GENETIC STUDIES ON ANTIGENS OF THE RODENT MALARIA PARASITE \textit{PLASMODIUM CHABAUDI}

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DECLARATION

I declare that this thesis is my own composition and that the research described is my own work.

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November, 1986
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ABSTRACT

Antigens of the rodent malaria parasite Plasmodium chabaudi were studied using monoclonal antibodies (mcabs). The mcabs were produced by fusing a mouse myeloma cell line with spleen cells from mice immunized with cloned isolates of the parasite, AS and CB.

The immunoglobulin heavy chain subclasses secreted by the hybridoma cell lines were determined by Ouchterlony double diffusion using commercially bought antisera.

The antigens with which the mcabs were reacting were analysed by Indirect Fluorescent Antibody test (IFA). They were also analysed by immunoprecipitation of metabolically labelled extracts of parasites followed by SDS-polyacrylamide gel electrophoresis and by Western blotting. Many of the mcabs reacted with a 250000 dalton protein synthesised in late trophozoites and schizonts.

Using these and other mcabs provided by Drs. D. Boyle and C. Hamers-Casterman, twelve different cloned isolates of P. chabaudi were screened using IFA. This survey revealed a great deal of antigenic diversity between isolates of the same species. This diversity was not correlated with the area of parasite origin; two isolates from the Congo were dissimilar and were similar to isolates from the Central African Republic.

A genetic cross was carried out between two isolates from the Central African Republic, AS and CB.
These two isolates were characterised by different isoenzyme and drug response markers as well as being antigenically distinct. Progeny of the cross were cloned and analysed. The antigenic profiles were the two parental forms only. This result was also found when cloned progeny from a different cross between AS and ER were analysed. These results show that the mcabs are reacting with different sites on one protein.

Immunoprecipitates of cross progeny were digested by a staphylococcal protease and the resulting peptides examined by SDS- polyacrylamide gel electrophoresis. The peptide maps obtained for the cross progeny were like the parental peptide maps. The 250kDa antigen appears to be the product of one gene which has many alleles.

The gene for the 250kDa antigen was inherited independently from another antigen gene recognised by a mcab. Both antigen genes were inherited independently from isoenzyme and pyrimethamine response markers.
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List of Abbreviations

ADA  Adenosine deaminase
BSA  Bovine Serum Albumin
CAR  Central African Republic
EDTA  Ethylenediaminetetra-acetate
FCA  Freunds Complete Adjuvant
FCS  Foetal Calf Serum
HAT  Hypoxanthine, aminopterin and thymidine
HPRT  Hypoxanthine phosphoribosyl transferase
IFA  Indirect Fluorescent Antibody test
kDa  kilo daltons
LDH  Lactate dehydrogenase
Mcab  Monoclonal Antibody
MFM  Methionine free medium (Eagles MEM)
PABA  Para-aminobenzoic acid
PAGE  Polyacrilamide Gel Electrophoresis
PBL  Peripheral Blood Lymphocytes
PBS  Phosphate Buffered Saline
PEG  Polyethylene Glycol
PFGE  Pulse field gel electrophoresis
PGD  6-phosphogluconate dehydrogenase
PMSF  Phenylmethylsulfonyl fluoride
PNG  Papua New Guinea
RPMI  RPMI 1640
RP15  RPMI with 15% FCS
SDS  Sodium Dodecyl Sulphate
TET  50mM Tris HCl pH8, 1mM EDTA, 0.5% Triton X-100
1 INTRODUCTION

1.1 The Problem of Malaria

Thirty years ago the Malaria Eradication Programme was launched by the World Health Organisation (WHO, 1957). It was based on the spraying of residual insecticides such as DDT on all indoor surfaces. This was intended to break the cycle of infection by killing female mosquitoes as they rested after a blood meal. The spraying programme was supported by the use of antimalarial drugs such as chloroquine to treat clinical cases of malaria.

However, early success led some countries to stop their spraying programmes prematurely. In most countries, small foci of infection remained and the disease spread back quickly into cleared areas. Also, the effectiveness of some insecticides was reduced. Some mosquitoes altered their behavior, others became physiologically resistant. New insecticides were used but they were more expensive. Pest control of crops also used insecticides and this helped the rapid development of resistance. (Chopin and Wassermann, 1981).

