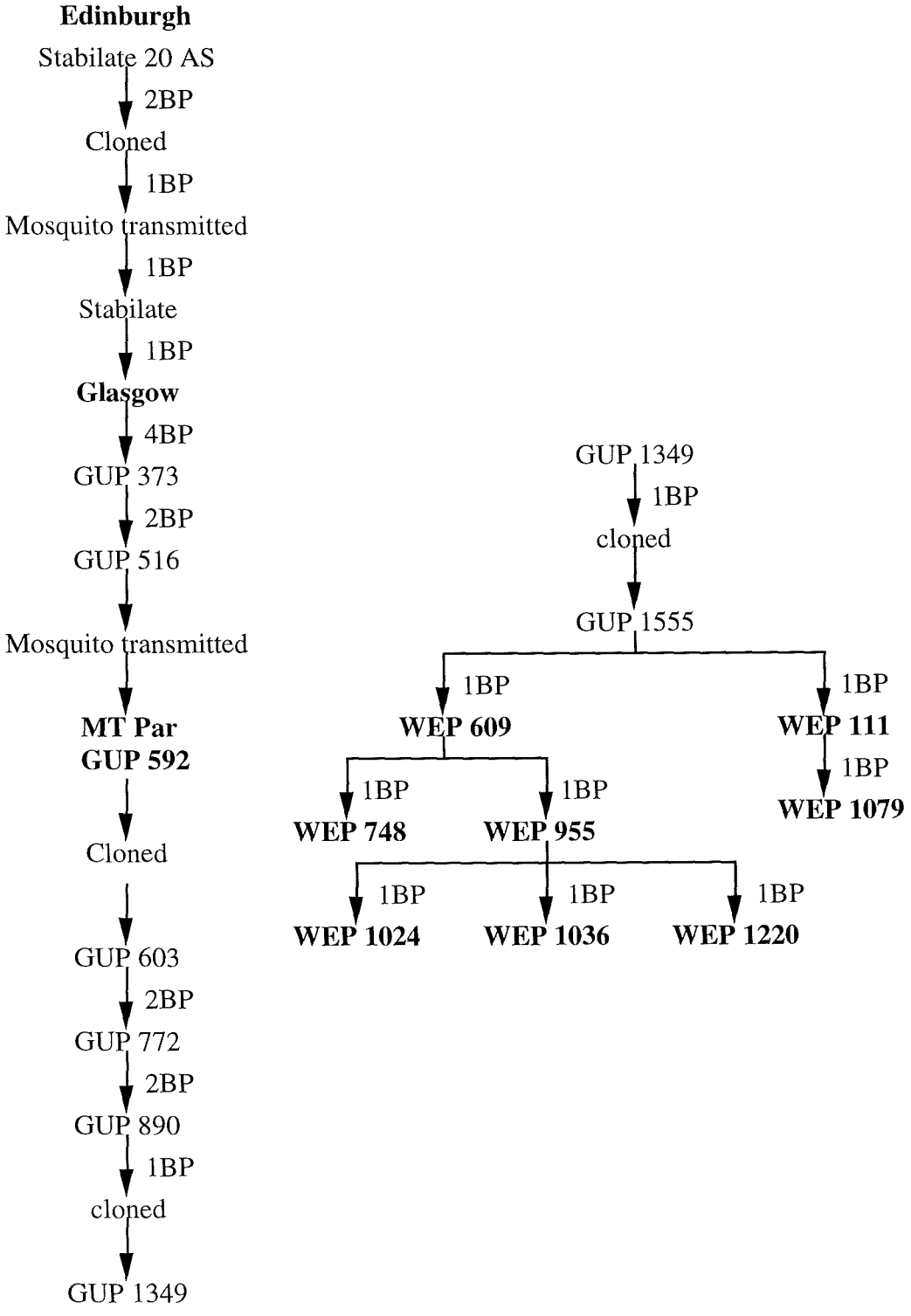
2.21.1 Preparation of DNA samples

Infected blood was collected under sterile conditions by cardiac puncture from 2 RL mice (>1 ml/mouse) for each variant type at a parasitaemia of ≥ 30%, and a RBC count performed by haemocytometry. The blood was passed through a prewetted 10 ml sterile Whatman CF11 powdered cellulose column to remove leucocytes (Beutler *et al.* 1976), washing through with prewarmed RPMI 1640 medium, and the filtrate washed. The pRBC were then cultured short term until schizonts developed (see 2.8). The pRBC were harvested from culture and washed x2 in PBS. The packed RBC were resuspended to 1ml in PBS and lysed by the addition of an equal volume of 0.15% saponin in PBS. The parasites were then washed x3 in excess PBS (200g for 10 min) and resuspended in PBS to the required concentration (final concentration in agarose of 5×10^8 or 2.5×10^9). An equal volume of 2% low melting point agarose (Sigma) in PBS at 42°C was added with gentle mixing, and the mixture pipetted quickly into moulds (Bio-Rad) prewarmed to 42°C. These were incubated for 20 min at 4°C to allow the agarose blocks to set. The blocks were gently removed from the moulds into PFGE lysis solution (see Appendix C) and incubated for 48 h at 42°C, with one change of solution, then stored at 4°C in lysis solution without proteinase K. Blocks thus made and stored may be kept for several years without noticeable degradation of DNA.

2.21.2 Electrophoresis

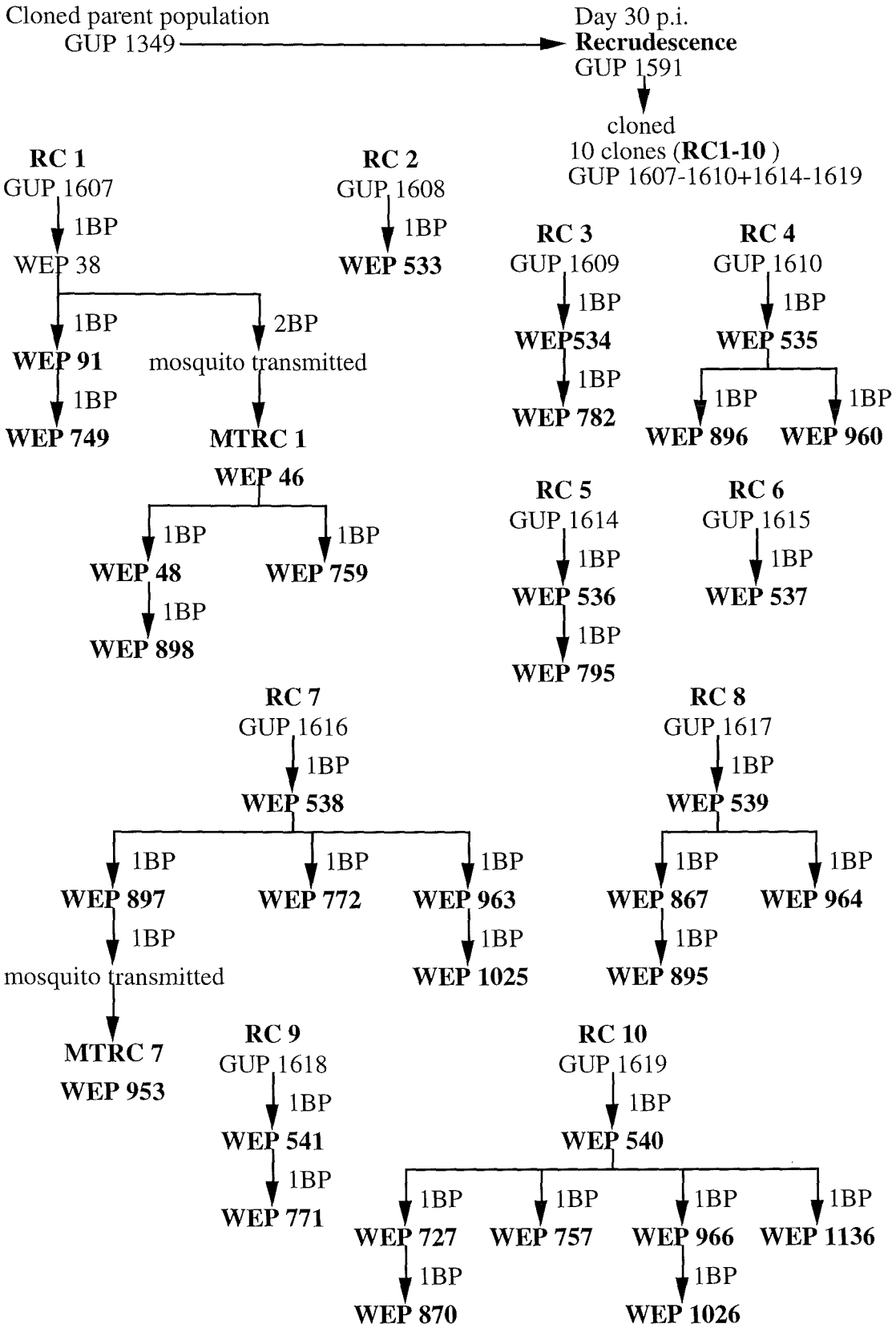
PFGE was performed using a contour-clamped homogeneous electric fields (CHEF) apparatus (CHEF-DR II system, Bio-Rad) (Chu *et al.* 1986; Vollrath & Davis 1987). DNA samples (approximately 0.25 of a 100 µl block) were loaded into the wells of a 100 ml agarose gel (IBI) (1% in 0.5x TBE) (see Appendix C). For 7 d electrophoresis, chromosomal grade agarose was used (Bio-Rad). The wells were topped up with low melting point agarose (1% in 0.5x TBE). Electrophoresis was carried out in 0.5x TBE at 12°C, either for a total of 72 h, with the first 24 h at 140V, 120 s switch time, then 24 h at 130V, 300 s switch time, and the final 24 h at 140V, 180 s switch time, or for a total of 168 h at 80V with switch time increasing from 180 s to 1000 s. DNA size markers of chromosomes of *Saccharomyces cerevisiae* , ranging in size from 0.2-2.5 Mb, and of *Hansenula wingei*, ranging in size from 1.05-3.13 Mb, were used. Bands were visualised by ethidium bromide staining for 15-20 min and examined on a UV transilluminator.

Fig. 2.1 History of *Plasmodium chabaudi chabaudi* AS strain parent populations. Derived from *Thamnomys rutilans* number 339 caught in the Central African Republic, March 1969.



GUP and WEP are stabilate reference codes. Bold print indicates stabilates used during this study. BP = blood passage.

Fig. 2.2 Derivation and stabilate history of recrudescent populations of *P. c. chabaudi* AS.



GUP and WEP are stabilate reference codes. Bold type indicates stabilates used in this study. BP = blood passage.

CHAPTER 3

ANTIGENIC VARIANTS OF *Plasmodium chabaudi* AS AND THE EFFECTS OF MOSQUITO TRANSMISSION: ANALYSIS BY LIVE IFAT

3.1 Introduction

Antigenic variation has been shown to occur in several species of malaria parasite. The size and nature of the possible repertoire of variant Ags available to the parasites has, however, only been appreciated very recently with the identification of the large and diverse *var* gene family for *P. falciparum* PfEMP1 (Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995), and is still not fully defined. In particular, little is known about the role that cyclical transmission through mosquitoes may play in determining the expression of these surface variant Ags.

The first indication of antigenic variation occurring in *Plasmodium* was reported by Cox (1959, 1962) in *P. berghei* infections. Antigenic variation is now considered to be a feature of most, if not all, malaria infections. Early studies were performed using uncloned parasites. In order to eliminate the possibility of minor populations of antigenically diverse parasites being present in the initial infecting population, however, it is now considered necessary to use clonal parasites, with which antigenic variation during malarial infection may be demonstrated unequivocally.

Different methods have been used to study the occurrence of antigenic variation during malarial infection. Some early studies compared resistance of animals to reinfection with parasites of the initial infecting parent population and with parasites from following recrudescences (Cox 1959; Voller & Rossan 1969 a & b). Antigenic variation in *P. knowlesi* was first identified using a schizont agglutination (SICA) test (Brown & Brown 1965). Later, an indirect fluorescence antibody test (IFAT) was used to identify variant populations of *P. knowlesi* (Hommel & David 1981; Barnwell *et al.* 1983b) and of *P. falciparum* (Hommel *et al.* 1983). More recently, modifications of previously utilised techniques have also been developed and used to study variant populations of *P. falciparum* : these include an immunogold-silver enhancement method (Hommel *et al.* 1991) and a mixed agglutination assay (Newbold *et al.* 1992; Roberts *et al.* 1992).

P. chabaudi was initially shown to undergo antigenic variation during the course of infection in NIH mice using a passive transfer system for analysis of variant populations (McLean *et al.* 1982 a & b). This distinguishes variant populations by the level of passive protection conferred by immune sera raised against homologous and heterologous parasite populations. Using this system, McLean *et al.* (1986a) examined parasite populations cloned from a recrudescence. The results of this, using immune sera raised against the infecting parent population, indicated that not only are recrudescences antigenically different from the infecting parent population, but also contain a mix of variant types. Subsequently, a method was developed for *in vitro* analysis of antigenically variant parasites of *P. chabaudi* using a triple layered IFAT on live pRBC (live IFAT) (McLean *et al.* 1986b). This method detects variant-specific Ags on the surface of late trophozoite/schizont-infected RBC. Immune sera [collected

on d 16/17 p.i., when passive protection was found to be optimal (McLean 1985)] were raised against the cloned parent and recrudescent parasite populations described in the passive transfer study (experiment 1) (McLean *et al.* 1986a). Using these sera and parasite populations in the live IFAT, four different variants could be detected from the recrudescence, all different from each other and from the parent. However, a +ve Ab titre of only 1:50-1:80 was observed using these sera, and six of the clones showed no reactivity with any of the immune (d 16/17) sera (Brannan *et al.* 1993).

In order to analyse further these variant parasite populations, hyperimmune serum was raised against each of the recrudescent clones, the cloned parent population and one of the recrudescent clones after mosquito transmission (MTRC 1). The first part of this chapter presents the results of testing this panel of hyperimmune sera against the parent and recrudescent clones in the live IFAT.

The sexual process which *Plasmodium* undergoes during transmission through mosquitoes can generate parasite diversity (Walliker *et al.* 1975, 1987). The effects of such processes on expression of antigenic variants seen in asexual erythrocytic forms are not clear, but merit investigation as an understanding of the role of MT in influencing such variation may be important both in terms of parasite biology and vaccine development. Voller & Rossan (1969a) described an apparent change in antigenic type of *P. cynomolgi bastianelli* upon cyclical transmission through mosquitoes. An alteration in antigenic type after MT of *P. knowlesi* has also been observed (Draper & Voller 1972). These early studies were, however, performed using uncloned parasite populations. McLean *et al.* (1987) reported the effects of MT of both uncloned and cloned antigenic variants collected from recrudescences of a previously cloned 'parental' type population of *P. chabaudi*, again using the passive transfer system to analyse variant populations. These experiments indicated a reversion to the parental type upon transmission through mosquitoes. It was suggested that antigenic variants of *P. chabaudi* AS strain may revert to a basic type after MT.

The second part of this chapter describes the results of MT of recrudescent cloned variant populations. Analysis by live IFAT using the panel of hyperimmune sera was performed on the cloned recrudescent population isolated following MT described previously by McLean *et al.* (1987) (MTRC 1), on another recrudescent population (RC 7) which had been transmitted successfully through mosquitoes (MTRC 7), and on the original parental parasite line obtained from Edinburgh post-MT but before any subsequent cloning (MT Par).

3.2 Live IFAT analysis of the parent and recrudescent clones using hyperimmune sera

The derivation of the *P. chabaudi* cloned parasite populations was described by McLean *et al.* (1986a) and is outlined in chapter 2. The parent clone and the 10

recrudescent clones (RC 1-10) were examined in the live IFAT (see 2.13) using hyperimmune sera raised against each of the populations and against MTRC 1 (see 2.9). Each parasite population was recovered from stabilate (see 2.3) and inoculated i.v. into mice kept in a RL cycle. In all cases, parasites examined in the live IFAT were no more than 2-3 blood passages from stabilate. For each combination of sera and parasites, the test was performed at least twice, and sera titred out to a +ve end point. The test was scored qualitatively on a +ve/-ve basis. Samples were scored -ve when no +ve fluorescence was observed on ≥ 3000 pRBC. Results were marked as very few +ve pRBC where approximately ≤ 5% +ve pRBC were observed as compared to the homologous serum results. The results of this analysis are shown in Table 3.1.

Hyperimmune sera were more successful in detecting variant Ags on the surface of pRBC compared to immune (d 16/17) sera, in so far as a homologous +ve result was obtained with all the hyperimmune sera (Table 3.1). In some cases this was to a very high titre, with no apparent decrease in the number of +ve pRBC with increasing serum dilutions. A certain level of cross reactivity between heterologous sera and recrudescent populations was, however, apparent. The hyperimmune serum raised against the parent population, however, was totally specific, and did not react with any of the recrudescent populations, though it was +ve only to a titre of 1:200. The hyperimmune sera raised against RC 4, 7 and 10 all gave a very high +ve serum titre against the homologous parasite populations of 1:10000; there was, however, some degree of cross reactivity at lower titres. With anti-RC 10 hyperimmune serum, this cross reactivity was minimal, with a very low titre of 1:10 +ve fluorescence against only three other cloned parasite populations. None of the hyperimmune sera raised against any of the other recrudescent clones showed any reactivity with RC 10. With anti-RC 7 hyperimmune serum, there was a +ve fluorescence against several other cloned populations, but in each case not to such a high titre as against the homologous parasites. There was no reactivity against RC 10, 8 and the parent. Hyperimmune sera raised against some of the other recrudescent clones did show a low level of reactivity with RC 7, but +ve fluorescence was never obtained with serum titres of > 1:100 in all instances. With hyperimmune serum raised against RC 4, there was a slightly greater degree of cross reactivity with some of the other recrudescent clones, but on no occasion did the serum give a +ve fluorescence to as high a titre as with the homologous parasite population. RC 4 did also show some cross reactivity with hyperimmune sera raised against other recrudescent clones, but again, not to as high a titre as with the homologous serum. Hyperimmune serum raised against RC 8 was specific for RC 8, but only to a +ve serum titre of 1:50 except for a very few pRBC of RC 5 showing +ve fluorescence at a titre of 1:10 with this serum.

From Table 3.1, it can be seen also that the other recrudescent populations, RC 1, 2, 3, 5, 6 & 9, to which hyperimmune sera were raised and tested in the live IFAT, all

showed similar levels of reactivity and cross reactivity with homologous populations and with each other, respectively. The effects of considering these as one variant type and therefore merging the results for these is shown in Table 3.2, along with the results for RC 4, 7, 8 and 10, and the hyperimmune sera raised against these. This condensed presentation helps to clarify the results of this analysis.

It can be noted from Table 3.1 that the hyperimmune serum raised against MTRC 1 reacted with all the cloned populations, though at a low serum titre. The parent, RC 1, 8 and 10 showed only a very few pRBC giving +ve fluorescence with this serum.

3.3 Mosquito transmission of recrudescent clones and analysis by live IFAT of parasite populations after mosquito transmission

In total, MT was attempted once each for RC 4 and RC 7, and twice each for RC 8 and RC 10. Of these, only RC 7 was transmitted successfully through mosquitoes and parasites collected from a mouse on which the infected mosquitoes had fed. In all other cases, oocysts were observed to develop, but either these failed to mature properly, or seemed to mature normally, but no sporozoites were seen in the mosquito salivary glands and mice failed to become infected when mosquitoes were fed onto them. The parasites collected from RC 7 transmitted through mosquitoes (MTRC 7), as well as MTRC1 and the original parental line post-MT but pre-cloning (MT Par) (see Figs. 2.1 & 2.2) were each tested in the live IFAT using the panel of hyperimmune sera. The results of this can be seen in Table 3.3. For both RC 1 and RC 7, the pattern of reactivity had altered significantly following transmission through mosquitoes. Both MTRC 1 and MTRC 7 showed a higher +ve serum titre (1:1000) with the hyperimmune serum raised against MTRC 1 than with any other hyperimmune sera. This was also the case for the uncloned parental parasite population, MT Par. Only hyperimmune sera raised against the parental clone and RC 8 showed no reactivity with any of the MT populations. RC 7 hyperimmune serum was +ve only to a titre of 1:100 against MTRC 7 and to a titre of 1:500 against MTRC 1. All other hyperimmune sera showed differing degrees of +ve fluorescence against the MT populations. With the exception of MTRC 1 hyperimmune serum, however, there were noticeably fewer +ve pRBC with all hyperimmune sera giving +ve fluorescence in the live IFAT against both MTRC 1 and 7, and also against MT Par.

3.4 Discussion

The results of the live IFAT using the hyperimmune sera indicate that cloned parasites derived from a recrudescence vary antigenically from the parent and from each other. This confirmed the results obtained with immune (d 16/17) sera previously reported (Brannan *et al.* 1993) and demonstrated the effectiveness of using the live IFAT method to detect antigenic variants of *P. chabaudi* . When the immune (d 16/17) sera were

used to analyse the recrudescent clones, four variant clones were identified by +ve fluorescence with homologous sera, all different from each other and from the parent. Testing with the hyperimmune sera confirmed this, showing these four recrudescent clones to be distinct from each other and from the parent. The other six clones also showed +ve fluorescence, unlike the case with the immune sera, where these all gave -ve results in the live IFAT. These six all appeared to be of a similar antigenic type. From this, a possible five antigenic types have been identified in the recrudescence, all distinct from the parent. This mix of antigenic types, determined using the live IFAT on clones of a recrudescence, confirms the results of McLean *et al.* (1986a), though with the passive transfer system, only three variant types could be identified definitely by their sensitivity to immune sera. The six antigenic types identified here probably do not represent the total repertoire of variants available to the parasite. In *P. knowlesi*, uncloned populations may consist of at least ten variants (Howard & Barnwell 1985) and in *P. falciparum*, ten variants were identified from the Indochina-1 strain (Hommel *et al.* 1991) and multiple variants have been shown to arise from a cloned population *in vitro* (Roberts *et al.* 1992). It is also apparent from the results of MT of *P. chabaudi* presented herein that other variants can appear. As a family of 50-150 *var* genes encodes the surface variant Ag PfEMP1 of *P. falciparum* (Baruch *et al.* 1995, Su *et al.* 1995), it may be that the number of antigenic types identified in the above studies is a conservative estimate of the true potential for antigenic diversity of variant Ags expressed on the surface of malaria-infected RBC.

Of the four most distinct recrudescent clones of *P. chabaudi* examined herein, hyperimmune sera to three, RC 4, 7 & 10, were of high homologous titres, though there was a certain degree of cross reactivity observed with different clones at low titres. RC 8 was distinct in that even with hyperimmune sera from mice infected six times, a titre of no higher than 1:50 could be obtained. It is possible that the variant Ag(s) on this parasite clone may be either poorly expressed or of low immunogenicity.

The cross reactivity observed between different clones with the hyperimmune sera may be due to the immunised mice being exposed to other variant types during the course of primary infection, which is allowed to clear completely before further reinfections. Recrudescences have, therefore, arisen which will contain parasites antigenically different from the immunising population. Subsequent reinfections, though cleared rapidly in the immune mice, may persist long enough for variants to arise, and increasing levels of specific Ab to the immunising population may possibly induce increased rates of antigenic variation by the challenge parasites. The induction of antigenic variation by Ab has been indicated by experiments with *P. knowlesi* (Brown 1973; Barnwell *et al.* 1983a). It is not known whether antigenic variation in *P. chabaudi* infections in mice is induced by Ab. The data presented in this chapter, whilst demonstrating that variation of Ags expressed at the surface of pRBC occurs

during the course of infection, give no indication of how these variants arise. However, intrinsic antigenic variation has been shown to occur spontaneously and at high rates early during the ascending primary parasitaemia in *P. chabaudi* infections (Brannan *et al.* 1994; chapter 6), and antigenic variants have been shown to be present during remission of the primary patent parasitaemia in mice infected with a cloned population of *P. chabaudi* (McLean *et al.* 1990), though not to the same extent as in the recrudescent populations. Whether these are all the same as the variants present in the recrudescence has not been determined. Variant parasites, having persisted through subpatency, may recrudesce due to the decline in the effector arm of the immune response observed to be associated with the appearance of a recrudescence (McLean *et al.* 1982b). It is clear, though, that immunised mice will have experienced to some degree, different parasite variants during the course of infection and reinfection.

It is also possible that these variant Ags belong to a family of Ags, similar in structure and perhaps possessing shared epitopes, in which case some level of cross reactivity could be expected to be observed in hyperimmunised mice. The case for a *P. chabaudi* variant Ag family is strengthened by results showing that *P falciparum* variant Ags are encoded by a large multi-gene family (Su *et al.* 1995).

The initial lack of success in transmitting recrudescent populations through mosquitoes was probably due, at least in part, to the time of year the experiments were undertaken. This was in June/July, when on some particularly hot days the extraneous temperature affected the regulation of the temperature in the insectary, causing it to rise to levels too high for parasite development in the mosquitoes (Walliker, personal communication). The optimal temperature for sporogony of *P. chabaudi* is 26°C (Killick-Kendrick 1971), and much above this will result in an absence of infective sporozoites present in the mosquito salivary glands. Success was finally achieved during the winter months. It is also possible that the failure of some recrudescent populations to be transmitted through mosquitoes is due to syringe passage of the parasites, albeit limited to the absolute minimum necessary. Repeated syringe passage can have a deleterious effect on infectivity of *Plasmodium* to mosquitoes (Landau & Boulard 1978). However, MT is a difficult procedure which is not guaranteed success simply by the very nature of working with the combination of live parasites, animals and insects.

Successful MT of recrudescent clones resulted in parasites which gave a different pattern of reactivity against the panel of hyperimmune sera in the live IFAT, indicating an alteration in antigenic type. This did not appear to be to the antigenic type of the cloned parental population used in this analysis. However, when the original parental population, obtained upon MT but prior to any subsequent cloning (MT Par), was tested against the panel of hyperimmune sera in the live IFAT, a similar pattern of reactivity to that seen with MTRC 1 & 7 was observed. Earlier studies (McLean *et al.* 1987)

comparing one of the same recrudescent parasite populations and its MT counterpart, using the passive protection assay, had indicated reversion to parental type. The apparent difference in results may be due to the different methods of analysis used. The passive protection assay used by McLean *et al.* measured the sensitivity of MT parasite populations to immune sera raised against the parent population. MT populations were found to be similar in sensitivity to the parental type, whereas the recrudescent population prior to transmission through mosquitoes was insensitive to the protective effect of the immune serum. This therefore implied an apparent reversion to parental type. The live IFAT analysis reported in this chapter detects expression of variant Ags on the surface of pRBC. These may not be the only Ags affected by MT, and indeed, there is no evidence as yet to equate the antigenic changes detected by the two assays, or even that the Ags concerned are located at the same site in/on the pRBC. It is, however, possible that MT may effect a reversion to a 'wild' or 'parental' type parasite, the properties of which are retained to some extent by clones derived from this. Indeed, the fact that the original MT parental population prior to cloning, and from which all parent populations are directly derived by cloning, shows a similar pattern of reactivity as the two MTRC populations is supportive of this notion. It is probable that these populations all show sensitivity to anti-parent sera using the passive transfer system. Subsequent cloning of this parental population, however, has resulted in the parental clone used in this analysis being of a different surface antigenic type, even though McLean *et al.* (1986a) had observed all clones of the parent to be sensitive to anti-parent sera. Cloning the parasites is performed by limiting dilution in mice, a process which takes 10-15 d, during which time it is possible that switching of surface variant Ags, as detected by live IFAT, could occur.

Previously, Voller & Rossan (1969a) reported a change in antigenic type after cyclical transmission of *P. cynomolgi bastianelli.* This was not to the parent type. It may, however, have been to a variant type occurring earlier in the infection of the monkey from which the parasites were obtained prior to transmission. Draper & Voller (1972) also noted an alteration in antigenic type of *P. knowlesi* after MT. The results described herein confirm these earlier findings.

Parasite populations obtained upon passage through mosquitoes, though not identical, appear to contain a mix of antigenic types. Although there is reactivity with hyperimmune sera against recrudescent populations, the higher titre reactivity seen with both MTRC populations against hyperimmune serum raised against MTRC 1 suggests the occurrence of a new antigenic type upon MT. This appears to be the predominant antigenic type present in the MT populations.

The apparent mix of antigenic types of *P. chabaudi* in the MT populations is perhaps analogous to *Trypanosoma brucei* infections, where tsetse-transmitted populations are found to contain a mix of antigenic types (Hajduk *et al.* 1981). For *T.*

brucei, this is due to a very high switching rate in fly-transmitted populations (Turner & Barry 1989). It may be that in *P. chabaudi*, a similar phenomenon caused by cyclical transmission accounts for the mix of antigenic types seen in mosquito-transmitted populations (Turner, personal communication).

Antigenic variation in malaria parasites is now an accepted phenomenon, and the use of *P. chabaudi* infections in mice as a model provides a means of studying this as it occurs *in vivo*. Whilst it is possible to study *in vivo* antigenic variation of *P. falciparum* in squirrel monkeys (Hommel *et al.* 1983), mosquito infection from this host remains difficult practically and is not routinely performed. Therefore, for determining the role of MT in influencing the expression of variant Ags, at present *P. chabaudi* provides the most suitable model.

Table 3.1 Titres of hyperimmune sera against cloned populations of *P. c. chabaudi* positive in the live IFAT.

POPULATION TESTED	HYPERIMMUNE SERA RAISED AGAINST											
	parent	MTRC 1	RC 1	RC 2	RC 3	RC 4	RC 5	RC 6	RC 7	RC 8	RC 9	RC 10
parent	**200**	*100	-	-	-	-	-	-	-	-	-	-
RC 1	-	*100	1000	100	100	100	100	100	100	-	1000	10
RC 2	-	100	1000	1000	100	100	1000	1000	100	-	1000	10
RC 3	-	100	1000	1000	1000	1000	1000	1000	1000	-	1000	-
RC 4	-	100	500	100	100	**10000**	100	500	100	-	100	-
RC 5	-	100	1000	100	100	100	500	500	100	*10	500	-
RC 6	-	100	1000	100	100	1000	500	1000	100	-	1000	-
RC 7	-	10	100	100	10	10	100	100	**10000**	-	100	-
RC 8	-	*50	-	-	-	-	-	-	-	**50**	-	-
RC 9	-	100	500	500	100	500	100	100	500	-	1000	-
RC 10	-	*100	-	-	-	-	-	-	-	-	-	**10000**

All values show reciprocal serum titres. *indicates very few positive pRBC.

Table 3.2 **Titres of hyperimmune sera against cloned populations of *P. c. chabaudi* positive in the live IFAT, grouping together those which show a similar pattern of reactivity.**

POPULATION TESTED	HYPERIMMUNE SERA RAISED AGAINST						
	parent	MTRC 1	RC 4	RC 7	RC 8	RC 10	Rest
parent	**200**	*100	-	-	-	-	-
RC 4	-	100	**10000**	100	-	-	100
RC 7	-	10	10	**10000**	-	-	100
RC 8	-	*50	-	-	**50**	-	-
RC 10	-	*100	-	-	-	**10000**	-
Rest	-	100	500	100	-	10	**1000**

All values show reciprocal serum titres. *indicates very few positive pRBC.

Table 3.3 **Positive titres in live IFAT of hyperimmune sera against cloned populations of *P. c. chabaudi*, and against these same populations and the original uncloned parent population after mosquito transmission.**

POPULATION TESTED	HYPERIMMUNE SERA RAISED AGAINST										
	parent	MTRC 1	RC 1	RC 2	RC 3	RC 4	RC 5	RC 6	RC 7	RC 8	RC 9	RC 10
RC 1	-	*100	1000	100	100	100	100	100	100	-	1000	10
MTRC 1	-	**1000**	**100**	**100**	**10**	**500**	**500**	**100**	**500**	**-**	**500**	**100**
RC 7	-	10	100	100	10	10	100	100	10000	-	100	-
MTRC 7	-	**1000**	**100**	**10**	**500**	**100**	**100**	**100**	**100**	**-**	**100**	**500**
MTPar (uncloned)	-	**1000**	**100**	**10**	**100**	**100**	**100**	**100**	**100**	**-**	**100**	**100**

All values show reciprocal serum titres. *indicates very few positive pRBC.

CHAPTER 4

THE BEHAVIOUR OF CLONED ANTIGENIC VARIANTS OF *Plasmodium chabaudi* AS *in vivo*

4.1 Introduction

Antigenic variation may be an important means by which malaria parasites evade the host's immune response, thereby allowing persistence of the asexual erythrocytic stages in the bloodstream of the semi-immune host. Little is known about whether expression of different variant Ags causes any differences in the biology or behaviour of the parasites *in vivo*. *P. chabaudi* AS in NIH mice provides an accessible model in which to study antigenic variation in malaria parasites *in vivo*. In this system, *P. chabaudi* displays a course of infection showing an acute primary patent parasitaemia followed by one, or sometimes two, patent recrudescences. Some parasite clones derived from a recrudescence have been shown to be antigenically different from the cloned infecting population and from each other (McLean *et al.* 1986a; Brannan *et al.* 1993; chapter 3).

A distinguishing character of *P. chabaudi* is the reported predilection for mature RBC (Landau & Boulard 1978). Carter & Walliker (1975) reported that in mice, normocytes were predominantly invaded, though during acute parasitaemia, when considerable reticulocytosis results, reticulocytes are also invaded (Carter & Walliker 1975; McDonald 1977; Jarra & Brown 1989). However, in chronic infections in $CD4^+$ T cell-depleted mice, a preference for reticulocytes has been observed (Taylor-Robinson & Phillips 1994c), and McNally *et al.* (1992) reported that *in vitro, P. c. chabaudi* displays a preference for reticulocytes.

This chapter describes some aspects of the behaviour of antigenically variant cloned populations of *P. chabaudi* examined *in vivo*. The courses of infection in NIH mice of the parent population and of four variant populations were examined in terms of the overall pattern, levels of peak parasitaemia and types of RBC invaded during the ascending parasitaemia. Further antigenic variation of these variant parasites was examined by live IFAT analysis of recrudescent populations collected during infections initiated by the variant parasite populations.

4.2 The course of infection of variant populations in mice

Groups of 6-7 mice were each infected i.v. with 1 x 10^5 pRBC/mouse of different antigenic types. These were the cloned parent population, RC 4, 7, 8 & 10. The course of infection of each of these is shown in Fig. 4.1. Parasitaemias of individual mice were monitored by examination of daily bloodsmears by optical microscopy and are expressed as the log of the geometric mean of each group. In all five cases, the course of infection of the primary patent parasitaemia followed an overall similar pattern and the rate of parasite growth early during the ascending parasitaemia was similar for those variant populations so analysed (see chapter 5).

Fig. 4.2 shows the peak primary parasitaemias observed in each of the groups. These are expressed as the median ± interquartile range of each group and statistical analysis was performed using the Kruskal-Wallis one-way analysis of variance. RC 10

peaked at a significantly lower parasitaemia ($P < 0.05$) of just less than 20%, compared to approximately 40% in the other groups. In mice infected with the parent or RC 10 parasites, peak parasitaemias occurred on d 9-10 p.i., and in mice infected with RC 4, 7 or 8, parasitaemias peaked on d 8-9 p.i. (Fig. 4.1). Parasitaemias of mice in all groups went subpatent around d 20 p.i., and in the parent, RC 4, 7 & 10 groups, the subpatent period was of a similar duration, with recrudescences appearing between d 25-30 p.i.. However, in the RC 8-infected group, recrudescences were not observed until after d 40 p.i., and then in only 3 of 6 mice in the group up to d 62 p.i., when the experiment was terminated.

The peak parasitaemias observed in the recrudescences are illustrated in Fig. 4.3. The peak recrudescence parasitaemias observed in the parent group were significantly greater ($P < 0.05$) than the peaks observed in the RC 4 & 7 groups. The peak recrudescences observed in the RC 10 group were higher than in the RC 4 & 7 groups, but lower than in the parent group, and were not significantly different from any of them ($P > 0.05$). RC 8 peak recrudescences could not be compared statistically due to the small sample size.

4.3 Reticulocytes during the early stages of infection

Reticulocytes were examined from d 7-11 p.i. on the Giemsa's stained thin blood smears used for monitoring the overall parasitaemias (see 4.2). This study was performed to assess the extent of reticulocytosis and parasite invasion of these cells and whether reticulocyte invasion had any bearing on the courses of infection of the variant parasite populations. The % of reticulocytes present and the % of reticulocytes infected were monitored for individual mice. Statistical analysis was performed using the Wilcoxin signed rank test to assess preference of invasion for each group, and reticulocyte % were compared on each day between groups using the Kruskal-Wallis one-way analysis of variance. The results of these analyses are presented in Figs. 4.4 - 4.6.

In all groups, the proportion of RBC comprising reticulocytes increased as the parasitaemia increased and then continued to increase, over the period examined, after the parasitaemia had peaked (Fig. 4.4). When comparisons were made between groups on each day (Fig. 4.5), all groups showed < 10% reticulocytes on d 7, but with the RC 7 group significantly lower than the parent group on this day ($P < 0.05$). On d 8, all groups still showed < 10% reticulocytes, but with the RC 10 group significantly lower than the RC 8 group ($P < 0.05$). By d 9, all groups showed an increase in reticulocytes to > 10%, with the RC 4 & 8 groups showing an increase to > 20% reticulocytes. The parent and RC 10 groups showed a reticulocyte % significantly lower than these groups ($P < 0.05$). Reticulocyte % in the RC 7 group was not significantly different from any of the other groups. Again, on d 10, reticulocyte % in the parent and RC 10 groups were significantly less than in the RC 4 & 8 groups, with the parent and RC 10 groups

showing around 20% reticulocytes, and RC 4 & 8 > 40%. By d 11, the % of reticulocytes in the RC 4, 7 & 8 groups had risen to >> 60%, with the RC 10 group slightly less but not significantly so. The parent group, however, still showed a significantly lower % of reticulocytes than the RC 7 & 8 groups ($P < 0.05$), but this was nevertheless > 40%.

From Fig. 4.4, it can be seen that as the % parasitaemia increases to a peak and then decreases, the % of reticulocytes infected follows a similar pattern, but at a lower % in all but the RC 10 group. In this group, the % of reticulocytes infected was greater than the % parasitaemia on d 7 & 8. By comparing the data for % parasitaemia and for % reticulocytes infected, no significant preference could be observed in the parent group, but in the RC 4, 7 & 8 groups, parasites showed a significant preference for mature RBC throughout the days examined ($P < 0.05$). For RC 10, a significant preference for reticulocytes was observed on d 8 ($P < 0.05$), but on d 9, 10 & 11, this was reversed, with a significant preference observed for mature RBC ($P < 0.05$).

In all groups, the % of parasites observed in reticulocytes increased over the period examined as the % of reticulocytes present increased (see Fig. 4.4). However, from Fig. 4.6, it can be seen that on d 7 p.i., > 5% of RC 10 parasites were within reticulocytes compared to around 1% of parasites in reticulocytes in all other groups. This was statistically significant ($P < 0.05$). On d 8, the % of RC 10 parasites in reticulocytes was significantly greater ($P < 0.05$) than for RC 4 & 8, but not any others. Through d 9-10, the % of RC 10 and parent parasites in reticulocytes increased more slowly than for the other groups, with significant differences being observed between the parent group and the RC 8 group on d 9, and between the RC 4 group and both the parent and RC 10 groups on d 10. By d 11, all groups showed approximately 30-40% of parasites in reticulocytes, with no significant differences observed between any of the groups.

4.4 Live IFAT analysis of recrudescences

Recrudescences were collected during the course of infection of each variant. These were then tested using the panel of hyperimmune sera in the live IFAT. The pattern of reactivity is shown in Table 4.1, with the reactivity of the cloned populations which had initiated the infections shown above for comparison (from Brannan *et al.* 1993; see also chapter 3). The parasite populations had all altered from the pattern observed in the cloned variant types which had initiated the infections. From these results, all the recrudescences appear to contain a mix of variant types, but recrudescences from infections with different variant types all showed very similar patterns of reactivity against the panel of sera.

4.5 Discussion

The results of this study show that the course of infection of *P. chabaudi* AS in NIH mice can differ for different cloned antigenically variant populations. The parasite clones used were all derived from a previously cloned parent population, which had undergone phenotypic antigenic variation during the course of infection (McLean *et al.* 1986a; Brannan *et al.* 1993). The differences observed *in vivo* between some of these antigenically variant populations possibly may reflect alterations in immunogenicity and/or functional characteristics associated with expression of antigenically variant molecules on the surface of pRBC. Indeed, expression of variant Ags has already been associated with cytoadherence of *P. falciparum*-infected RBC *in vitro* (Magowan *et al.* 1988), with sequestration of *P. c. chabaudi* and of *P. fragile in vivo* (Gilks *et al.* 1990; Handunnetti *et al.* 1987) and with virulence of *P. knowlesi* (Barnwell *et al.* 1983b). Antigenic variation of *P. falciparum in vitro* has also been shown to be associated with alterations in adhesive phenotypes (Roberts *et al.* 1992).

The most striking differences in the courses of infection are the consistent and significantly lower peak parasitaemia observed in RC 10-infected mice, and the later/lack of recrudescence in RC 8-infected mice. Although this chapter describes the courses of infection followed only once, the group sizes were all sufficiently large to ensure the validity of the statistical analysis and the main differences observed between the courses of infection were also observed during other experiments. The lower peak primary parasitaemia in the RC 10 group was not due to an intrinsically lower rate of growth (see chapter 5) and was observed, without exception, in other experiments. This may be due to this variant being significantly more immunogenic than the other variants. This has not been demonstrated to be the case in terms of provoking a specific Ab response in hyperimmunised mice, where the same serum titre is attained against RC 4, 7 & 10 in the live IFAT (Brannan *et al.* 1993; chapter 3). However, RC 10 may provoke a greater level of non-specific, non-Ab responses compared to other variants, or RC 10 parasites may be more susceptible to immune effector mechanisms such as the production of NO, known to occur around the peak of the primary parasitaemia and to contribute to the control of this phase of *P. chabaudi* infections (Taylor-Robinson *et al.* 1993, 1996).

The preference for reticulocytes exhibited by RC 10 early in infection could possibly impose a limitation on the growth of these parasites, but this preference is transitory and by peak parasitaemia the preference is for normocytes, similar to that for the other groups. However, the differences in reticulocyte invasion of RC 10 compared to the other parasite clones may possibly play some role in the lower peak parasitaemias observed in mice infected with RC 10. *P. berghei, P. yoelii* and *P. c. chabaudi*-infected reticulocytes are reported to be more immunogenic than similarly infected normocytes (Poels *et al.* 1977; Jarra & Brown 1980; Jayawardena *et al.* 1983; Schetters

et al. 1986). Therefore, the increased level of reticulocyte invasion by RC 10 parasites early in infection, small as it may appear, may induce an increased or more rapid immune response which prevents the parasitaemia in these mice from reaching the levels observed in infections with other variants.

The difference in the onset of a recrudescence in RC 8-infected mice compared to the other groups is of interest, especially in light of the results of Gilks *et al.* (1990), where the apparent loss of expression of variant surface Ags, and therefore the loss of ability of the parasite to undergo antigenic variation, resulted in a lack of recrudescence. Herein, it was possible to raise hyperimmune serum to a titre of only 1:50 against RC 8 surface Ags in a live IFAT following identical immunisation procedures, which, for other variants, achieved much higher titres (Brannan *et al.* 1993). Therefore, either the variant Ags expressed by RC 8 are of much lower immunogenicity or are expressed at much lower levels. The results of the live IFAT analysis of the recrudescences show clearly, however, that antigenic variation can still occur in the RC 8 population. The reasons for the late/lack of recrudescence in RC 8-infected mice are thus not clearcut. It may be that there is a lack of expression of variant Ags, and thus an inability to undergo antigenic variation, in all but a few of RC 8 parasites. Recrudescences, if appearing at all, would therefore occur considerably later because the longer time taken for the few surviving variants to multiply to detectable levels. If those parasites in the population able to undergo antigenic variation are indeed present at a low frequency, some mice may have either received an inoculum containing no such parasites, resulting in no recrudescence, or an inoculum containing so few parasites that recrudescences develop even later, beyond the timescale of the experiment. Alternatively, where no recrudescences were observed, it is possible that recrudescences were present at very low levels, below the limit of detection by examination of Giemsa's stained thin blood smears by optical microscopy (sensitivity approximately 1 pRBC in 10000 RBC). Other means of analysis would need to be used to ascertain whether parasites were still present in the mice. Sub-inoculation of blood from the infected mice into naive mice and monitoring these for patent parasitaemia would be a sensitive if rather laborious method, which could determine the presence of pRBC not detected by examination of bloodsmears from the initial infections. This has been used to detect the final clearance of pRBC from the bloodstream of infected mice (eg. Gilks *et al.* 1990). Amplification of parasite genomic DNA by the polymerase chain reaction (PCR) is a recent molecular technique for detecting very low numbers of parasites in the bloodstream of infected animals (Snounou *et al.* 1992; Tirasophon *et al.* 1994), and which could be applied to such investigations.

Recrudescences in mice infected with the parent population showed a higher peak parasitaemia than in the other infections, significantly so compared to RC 7 & 8. This may be due to the fact that in the parent infections, there is no cross reactivity between

the infecting population and the recrudescent parasites in terms of surface Ags, as determined by the live IFAT analysis, thereby allowing the recrudescent parasitaemia to reach a higher level before immune mechanisms effect remission to subpatency. In infections with the other recrudescent populations, there is a certain level of cross reactivity between the infecting population and the recrudescent populations, as determined by live IFAT analysis. Therefore, the immune response, having been primed to these common surface Ags, may be able to react more rapidly to the recrudescent parasites, effecting a lower recrudescent peak than in the parent infections.

The fact that the variant populations, derived as antigenic variants of the parent population, can undergo further antigenic variation indicates that this process is not a single event in the course of infection of *P. chabaudi*, and that in a natural host-parasite combination, subsequent recrudescences will consist of further antigenic variants, as has been observed to occur in *P. fragile* infections in toque monkeys (Handunnetti *et al.* 1987).

Reticulocytes were examined during the ascending parasitaemia to investigate any involvement in the different peak parasitaemias observed. Assessment of reticulocyte numbers was by examination of Giemsa's stained thin bloodsmears. Slides were not coated with brilliant cresyl blue to stain residual nucleic acid in reticulocytes, due to the slides being prepared without the intention of examining reticulocytes, which was subsequently decided upon. However, identifying reticulocytes without this counterstaining is straightforward, and comparable results are obtained both with or without brilliant cresyl blue (Taylor-Robinson, personal communication). Indeed, the type of Giemsa's stain used is more important and can affect such results considerably (Taylor-Robinson & Phillips 1993b). The results obtained here for reticulocyte % in the RBC population during *P. chabaudi* infection appear to correspond to those of Jarra & Brown (1989) and Taylor-Robinson & Phillips (1993b) using similar Giemsa's staining. However, in neither of these studies did they report a % of infected reticulocytes > the % parasitaemia, as has been observed for RC 10 early during infection. This preference for reticulocytes seen in RC 10 infections, be it only transitory, indicates that the preference for mature RBC *in vivo* reported for *P. chabaudi* (Carter & Walliker 1975; Landau & Boulard 1978) may not always be uniform, as demonstrated by the results of Taylor-Robinson & Phillips (1994c) and may explain the preference for reticulocytes observed *in vitro* by McNally *et al.* (1992).

That cloned variant populations of *P. chabaudi*, derived from a previously cloned population, can differ significantly and consistently in the course of infection is of importance in interpreting, comparing and extrapolating results obtained from murine malaria models. The particular surface variant Ags expressed by malaria parasites may influence the outcome and severity of infection, and thereby emphasises the complexity of the host-parasite relationship.

Figure 4.1 The course of infection in groups of mice infected with antigenically variant populations of *P. chabaudi* AS.

1 x 10^5 pRBC/mouse i.v. on d 0. **a**. parent; **b**. RC 4; **c**. RC 7; **d**. RC 8; **e**. RC 10.