Parasites were also shown to have developed to antimalarials resistance. In 1961, Moore and Lanier reported cases of *P.falciparum* in Colombia with abnormal reactions to chloroquine. Within three years resistance to chloroquine was found in Brasil, Cambodia, Thailand and Vietnam (Peters, 1970). It also spread to East Africa (Fogh et al, 1979, Onori et al, 1982). In Thailand, many
parasites have multiple drug resistance (Thaithong et al, 1983).

Although the original schedules for eradication were found to be unrealistic, in the revision of the programme in 1969 (WHO, 1969) global eradication was still considered to be the ultimate goal to be pursued. The methodology of malaria eradication was to be reviewed to develop alternative methods which were simpler and less expensive. In 1976 the Special Programme for Research and Training in Tropical Diseases was launched (Wernsdorfer, 1977). It selected six diseases for concentrated attack including malaria. The objectives of the programme are to encourage research and development of new and improved tools for the control of disease and also to strengthen the research capability of the tropical countries where these diseases are prevalent.

1.2 Malaria Life Cycle

Malaria parasites were first observed in the blood of a patient by Laveran in 1880. Since then, the rest of the complex life cycle of human and many animal species of malaria parasite has been described. The work in this study has been carried out on the rodent malaria parasite *P.chabaudi*. It was first described by Landau (1965) and redefined by Carter and Walliker (1975).

Figure 1 shows a diagram of the life cycle of *P.chabaudi*. The cycle starts when a female mosquito takes a blood meal. Malaria gametocytes are ingested (1).
into the midgut, in which the macrogametocyte transforms into the female gamete and the microgametocyte undergoes exflagellation (2). This process releases male gametes which fertilise the female gametes (3). A motile ookinete is formed (4 & 5) which penetrates the wall of the midgut. This develops into an oocyst which takes 8 to 15 days to mature (6-9). When mature, the oocyst ruptures (10) liberating sporozoites which migrate to the salivary glands (11) When the mosquito takes another blood meal, the sporozoites are injected into the blood stream of the mammalian host.

The sporozoite circulates in the blood (12) until it reaches the liver where it enters a hepatocyte. It develops into an exoerythrocytic schizont. This takes more than 50 hours to mature (13-15) before rupture and release of many exoerythrocytic merozoites (16) into the blood stream.

These merozoites invade erythrocytes (17) and undergo further cell division (18-21) to form erythrocytic schizonts. *P.chabaudi* schizonts contain 6 to 8 merozoites which are released by cell rupture allowing the merozoites to reinvade other red blood cells. Development of the erythrocytic stages of *P.chabaudi* is synchronous, with each cycle taking 24 hours. The parasite preferentially invades mature erythrocytes. Some merozoites on reinvasion develop into gametocytes (1) which circulate in the blood stream ready to be ingested by a mosquito.
Figure 1. Life cycle of rodent malaria. From Landau and Boulard (1978).
1.3. Vaccine Studies

Apart from research into the development of insecticides and antimalarial drugs there have been many investigations into the immune response to malaria with a view to producing a vaccine. Reviews on this subject are by Cohen (1979), Desowitz & Miller (1980), Playfair (1980), Deans & Cohen (1983), WHO (1984) and Bruce-Chwatt (1985).

The idea that a vaccine could be produced against malaria was encouraged by the studies of Cohen et al (1961), who showed that antibodies are important in suppressing infection of *P. falciparum* and *P. malariae*. These workers gave immune adult gamma globulin to twelve Gambian infants with severe clinical malaria. They observed that the symptoms of the disease were reduced and the parasitaemias reduced to very low levels. They found no alteration on parasite morphology during treatment. Since only immature parasites are detectable in peripheral blood, they deduced that the antibody was acting upon mature schizonts or free merozoites. This West African serum was also found to be protective against East African *P. falciparum* by McGregor et al (1963), showing that at least some of the antigens which induce a protective response are shared by separated populations of parasites. Miller et al (1975) have shown that immune serum from chloroquine-cured rhesus monkeys infected with *P. knowlesi* causes aggregation of merozoites in vitro. They found that antibody inhibited parasite penetration.
of erythrocytes, possibly by merozoite agglutination.

Although current studies are aiming at the production of a vaccine with defined antigens, vaccination studies on malaria using various parasite preparations have been carried out for many years. These studies have been in two main areas, (1) immunity to sporozoites, and (2) immunity to the erythrocytic stages, and these are discussed separately here.