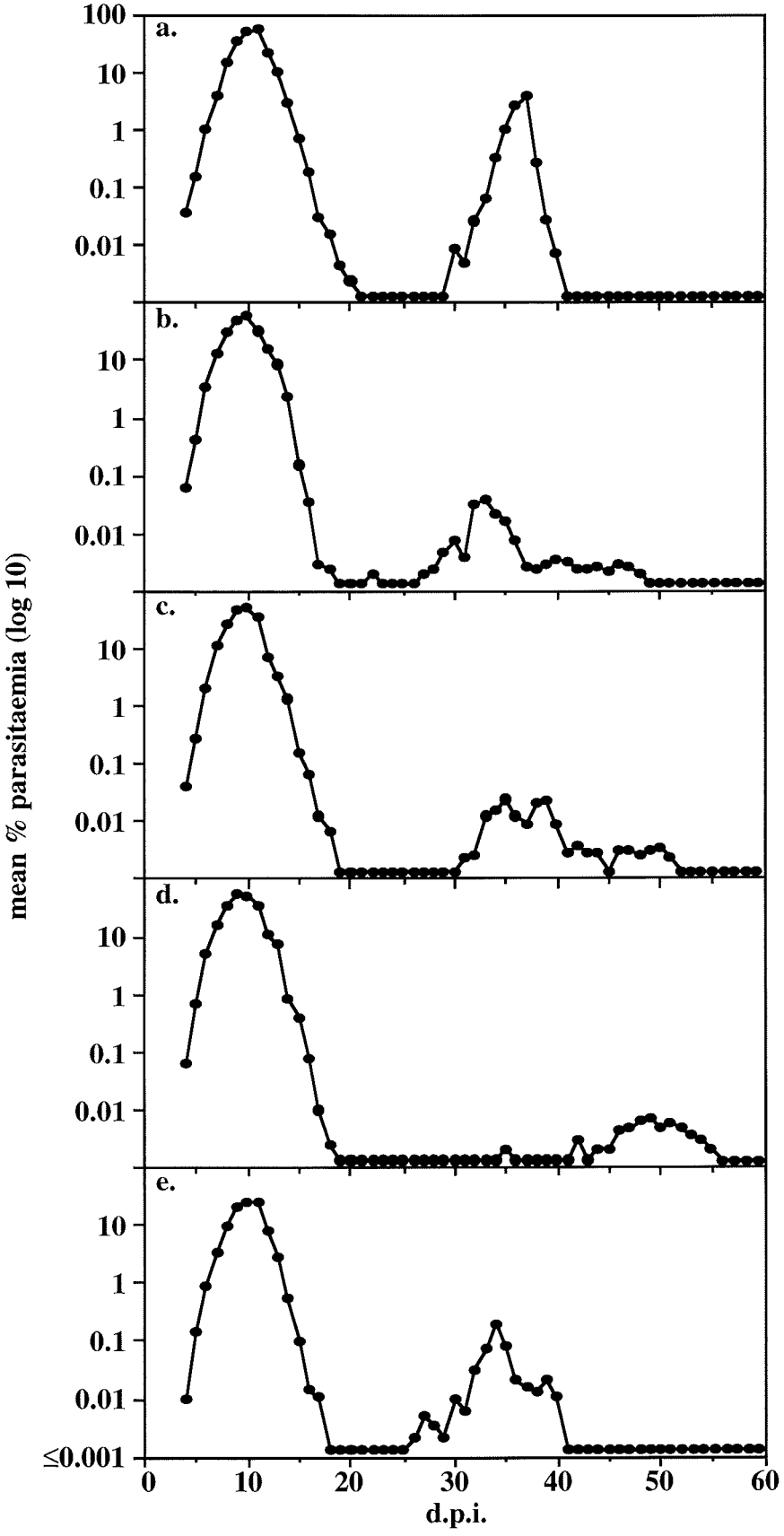


Figure 4.2 Primary peak parasitaemias in groups of mice infected with antigenically variant populations of *P. chabaudi* AS.

1 x 10^5 pRBC/mouse i.v. on d 0. Median peaks ± interquartile ranges.

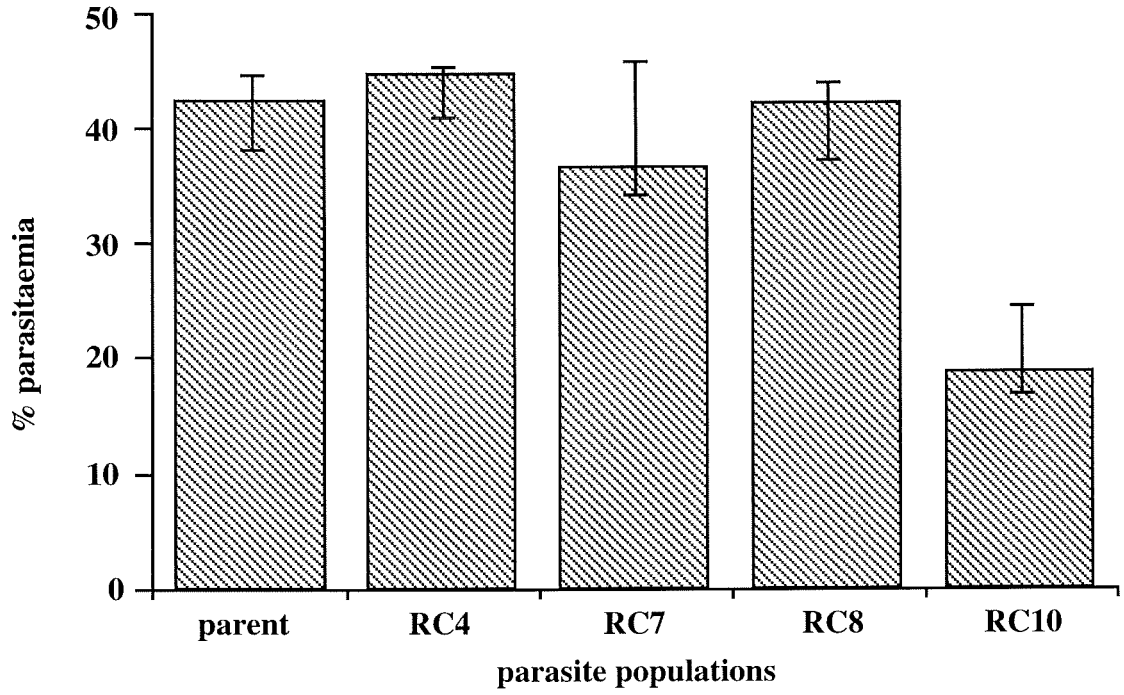


Figure 4.3 Recrudescent peak parasitaemias in groups of mice infected with antigenically variant populations of *P. chabaudi* AS.

1 x 10^5 pRBC/mouse i.v. on d 0. Median peaks ± interquartile ranges.

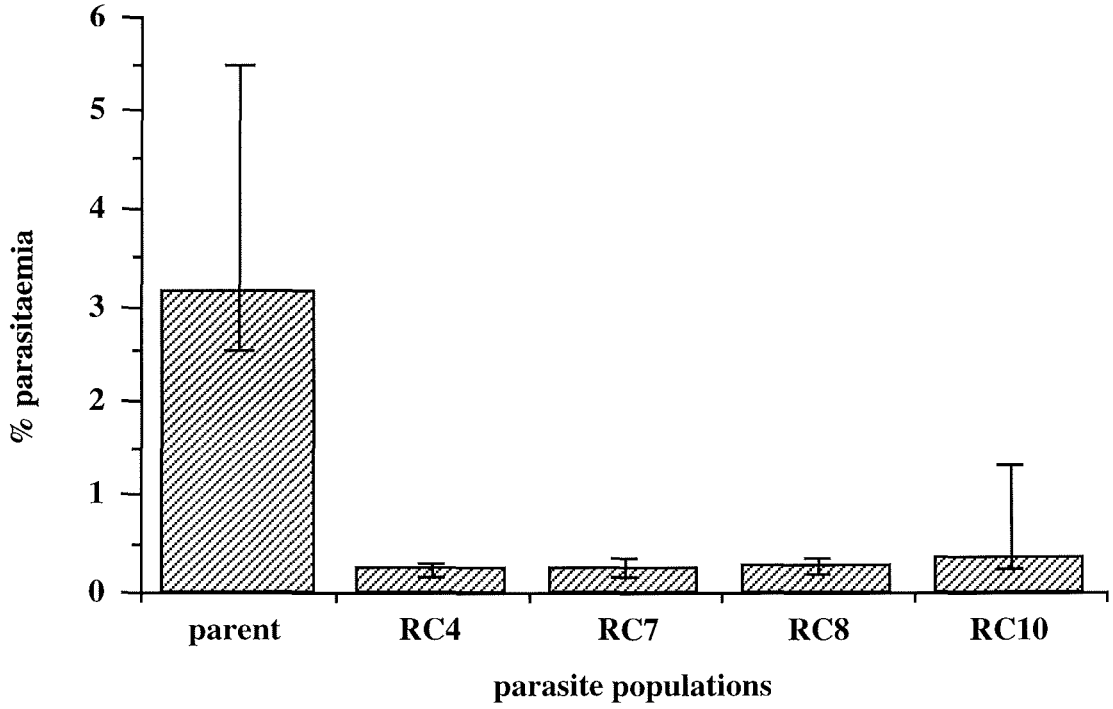


Figure 4.4 RBC invasion in groups of mice infected with variant populations of *P. chabaudi* AS .

1 x 10^5 pRBC/mouse i.v. on d 0. **a**. Parent; **b**. RC 4; **c**. RC 7; **d**. RC 8; **e**. RC 10. Lines represent median % for parasitised erythrocytes (—●—), parasitised reticulocytes (—○—), reticulocytes (—■—) and parasitised reticulocytes as a % of total parasitised erythrocytes (—□—).

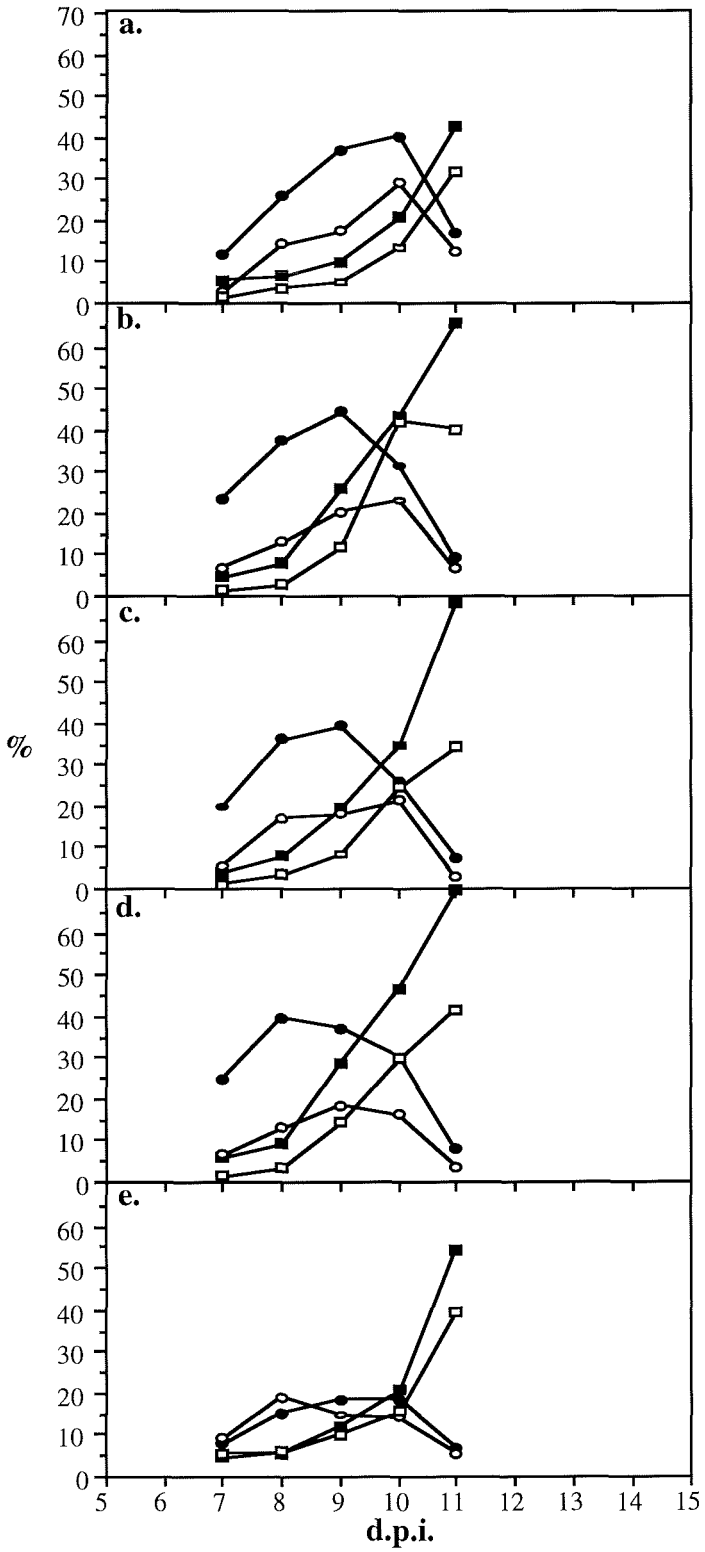


Figure 4.5 Comparison of reticulocytosis on d 7-11 p.i. in groups of mice infected with antigenically variant populations of *P. chabaudi* AS.

1 x 10^5 pRBC/mouse i.v. on d 0. Parent (□), RC 4 (▨), RC 7 (□), RC 8 (▨) and RC 10 (▨). Median % ± interquartile ranges.

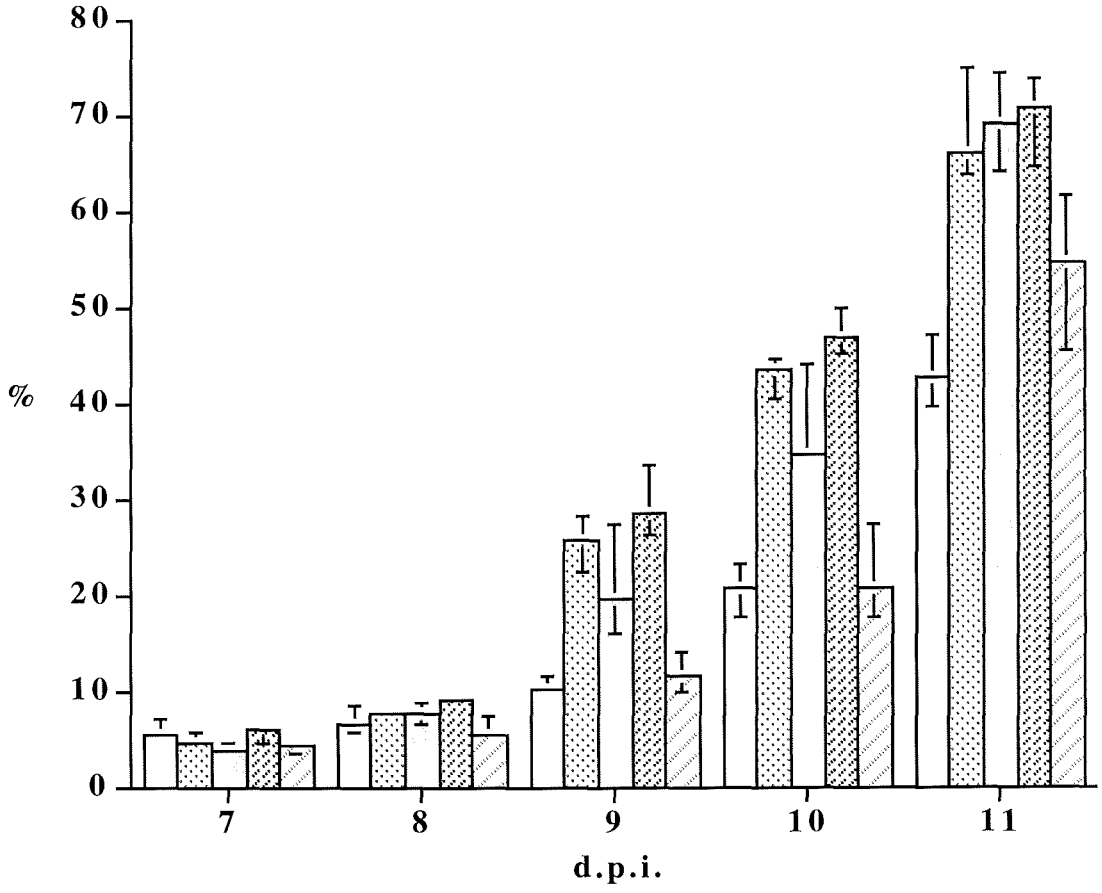


Figure 4.6 **Comparison of parasitised reticulocytes as a % of total pRBC on d 7-11 p.i. in groups of mice infected with antigenically variant populations of *P. chabaudi* AS .**

1 x 10^5 pRBC/mouse i.v. on d 0. Parent (□), RC 4 (▨), RC 7 (□), RC 8 (▨) and RC 10 (▨). Median % ± interquartile ranges.

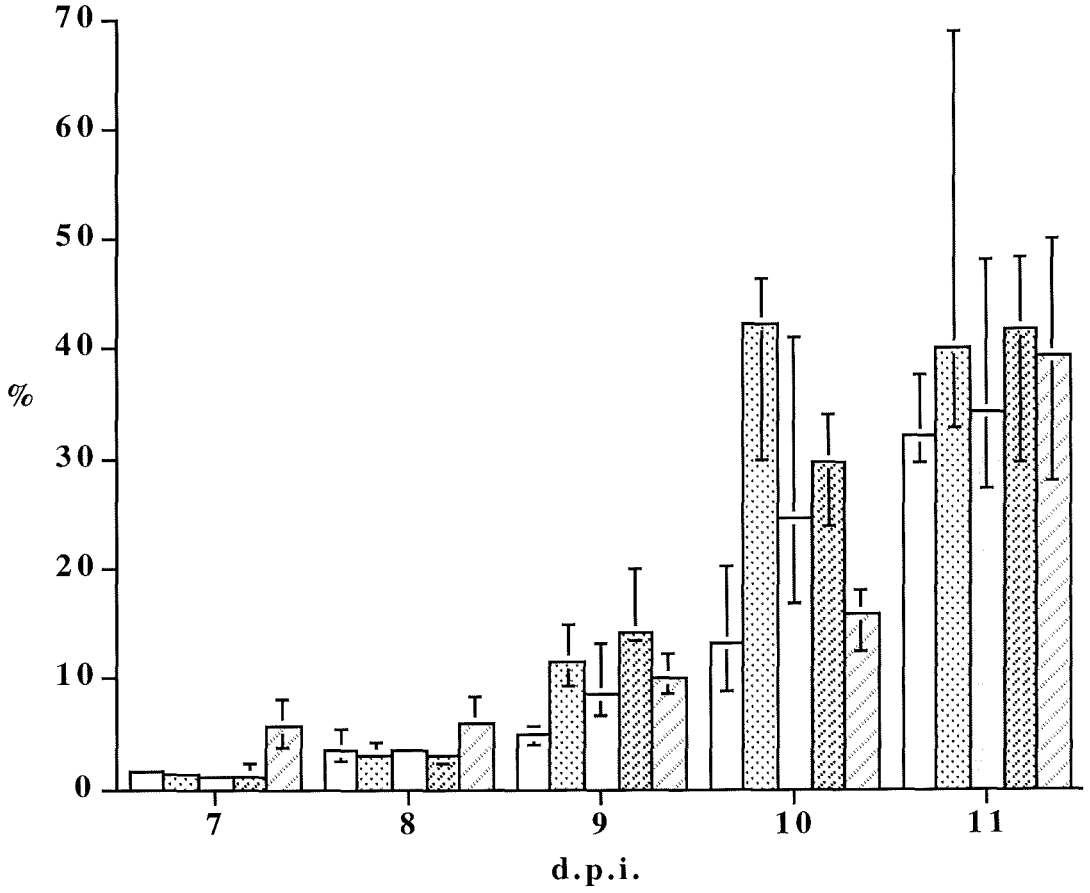


Table 4.1 Live IFAT analysis of recrudescences of infections with antigenically variant populations of *P. chabaudi* compared with the reactivity of the infecting population.

POPULATION TESTED	HYPERIMMUNE SERA RAISED AGAINST											
	parent	MTRC 1	RC 1	RC 2	RC 3	RC 4	RC 5	RC 6	RC 7	RC 8	RC 9	RC 10
parent	200	*100	-	-	-	-	-	-	-	-	-	-
parent d31	-	**100**	**500**	**100**	**1000**	**100**	**500**	**500**	**100**	***10**	**500**	***10**
RC 4	-	100	500	100	100	10000	100	500	100	-	100	-
RC 4 d31	-	**500**	**500**	**100**	**1000**	**500**	**100**	**500**	**100**	***10**	**500**	***10**
RC 7	-	10	100	100	10	10	100	100	10000	-	100	-
RC 7 d33	-	**500**	**500**	**100**	**1000**	**100**	**500**	**500**	**100**	***10**	**500**	***10**
RC 8	-	*50	-	-	-	-	-	-	-	50	-	-
RC 8 d47	-	**500**	**500**	**100**	**1000**	**100**	**500**	**500**	**100**	***10**	**500**	***10**
RC 10	-	*100	-	-	-	-	-	-	-	-	-	10000
RC 10 d31	-	**500**	**500**	**100**	**1000**	**100**	**500**	**500**	**100**	***10**	**500**	***1000**

All values show reciprocal serum titres. *indicates very few +ve pRBC.

CHAPTER 5

ANALYSIS OF ANTIGENIC VARIATION RATES OF *Plasmodium chabaudi* AS *in vivo*

5.1 Introduction

Chronicity of infection is an important contributor to malarial pathogenesis (reviewed by Howard 1988; Terry 1988) and is due, at least in part, to antigenic variation, an immune evasion mechanism which is also a feature of several other parasitic protozoa and bacteria (see 1.5.2). As described previously (1.5.2), malaria parasites have been shown to undergo antigenic variation in several host-parasite combinations and it is now accepted to be a feature of most, if not all, malaria parasites.

A determination of the rate of antigenic variation is of interest because it pertains directly to the nature of the host-parasite interaction. Parasite populations undergoing antigenic variation interact with the immune system such that individual variant Ag types (VATs), and VAT-specific Ab, are detected in linear sequence in an infection (e.g. Handunnetti *et al.* 1987). This pattern has been observed in a wide variety of systems that undergo antigenic variation (reviewed by Borst 1991; Turner 1992). If switching occurs at a low rate, then a straightforward relationship can be envisaged involving 'pacing' of the parasite switching rate with development of VAT-specific immune responses of the host. If antigenic variation occurs at a high rate, however, this view cannot be correct and a more complex functional strategy for immune evasion has to be envisaged. The rate of antigenic variation of *P. falciparum* in *in vitro* culture has been reported to be very high, at up to 2% per generation (Roberts *et al.* 1992).

A possible explanation for a more complex strategy for antigenic variation may lie in the association between the cytoadherence/sequestration phenotype, which allows avoidance of immune clearance in the spleen of infected hosts (see 1.5.2), and antigenic variation (Roberts *et al.* 1993). An association between these two evasion mechanisms has been demonstrated in *P. falciparum* (Biggs *et al.* 1992; Roberts *et al.* 1992) and in *P. chabaudi* (Gilks *et al.* 1990). It may be necessary to consider the functions of both mechanisms in combination. As sequestration of *P. falciparum* in the brain is associated with the pathology of human cerebral malaria (MacPherson *et al.* 1985), the rate of antigenic switching may also be important because of its potential effect on the severity of disease, not only in terms of parasitaemia and chronicity of infection, but also in terms of the cerebral pathology that may be induced.

Experiments with *P. falciparum* are essentially restricted to *in vitro* models, as there is a lack of suitable and available laboratory hosts for *in vivo* studies. *P. chabaudi* infection in mice, therefore, has become a recognised model in which to study host-parasite processes of malarial infection *in vivo* (Long 1988) and antigenic variation has been shown to occur during *P. chabaudi* infections (McLean *et al.* 1982 a & b, 1986; Gilks *et al.* 1990; Brannan *et al.* 1993). The characteristic infection (see 1.7), a synchronous asexual erythrocytic cycle with schizonts sequestering to deep tissue capillaries and with VATs expressed on late-trophozoite/schizonts, likens *P. chabaudi* to *P. falciparum* in several key aspects of its biology. This model has been used,

therefore, to measure *in vivo* rates of antigenic switching for individual VATs. These VAT-specific rates were then summed to derive a minimum estimate for the overall rate of antigenic variation. The results of these studies are presented in this chapter.

5.2 Estimation of the rate of switching

To determine the rates of switching on of 3 minor VATs in the first wave of parasitaemia, in each of two experiments, groups of 30 mice were infected i.v. with 1 x 10^5 pRBC/mouse of the parent population. The courses of infection were followed by examination of Giemsa's stained thin blood smears by optical microscopy to determine the size of the parasite populations and to indicate when they were growing exponentially. When exponential growth was observed, this showed that on successive days the populations differed by one erythrocytic cycle (the duration of the cycle being 24 h for *P. chabaudi*) and immune-mediated killing was essentially absent. At each of two time points, t_A and t_B, during the phase of exponential growth (d 5 & 6 p.i.), 6 mice were selected randomly from each group. Although only 6 mice were used at each of the time points, the large size of the initial groups was necessary to ensure and confirm that growth rates were representative in the randomly selected mice. RBC were collected by cardiac puncture for immunogold labelling and silver staining (IGSS) analysis (see 2.16) as described for *P. falciparum* pRBC (Hommel *et al.* 1991). By this method, the prevalences of VATs were determined using hyperimmune sera at dilutions specific for individual VATs (Brannan *et al.* 1993; see chapter 3). Preliminary experiments, the results of which have not been included in this chapter, suggested that during this phase of exponential growth, the parasite population consists of a 'parent' VAT and several 'minor' VATs. The prevalence of each VAT, P, was based on counts, n, of 25000-30000 pRBC/group of 6 mice/day, with approximately equal numbers of pRBC counted for each mouse. 95% confidence limits, L, of these prevalence values were estimated from $L = 2\sqrt{(pq/n)}$, where $q = 1\text{-}p$ (Snedecor & Cochran 1967).