1.3.1. Immunity to Sporozoites

Mulligan et al (1941) first showed that inactivated sporozoites could induce immunity to *P. gallinaceum*. Nussenzweig et al (1972) reviewed vaccination studies against mammalian malarials using sporozoites and concluded that in all cases irradiated sporozoites could be used as effective vaccines. However, Collins & Contacos (1972) found that they were ineffective in the simian malaria *P. cynomolgi*. The main problem of using irradiated sporozoites as a vaccine was that they had to be given intravenously. However, Orjih et al (1981) inoculated irradiated *P. berghei* sporozoites intramuscularly into young and adult mice and found good protection in the young mice. There was some protection in the adult mice but not as great as with intravenous injection. Clyde et al (1975) and Rieckman et al (1979) allowed X-irradiated infected mosquitos to feed on human volunteers over an extended period of time. Both studies found that this induced protection against homologous and heterologous
P. falciparum challenge but it lasted only a few months.

Clyde et al (1975) found that the protection was species specific and not effective against P. vivax challenge although immunity to P. vivax could also be induced by this method. Nussenzweig et al (1967 & 1969) found that sporozoite immunity to P. berghei is stage specific and had no effect on the course of infection when challenged with erythrocytic stages of the parasite. Since the symptoms of the disease are caused by the erythrocytic stages of the parasite a vaccine would have to be effective against them.

1.3.2. Immunity to Erythrocytic Stages

Brown et al (1970) immunised rhesus monkeys against P. knowlesi using dead parasites, given with Freund's Complete Adjuvant (FCA). Two out of three monkeys survived the initial parasitaemia after challenge and rapidly cleared their infection. Their immunity was also effective against another strain of P. knowlesi but not against another species of Plasmodium. Schenkel et al (1973) used a non viable lyophilized antigen preparation from blood stage antigens of P. knowlesi, and compared its effect on the infection in monkeys when given in combination with FCA, BCG or nucleotides and aluminium hydroxide as an adjuvant. The only effective combination had FCA as the adjuvant. Mitchell et al (1974) used fresh merozoites and mature schizonts of P. knowlesi with FCA to
inoculate rhesus monkeys. Immunity against challenge with the same variant was induced in both systems although only the merozoite preparation induced immunity to a different variant. Wellde et al (1979) immunised three Aotus monkeys with *P. falciparum* blood forms which had been irradiated. No adjuvant was used and two of the monkeys survived for more than 180 days after challenge with fresh parasites.

1.4. Identification of Individual Antigens

1.4.1 Identification by Immune Serum

Large amounts of antigen are required in immunization studies. Sporozoites cannot be cultured and their isolation from infected mosquitoes is time consuming. Although the erythrocytic stages of *P. falciparum* can be cultured not all isolates grow well. Those that do grow have a low level of multiplication and lose their synchronicity. Apart from the problems of obtaining enough material for immunization, parasite preparations could also include unwanted material such as salivary glands or red blood cell membranes which might induce autoimmune reactions in the recipient. Vaccines ideally require well-defined, pure malaria antigens. Thus it is important to discover which antigens induce a protective response and which induce an ineffective response or even one which is harmful to the host.

Reese et al (1981) immunoprecipitated metabolically labelled proteins of *P. falciparum* with immune human
sera which inhibited parasite growth in vitro and compared these with proteins precipitated by non-inhibitory sera. They found proteins of 200kDa, 75-80kDa and 45kDa precipitated only by the inhibitory sera. Perrin et al (1981a) obtained similar results but identified two additional antigens of 140kDa and 110kDa. However, Brown et al (1982) found only one protein of 96kDa specifically precipitated by inhibitory immune serum. Schmidt-Ullrich & Wallach (1978) prepared a hyperimmune serum in rhesus monkeys against \textit{P. knowlesi} and identified three proteins specific to the membranes of parasitised erythrocytes.

1.4.2 Identification Using Monoclonal Antibodies

Although the above methods enable antigens to be identified, they do not aid their isolation for use in vaccination studies. The advent of monoclonal antibody (mcab) technology has enabled many antigens to be identified and purified. Kohler & Milstein (1975) produced continuous cultures secreting antibody of defined specificity to sheep red blood cells using Sendai virus as the fusing agent. PEG as the fusing agent was used in the production of antibodies to major histocompatibility antigens (Galfre et al., 1977) and is commonly used in many protocols. The wide applications of mcabs have been seen in their use in many fields of medical research from cancer and tumours to viral and bacterial diseases (Lennox, 1984). They have been made against many parasites including malaria,
trypanosomes, schistosomes and leishmania (Cohen, 1984). Within the field of malaria research, mcabs have been used to study different parts of the life cycle; sporozoites, gametes and blood forms of several different species of *Plasmodium*. Their great advantage over previously available reagents has been their monovalent reactions to identify individual components out of the complex mixture of antigens which makes up the parasite.