The data for the sizes of the parasite populations and prevalences of each VAT at time points t_A and t_B were used to calculate VAT-specific switching rates for several minor VATs simultaneously. For each VAT, the size of the parasite population expressing a minor VAT was calculated at t_A and t_B as H_A and H_B, respectively, from the mean total parasitaemia (N) and the prevalence (P) of that VAT. The size of H_B was assumed to be dependent on two components: growth of H_A and switching to that VAT by parasites expressing other VATs (N-H) during the time period $t_{(A\text{-}B)}$. As the population was growing exponentially between the two timepoints (Fig. 1), it was possible to estimate a theoretical population size of H_B, $\hat{H}_B$ that could be attributed exclusively to growth of H_A. The number of parasites that have switched to expression of the minor VAT at t_B, S, is therefore given by H_B-$\hat{H}_B$. The rate of switching per schizogonous event, σ, is given by $S/(N_A\text{-}H_A)$.

These values of σ are, formally, the sum of the rate at which a VAT is switched on minus the rate at which the subpopulation expressing that VAT is switched off. Switching rates, however, have been measured in VATs at low prevalence and therefore this second rate is presumed to be negligible.

A minimum estimate of the overall switching rate was obtained by summation of the individual rates for the minor VATs examined.

5.3 Courses of infection and growth rates

The course of infection for the primary patent parasitaemia observed in each experiment is shown in Fig. 5.1. These are similar to each other and similar to the courses of infection observed in other experiments (e.g. see chapter 4). In both experiments, parasites grew exponentially over the time period examined for switching rates (Fig. 5.2). Growth rates of these exponentially increasing parasite populations were determined by least squares regression analysis on the data for d 4-6 in each experiment and is also shown in Fig. 5.2. The R^2 values indicate a good straight line fit for the data on these days and the slope values are similar for each experiment. However, as can be seen from Fig. 5.2, by d 7 p.i. in both experiments, parasite growth was slowing down. These growth rates are within the usual range observed for growth of *P. chabaudi* AS in NIH mice, examples of which are shown in Fig. 5.3, and which are from an experiment outlined in chapter 4. From these, it can be seen that the growth rates for different VATs are similar when each is used as the infecting population and that all grew exponentially over the time period examined.

5.4 IGSS

The +ve staining obtained using VAT-specific antisera and the IGSS technique to detect minor VATs in a *P. chabaudi* infection is shown in Fig. 5.4. Parasites were visualised in RBC by Giemsa's staining. pRBC recognised by a variant-specific antiserum were visualised by black silver granules covering the surface of the RBC. pRBC not recognised by an antiserum showed no such silver staining, suggesting that each of the 3 antisera that were used labelled in a VAT-specific manner.

5.5 Switching rates

In experiment 1, the switching rates for 3 minor VATs were estimated and in experiment 2 the switching rates for 2 of these minor VATs were again estimated. Prevalence values for specific minor VATs and parasitaemia data are shown in Table 5.1, with the estimates of VAT-specific switching rates calculated from these parameters. In both experiments, the prevalences of the minor VATs were < 1% and increased from t_A to t_B. Such increases have been detected only because very large

numbers of pRBC were counted. A comparison of the minor VAT population size, H_B and theoretical populations size, $\hat{H}_B$, due only to growth of H_A, indicated that a substantial proportion of the increase in size was not due to growth alone, but due to switching. The number of switches, S, accounts for between 9-47% of the increase in population size, depending on the particular VAT.

The data in Table 5.1 show that switching rates to individual VATs can be high. RC 4, for example, is switched on approximately 1 in every 100 times that a parasite undergoes schizogony. The estimates of VAT-specific switching rates were reproducible, as demonstrated by comparisons of the results of the two experiments, where there is a less than twofold difference between the values for RC 4 and RC 10. This indicates that the parasitaemia and prevalence values have been measured with sufficient accuracy to give confidence in the results. Switching rates may vary depending on the VAT being switched on: in both experiments, RC4 was switched on at a threefold higher rate than RC10, and in experiment 1, RC 7 was switched on at a sixfold lower rate than RC 10. These rates are represented graphically in Fig. 5.5.

Estimates for the overall minimum rate of antigenic variation are shown in Table 5.2. These are obtained by summation of the VAT-specific switching rates shown in Table 5.1 for each of the two experiments. Given that other unidentified minor VATs will have been present, but for which switching rates have not been determined in these experiments, the overall rate will be higher than these estimates. The results in Table 5.2 show that antigenic switching occurred at a high overall minimum rate and at least 1 in 80 malaria parasites underwent antigenic variation at each round of schizogony.

5.6 Discussion

The results of the experiments described in this chapter show that antigenic variation occurs at high rates during *P. chabaudi* infection. This is the first study to measure switching rates for malaria parasites *in vivo* and the first to measure rates for individual VATs. These values were measured directly as rates of switching on of minor VATs.

During the phase of exponential growth of the first wave of parasitaemia, at least 4 different VATs were present; the parent, and 3 minor VATs. These minor VATs were detected using the IGSS method with hyperimmune sera specific for each VAT. This method has been found to be as sensitive as the phycoerythrin-based staining method used in the live IFAT (Brannan *et al.* 1993; chapter 2; unpublished observations). The IGSS method, however, has two advantages which enabled such a study of antigenic switching to be performed. Firstly, unlabelled late trophozoites/schizonts can be more readily detected, and secondly, the preparation of permanent slides allowed large numbers of pRBC to be counted. A large sample size was necessary to ensure prevalence values of acceptable accuracy for estimating switching rates. The method used to determine the rate of antigenic switching is similar

to that previously used to measure switching rates in *Trypanosoma brucei* (Turner & Barry 1989). However, there are differences between the two methods of analysis which are due to the differences in biology between the two parasites. In malaria parasites, variable Ags are expressed on the surface of late trophozoite/schizonts (reviewed by Hommel & Semoff 1988) and therefore the switch rate is expressed as a rate per schizont. Also, schizogony is synchronous and occurs in *P. chabaudi* every 24 h; therefore, difference rather than differential equations have been used.

One underlying assumption for the method used herein to calculate switching rates is that the rate of growth is the same for all VATs. The fact that the VATs detected as minor VATs in this study were all found to grow at similar rates when injected into mice at a standard infection inoculum of 10^5 pRBC/mouse (Fig. 5.3) supports this assumption. These results also confirm that the differences observed in the increase in prevalence of these minor VATs were not due to differential growth rates, but rather were due to differential rates of switching on of these VATs.

A second underlying assumption is that specific immune effector mechanisms will not have significantly affected the population growth during the period of analysis. The regression analyses show a good straight line fit over the period of the experiments and therefore support this assumption. Other supporting evidence that this is the case comes from the observation that at this stage of infection, fluorescent Ab titres are not significantly above background levels and that serum taken from mice at this stage of infection could not passively transfer protection (McLean *et al.* 1982a). However, there is likely to have been some activation of non-specific immune mechanisms.

The minimum estimates obtained for overall switching rates of 1.2-1.6% are of a similar order to the 2% switching rate reported for *P. falciparum in vitro* (Roberts *et al.* 1992). This is confirmation, therefore, that the high rate seen *in vitro* can occur *in vivo*. In both cases, it is the spontaneous rate of antigenic variation which has been measured. It has been reported that variant-specific Ab to *P. knowlesi* can induce antigenic variation (Brown 1973; Barnwell *et al.* 1983 a & b). If induction occurs in other species, then there is the potential for these rates to be modified as an infection progresses. There is as yet, however, no evidence for Ab induction of antigenic variation in any species other than *P. knowlesi*. Antigenic variation has been shown to occur spontaneously in *P. falciparum in vitro* (Biggs *et al.* 1991; Roberts *et al.* 1992) and in *P. fragile* in the absence of immune pressure *in vivo* (Handunnetti *et al.* 1987).

The switching rates measured in malaria parasites are consistent with rates of antigenic variation measured in other organisms, for example, tsetse fly-transmitted *T. brucei* (Turner & Barry 1989) and *Borrelia hermsii* (Stoenner *et al.* 1982), where switching rates of up to 10^{-2} and 10^{-3} respectively, have been reported. High rates of switching, considerably in excess of commonly observed rates of spontaneous gene

rearrangements, appear to be a standard feature of systems of antigenic variation (Turner 1992).

The results for individual VATs show that switching on of different VATs occurs at different rates. Conceptually, the rates of switching between VATs may be determined by the VAT which is being switched off, the VAT which is being switched on, by both or by neither. Since the results of this study demonstrate that different VATs are switched on at different rates, this last possibility cannot apply and, at least in part, the VAT being switched on regulates the rate of switching. Whether the VAT being switched off also influences the switching rate has not yet been investigated and is outwith the scope of this study. Investigation of this would be complicated, however, by the difficulty of distingushing between switching off of a VAT and immune clearance of the same VAT.

Differential rates of switching between VATs as observed here can lead to hierarchical expression of VATs in an infection. This is a feature of systems of antigenic variation (Borst 1991; Turner 1992) and has been shown in *P. fragile* infections in toque monkeys (Handunnetti *et al.* 1987). A hierarchy of expression of VATs is necessary for antigenic variation to function in immune evasion, such that different VATs are presented to the immune system at different times (Turner 1992). However, the high rates of antigenic variation observed here for *P. chabaudi* result in more than one VAT being presented to the immune system at any one time. Therefore, the interaction with the host's immune sytem may be more complex than evasion of an individual VAT-specific Ab response. However, due to the hierarchical switching, there will be quantitative differences in the VATs present at any one time, and it may be that a threshold level of a particular VAT is necessary for an effective VAT-specific Ab response to be generated, as has been demonstrated in *T. brucei* (Seed & Sechelski 1988). Alternatively, such rapid rates of antigenic variation may hinder the maturation of VAT-specific immune responses. Either way, antigenic variation will increase the longevity of infection and should therefore facilitate transmission of the parasites from mammal to mosquito.

Antigenic variation has been shown to be linked to a second immune evasion mechanism of malaria parasites, that of cytoadherence/sequestration. This has been demonstrated in both *P. falciparum* (Magowan *et al.* 1988; Roberts *et al.* 1992; Biggs *et al.* 1992) and *P. chabaudi* (Gilks *et al.* 1990). This has so far not been demonstrated with the VATs used in this study (see chapter 6) but it is likely that alternative means of studying cytoadherence/sequestration would detect some link as it is the same parasite strain used by Gilks *et al.* (1990). By mediating sequestration, expression of variant Ags allows schizont-infected RBC to avoid passage through, and thereby immune clearance in, the spleen of infected hosts. Sequestration can also cause considerable pathology, such as cerebral malaria (MacPherson *et al.* 1985). The hierarchical

expression of VATs implies that there may be an equivalent hierarchical expression of cytoadherence phenotypes. Given the importance of sequestration in causing cerebral pathology in *P. falciparum* infection, this study suggests that determining the rate of change of cytoadherence phenotypes and the linkage in rates for antigenic variation and cytoadherence requires investigation.

Figure 5.1 **Courses of infection of *P. chabaudi* in mice for experiments to estimate switching rates of antigenically variant parasites.**

1 x 10^5 pRBC/mouse i.v. on d 0. **a.** experiment 1; **b.** experiment 2.

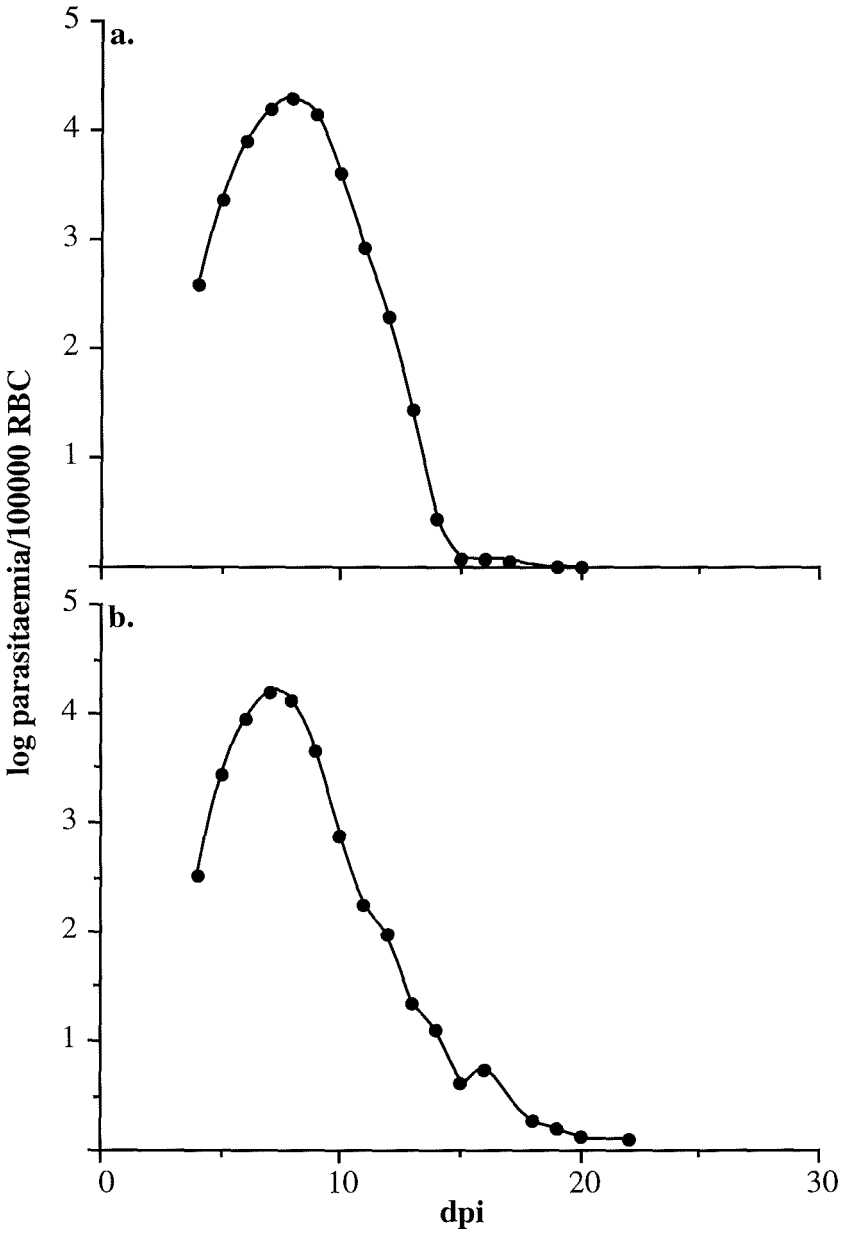


Figure 5.2 **Growth curves for *P. chabaudi* population from d 4-7 p.i. in mice in each of two experiments: regression analysis for d 4-6.**

a. experiment 1; **b.** experiment 2.

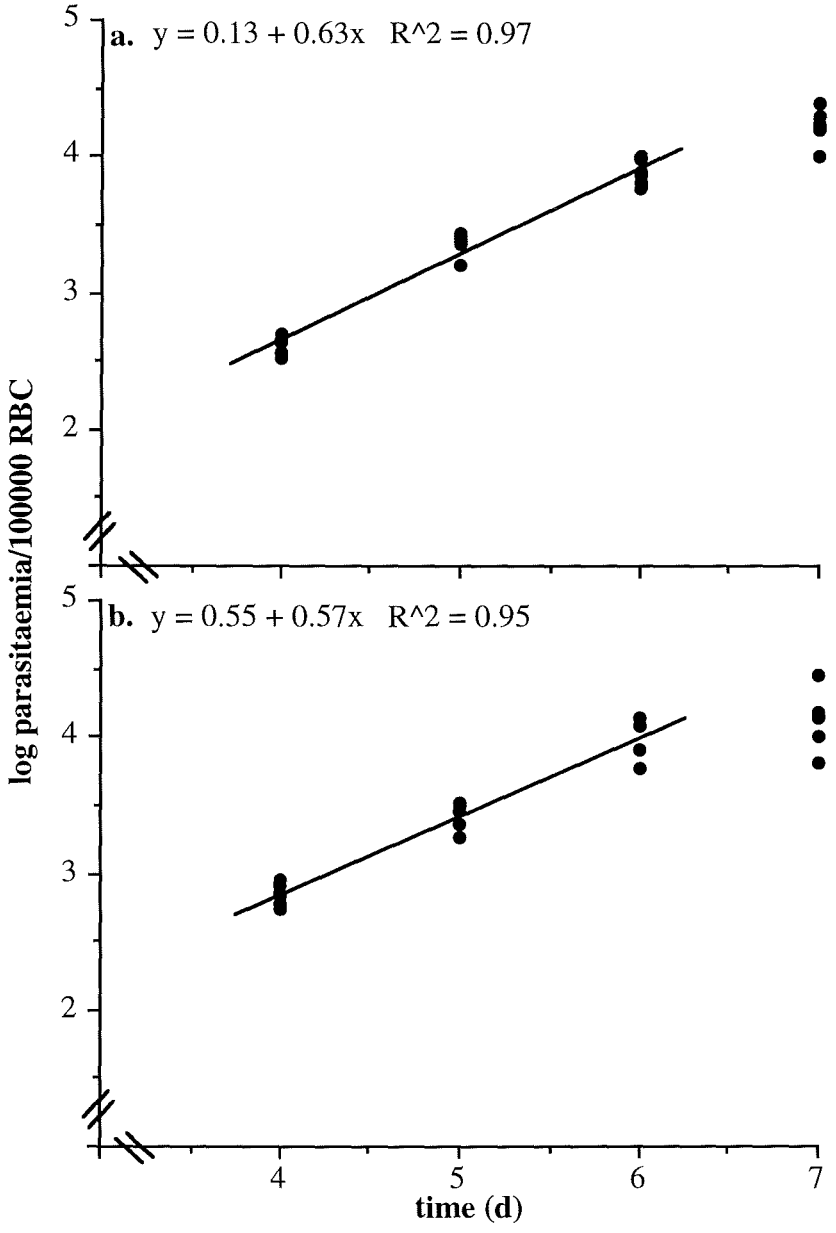


Figure 5.3 **Growth curves for *P. chabaudi* variant populations from d 4-7 p.i.: regression analysis for d 4-6.** **a.** Parent; **b.** RC 4; **c.** RC 7; **d.** RC 10.

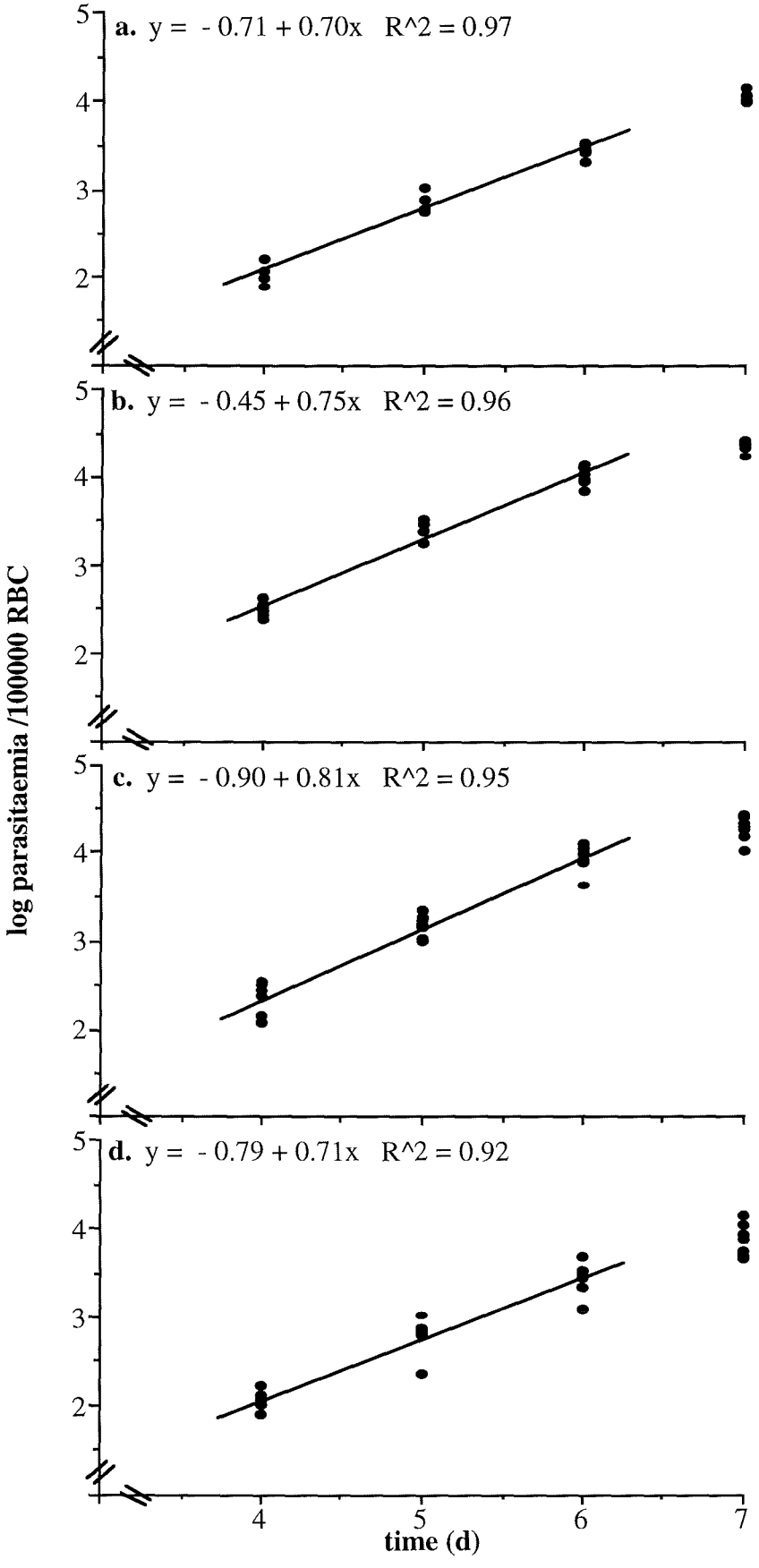


Figure 5.4 ***P. chabaudi*** **-infected blood showing immunogold-silver staining for schizont-infected RBC recognised by VAT-specific hyperimmune sera.**

a. RC 4; **b.** RC 7; **c.** RC 10. The surface variant Ags recognised by the antisera are detected by darkly stippled cells within which a late trophozoite/schizont may be visible (arrow), compared with pRBC not expressing the VAT recognised by the antisera (arrowhead).

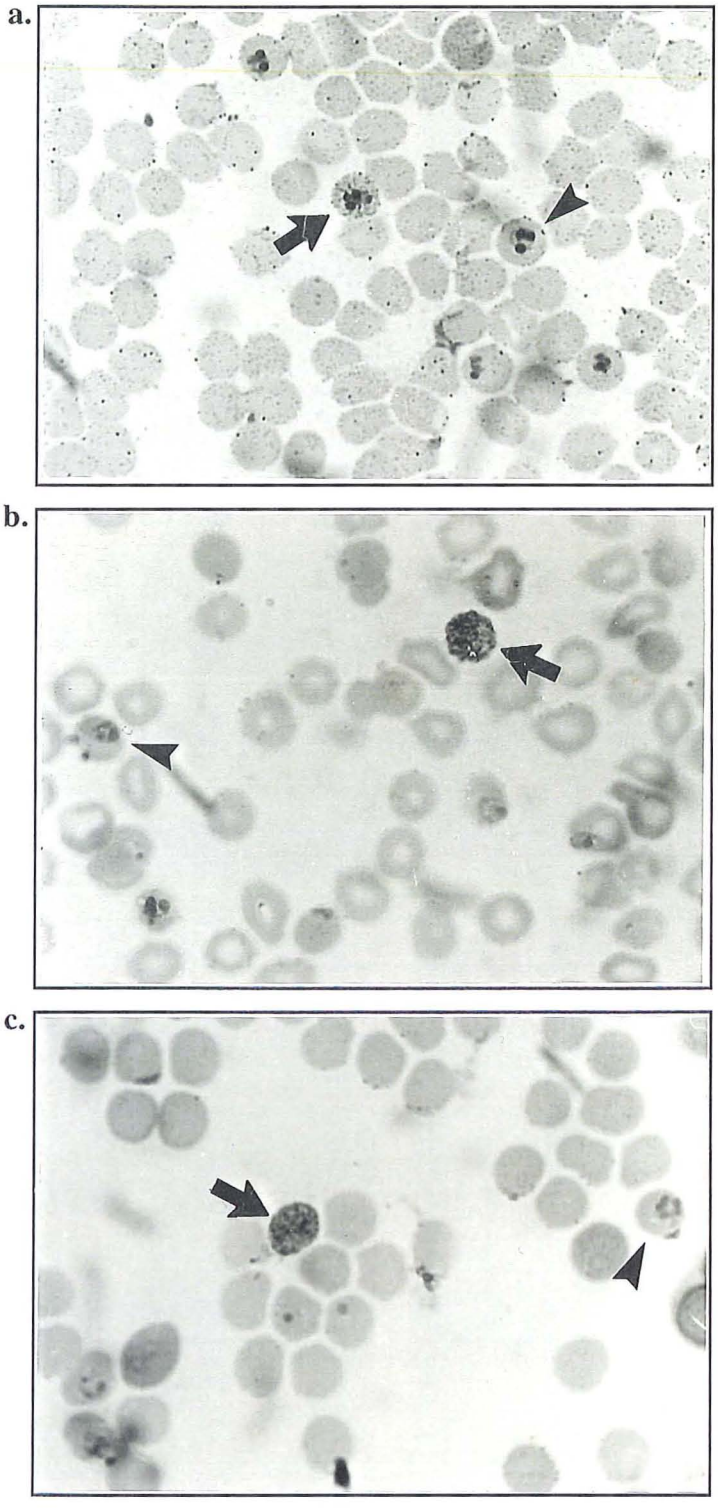


Table 5.1 Results of experiments to determine the rates of switching to individual VATs.