Yoshida *et al* (1980) made a hybridoma cell line against sporozoites of *P.berghei*. Antibodies secreted by the cell line bound to a protein round the surface membrane of the sporozoites. When sporozoites were incubated with antibody *in vitro* they lost their infectivity to mice. When antibody was given to mice they were resistant to sporozoite challenge (Potocnjak *et al.*, 1980). Using the same mcab, Hollingdale *et al* (1982) could inhibit the entry of sporozoites into cultured embryonic lung cells. Inhibitory mcabs to the sporozoites of *P.knowlesi* were made by Cochrane *et al* (1982). Sporozoites were incubated with antibody for 60mins before being injected into rhesus monkeys. Five mcabs could inhibit infectivity completely. Sporozoites incubated with normal mouse serum gave rise to parasitaemias in the monkeys 7 days after the injection.

Nardin *et al* (1982) made mcabs against the sporozoites of the human malarias *P.falciparum* and *P.vivax*. The mcabs were tested for their ability to neutralise the infectivity of sporozoites by incubating
the sporozoites with various dilutions of antibody prior to injecting into splenectomised chimpanzees. The *P.falciparum* control chimpanzees developed parasitaemias about 14 days after injection. The experimental animals did not develop parasitaemias until after day 21, and one had not shown any parasites when monitoring stopped on day 61. The experiments with *P.vivax* sporozoites showed that the mcabs could delay patency of infection and in one out of five chimps no parasites were seen up to day 79 when the experiment ended. Since primates are scarce, these *in vivo* experiments were correlated with *in vitro* inhibition by Hollingdale et al (1984). The mcabs of Nardin et al (1982) were tested for their ability to inhibit invasion of cultured human hepatoma cells by sporozoites. Mcabs against *P.falciparum* sporozoites could inhibit their entry into the hepatoma cells. *P.vivax* sporozoites were inhibited by mcabs raised against them.

Many different groups have produced mcabs against antigens of erythrocytic stages of *P.falciparum*, *P.knowlesi*, *P.chabaudi* and *P.yoelii*. Despite being produced against different species of malaria, a class of proteins with which some of the mcabs reacted was found to exhibit similar properties (Newbold, 1984). The Mr of these proteins ranged from 190K in *P.falciparum* to 230K in *P.yoelii* and 250K in *P.chabaudi* and *P.knowlesi*. They were synthesised late in the erythrocytic cycle and had a characteristic
immunofluorescence pattern in fixed preparations reacting with mature schizonts and free merozoites. Although the mcabs did not react with different species, a polyvalent mouse serum raised against the purified P. yoelii protein reacted with the proteins of P. falciparum and P. chabaudi (Holder et al., 1983). When animals were given mcab or the purified protein, with which the mcab reacted, they were resistant, in varying degrees, to challenge with blood form parasites (Freeman et al., 1980; Epstein et al., 1981; Boyle et al., 1982; Hall et al., 1984a).

Other antigens which may be important in protective immunity have also been identified by mcabs. For example, Deans et al. (1982) made rat mcabs against P. knowlesi merozoites and identified a surface antigen of 66kDa. Fab fragments of the mcabs inhibited invasion of erythrocytes by merozoites. Perrin et al. (1981b) produced mcabs against P. berghei and P. falciparum which inhibited the growth of P. falciparum in culture. The mcabs identified P. falciparum antigens of 41kDa and 96 and 36kDa. Stanley et al. (1985) made mcabs to a 56kDa glycoprotein on the surface of P. falciparum merozoites; there was no evidence, however, that this antigen was involved in immunity. Miller et al. (1984) produced several mcabs against P. knowlesi. These were shown to be inhibitory and react to with a protein of 140kDa. Taylor et al. (1981) produced mcabs to P. yoelii. Some were stage specific, some were species specific, others cross reacted with other rodent malaria
species and some even cross reacted with rodent, primate and avian malarial species.

Although a large number of different antigens are implicated by mcabs to be involved in protective immunity, not all are likely to be incorporated in a vaccine. Newbold (1984) discusses this point and the problems in using animal model and culture facilities to study the problem. Brown et al (1983) have shown that there is no correlation between the known immune status of the donor of serum and the ability of the serum to inhibit growth of \textit{P.falciparum} parasites \textit{in vitro}.