The mean parasitaemia, N, is given as log number of 100000 RBC and the prevalence, P, is expressed as mean ± 95% confidence limits.

Experiment	VAT	Time point	Mean parasitaemia, N (%)	Prevalence of minor VAT, P (%)	Minor VAT population size, H	Theoretical size of minor VAT population due only to growth, $\hat{H}$	Number of switches, S	Rate of switching/ schizont/ day, σ
1	RC 4	*A*	2.35	0.464 ± 0.085	1.09×10^{-2}		2.2×10^{-2}	9.2×10^{-3}
		B	7.93	0.737 ± 0.100	5.84×10^{-2}	3.68×10^{-2}		
1	RC 7	*A*	2.35	0.128 ± 0.047	3.01×10^{-3}		1.0×10^{-3}	4.3×10^{-4}
		B	7.93	0.142 ± 0.042	1.12×10^{-2}	1.02×10^{-2}		
1	RC 10	*A*	2.35	0.121 ± 0.043	2.86×10^{-3}		6.8×10^{-3}	2.9×10^{-3}
		B	7.93	0.207 ± 0.052	1.64×10^{-2}	9.65×10^{-3}		
2	RC 4	*A*	2.73	0.367 ± 0.073	1.00×10^{-2}		3.4×10^{-2}	1.3×10^{-2}
		B	10.37	0.697 ± 0.096	7.22×10^{-2}	3.80×10^{-2}		
2	RC 10	*A*	2.73	0.130 ± 0.042	3.53×10^{-3}		1.1×10^{-2}	4.0×10^{-3}
		B	10.37	0.233 ± 0.056	2.42×10^{-2}	1.34×10^{-2}		

Figure 5.5 **Rates of switching on of variant parasites of *P. chabaudi* calculated from d 5 and d 6 p.i. prevalence counts.**

a. experiment 1; **b.** experiment 2.

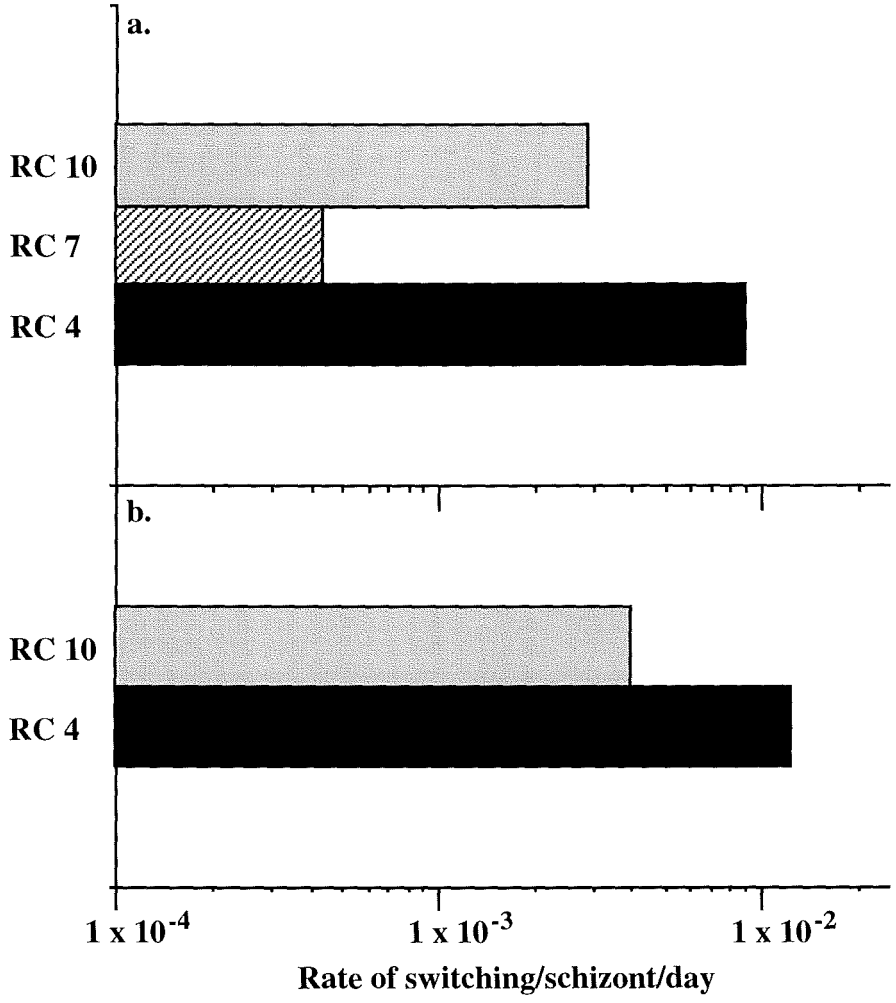


Table 5.2 **Minimum estimates of the overall rate of antigenic variation in two experiments.**

Experiment	Number of VAT combinations	Summed values of σ
1	3	1.25×10^{-2}
2	2	1.65×10^{-2}

CHAPTER 6

SEQUESTRATION *in vivo*, CYTOADHERENCE *in vitro* AND MOLECULAR KARYOTYPING: A COMPARISON OF ANTIGENICALLY VARIANT POPULATIONS OF *Plasmodium chabaudi* AS

6.1 Introduction

P. c. chabaudi in mice and in thicket rats exhibits a synchronous, 24 h asexual erythrocytic cycle, usually undergoing schizogony at around midnight (Landau & Boulard 1978). Normally, shortly before schizogony, most RBC infected with late stage *P. chabaudi* parasites undergo withdrawal from the peripheral circulation, although some schizonts do remain circulating. Sequestration occurs mostly in the liver in murine infections (Cox *et al.* 1987; Gilks *et al.* 1990). Sequestration of late stage parasites also occurs in *P. falciparum* infections, though the major sites of sequestration differ from those of *P. chabaudi* and peripheral withdrawal of schizonts is usually complete (see 1.5.3).

Sequestration of malaria parasites *in vivo* occurs as a result of cytoadherence of pRBC to vascular endothelial cells in various tissues. In *P. falciparum* infections in humans, this can include cytoadherence to endothelial cells lining post capillary venules in the brain, and is causally linked to cerebral malaria (MacPherson *et al.* 1985). Several model systems have been used to study cytoadherence of *P. falciparum*-infected RBC, including primary cultures of human umbilical vein endothelial cells (Udeinya *et al.* 1981) and a variety of human cell lines expressing receptors for adherence (Schmidt *et al.* 1982; Panton *et al.* 1987; Ockenhouse & Chulay 1988). Cells transfected with genes for adherence receptors have also been used for adherence studies (Berendt *et al.* 1989; Oquendo *et al.* 1989; Hasler *et al.* 1993), as have purified proteins (Roberts *et al.* 1985; Barnwell *et al.* 1989; Ockenhouse *et al.* 1989; Hasler *et al.* 1990; Ockenhouse *et al.* 1992a). An *in vitro* model for *P. chabaudi* cytoadherence using mouse cell lines has also been described (Cox *et al.* 1987).

Gilks *et al.* (1990) reported a link between sequestration and expression of variant Ags in *P. chabaudi*, having isolated a parasite clone which did not apparently express surface variant Ags, did not recrudesce and did not sequester over the period of schizogony. *In vitro* cytoadherence has been correlated with expression of surface variant Ags in *P. falciparum* (Magowan *et al.* 1988; reviewed by Howard *et al.* 1990), and antigenic variation of *P. falciparum* has been shown to be associated with changes in cytoadherence phenotypes of parasites *in vitro* (Roberts *et al.* 1992; Biggs *et al.* 1992).

Given such evidence linking antigenic variation/variant Ags with parasite sequestration and cytoadherence, it was considered of interest to examine the variant parasite populations used in this study in terms of their sequestration *in vivo* and their cytoadherence *in vitro*. The results of these studies are presented in this chapter.

Until very recently (and at the time the work described herein was performed), little was known about antigenic variation in *Plasmodium* at the chromosomal level. However, with the identification of the *var* gene family encoding variant Ags involved in alterations of antigenic and cytoadherent phenotypes of *P. falciparum* pRBC (Baruch

et al. 1995; Smith *et al.* 1995; Su *et al.* 1995), our knowledge of this area is set to expand. This progress in our understanding of antigenic variation in malaria parasites has been aided by advances in methods of studying biological events at the molecular level. One such technique, that of pulsed field gel electrophoresis (PFGE) (Schwartz & Cantor 1984), revolutionised the study of *Plasmodium* chromosomes, finally enabling the separation of fourteen chromosomes of *P. falciparum* (Kemp *et al.* 1987; Wellems *et al.* 1987; Gu *et al.* 1990), which is consistent with electron microscope observations of fourteen kinetochores (Prensier & Slomianny 1986). In *P. falciparum*, homologous chromosomes in independently collected isolates can vary in size (Kemp *et al.* 1985; Van der Ploeg *et al.* 1985). Such size polymorphisms occur frequently in natural malarial infections, and can involve deletions (Corcoran *et al.* 1986). Size variations can also occur *in vitro*, usually due to deletions, as has been shown in a study of chromosomes of a cloned *P. falciparum* line (Corcoran *et al.* 1988). Subtelomeric deletions have been shown to be responsible for the loss of expression of several *P. falciparum* Ags including knob-associated histidine-rich protein (KAHRP) (Corcoran *et al.* 1986; Culvenor *et al.* 1986; Ellis *et al.* 1987) and ring-infected erythrocyte surface Ag (RESA) (Cappai *et al.* 1989) and a correlation has been noted between subtelomeric deletions and loss of cytoadherence *in vitro* of *P. falciparum* (Biggs *et al.* 1989). The *var* genes of *P. falciparum* have also been newly located by PFGE to the subtelomeric region at the end of most malarial chromosomes (Rubio *et al.* 1996).

The chromosomes of *P. chabaudi* have been less intensively studied, but also number fourteen (Sheppard *et al.* 1989). As in *P. falciparum*, chromosomal size variations have been found in different isolates of *P. chabaudi* (Langsley *et al.* 1987; Sharkey *et al.* 1988). The variant parasite populations used in this study were found to display differences in the course of infection (see chapter 5) and preliminary investigations of *in vitro* cytoadherence suggested a possible difference between the variant populations in their cytoadherence properties *in vitro*. As there is a correlation between loss of cytoadherence and subtelomeric deletions in *P. falciparum* (Biggs *et al.* 1989), and given the link between antigenic variation and differences in cytoadherence (Roberts *et al.* 1992; Biggs *et al.* 1992), it was considered of interest to examine the chromosomes of the variant *P. chabaudi* populations by PFGE. The results of this molecular karyotyping are also presented in this chapter.

6.2 Sequestration *in vivo* of antigenically variant populations

Groups of 5 mice were each infected with a parasite population (10^5 pRBC/mouse). Parent, RC 1, 4, 7, 8 and 10 parasite populations were included in this study. Mice were kept either in NL or RL, with examination of sequestration in parent and RC 8 parasites repeated in mice kept in both light cycles. There were no differences in the course of infection observed between NL and RL infections. Sequestration was

examined when parasitaemias before schizogony were between 10-20%, except in one case (parent RL), where the mean parasitaemia was slightly > 20%. Peripheral withdrawal over the period of schizogony of variant populations was examined by taking hourly blood smears from usually 3 h before schizogony was expected to occur and continuing for 6-8 h in total. Differential parasitaemia counts in ≥ 1000 RBC were recorded from these. Blood smears were taken starting at least 2 h before schizogony was expected to occur. Parasites were identified as ring stages, trophozoites or schizonts, with parasites counted as schizonts when at least two separate nuclei could be distinguished clearly. The total parasitaemia at each time point constitutes the sum of the parasitaemias for each parasite stage. These parasitaemias are shown in Figs. 6.1-6.6.

In all cases, peripheral withdrawal during schizogony was associated with a transient fall in the total parasitaemia. Trophozoites numbered 100% of parasites initially, decreasing as the % of parasites constituting ring stages increased over the period of schizogony, until ring stages numbered approximately 100% of parasites 6-8 h later. Numbers of schizonts seen in the peripheral circulation remained low, though these parasite stages were never completely absent, over the period of schizogony. Differential parasitaemia counts were not performed at 1000 h and 1100 h in RC 7 infections, but from the subsequent counts, it is apparent that the pattern is the same with this parasite population as for the other infections.

For the parent parasite population, in NL (Fig. 6.1a), a drop in total parasitaemia was observed at 2000 h which was of approximately 30% of the starting parasitaemia that day. Similarly, in RL (Fig. 6.1b), a fall of approximately 30% in total parasitaemia was observed at 1200 h. With RC 1 parasites in NL (Fig. 6.2), a drop in total parasitaemia of approximately 30% was observed at 2000 h. With RC 4 parasites in RL (Fig. 6.3), the drop in total parasitaemia was > 50%, the lowest total parasitaemia being observed at 1300 h. RC 7 in RL (Fig. 6.4) exhibited a drop in total parasitaemia of approximately 30%, observed at 1100 h. With RC 8 in NL (Fig. 6.5a), only a 25% drop in total parasitaemia was observed, occurring at 2000 h. However, in RL (Fig. 6.5b), a drop of approximately 50% in total parasitaemia was observed, occurring at 1200 h. RC 10 parasites in RL (Fig. 6.6) exhibited a drop in total parasitaemia of > 50%, observed at 1200 h. There was no correlation between the decrease in total parasitaemia and the number of schizonts present in the peripheral circulation in mice, and similar levels of circulating schizonts were observed in all the variant parasite infections examined.

6.3 Cytoadherence *in vitro* of antigenically variant populations

Cytoadherence *in vitro* of variant parasite populations was compared by performing binding assays as described by Cox *et al.* (1987), initially using a mouse lung

endothelial cell line, B10 D2, and subsequently also with other fibroblast-like cell lines, 3T3 and 3T3 A31 (see 2.15). Statistical analysis was performed using Student's *t* test to compare initial and final parasitaemias, and analysis of variance for comparison of results between parasite populations and between cell types. In the initial experiments using B10 D2 cells (Table 6.1 and Fig. 6.7), the % of bound RBC parasitised was significantly different from the initial parasitaemia of blood infected with parent, RC 1, RC 4 and RC 7 parasites. With RC 8-infected blood, there was no significant difference between the % of bound RBC parasitised and the initial parasitaemia. However, in these binding assays, no significant differences were observed between any of the parasite populations tested in the increase from the initial parasitaemia (Fig. 6.7). The overall level of binding of pRBC in all these tests was low, ranging from the highest of 64 pRBC/500 cells to the lowest of 1 pRBC/500 cells. There was a background level of binding of nRBC in all test assays which was not significantly different from the level of nRBC binding in unparasitised blood control assays.

In the follow-up set of binding assays using the three different cell types the starting parasitaemia was adjusted to 7% with nRBC. The results of these are shown in Table 6.2. For all the parasite populations tested, parent, RC 4, 7, 8 and 10, with each of the cell types the % of bound RBC parasitised was significantly greater than the initial parasitaemia. There were no significant differences between the three cell types used with regard to the final % of bound RBC parasitised and the cell types did not affect the % binding of the different parasite populations. The % of bound cells parasitised differed significantly between RC 4 and all other parasite populations tested and between RC 7 and all other parasite populations tested. There were no significant differences between parent, RC 8 and RC 10 in this respect. The absolute levels of binding of both pRBC and nRBC were variable, but there was overall a strong positive correlation between the number of pRBC/500 cells and the number of nRBC/500 cells. The numbers of pRBC/500 cells in each test were compared and there was no significant difference overall between the cell types used. There were significant differences between the parasite populations, with the number of pRBC/500 cells differing significantly between each of the parasite populations except between the parent and RC 4 and between the parent and RC 7. There was also significant interaction between the parasite populations and the cell types, indicating that the binding levels of pRBC/500 cells to different cell types differed for different parasite populations. This is illustrated in Fig. 6.8. For the levels of binding of unparasitised RBC/500 cells, there was no difference between the cell types and no significant interaction between the cell types and the parasite populations. There were, however, significant differences between each of the parasite populations tested except between RC 4 and RC 10 and between RC 4 and RC 8.

6.4 Molecular karyotyping of antigenically variant populations

Chromosomes of variant parasite populations were compared by PFGE using a CHEF apparatus. DNA was prepared from parent, RC 4, 7, 8 and 10 parasite populations as described (see 2.21). Two different sets of running conditions were used to display optimal separation of the chromosomes. Firstly, a 3 d run, allowing better separation of the smaller chromosomes (Fig. 6.9) and secondly a 7 d run, allowing better separation of the larger chromosomes (Fig. 6.10). Yeast chromosomes were used as size markers as indicated, and in addition, DNA prepared from *P. chabaudi* AS independently in Edinburgh was used as a control (3CQ). In Fig. 6.9, in all tracks loaded with parasite DNA, ten bands are clearly visible, ranging in size from over 2200 Kb to less than 700 Kb. There appear to be no differences in chromosome sizes between any of the variant parasite populations analysed and these all appeared to have a similar chromosome banding pattern to the control parasite 3CQ. In Fig. 6.10, the larger chromosomes have been separated out more and eleven bands are clearly visible in all tracks loaded with parasite DNA. These range in size from greater than 3500 Kb to less than 850 Kb. Again, there are no apparent differences between any of the variant parasite populations analysed and these all appear to have a similar banding pattern to that of the control parasite 3CQ.

6.5 Discussion

The results of the detailed study of differential parasitaemias indicate that all the recrudescent parasite populations studied exhibited withdrawal of late stage parasites from the peripheral circulation during schizogony. However, this was never complete, with low numbers of schizonts present in the peripheral circulation of infected mice over the time when schizogony occurred. In RL infections, the transient drop in parasitaemia associated with sequestration during schizogony occurred around midday, the slightly different times (± 1 h) considered to be due to differences in the time of recovery from stabilate. In NL infections, this drop in parasitaemia was observed earlier than expected, at 2000 h. This was probably in part due to the time of recovery from stabilate, which was approximately 4-5 h earlier than the corresponding time for RL parasites. This could have been avoided by recovering the stabilate later, but the timing of schizogony around midnight was not necessary for the aims of the experiments. External daylight may also have been a factor, as, although artificial lighting was controlled to have NL and RL as exact opposites, mice kept in NL were also exposed to natural light and therefore affected by longer days. This was not ideal, as at the time of year the NL infections were studied (April), day length was longer than 12 h. The animal house windows were subsequently blacked out to eliminate external light. However, the sequestration studies were not all repeated as the main objectives of the experiments were not affected by this uncontrolled variable.

In all infections studied, the pattern of overall and differential parasitaemias over the period of schizogony were similar to those observed for *P. chabaudi* by Cox *et al.* (1987) and Gilks *et al.* (1990) for sequestering parasites. There were some differences between variant populations in the mean level of decrease observed in total parasitaemia, but the decreases were not consistent between groups of mice infected with the same variant population. A possible explanation for this may be differences between the time at which the maximum decreases occurred and the time at which parasitaemias were recorded in some groups. These variations in the level of decrease in total parasitaemia may also have been due to differences between groups of mice, experiments having been performed at different times. The particular variant Ags expressed and the differences in the level of expression/immunogenicity of these antigens, as measured by Ab titres in the live IFAT (see chapter 3), did not seem to affect sequestration. The total % parasitaemia of RC 8 (low titre) dropped just as much as those of RC 4, 7 and 10 (high titre); levels of circulating schizonts were also similar.

The results of the *in vitro* binding assays comparing variant parasite populations demonstrated that binding was preferential for pRBC compared to nRBC for all variant types studied. However, in the initial experiments with B10 D2 cells, preferential binding of pRBC was observed with blood infected with parent, RC 1, 4 and 7. No preferential binding of pRBC from blood infected with RC 8 was observed. The follow-up experiments with the three different cell types did show preferential binding of all the populations studied, including RC 8, with no difference between the cell types. The reason for this discrepancy between the two sets of experiments is not entirely clear. In the first set of experiments using the B10 D2 cell line, however, initial parasitaemias were not adjusted with nRBC to a uniform parasitaemia; therefore, comparisons between the parasite populations in terms of the % bound cells parasitised and the numbers of pRBC/500 cells could not be made. The different starting parasitaemias may also have affected the results. For these reasons, further repeats of binding assays performed exactly as in the first set of binding assays were not performed, which would have been necessary for final conclusions to be drawn from the results obtained. However, initial results with the B10 D2 cells did appear to indicate that RC 8 pRBC did not show preferential binding and other parasite populations did, in that the % of bound RBC parasitised was significantly different from the starting parasitaemia. When these results were examined by comparing the increases in parasitaemia after binding, however, no significance difference could be found between the parasite populations. These results were, therefore, difficult to interpret.

In the subsequent set of experiments, appropriate modifications were made. The starting parasitaemia was adjusted to 7% in all cases and a greater number of binding assays was performed with each parasite population, thereby allowing a more satisfactory analysis of the results. In this set of experiments, using the three different

cell lines, binding was specific for pRBC with all parasite populations studied. The differences between the parasite populations in the % of bound cells parasitised is difficult to interpret separately from the absolute levels of binding of pRBC. Nevertheless, there appears to be a higher specificity of binding of pRBC of the parent, RC 8 and 10 compared to RC 7 and 4 and of RC 7 compared to RC 4. This is not affected by the different cell types. However, the level of binding is different for different parasite populations. These differences do not seem to relate to the specificity of binding, but there is a strong correlation between the level of binding of pRBC and the level of binding of nRBC. This may well reflect the thoroughness of washing to remove unbound RBC or a difference in the binding capacity of the cell lines from assay to assay, perhaps dependent on how rapidly the cells are dividing, the stage of division of the cells, or the cell density. Such reasons may account also for the different patterns of binding of parasite populations with different cell types.

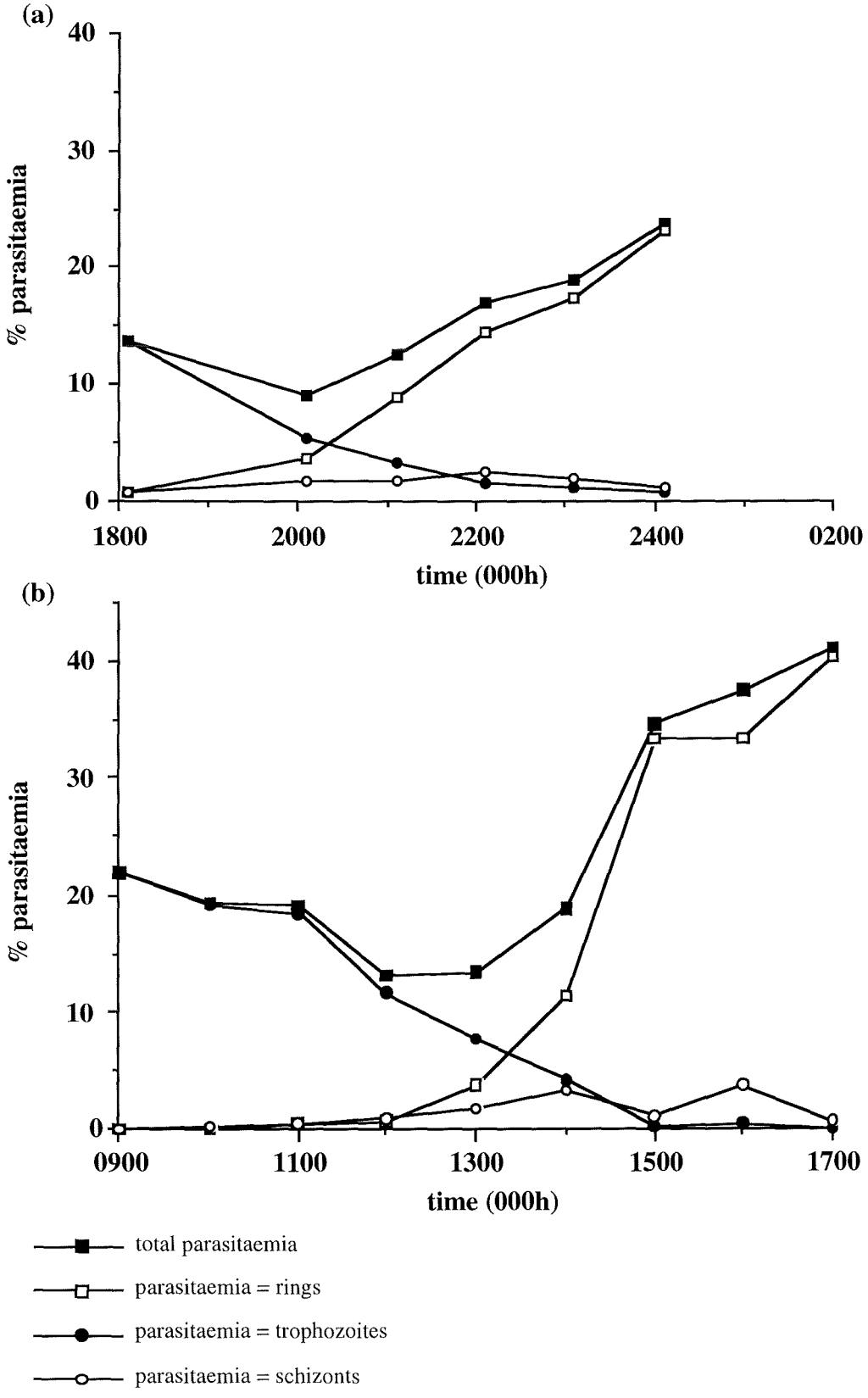
Comparing cytoadherence *in vitro* of variant populations was, overall, not very satisfactory. Levels of binding were usually low, especially compared to the levels observed with *P. falciparum* in similar binding assays with C32 melanoma cells (Schmidt *et al.* 1982), and there was often a high level of variability between individual binding assays using the same parasite population, even where these were performed at the same time. For practical reasons, binding assays could not all be performed at once, and although every effort was made to standardise the assays, differences between the parasite stages used in the assays, in the thoroughness of washing and in the density of the adherent cell lines on the Petri dishes may all have contributed to this variability. Further investigations would be necessary to ascertain the receptors on the cells responsible for the specific binding. Differences in expression of these between the cells types, whether the levels of expression are affected by cell division etc. would all be of interest in the interpretation of such binding assays. Alternatively, purified host cell receptors could be used in binding assays. Whether or not there are real differences between the variant parasite populations in terms of their cytoadherence could then perhaps be elucidated.

In vivo, it appears that sequestration of *P. chabaudi* is due to cytoadherence mainly to endothelial cell lining the liver sinusoids (Cox *et al.* 1987), though binding to Kuppfer cells in the liver has also been reported (Gilks *et al.* 1990) but this is more likely to be an immune clearance mechanism than sequestration. It would therefore be preferable to perform binding assays using liver endothelial cells. The specificity and capacity of binding of pRBC to these cells may be greater than to the cell lines used in this study, and the receptors responsible for the binding may be different. Furthermore, the capacity of sera raised against variant antigens to inhibit specific binding would be of considerable interest. It is, however, outwith the scope of this study to investigate these possibilities.

Molecular karyotyping of the antigenically variant populations by PFGE separated 10 or 11 chromosomal sized bands. The results were similar to those previously observed for *P. chabaudi* chromosomes with the running conditions used (J. Carlton, personal communication). Some of the bands represent more than one chromosome; the intensity of staining is increased due to the greater amount of DNA present in these bands. In Fig. 6.9, counting the chromosomes from the smallest up, 1 + 2 = 1 band, 3-7 are each represented by one band, 8 + 9 = 1band, 10 = 1band, 11 + 12 = 1 band and 13 + 14 = 1 band. With both of the running conditions used, the variant populations all appeared to have the same number and sizes of chromosomes. It was of interest to compare the molecular karyotypes of the variant populations as chromosome polymorphisms have been detected between different isolates of *P. chabaudi* (Sharkey *et al.* 1988), probably representing antigenic diversity between isolates. All the variants examined herein have the same karyotype, demonstrating that they are from the same original parasite population and confirming that the differences in variant Ags are due to true phenotypic antigenic variation.

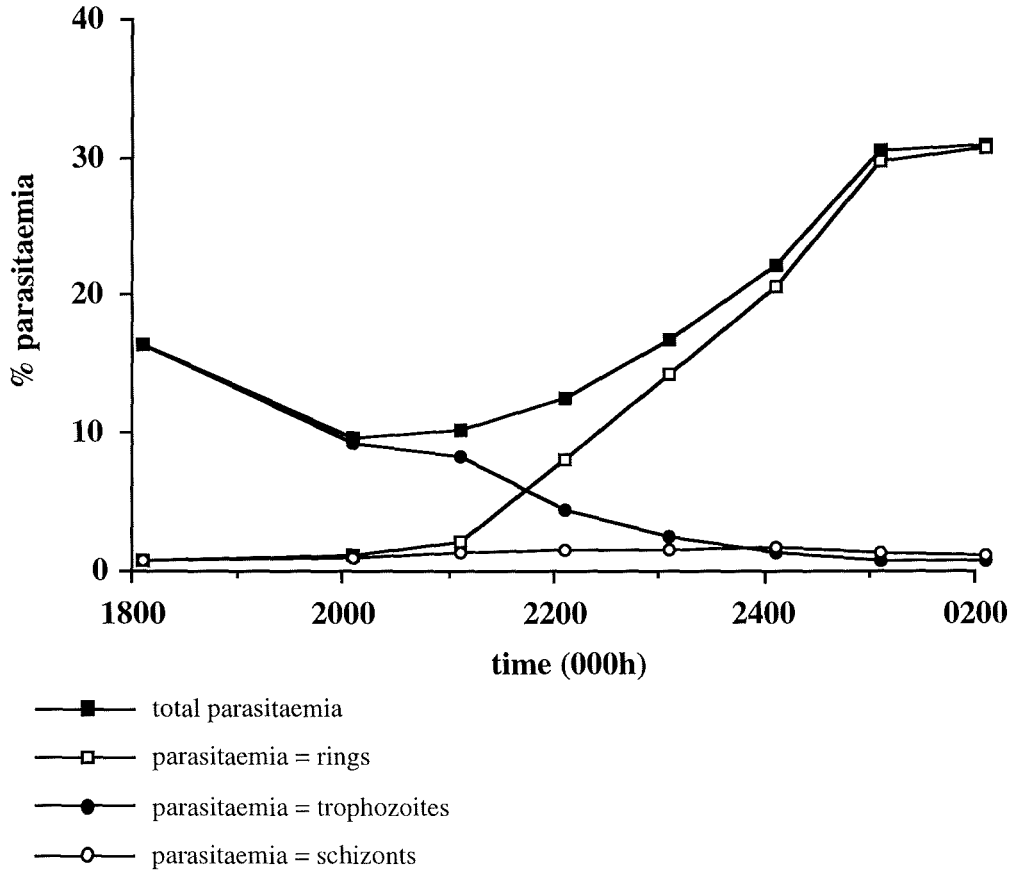
The variant parasite populations included in this study do apparently show some differences in their cytoadherence properties *in vitro*, but all exhibit cytoadherence *in vitro* and sequestration during schizogony *in vivo*. No chromosomal size variations could be observed between them by PFGE. From these observations, it appears that chromosomal rearrangements such as deletions, as described for *P. falciparum* (Biggs *et al.* 1989), have not occurred in any of the parasite populations leading to loss of cytoadherence or lack of sequestration. From initial results with RC 8, which reacted with homologous hyperimmune serum to a titre of only 1:50 in the live IFAT, and infections of which showed a lack or late onset of recrudescence, it appeared that this variant population bore similarities to a parasite population identified by Gilks *et al.* (1990). However, the results presented in this chapter demonstrate that RC 8 does sequester *in vivo* and also exhibits cytoadherence *in vitro*. This parasite population is not, therefore, similar to that described by Gilks *et al.* in this respect. It is likely that antigenic variation in *P. chabaudi* leads to differences in cytoadherence phenotypes as in *P. falciparum* (Roberts *et al.* 1992), but an improved method of studying cytoadherence *in vitro* for *P. chabaudi* would be necessary before this could be demonstrated conclusively.

Figure 6.1 Peripheral withdrawal during schizogony of parent parasite population: differential parasitaemias from tail-blood smears taken from mice in (a) NL and (b) RL.



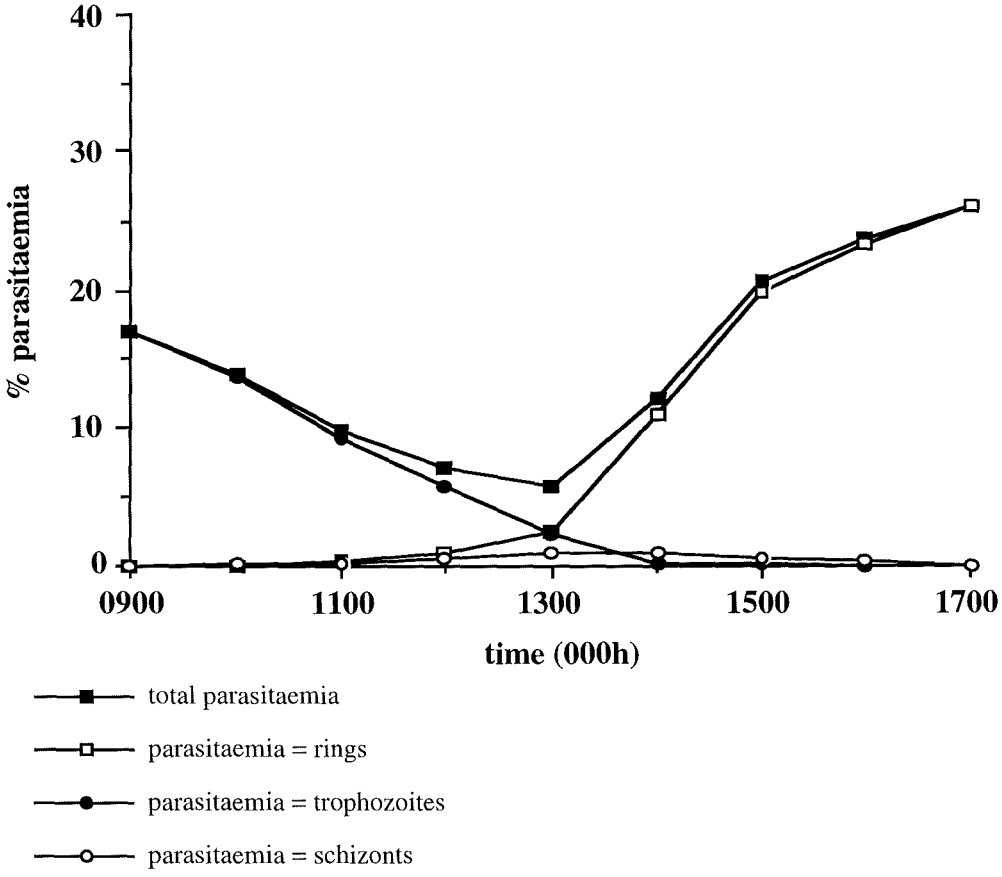
Each point represents the arithmetic mean parasitaemia of 5 mice.

Figure 6.2 Peripheral withdrawal during schizogony of RC 1 parasite population: differential parasitaemias from tail-blood smears taken from mice in NL.



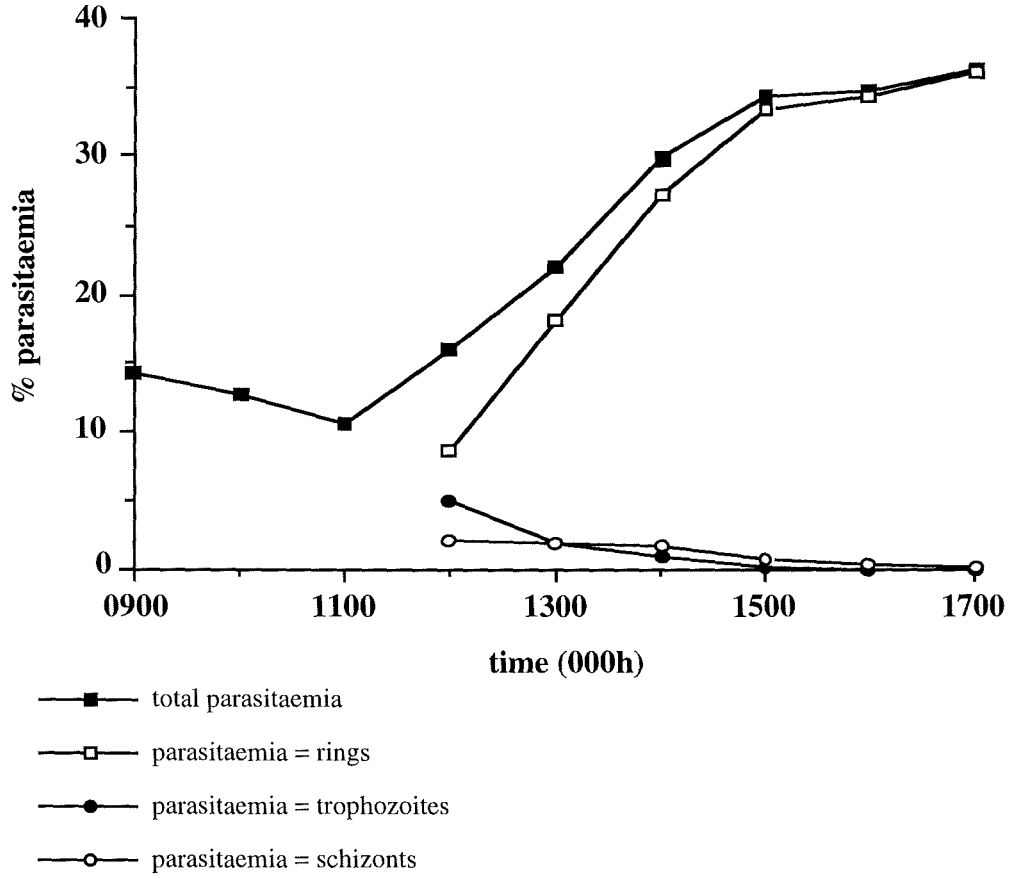
Each point represents the arithmetic mean parasitaemia of 5 mice.

Figure 6.3 Peripheral withdrawal during schizogony of RC 4 parasite population: differential parasitaemias from tail-blood smears taken from mice in RL.



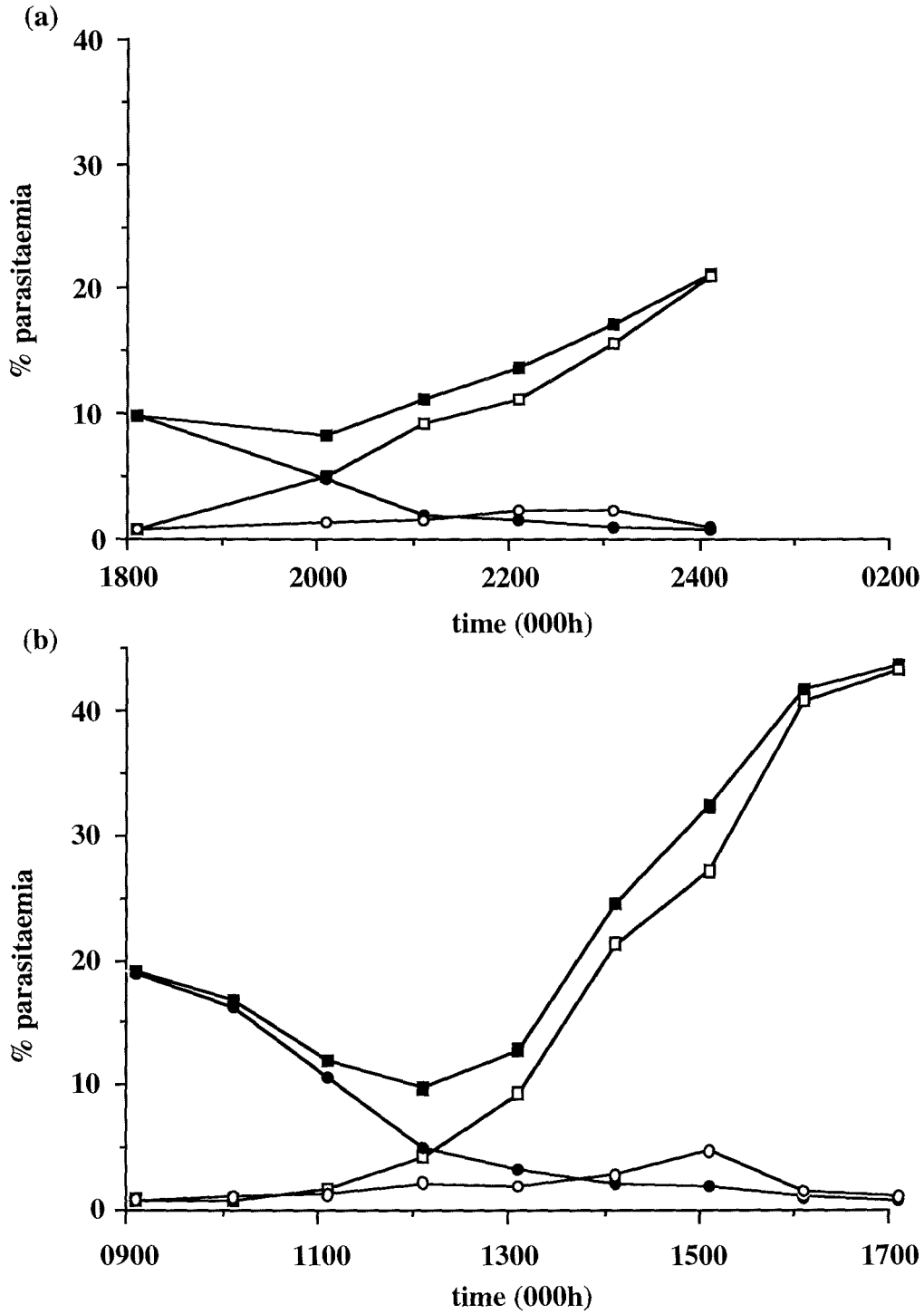
Each point represents the arithmetic mean parasitaemia of 5 mice.

Figure 6.4 Peripheral withdrawal during schizogony of RC 7 parasite population: differential parasitaemias from tail-blood smears taken from mice in RL.



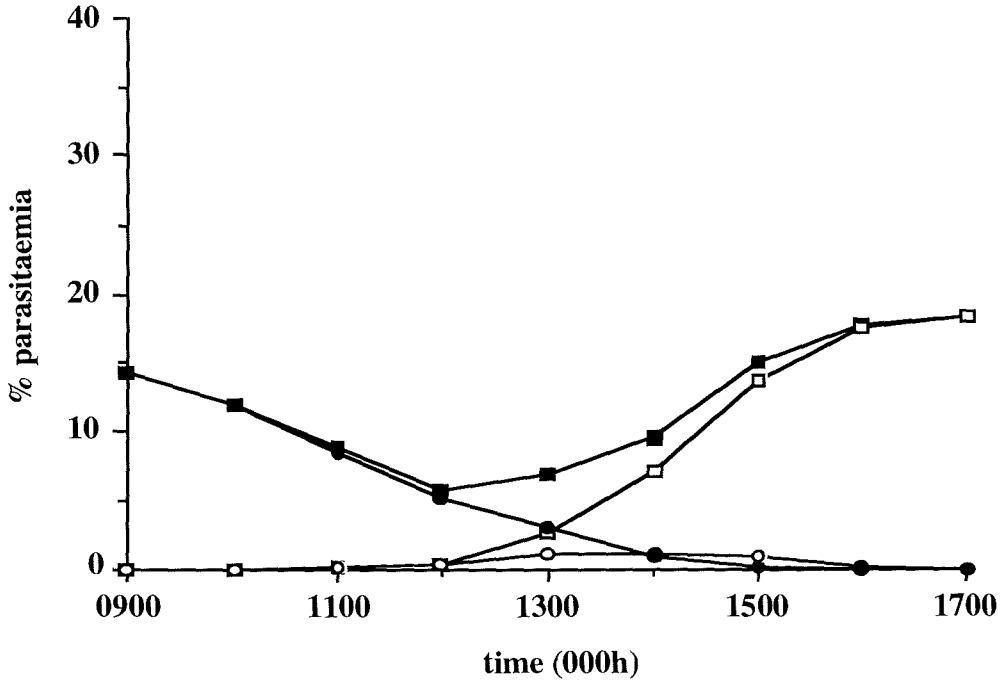
Each point represents the arithmetic mean parasitaemia of 5 mice.

Figure 6.5 Peripheral withdrawal during schizogony of RC 8 parasite population: differential parasitaemias from tail-blood smears taken from mice in (a) NL and (b) RL.



Each point represents the arithmetic mean parasitaemia of 5 mice.

Figure 6.6 Peripheral withdrawal during schizogony of RC 10 parasite population: differential parasitaemias from tail-blood smears taken from mice in NL.



Each point indicates the arithmetic mean parasitaemia of 5 mice.

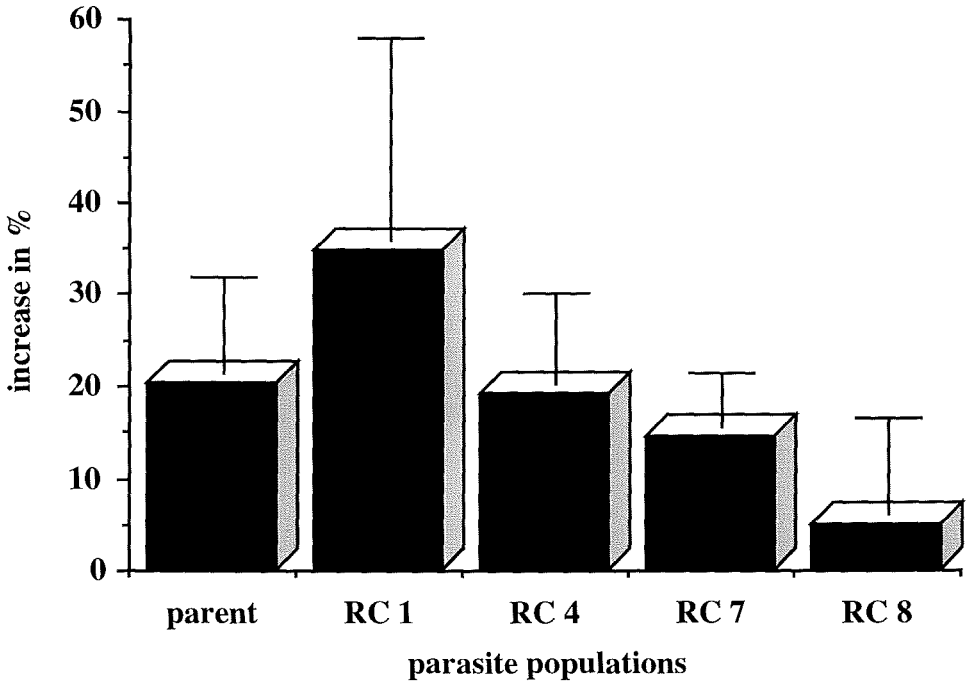
Table 6.1 A comparison of *in vitro* cytoadherence of variant parasite populations using B10 D2 cell line.

Parasite population	n	initial parasitaemia (% ± S.D.)	% bound RBC parasitised (mean ± S.D.)	no. pRBC/500 cells (mean ± S.D.)	no. nRBC/500 cells (mean ± S.D.)
parent	4	9.70 ± 1.77	*29.90 ± 12.19	28.02 ± 26.22	52.30 ± 30.71
RC 1	4	21.65 ± 11.37	*56.58 ± 15.16	22.75 ± 14.68	14.50 ± 3.00
RC 4	4	18.00 ± 6.28	*37.24 ± 12.12	16.52 ± 8.93	25.81 ± 7.04
RC 7	3	13.91 ± 4.68	*30.50 ± 7.09	14.95 ± 11.45	30.25 ± 16.16
RC 8	3	11.97 ± 2.45	16.99 ± 12.79	5.31 ± 4.01	30.82 ± 31.74
nRBC control	14	-	-	-	26.52 ± 26.55

n = number of binding assays, 500 B10 D2 cells counted/assay.

* indicates significant difference in % bound RBC parasitised compared to initial parasitaemia, $p < 0.05$.