1.4.3 Identification of Variant Antigens

IFA has been used to identify variant antigens recognised by many mcabs. This technique has many advantages. Firstly it can give rapid results, within 2 hours. Secondly, it can give information about the location of the antigen. For example, in different species of \textit{Plasmodia} characteristic IFA patterns are seen for the high molecular schizont antigen (Holder et al, 1983) and the rhoptry antigen (Oko et al, 1984, Howard et al, 1984, Schofield et al, 1985). Thirdly, the number of parasites reacting per field can indicate stage specificity and can identify minority parasite populations, down to less than 1% (McBride et al, 1982) in a mixed infection.

A panel of mcabs raised against two Thai isolates were used to screen a number of isolates of \textit{P.falciparum} from different areas (McBride et al,}
1982 & 1985). These studies showed a great deal of antigenic diversity between the isolates tested with no clear regional differences identified. On the basis of the IFA reactions of mcabs specific for one antigen, isolates could be allocated into one of seven groups or serotypes. This antigen ranged in size between 195kDa to 200kDa in different clones as shown by immunoprecipitation (McBride et al., 1985). Immunoprecipitation of this antigen in newly isolated parasites from the Gambia with these mcabs also showed that it varied in size in different isolates (Howard, et al., 1986).

Antigenic diversity of *P. falciparum* was also shown by Schofield et al. (1982) using mcabs raised against two isolates from Papua New Guinea (PNG). These mcabs reacted with all PNG isolates tested but did not react with isolates from Thailand, Ghana, the Netherlands or Nigeria.

1.5. Molecular Studies on Antigens

Once the proteins involved in host protective immunity have been identified, the next steps are the cloning of parasite genes, followed by the expression and identification of the genes encoding the proteins. The protective epitope may then be produced by genetic engineering methods or by chemical synthesis.

The first malaria gene to be cloned and sequenced was the circumsporozoite protein of *P. knowlesi*. Ellis et al. (1983) isolated a cDNA clone which expressed a
fusion polypeptide. This reacted with mcabs to the circumsporozoite protein. The polypeptide was composed of twelve amino acids repeated twelve times. A repeating unit was also found in the structure of the circumsporozoite protein gene of *P. falciparum* (Dame et al., 1984 and Enea et al., 1984).

This repeating unit comprised of four amino acids. Peptides comprising of a number of copies of the repeat were expressed in *E. coli* and injected into mice. The resulting sera reacted with live sporozoites and blocked the invasion of human hepatoma cells by sporozoites (Young et al., 1985). Synthetic peptides which encoded several tetrapeptide repeats were also synthesised (Zavala et al., 1985 and Ballou et al., 1985). The peptides were coupled to carriers and injected into mice and rabbits. Both groups found their sera could inhibit the invasion of hepatoma cells by sporozoites *in vitro.*

The erythrocytic stages synthesise many more proteins than the sporozoites and many groups are involved in producing gene libraries expressing blood stage antigens. Kemp et al. (1983) were the first to produce a *P. falciparum* cDNA library and screen it with immune human sera which was known to inhibit parasite growth *in vitro.* The first antigen they identified was an S-antigen. Because of the great amount of diversity of S-antigens (Wilson et al., 1969, Anders et al., 1983) it is unlikely to be a candidate in the search for a vaccine although Saul et al., (1985) showed a mcab
against an S-antigen could inhibit growth \textit{in vitro}.

A \textit{P.falciparum} protein of Mr 190000 was purified using mcabs by Hall et al., (1984a). Three Saimiri monkeys were immunized with purified protein with FCA. Two of the monkeys recovered from the parasitaemia arising from challenge. The parasites used in the challenge were from a different strain than was used to purify the protein. Data from mcab reactivity showed that the protein had constant and variable regions and the immunization data suggested that the constant part of the protein may have protective value. The purified protein was also used to raise a polyclonal rabbit serum for screening their cDNA library made from the Thai isolate K1. They isolated a clone containing 300 base pairs of the gene. A further part of the gene was obtained by Cheung et al., (1985), who used the isolate SGE2 from Zaire. The whole of the protein from the Wellcome West African isolate was sequenced by Holder et al (1985).

The entire K1 sequence was reported by Mackay et al (1985) and the sequence for an isolate from PNG, MAD 20, was reported by Tanabe et al (1986) who also compared the sequences which have been identified. Apart from the N-terminal signal peptide and the C-terminal region which is rich in hydrophobic amino acids they were able to divide the gene into blocks. Some of the blocks are highly conserved with greater than 87% of the amino acids homologous between the isolates. In some blocks the sequences appear to have diverged and have
less than 40% homology. The remaining blocks appear to be conserved sequences with patches of non-homologous residues. It is not yet known which of the blocks encode the epitopes which induce the protective response shown by Hall et al (1984a). However, since they were able to protect against a heterologous challenge it is probably in one of the conserved regions.

Coppel et al (1984) identified an antigen of *P. falciparum* on the surface of ring-infected erythrocytes. Perlmann et al (1984) showed that antibodies to the same antigen inhibited parasite growth *in vitro*. The sequence of the protein revealed two regions of repetitive sequence. 14 Aotus monkeys were immunized with recombinant polypeptide in FCA and 9 monkeys recovered from challenge (Collins et al, 1986).

Koenen et al (1984) isolated a clone of an antigen on the membrane of erythrocytes infected with mature parasites not rings. This protein was composed of 9 amino acid repeats. Stahl et al (1985) identified another clone recognised by human immune serum. This protein had repeats of 6 amino acids interspersed with regions containing both acidic and basic amino acids. Antibodies to this protein react with mature and immature parasites but not the surface of the erythrocyte.

Hope et al (1984) found cross-reaction between sporozoites and blood stages using a mcab. When they sequenced the gene from the erythrocytic stage, they
found a sequence of 15 amino acids which had homology to the tandemly repeated tetramer of the circumsporozoite protein (Hope et al., 1985). The common epitope probably lies within this small sequence.

Several other genes of malaria parasite have been sequenced (Kemp et al., 1986, Scaife et al., 1986). The most notable feature of many of the genes so far has been the presence of repeated units. The function of these is not yet known. The sequence of the repetitive units of proteins expressed in the erythrocytic stage shows a great deal of diversity. Often repeated units do not cross-react with other isolates although non-repetitive parts of the same protein do cross-react (Cowman et al., 1985).

Molecular vaccines do have certain limitations (Mitchell, 1984). There is the requirement of adjuvants or antigen delivery systems. There is also the problem that a vaccine of restricted composition could select parasites which do not express the antigen. Guidelines have been published for the field trials of the vaccines (WHO 1986).

1.6. Genetics of Malaria Parasites

There are many species of malaria, four of which infect humans. Although each species is distinct, there is a great deal of variation within species. Studies of the genetics aim to show the mechanisms through which this variation arose and how it spreads. Until recently it was not possible to study the genetics of human
malarias so the convenient model of the rodent malarias was used. Reviews on malaria genetics are by Beale (1980) and Walliker (1983).

The method used for carrying out genetic crosses is to allow mosquitoes to ingest a blood meal containing gametocytes of two cloned lines. When mature sporozoites are present in the salivary glands the mosquitoes are fed on an uninfected host. The resultant infection will contain a mixture of the progeny of self and cross fertilization. Individual progeny are cloned by diluting infected blood and injecting a single parasites into a host.

The fundamental question of ploidy was one of the first problems studied by genetics. Variant forms of parasite enzymes revealed by electrophoresis were shown to be stable markers. When Rosario (1976) crossed two lines of *P. chabaudi* which differed in two enzymes, four types of progeny were produced. Two were the parental types and two were new combinations of the enzyme forms. The conclusion was that the blood forms were haploid. If they were diploid then three types of progeny would be predicted from the cross, the two parental types and one hybrid with both types of each enzyme.

The use of enzymes enabled the identification of species and sub-species of malaria (Carter 1978). Genetic studies in the laboratory have shown that interbreeding within the species *P. yoelii* can occur (Knowles et al., 1981 and Lainson 1982 & 1983a). If
populations in the wild can interbreed freely then characters such as drug resistance and variant antigens will have no barrier to their rapid spread.

Crosses between parasite lines of *P.yoelii* and *P.chabaudi* resistant and sensitive to pyrimethamine showed segregation of the pyrimethamine response and recombination with isoenzyme markers (Walliker et al., 1973 and 1975). The conclusion was that resistance was probably due to a single mutation. Pyrimethamine resistance in *P.falciparum* arose rapidly in areas where the drug was used, indicating that the resistance had a similar mechanism in rodent and human malarias.