Figure 6.7 **A comparison of *in vitro* cytoadherence to B10 D2 cell line of variant parasite populations: increase from initial parasitaemia in % bound cells parasitised**



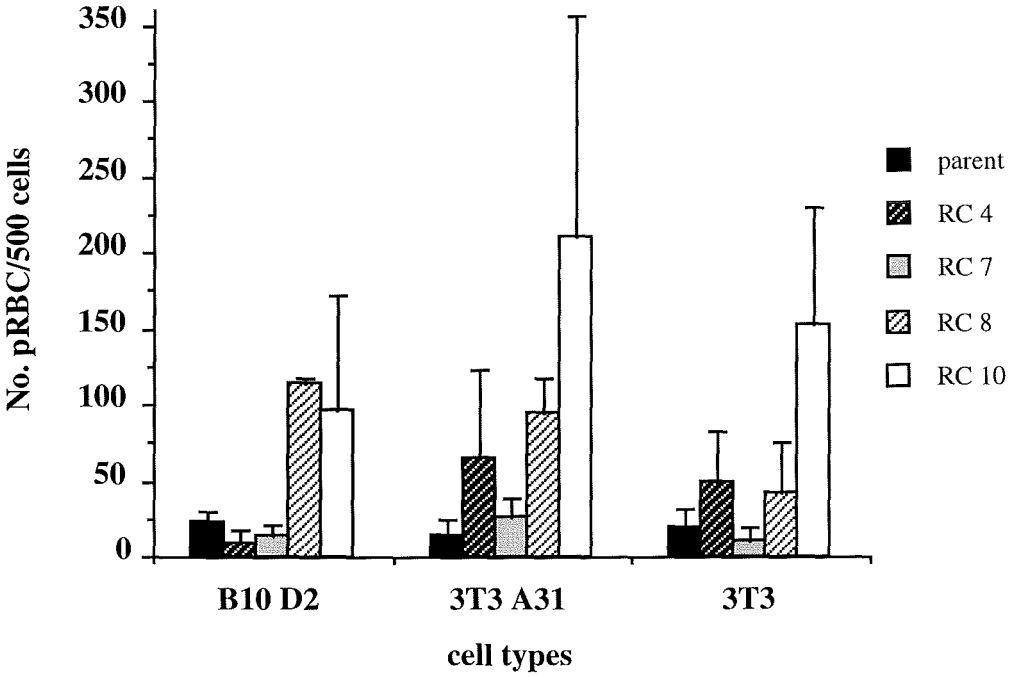
Each bar represents the arithmetic mean increase from initial parasitaemia, error bars = S.D.

Table 6.2 A comparison of *in vitro* cytoadherence of variant parasite populations using different cell lines.

Parasite population	Cell line	n	% bound RBC parasitised (mean ± S.D.)	no. pRBC/500 cells (mean ± S.D.)	no. nRBC/500 cells (mean ± S.D.)
Parent	B10 D2	4	*74.64 ± 13.97	22.00 ± 8.41	9.00 ± 6.32
	3T3 A31	8	*63.17 ± 9.06	14.00 ± 10.53	7.50 ± 4.99
	3T3	7	*76.14 ± 13.77	18.71 ± 12.12	8.00 ± 8.33
RC 4	B10 D2	4	*51.12 ± 8.47	9.00 ± 8.04	10.50 ± 12.39
	3T3 A31	7	*45.99 ± 8.96	64.29 ± 58.13	76.43 ± 71.90
	3T3	8	*42 61 ± 8.01	50.00 ± 32.11	65.50 ± 39.61
RC 7	B10 D2	4	*54.76 ± 7.24	13.50 ± 6.76	10.50 ± 3.00
	3T3 A31	7	*57.83 ± 8.99	25.82 ± 12.78	21.52 ± 16.91
	3T3	8	*48.67 ± 13.89	10.50 ± 8.09	13.50 ± 12.21
RC 8	B10 D2	4	*90.28 ± 2.26	113.50 ± 3.42	12.25 ± 2.99
	3T3 A31	8	*74.26 ± 12.02	94.50 ± 22.95	32.63 ± 16.23
	3T3	8	*59.73 ± 19.92	42.50 ± 33.21	20.63 ± 6.52
RC 10	B10 D2	8	*78.66 ± 17.37	96.5 ± 74.81	75.25 ± 122.98
	3T3 A31	12	*69.96 ± 20.21	211.58 ± 145.42	84.58 ± 76.39
	3T3	12	*71.10 ± 10.42	152.83 ± 77.46	69.42 ± 64.54

n = number of binding assays performed, ≥ 500 cells counted/assay. * indicates significant difference in % bound RBC parasitised when compared to initial parasitaemia of 7 %.

Figure 6.8 **A comparison of *in vitro* cytoadherence of variant parasite populations using different cell lines: the difference between cell types in the number of bound pRBC/500 cells is different for each variant parasite population.**



Each bar represdents the arithmetic mean number of pRBC/500 cells, error bars = S.D.

Figure 6.9 Fractionation of *P. chabaudi* AS variant parasite chromosomes by pulsed field gel electrophoresis.

Chromosomes separated in a CHEF apparatus, 3d run (see 2.21). Track Y =Yeast chromosomes*S. cerevisiae*, with approximate sizes (Kb) indicated on left. Other tracks are chromosomes of *P. chabaudi* variant populations as indicated. 3CQ= chromosomes of *P. chabaudi* AS population as used and prepared in Edinburgh.

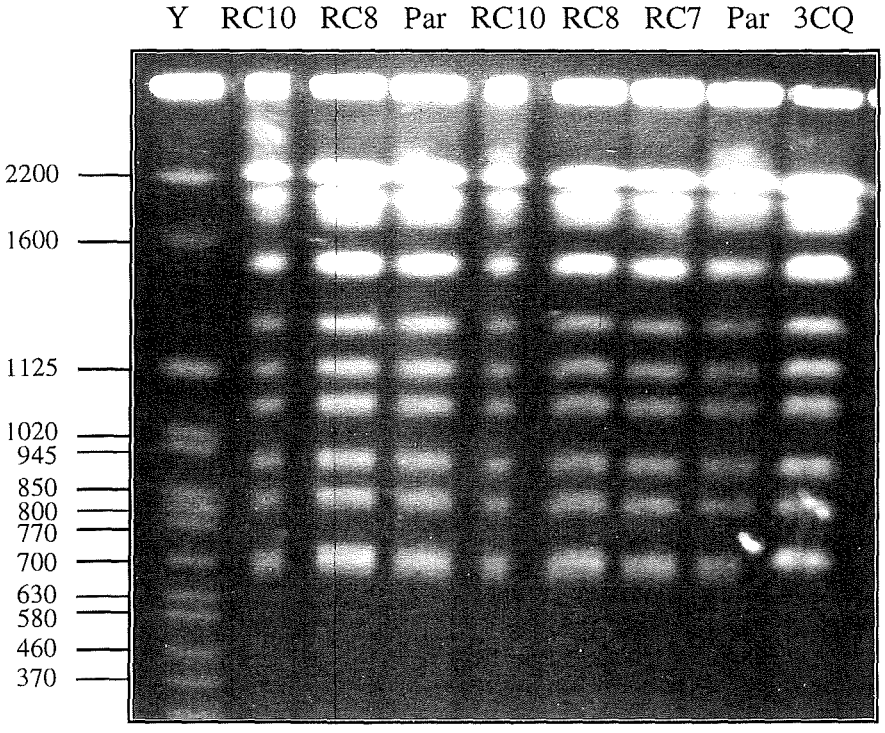
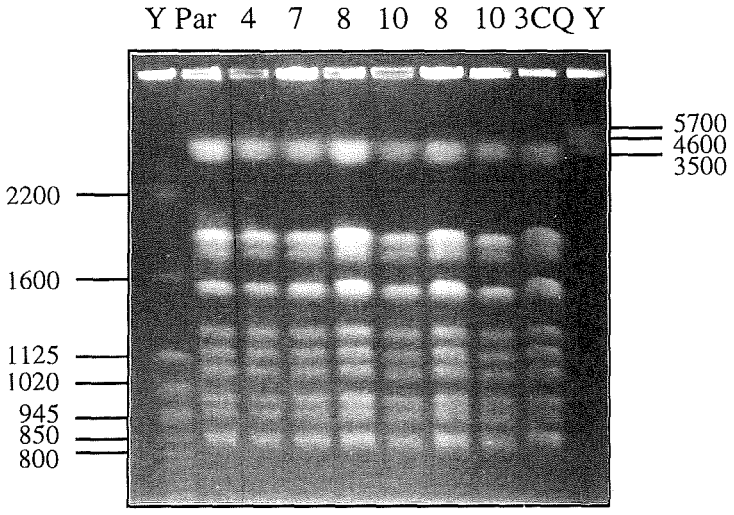


Figure 6.10 Fractionation of *P. chabaudi* AS variant parasite chromosomes by pulsed field gel electrophoresis.

Chromosomes separated using a CHEF apparatus, 7d run (see 2.21). Track Y left hand side = Yeast chromosomes *S. cerevisiae*, with approximate sizes (Kb) indicated on left. Track Y on right hand side = Yeast chromosomes *S. pombe*, with approximate sizes (Kb) indicated on right. Other tracks are chromosomes of *P. chabaudi* variant populations as indicated. 3CQ = chromosomes of *P. chabaudi* AS population as used and prepared in Edinburgh.



CHAPTER 7

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST SURFACE VARIANT ANTIGENS OF *Plasmodium chabaudi* AS

7.1 Introduction.

The production of monoclonal antibodies (mAbs) was first described by Köhler & Milstein in 1975, since when mAbs have become a powerful tool in many areas of biological research.

In malaria research, as in other areas of parasite immunology, mAbs have proved extremely useful in the preparation of purified reagents, (e.g. cytokines, specific antisera and adhesion molecules) and in the identification of antigenic determinants involved in anti-parasite reactions. Such uses of mAbs have led to improved sero-diagnosis and have facilitated sero-epidemiological studies, the elucidation of mechanisms of resistance and disease and the identification and preparation of Ags for use in potential vaccines. MAbs have been developed against a range of malarial Ags from all stages of the parasite life cycle (reviewed by Phillips & Zodda 1984). For instance, mAbs have been described which inhibit growth *in vitro* of *P. falciparum* (Perrin *et al.* 1981; Schofield *et al.* 1982; Myler *et al.* 1982) and of *P. knowlesi* (Epstein *et al.* 1981; Deans *et al.* 1982) and mAbs have been found to be protective *in vivo* against *P. yoelii* (Freeman *et al.* 1980; Holder & Freeman 1981), *P. berghei* (Potocnjak *et al.* 1980) and *P. chabaudi* (Boyle *et al.* 1982). MAbs have been used to identify, for example, epitopes shared between different parasite stages of *P. falciparum* (Hope *et al.* 1984; Szarfman *et al.* 1988), repeated epitopes of the CS proteins of *P. falciparum* and of *P. vivax* common to different isolates within each species (Zavala *et al.* 1985), cross-reactive bloodstage Ags between *P. chabaudi, P. falciparum, P. vivax* and *P. cynomolgi* (Wanidworanun *et al.* 1989), the *P. cynomolgi* complex (Kamboj *et al.* 1988) and both cross-reactive and species-specific Ags of *P. chabaudi* and *P. yoelii* (Holmquist *et al.* 1986). MAbs have also been used to demonstrate considerable Ag diversity in *P. falciparum* (McBride *et al.* 1982). Such findings have important implications for vaccine design and development, and serve to illustrate the usefulness, versatility and potential of mAbs in malaria research.

In the *P. chabaudi*-mouse model used herein to study antigenic variation in malaria parasites, the production of mAbs against surface variant Ags of *P. chabaudi* could potentially facilitate immunochemical characterisation of any given variant Ag and identification of the gene(s) encoding such Ags. These genes would likely be the *P. chabaudi* homologue of the newly described multigene family, *var*, encoding PfEMP1 of *P. falciparum* (Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995). Variant populations of parasites could be purified more easily, allowing studies of variant parasite populations *in vivo* and *in vitro* to be performed without the initial presence of minor populations possibly affecting the results. Cross-reactivity of hyperimmune sera with other Ags and with each other, demonstrated in chapter 3, could be overcome, and the limited availability of such hyperimmune sera would cease to be a problem if mAbs against variant Ags were to become available. With such possibilities, it was

considered worthwhile to prepare mAbs against variant parasite populations. This chapter describes the results of this work and the use of a mAb in the live IFAT and in Western blot analysis of variant populations.

7.2 Fusions and hybridoma growth

In all, eight fusions were performed as described in chapter 2. Four were with spleen cells from mice immunised with parent population parasites, and two each with spleen cells from mice immunised with RC 7 parasites or RC 10 parasites. The outcome of these is shown in Table 7.1 and the end point of hybridoma culture following the fusions is detailed in Table 7.2. All fusions resulted in hybridomas growing, with usually a high % of wells +ve for hybridoma growth. Medium was taken from these wells and tested in the live IFAT with homologous parasites for Ab against Ags on the surface of pRBC. However, the % of hybridoma-containing wells +ve in the live IFAT was low. The exception was in fusion 3, where > 10% of hybridoma wells gave a +ve result in the live IFAT. In fusion 2, where a low % of wells was +ve for hybridoma growth, no wells gave a +ve result in the live IFAT. Where a well was found to contain Ab giving a positive result in the live IFAT, the hybridoma cells were cultured with the aim of expanding and cloning the cells to produce a mAb (see chapter 2). Unfortunately, although the initial fusions met with a degree of success, the continued propagation of the hybridomas proved problematic. With hybridomas from fusions 1 and 3, the cells did not grow very much from the numbers originally observed and did not survive for more than a few days in the original 96 well plates. The hybridomas from fusions 4 and 5 were moved up to 24 well plates more quickly in an attempt to avoid the accumulation of debris from dead cells in the 96 well plates. However, the medium soon became -ve in the live IFAT and the cells ceased growing and died.

With hybridomas from fusion 6, the cells were again moved up quickly to 24 well plates. There were additional problems with fungal contamination at this time, mostly in the 96 well plates. Seven of the hybridoma cultures remained +ve in the live IFAT both in 24 well plates and in 6 well plates and were cloned by limiting dilution. When possible, these hybridoma cultures were frozen in liquid N_2 as stabilates. The success of cloning hybridoma cells is detailed in Table 7.3. A reasonable % of wells were +ve for hybridoma growth after these clonings, given a dilution of 0.5 cells/well, but all were -ve in the live IFAT. The cultures of the uncloned hybridoma cells from all but three of the wells originally +ve in the live IFAT had subsequently become -ve in the live IFAT and were terminated. The remaining three were cloned again, but two were lost to fungal contamination. The remaining positive, hybridoma 1, 9B was cloned a second time from a 25 ml flask, yielding a good % of wells +ve for hybridoma growth but all -ve in the live IFAT. A third cloning, from hybridomas recovered from stabilate and cloned as soon as possible from a 24 well plate, again yielded a good % of

hybridoma wells. Live IFAT screening revealed one +ve well. This was expanded and also re-cloned directly from the 96 well plate. From this , a very low % of wells were +ve for hybridomas, but all were +ve in the live IFAT. These were expanded, frozen in liquid N_2 as stabilate and also injected into pristane-primed mice for production of ascites (see chapter 2). Tissue culture S/N was also collected for mAb and frozen at -20°C.

From fusion 8, for practical reasons, only two plates were screened initially for Ab. Two of the other plates were frozen at -70°C (see chapter 2), whilst the third plate was lost to fungal contamination. Hybridomas from the two plates screened, which were found to be +ve in the live IFAT, were cloned directly from 96 well plates in an attempt to avoid fungal contamination and overgrowth of the Ab-secreting cells desired by other hybridomas. From the cloning, the % of wells +ve for hybridomas was moderate in three and low in two, given a dilution of 0.5 cells/well. However, all the wells were -ve in the live IFAT and were terminated. The uncloned hybridoma cells which were moved up to 24 well plates were unfortunately lost to fungal contamination. The remaining two 96 well plates were recovered from frozen but fungal contamination continued to be a problem and the cultures were terminated before any further screening could be performed.

7.3 Antibody isotyping by Ouchterlony double diffusion

Ascitic fluid of mAb 1, 9B was used for isotyping by Ouchterlony double diffusion as described in chapter 2. The results of this are shown in Fig. 7.1. When the ascitic fluid was used neat, Abs of isotypes IgM and IgA, present at low concentrations in the ascitic fluid, were detected. This is indicated by the precipitation lines between the wells containing the anti-μ and anti-IgA Abs and the centre well, with the position of the lines very close to the centre well indicative of the low concentration of these Abs. These were not observed when the ascitic fluid was diluted, even just to 1/10. The mAb in the neat ascitic fluid was present at too high a concentration to be precipitated by the corresponding anti-Ig, but at dilutions of 1/10, 1/100 and 1/1000, a precipitation line could be seen between the centre well and the well containing anti-IgG_1. The mAb was therefore determined to be of the IgG_1 isotype.

7.4 Live IFAT analysis of mAB 1, 9B

Ascitic fluid from a mouse injected with hybridoma 1, 9B, secreting a mAb against a surface Ag of parent-infected RBC, and serum from this mouse and also from two other similarly treated mice, were tested in the live IFAT against parent parasites and against RC 10. The results of this are shown in Table 7.4. The ascitic fluid and all three sera gave a +ve fluorescence against the parent in the live IFAT and were all -ve against RC10 at all dilutions tested.

7.5 Western blot analysis of parent parasite population

Crude Ag preparations were made from the parent parasite and RC 10 populations and SDS-PAGE performed, followed by transfer onto nitrocellulose and western blotting (chapter 2). The nitrocellulose strips were probed with anti-parent and anti-RC 10 hyperimmune sera and also with mAb 1, 9B both as hybridoma cell culture S/N (neat) and as ascitic fluid (1/100). NMS was used as a negative control. The results of this Western blotting are not shown. However, to summarise, the hyperimmune sera identified many bands in the Ag preparations, but with no readily identifiable, consistent differences between the bands detected by the anti-parent hyperimmune serum and the bands detected by the anti-RC 10 hyperimmune serum with either of the Ag preparations. The cell culture S/N did not identify any bands at all from either the parent Ag preparation or the RC 10 Ag preparation. The ascitic fluid revealed two bands, from both the Ag preparations, but these were also the only bands revealed by the NMS controls. No other bands were visible in the strips probed with the ascitic fluid.

7.6 Discussion

Within the last year or so, Baruch *et al.* (1995) have described the specificity of antisera generated against recombinant protein fragments of two related *var* genes for the PfEMP1 molecule of the particular *P. falciparum* strain to which the sera were raised. To date, however, no published report has described mAbs against surface variant Ags of *Plasmodium.*, raised either against recombinant fusion proteins or whole molecule native Ags. The successful production of one such mAb against *P. chabaudi*, described herein, is therefore novel.

The fusions performed in order to generate *P. chabaudi*-specific mAbs were of varying success. In terms of hybridoma formation, they were viewed as successful, but in the majority of cases, the number of hybridoma wells identified as producing an Ab of interest was low. There are a number of possible reasons for this: the frequency of B cells in the spleens of immunised mice producing Ab against surface variant Ags may have been low; hybridomas producing other Abs rapidly outgrew hybridomas producing Abs of interest; B cells secreting Abs of interest produced unstable hybridomas which either stop growing or stop secreting Ab; a combination of Abs against more than one epitope of variant Ags may sometimes be necessary to give a positive fluorescence; the conditions used for cell fusions in some way selected against successful fusions with B cells secreting the Abs of interest. A combination of some or all of these factors is likely to account for the low number of wells found to be producing Abs against surface variant Ags. Hyperimmune sera produced a high titre of Ab against some of the variant Ags, but these sera also contained Ab of unknown titres against many other malarial Ags, and the variant Ags, although seemingly

immunodominant, constitute only a very small proportion of parasite Ags presented to the mouse immune system. It is, therefore, more than likely that the B cells secreting Ab against the variant Ags are of low frequency. The progressive loss of Abs giving positive fluorescence from the medium of hybridoma cultures which were apparently growing well may indicate the presence of other faster growing hybridomas or that Ab secretion and/or cell multiplication may stop in some hybridomas (Harlow & Lane 1988). It may well be that such hybridomas producing Abs against variant Ags are often inheritantly unstable. Screening of hybridomas for Abs against variant Ags was performed by live IFAT, and was very labour intensive. By this method, there was no practical means by which combinations of Ab could be screened, unless several wells were pooled and screened together instead of individually. The pooling of culture S/N, however, may have had the effect of diluting out the desired Abs, resulting in none of the wells being identified as positive for Abs against variant Ags, which, when screened separately, may have been. An alternative screening method which would be less labour intensive and therefore more versatile for screening combinations of wells would greatly facilitate preparation of mAbs against variant Ags. There is no way of knowing if the procedure used for cell fusions may result in some B cells being selected against or if some B cells (those of interest?) are resistant to fusion with myeloma cells (Goding 1996).

The difficulties encountered in subsequent propagation of hybridomas can also be explained by the above reasons. With increasing experience, cloning was performed as early as possible to circumvent overgrowth with other hybridomas. Unfortunately, as the actual fusions became more successful, with greater numbers of hybridomas being produced and with more than one colony per well, this in itself became problematic. The hybridomas which screened positive for Ab against variant Ags tended to be fairly slow-growing, which increased the need for immediate cloning. Fungal contamination was, at times, an overwhelming problem, even with amphotericin B added routinely to the culture medium and with nystatin added in attempts to arrest fungal growth. Bacterial contamination never occurred in any of the myeloma or hybridoma cultures; it is difficult to know what additional measures could have been taken to prevent such fungal contamination.

Despite the problems encountered, the methodology followed ultimately did prove successful, if to a limited extent, in that a mAb was produced. This was against a surface Ag of the parent parasite population, was of the IgG_1 Ab isotype and is believed to be variant-specific. When tested against RC 10 in the live IFAT, no positive fluorescence was observed. It is not known if there is any reactivity against other variant types. This would require testing in order to clarify the variant specifity of the mAb. In preliminary attempts to characterise the variant Ag recognised by the mAb, Western blotting was performed using the mAb both as hybridoma cell culture S/N and

as ascitic fluid (results not shown). In neither case were any bands detected specifically by the mAb. The transfer of Ags was successful, as judged by probing strips with hyperimmune sera, and by staining the gels post-tranfer. It can therefore be concluded that the Ag was not present on the nitrocellulose in a form recognised by the mAb. SDS-PAGE, whilst separating proteins in relation to their MW, destroys the 3-dimensional structure of proteins (Fenton 1993). The ionic detergent SDS eliminates both the native charge and structure of proteins, and when used in conjunction with a reducing agent, such as 2-mercaptoethanol, proteins become -vely charged linear molecules. It is likely that this is why no bands were detected by Western blotting with the variant-specific mAb, which is most probably against a conformational epitope.

The potential of mAb production against native variant Ags of *P. chabaudi* has not been fully realised in the work outlined in this chapter. This approach to the study of malarial variant Ags does, however, still hold possibilities and, as there is no apparent reason for it not proving successful, is both valid and of relevance to the comparable *P. falciparum* studies currently undertaken. The fact that hybridomas producing Abs giving positive fluorescence in the live IFAT can be identified and a mAb can be produced is testament to the potential value of the approach. However, as there are as yet no published reports of mAbs being produced against these surface variant Ags of late-stage malaria parasites, it is possible that these Ags and the nature of the Ab response to such Ags in some way precludes the routine applicability of this approach and the ready availability of mAbs so generated.

Future studies using the *P. chabaudi*-specific mAb generated by the protocol described in this chapter may provide information on the relationship between antigenic variation, sequestration and cytoadherence of malaria-infected RBC. Notably, it would be of interest to examine the effect of the mAb on the binding of parent population pRBC to different cytoadherence receptors known to mediate adherence to endothelial cells. By comparison, it is known that the antisera recently raised to recombinant peptides of PfEMP1 block the binding of *P. falciparum* to CD36 but not to TSP (Baruch *et al.* 1995).

Table 7.1 Success of fusions performed using spleen cells from mice immunised with variant parasite populations.

Fusion	Parasite population raised against	no. of wells +ve for hybridoma growth	% of wells +ve for hybridoma growth	no. of wells +ve in LIFAT	% of hybridoma wells +ve in LIFAT
1	parent	202/472	42.8	6	3.0
2	RC 7	56/464	12.1	0	0
3	RC 10	68/288	23.6	7	10.3
4	RC 7	283/464	61.0	5	1.8
5	parent	116/224	51.8	5	4.3
6	parent	464/464	100	11	2.4
7	parent	n.d.	-	-	-
8	RC 10	464/464	100	5 from 2 plates	2.7

Table 7.2 End point of hybridoma cultures from fusions for production of mAb against variant surface antigens

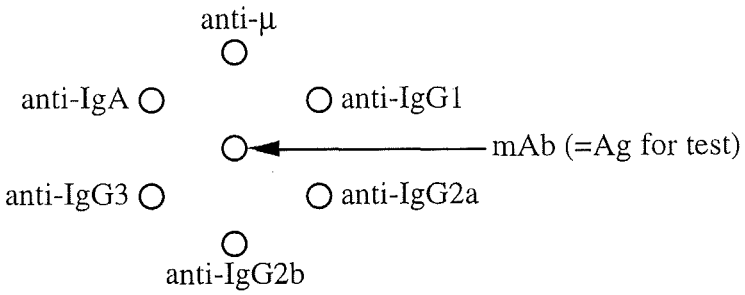
Fusion	Parasite population raised against	End point of culture
1	parent	96 well plates: cells failed to grow
2	RC 7	24 well plate: all -ve in LIFAT; cultures terminated
3	RC 10	96 well plates: cells failed to grow
4	RC 7	24 well plate: cells failed to grow
5	parent	24 well plate: cells failed to grow
6	parent	2nd cloning and ascites production from 1 clone
7	parent	overwhelming fungal contamination before screening commenced
8	RC 10	1st cloning: cells failed to grow

Table 7.3 Success of cloning by limiting dilution of hybridoma cells (0.5 cells/well).