The results of chloroquine resistance studies showed a quite different picture. When a *P.chabaudi* line resistant to a high level of chloroquine was crossed to a sensitive line, a range of levels of resistance was found in the resulting clones (Padua 1981). The conclusion from this was that the high level of resistance was due to an accumulation of several mutations at different loci. The use of chloroquine as a prophylactic probably provided ideal conditions for the selection of resistance to the drug in *P.falciparum*.

The first study of the inheritance of antigens of malaria parasites was by Panton *et al.* (1984). Using crossed-immunoelectrophoresis they identified an antigen of *P.yoelii*, Py1, whose quantity and electrophoretic mobility differed between lines. Results from a cross showed that the quantity of the antigen correlated with the virulence of the parasites. The electrophoretic
mobility of Pyl segregated independently of the virulence character. The two antigenic markers also recombined with isoenzyme and drug sensitivity markers.

Although more characters of *P. falciparum* such as variant proteins identified by 2D-PAGE (Fenton et al, 1985) and mcabs (McBride et al., 1982) have been added to the isoenzymes and drug response markers, no linkage groups have been identified. Sinden (1978) using cytological data suggested a haploid number of 10 chromosomes. Pulse field gel electrophoresis (PFGE) used by Kemp et al (1985) gave an estimate of at least 8 chromosomes in *P. falciparum*. The same technique has separated at least 10 chromosomes in *P. chabaudi* (A. Sharkey, unpublished).

Studies on the variation of characters when added to the knowledge of how these characters are inherited, are very important in control measures. Studies on the genetic basis of drug resistance have shown how drug resistant mutations could arise and spread through the parasite population which is largely interbreeding. In the development of a vaccine, studies on the genetics of antigens could provide information enabling predictions to be made about the likelihood of new forms of antigen arising and spreading through the population as a result of the selection pressure.

1.7. Aims of This Work

Many groups of research workers are concentrating on identifying and cloning the genes of protective
antigens of malaria with a view to producing a vaccine. However, very little is known about the ability of malaria parasites to generate new antigens. Antigenic variation in *P. knowlesi* (Brown & Brown, 1965, Howard et al., 1983), *P. chabaudi* (McLean et al., 1982) and *P. falciparum* (Hommel et al., 1983) was identified using sera. Using mcabs, McBride et al. (1982) and Schofield et al. (1982), have shown antigenic diversity of individual antigens within *P. falciparum*. Mackay et al. (1985) and Schwartz et al. (1986) have shown diversity of an antigen at the DNA level. It is important to study how new forms of antigens can arise so that the response of the parasite population to the selection pressure, which a vaccine would induce, could be predicted. Genetic studies of *P. falciparum* were not possible until recently. Rodent malarias are useful models with which to study the genetics of the parasites (Walliker, 1983). Panton et al. (1984) used crossed-immunoelectrophoresis to study the genetics of *P. yoelii* antigens.

The aims of this study were

1) to identify variant antigens in the rodent malaria species *P. chabaudi*. For this part of the project, mcabs were prepared from mice infected with *P. chabaudi* and screened against a variety of cloned parasite lines using IFA. This was expanded by the use of mcabs from other laboratories also made against *P. chabaudi*. 
2) to characterise the antigens with which the mcabs reacted by immunoprecipitation of metabolically labelled parasite proteins and digestion of the immunoprecipitates by a protease. Blotting of proteins on nitrocellulose membranes was also used.

3) to study the genetics of the variant antigens in crosses between clones differing in these antigens. This was to determine whether diversity was due to allelic variation of a single gene or diversity of genes at different loci. It was also to determine if there was any linkage between antigen markers and isoenzymes and pyrimethamine response.
2 MATERIALS AND METHODS

2.1 Laboratory Hosts

2.1.1 Rodents

Most of the mice used in this study were bred and housed in the Department of Genetics, University of Edinburgh. Some C57BL mice and white rats were obtained from the University of Edinburgh Centre for Laboratory Animals.

C57BL mice were used for all routine parasite passages and preparations. Mixed outbred strains of mice were used in cloning experiments. All hybridoma work was carried out using Balb/c mice.

Rodents were supplied with a diet of pelleted rat cake (B.P. 1). Drinking water was supplemented with 0.05% w/v para-aminobenzoic acid (PABA) to aid parasite growth (Jacobs, 1964).

In experiments requiring parasites in the late stages of the erythrocytic cycle, mice were kept in a reversed light regime with illumination between 5.30pm and 8.30am; under these conditions, schizogony of *P. chabaudi* occurred during the daytime.