Fusion	Parasite population raised against	hybridoma I.D.	Stage of culture at which cloning performed	no. of wells +ve for hybridoma growth	% of wells +ve for hybridoma growth	LIFAT results (% +ve)
6	parent	1, 3C	6 well plate	24/96	25.0	all -ve
6	"	1, 4D	"	26/96	27.1	"
6	"	1, 9B	"	34/96	35.4	"
6	"	2, 11B	"	32/96	33.3	"
6	"	3, 3F	"	36/96	37.5	"
6	"	3, 7D	"	14/96	14.6	"
6	"	3, 12D	"	28/96	29.2	"
6	"	1, 9B	25ml flask	307/576	53.3	"
6	"	1, 9B	24 well plate	104/192	54.2	1 +ve 2, 3G (0.96%)
6	"	3, 3F	"	n.d.	-	-
6	"	3, 12D	"	n.d.	-	-
6	"	1, 9B cl.2, 3G	1st cloning, 96 well plate	7/192	3.6	7/7 +ve (100%)
8	RC 10	1, 3F	96 well plate	26/96	27.1	7/26 +ve (26.9%)
8	"	1, 6A	"	23/96	24.0	all -ve
8	"	1, 7G	"	30/96	31.3	"
8	"	1, 8H	"	11/96	11.5	"
8	"	2, 3D	"	5/96	5.2	"

Fig. 7.1 Antibody isotyping of anti-parent mAb 1, 9B by Ouchterlony double diffusion

a. Pattern of anti-Ig Ab in wells



b. Results using mAb ascitic fluid

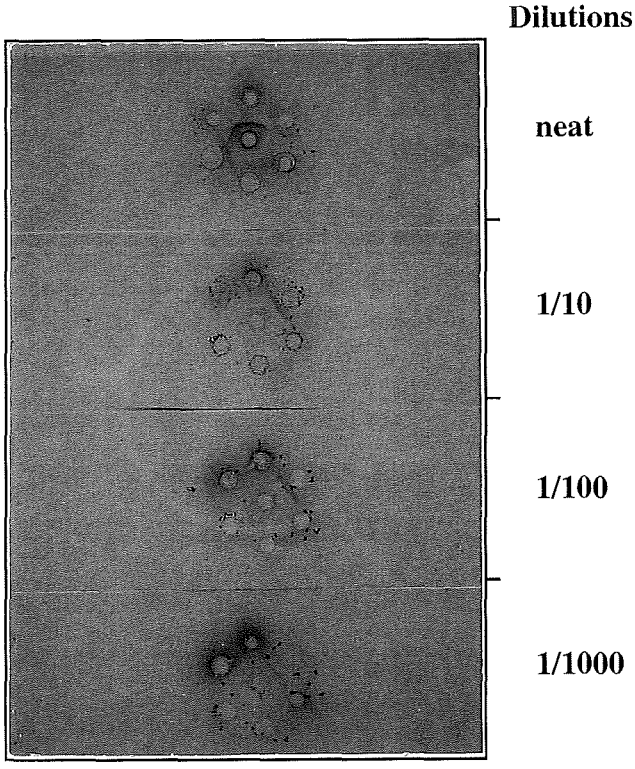


Table 7.4 **Reactivity of mAb 1, 9B in the live IFAT against parent and RC 10 parasite populations using ascitic fluid and serum from mice injected with 1, 9B hybridoma cells.**

dilution	ascitic fluid		serum 1		serum 2		serum 3	
	parent	RC 10	parent	RC 10	parent	RC 10	parent	RC 10
1/10	+	-	+	-	+	-	+	-
1/50	+	-	+	-	+	-	+	-
1/100	+	-	+	-	+	-	+	-
1/1000	+	-	+	-	+	-	+	-

CHAPTER 8

GENERAL DISCUSSION

The use of *P. chabaudi* in mice as a model in which to study host-parasite interactions of malaria infections is already well established. The similarities of *P. chabaudi* to *P. falciparum* mentioned previously (see 1.7) and its accessibility in a laboratory situation makes it the model of choice for many studies. The *P. chabaudi*-mouse model has been used extensively in studies on immune responses to malaria infection (reviewed by Taylor-Robinson 1995). It has also been used, to a lesser degree, in studies on antigenic variation (McLean *et al.* 1982b, 1986a, 1987, 1990; Gilks *et al.* 1990), sequestration (Cox *et al.* 1987; Gilks *et al.* 1990; Dennison & Hommel 1993) and cytoadherence (Cox *et al.* 1987). Although due care must be taken in extrapolating results from the *P. chabaudi*-mouse model to *P. falciparum* infections in humans (Butcher 1996), as a tool for gaining knowledge of basic mechanisms of immunity and immune evasion in malaria infections, it is of immense value.

The need for a fuller understanding of the host-parasite relationship in malaria is still apparent. Malaria continues to be a highly prevalent disease causing much morbidity and mortality (see 1.1), despite implementation of various control measures (reviewed by Institute of Medicine 1991; Targett 1991). This is, in part, due to the inadequacy of resources available in malarious countries for treatment and control of malaria, but the development of drug resistance, and immune evasion strategies employed by the parasites, are also contributing factors. The need for more effective control measures, most importantly an effective vaccine, is therefore paramount. Rational approaches to therapeutics and to vaccine design and development may be facilitated by the identification and understanding of interactions between malaria parasites and immune mechanisms, including immune evasion by the parasites.

One such immune evasion strategy is antigenic variation. The aim of the work described in this thesis was to increase the knowledge and understanding of antigenic variation in malaria parasites, specifically *P. chabaudi*, but with the possible applicability of results to, and validation of findings from, other plasmodia, especially *P. falciparum*.

Initial experiments were performed as a continuation of previous work in Professor Phillips' laboratory (McLean *et al.* 1986 a & b; Brannan *et al.* 1993). The results of these experiments, which identified *P. chabaudi* cloned variant populations derived from a recrudescence and variant-specific hyperimmune sera, formed the basis, and provided the tools required, for subsequent studies. The biological properties of different VATs could thus be compared *in vivo* and *in vitro*, and the hyperimmune sera used to examine the expression of VATs after MT and during infection.

All the recrudescent clones were different from the parent, and some were different from each other. In total, six VATs, including the parent, were identified by the live IFAT analysis using a panel of hyperimmune sera (see chapter 3). This confirmed and extended previous analyses of these populations using a passive transfer

system (McLean *et al.* 1986a) and using immune sera collected on d 16 & 17 p.i. in the live IFAT (Brannan *et al.* 1993). It is likely that these VATs represent members of a family of Ags encoded by a super gene family, as has been shown for the variant Ag molecule in *P. falciparum* , PfEMP1 (Baruch *et al.* 1995; Su *et al.* 1995).

Analysis of two of the cloned recrudescent populations after MT using the hyperimune sera in the live IFAT indicated an alteration in VAT from the original RC populations (see chapter 3). There was a change in the predominant VAT to a new type, but also an apparent mix of VATs. This mix of VATs may be due to a very high switching rate in mosquito-transmitted populations, as has been indicated for tsetse-transmitted trypanosomes (Turner & Barry 1989). A change in VATs after MT is not surprising, especially if the genes for variant Ags are distributed throughout the genome, as has been found for the *var* genes in *P. falciparum* (Su *et al.* 1995; Peterson *et al.* 1995). The repertoire would be reshuffled by reassortment and recombination during meiosis. Such rearrangements may in some way prime the parasites for rapid switching. The reversion to a basic or parental type upon cyclical transmission, which is possibly indicated by there being the same new predominant VAT in all three MT populations examined, may effect this, by this VAT being one which switches off at a high rate. This rapid switching and mix of VATs would be advantageous to the parasites, especially upon tranfer to a semi-immune host, likely in endemic areas, by allowing the survival of some parasites and rapid switching to other VATs possibly not already experienced by the host. It would thus be of interest to measure switching rates and parasite survival of MT populations in both naive and semi-immune mice. At present, this type of study is really only feasible in the *P. chabaudi*-mouse model.

The observed differences between the courses of infection of different variant populations (see chapter 4) may reflect functional differences among the parasites and/or, possibly related to this, differences in the immune responses that they elicit. Measurement of some indices of the immune response during infections of different variant populations, such as VAT-specific Ab levels and isotypes, and NO production, would be of interest and may give an indication of the latter. The results presented in chapter 4 are of importance in demonstrating that infections with different VATs may exhibit differences in the severity and duration of disease, possibly unrelated to differences in cytoadherence phenotype observed for different VATs in *P. falciparum* (Roberts *et al.* 1992).

Antigenic variation in *P. chabaudi* is shown to occur at high rates, 1.6%/schizont/day, *in vivo* (Brannan *et al.* 1994; see chapter 5). This is in line with rates of antigenic variation of 2% per generation reported for *P. falciparum in vitro* (Roberts *et al.* 1992). The results in chapter 5 showing differential rates of switching on of individual VATs provide experimental support for an explanation as to why switching rates should be so high. Antigenic variation functions to facilitate

transmission of parasites from mammal to mosquito by enabling evasion of the host immune response by parasites and thereby increasing the longevity of infection. To do this, VATs are expressed in a hierarchical sequence which is achieved by regulating the rates of switching between individual VATs. As differential switching rates must occur at rates higher than those for background recombinational events (typical *per capita* rate values are approximately 10^{-6}), the inevitable consequence of the requirement for differential switching rates between VATs is that the overall rate of switching is high.

Differential switching rates in malaria parasites have not as yet been demonstrated in any other study. Differences in the frequency of appearance of some VATs in cloned cultures of *P. falciparum* may reflect differential rates of switching for these VATs (Smith *et al.* 1995), while the sequential appearance of VATs in *P. fragile* infections (Handunnetti *et al.* 1987) may be reasonably expected to reflect differential switching rates, with those VATs appearing earlier likely to have higher switching rates than those appearing later. A mechanism enabling differential switching rates, as shown for *P. chabaudi* in chapter 5, is the primary candidate for causing hierarchical expression of VATs, which is a diagnostic feature of systems of antigenic variation (Borst 1991; Turner 1992), and thus probably occurs in all plasmodia in which antigenic variation occurs.

The rate of switching may be determined, if only theoretically, by the VAT being switched off, the VAT being switched on, by both or by neither. The latter possibility cannot apply, as this work has shown that, at least in part, the VAT being switched on regulates the rate of switching. What was not examined, and what could perhaps prove more difficult to determine, is whether the VAT being switched off also influences switching rates. One possible way of examining this would be to measure rates of switching on of minor VATs during infections initiated by different VAT populations. It can be assumed that the main direction of switching between VATs is from the major VAT to the minor VATs, and therefore a comparison of rates of switching on of minor VATs in different infections may indicate whether the major VAT being switched off plays a part in determining switching rates. This would give only indirect evidence, but to measure rates of switching off of VATs directly would be very difficult due to problems of distinguishing between switching and immune clearance.

A high rate of antigenic variation (Roberts *et al.* 1992; Brannan *et al.* 1994; see chapter 5) pertains directly to the nature of the host-parasite relationship. A complex functional relationship must exist, as opposed to a straightforward pacing of the switching rate with the immune response if rates were low. One explanation for a more complex strategy may lie in the association between antigenic variation and cytoadherence/sequestration of pRBC. A clear association between these two evasion mechanisms has been shown in *P. falciparum* (Biggs *et al.* 1992; Roberts *et al.* 1992)

and in *P. chabaudi* (Gilks *et al.* 1990), and therefore it may be necessary to consider the functions of both mechanisms in combination.

The *P. chabaudi*-mouse model has the potential to be used for studies examining antigenic variation and cytoadherence/sequestration in combination, and interactions with the host immune system. Chapter 6 describes work performed to examine the sequestration *in vivo* and cytoadherence *in vitro* of different variant populations. The results presented show that peripheral withdrawal during schizogony occurs, as other studies have shown (McDonald & Phillips 1978; Cox *et al.* 1987; Gilks *et al.* 1990), but also show no differences in the extent of withdrawal between different variant populations. However, no investigation of the sites of sequestration of these populations was undertaken. This would be of interest, as would whether the extent and site of sequestration of different variant populations differs during infection, or in response to the artificial induction/introduction/blockade of immune stimuli , such as Abs and/or cytokines. Immune serum can reverse sequestration of *P. falciparum* in *Saimiri* monkeys and cytokines such as TNF, IL-1 and IFN-γ can induce ICAM-1 expression on endothelium *in vitro* and *in vivo* (Pober *et al.* 1986; Munro *et al.* 1989; Petzelbauer *et al.* 1993). Expression of VCAM-1 and E-selectin is also induced by TNF and IL-1 (reviewed by Pigott & Power 1993). *P. chabaudi* pRBC can also be induced to sequester in the brains of mice during mixed infections with *P. berghei* (Dennison & Hommel 1993; Hommel 1993), indicating that induction of receptor expression and alterations in sequestration patterns of *P. chabaudi* can be achieved, and is worthy of investigation.

Sequestration and expression of variant Ags during crisis in *P. chabaudi* infections would be another possible avenue of research. Is the rapid clearance of pRBC due to the breakdown of such evasion strategies? A peak of NO production occurs around the time of crisis in *P. chabaudi* infections (Taylor-Robinson *et al.* 1993, 1996). Physiological levels of NO have a cytostatic effect on mature *P. falciparum* pRBC *in vitro* (Balmer *et al.* 1995; Taylor-Robinson 1997). If this then halts transportation to the pRBC surface and expression of variant Ags, then sequestration may be prevented, allowing clearance of pRBC in the spleen. In a parental infection, during crisis and remission of the parasitaemia, expression of the major VAT could not be detected by IGSS with anti-parent hyperimmune sera and only low levels of any VAT could be detected (L.R. Brannan, unpublished observations). Alternatively, does the disappearance and non-reappearance of the major VAT represent selective clearance of this VAT during crisis, and/or its selective clearance during the re-emergence of parasites after crisis and remission? If clearance of pRBC during crisis and remission is non-specific, then all VATs present (major and minor) will be cleared indiscriminately. Preliminary results indicate that this may not be the case. Low levels of minor VATs could still be detected during crisis and remission. However, there was

no increase in the number of pRBC detected expressing minor VATs commensurate with the loss of detectable expression of the major VAT at crisis (L.R. Brannan, unpublished observations). A further possibility is that switching mechanisms may be interfered with during crisis, with the major VAT being switched off but with no switching on of other VATs. There may also be an inability to switch on the parent VAT again after crisis and remission, leading to its non-reappearance. The detection of VATs in such studies is limited by the lack of availability of a full range of VAT-specific reagents. However, even with those reagents presently available, some of the possibilities raised here could be investigated. The host-parasite interactions that take place during crisis with regard to antigenic variation and sequestration certainly warrant further study, and the *P. chabaudi*-mouse model provides a vehicle for such investigations.

The results of the *in vitro* cytoadherence assays presented in chapter 6 showed preferential binding of pRBC in all variant populations studied, with some differences in the specificity of binding between different variant populations. The levels of binding in these assays are similar to those reported by Cox *et al.* (1987), but are low compared to the levels of binding to C32 melanoma cells reported for *P. falciparum* in similar binding assays (Schmidt *et al.* 1982). The development of an improved *in vitro* cytoadherence assay, perhaps using a different cell type, may facilitate further study of the link between different VATs and cytoadherence. As the liver is the major site of sequestration of *P. chabaudi* (Cox *et al.* 1987), the possibility of an assay based on liver sinusoidal cells should be considered. Experiments to determine the effects of VAT-specific immune or hyperimmune sera or of mAbs on cytoadherence *in vitro* would be of interest, perhaps giving additional indications of the involvement of variant Ags in cytoadherence of pRBC. The identification of the host receptors mediating cytoadherence and sequestration of *P. chabaudi* would be beneficial, and the use of purified host receptors in cytoadherence assays may allow further comparisons of VATs in their ability to cytoadhere. Expression of such receptors could be investigated in different sites and at different times during infections in mice, and compared between infections with different variant populations, in parallel with studies of parasite sequestration. The possible rosetting properties of the different VATs also await examination.

Recent technological advances have enabled the manipulation of the immune system in mice, thereby allowing detailed dissections of immune responses to various pathogens, including malaria parasites (reviewed by Taylor-Robinson 1995). Such an inductive approach is obviously not possible in humans, for which malaria field studies are purely deductive in nature. The ability to artificially knock out or induce components of the immune system can be employed to investigate the effects of such immune factors on antigenic variation and the expression of variant Ags by malaria

parasites. In experiments in which mice were depleted of B cells or Th2 cells and infected with *P. chabaudi*, a chronic bloodstream infection was observed, in which the predominant VAT remained unchanged (Taylor-Robinson & Phillips 1996). A change in the major VAT was observed only when B cells and Th2 cells were present together. As Th2-derived cytokines regulate B cell differentiation and hence Ab production, Th2 cells may play a role in influencing the major VAT present during bloodstream infection. These results also indicate that VAT-specific Ab has a role in influencing antigenic variation in malaria parasites. It is probably via a selective process, rather than the induction of antigenic variation indicated for *P. knowlesi* (Brown 1973), as intrinsic antigenic variation occurs during the ascending parasitaemia, when immune-mediated killing is essentially absent (Brannan *et al.* 1994; see chapter 5). Increases in rates of antigenic variation due to extrinsic factors, such as Ab, however, cannot be ruled out.

The biochemical and genetic basis of antigenic variation in *P. chabaudi* remains to be elucidated. In *P. falciparum*, the variant molecule, PfEMP1, and the *var* gene family encoding this molecule, have now been identified (Leech *et al.* 1984; Baruch *et al.* 1995; Su *et al.* 1995). An open transfer of information and the availability of molecular probes could facilitate identification of the homologous *P. chabaudi* variant molecule and gene(s) encoding this molecule. Obviously, the resources available for research into antigenic variation in *P. falciparum* are significantly greater than those available for the equivalent research into antigenic variation in *P. chabaudi*. This is as it should be, but given the potential for host immune responses to influence variant Ag expression, antigenic variation, and sequestration, research on *P. chabaudi* should continue to be considered worthy of support.

APPENDICES

APPENDIX A

Buffers

Phosphate-buffered saline (PBS)	
Stock	60.0 g Na_2HPO_4. $12H_2O$ 13.6 g NaH_2PO_4. $2H_2O$ 8.5 g NaCl Made up to 1 l with ddH_2O
Buffer	40 ml stock, made up to 1 l with 0.9% saline and adjusted to pH 7.2
Giemsa's phosphate buffer	3.0 g Na_2HPO_4 0.6 g KH_2PO_4 Made up to 1 l with ddH_2O and adjusted to pH 7.4
Barbitone buffer	12.0 g barbital sodium (5'5 sodium diethylbarbiturate) 4.4 g barbital (5'5 diethylbarbituric acid) 0.15 g merthiolate Made up to 1 l with ddH_2O and adjusted to pH 8.2
SDS-PAGE sample buffer	400 µl 10% w/v SDS 200 µl 1M Tris HCl pH 6.8 200 µl 2-mercaptoethanol 100 µl glycerol 100 µl 0.1% w/v bromophenol blue 1 ml ddH_2O
SDS-PAGE running buffer	25 mM Tris base 192 mM glycine 0.1% w/v SDS
Tris-glycine/SDS transfer buffer	43.26 g glycine 9.09 g Tris 3.0 g SDS 600 ml methanol Made up to 3 l with ddH_2O, adjusted to pH 7.4 and stored at 4°C

Wash buffer	
10x stock	90.0 g NaCl 12.11 g Tris Made up to 1 l with ddH_2O and adjusted to pH 7.2
For use	100 ml stock 900 ml ddH_2O 0.5 ml (0.05%) Tween-20
AP buffer	100 mM NaCl 5 mM $MgCl_2$ 100 mM Tris Made up to 1 l with ddH_2O and adjusted to pH 9.5

APPENDIX B

Media

RPMI 1640

Stock	10.39 g RPMI 1640 powdered medium (with L-glutamine) (Gibco) 5.94 g HEPES (Sigma) Made up to 960 ml with ddH_2O, filter-sterilised and adjusted to pH 7.2
Malaria Incomplete RPMI	100 ml stock RPMI 4.2 ml 5% w/v $NaHCO_3$ (filter-sterilised) 0.25 ml gentamycin sulphate (Sigma)
Cell Culture Incomplete RPMI	85 ml stock RPMI 11 ml L-glutamine (Gibco) 5.5 ml 3.5% w/v $NaHCO_3$ (filter-sterilised) 0.55 ml 0.1 M 2-mercaptoethanol 22 ml fungizone (Gibco) 2.2 ml gentamycin sulphate (Sigma)

Complete RPMI

Both complete media contained 5-10% FCS (Gibco), unless otherwise stated.

Sterile FCS was heat-inactivated at 56°C for 30 min and stored at -70°C until use.

HT

100x Stock	0.136 g hypoxanthine 0.039 g thymidine Made up to 100 ml with ddH_2O at 70-80°C, filter-sterilised and stored at -20°C

HAT

50x Stock	100 ml HT stock 10 ml 1000x aminopterin stock (17.6 mg aminopterin in 80 ml ddH_2O) 90 ml ddH_2O, filter-sterilised and stored at -20°C

OPI

100x Stock	1.5 g oxaloacetate 0.5 g sodium pyruvate 2000 i.u. bovine insulin (Sigma) Made up to 100ml with ddH_2O, filter-sterilised and stored at -20°C

Ham's F-10

Stock	9.8 g Ham's F-10 powdered medium (Gibco) 5.96 g HEPES (Sigma) 1.20 g $NaHCO_3$ Made up to 1 l with ddH_2O, filter-sterilised and adjusted to pH 7.4
Incomplete Ham's F-10	100 ml Ham's F-10 1.0 mg (100,000 i.u.) penicillin-G (Sigma) 2.0 mg (200,000 i.u.) streptomycin sulphate (Sigma)
Complete Ham's F-10	95 ml Incomplete Ham's F-10 5 ml FCS (Gibco)

Dulbecco's Modified Eagle's Medium (DMEM)

Stock	9.70 g DMEM powdered medium (with Earle's salts, amino acids & L-glutamine) (Gibco) 5.94 g HEPES (Sigma) 2.20 g $NaHCO_3$ Made up to 1 l with ddH_2O, filter-sterilised and adjusted to pH 7.2
Incomplete DMEM	100 ml DMEM 1.0 mg (100,000 i.u.) penicillin-G (Sigma) 2.0 mg (200,000 i.u.) streptomycin sulphate (Sigma)
Complete DMEM	90 ml Incomplete DMEM 10 ml FCS (Gibco)

APPENDIX C

Miscellaneous Reagents

Sorbitol-glycerol	380 g glycerol (Sigma) 39 g sorbitol (BDH) 6.3 g NaCl
Ouchterlony slides	0.5 g agar (Difco) dissolved in 100 ml dH_2O (100°C waterbath). Agar solution pipetted onto clean, dry slides. Slides dried and stored at RT until required.

N.B. Pre-coating slides with a weak agar solution enables the final agar gel to be held in place during the Ouchterlony double diffusion washing procedure (see 2.14.12).

Coomassie Brilliant Blue stain	0.1% w/v Coomassie Brilliant Blue R-250 (Sigma) 25% v/v methanol 10% v/v glacial acetic acid 1% v/v glycerol

SDS-PAGE

Solution A	0.5 M HCl 3 M Tris base 15 mM TEMED
Solution B	0.5 M HCl 0.5 M Tris base 30 mM TEMED
Solution C	Protogel™ (30% acrylamide, 0.8% bisacrylamide) (Bio-Rad)

Separating gels (x2)

5% w/v acrylamide	5.0 ml solution A 3.0 ml solution C 18.0 ml dH_2O 0.2 ml SDS 0.2 ml ammonium persulfate

25% w/v acrylamide	5.0 ml solution A 15.0 ml solution C 6.0 ml dH_2O 0.2 ml SDS 0.2 ml ammonium persulfate
Stacking gels (x2) 3% w/v acrylamide	1.9 ml solution A 2.5 ml solution C 10 ml dH_2O 0.15 ml SDS 0.15 ml ammonium persulfate
<u>NBT/BCIP</u> NBT Stock	0.5 g NBT (Sigma) 10 ml 70% dimethylformamide
BCIP Stock (kept in the dark)	0.5 g BCIP (Sigma) 10 ml 100% dimethylformamide
For use	66 µl NBT stock 33 µl BCIP stock 9.901 ml AP buffer
<u>PFGE lysis solution</u>	0.5 M EDTA (BDH) 1% *N*-lauryl sodium sarcosinate (Sarkosyl) (Sigma) 5 mg/ml proteinase K (Sigma) Made up to 10 ml with dH_2O and adjusted to pH 8.0
<u>TBE</u> 10x Stock	108 g Tris base 54 g boric acid 8.35 g disodium EDTA Made up to 1 l with dH_2O and adjusted to pH 8.5
For use (0.5x TBE)	50 ml 10x stock 950 ml dH_2O

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