2.1.2 Mosquitoes

*Anopheles stephensi* were maintained in a 12 hour light, 12 hour dark cycle at 24-26°C with 80% relative humidity. They were supplied with 10% glucose solution supplemented with 0.05% PABA.
2.2 Parasites

2.2.1 Definitions

In this work parasites are described as isolates and clones, the definitions of these terms are as follows.

Isolate. A sample of parasites collected from a naturally infected host on a single occasion. Parasites within the sample are not necessarily genetically homogeneous.

Clone. Genetically homogeneous parasites which are derived from a single cell by asexual division.

2.2.2 Parasite Lines

Most of the parasites used in this work (Table 1) were from a collection of *P.chabaudi* described by Carter and Walliker (1975) and Carter (1978) which is maintained in Edinburgh in liquid nitrogen. Two additional lines were EF (Lainson, 1983b) and ER which was sent to this laboratory by C. Hamers-Casterman as an isolate designated 56L. All parasites had been cloned by limiting dilution before this study (2.2.5.).

The main parasites used in this work were AS, CB and ER which had been isolated in the Central African Republic (CAR). The CB line used was a pyrimethamine resistant line, which was selected by D. Walliker by treating infected mice with 30mg/kg pyrimethamine over four days and then cloning the surviving parasites by limiting dilution. AS and ER were both sensitive to pyrimethamine.
<table>
<thead>
<tr>
<th>Line</th>
<th>Country of Origin</th>
<th>Species &amp; sub-species</th>
<th>Isolate</th>
<th>Host Species</th>
<th>Date of Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>C.A.R.</td>
<td>P.c.chabaudi</td>
<td>1AS</td>
<td>T.rutilans</td>
<td>1969</td>
</tr>
<tr>
<td>CB</td>
<td>C.A.R.</td>
<td>P.c.chabaudi</td>
<td>2CB</td>
<td>T.rutilans</td>
<td>1970</td>
</tr>
<tr>
<td>ER</td>
<td>C.A.R.</td>
<td>P.chabaudi</td>
<td>56L</td>
<td>T.rutilans</td>
<td>1965</td>
</tr>
<tr>
<td>AF</td>
<td>C.A.R.</td>
<td>P.c.chabaudi</td>
<td>16AF</td>
<td>T.rutilans</td>
<td>1969</td>
</tr>
<tr>
<td>AL</td>
<td>C.A.R.</td>
<td>P.c.chabaudi</td>
<td>1AL</td>
<td>T.rutilans</td>
<td>1969</td>
</tr>
<tr>
<td>AQ</td>
<td>C.A.R.</td>
<td>P.c.chabaudi</td>
<td>1AQ</td>
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<td>1969</td>
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<tr>
<td>BE</td>
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<td>P.c.chabaudi</td>
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<td>T.rutilans</td>
<td>1969</td>
</tr>
<tr>
<td>DK</td>
<td>Congo</td>
<td>P.c.adami</td>
<td>408XZ</td>
<td>T.rutilans</td>
<td>1972</td>
</tr>
<tr>
<td>DS</td>
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<td>P.c.adami</td>
<td>556KA</td>
<td>T.rutilans</td>
<td>1970</td>
</tr>
<tr>
<td>EF</td>
<td>Cameroun</td>
<td>P.chabaudi</td>
<td>Biboto36</td>
<td>Hylomycus</td>
<td>1973</td>
</tr>
</tbody>
</table>
2.2.3. Estimation of Parasitaemia

Thin films of tail blood were fixed with methanol. They were then stained with 10% Giemsa's stain in distilled water (pH 7.2) for 45 minutes or with 50% Giemsa for 5 minutes. Parasitaemias were expressed as a percentage of the number of parasites in 1000 red blood cells.

2.2.4. Passaging of Parasites

Routine passages of parasites used citrate saline (0.9% NaCl, 1.5% Na citrate, pH 7.2) as the diluent of parasitised blood. The inoculum was given through the peritoneum.

When a predetermined number of parasites per inoculum was required, the number of red blood cells per ml of blood was determined using a Coulter Counter. The product of this and the parasitaemia gave the number of parasites per ml. Dilution steps using 50% foetal calf serum (FCS) in RPMI medium 1640 were carried out until the required number of parasites per inoculum of 0.1ml was achieved.

2.2.5. Limiting Dilution Cloning of Parasites

To minimize the number of erythrocytes containing more than one parasite, mice with parasitaemias of 1-2% were used as donors. A sample of infected blood was serially diluted in 50% FCS in RPMI until there was 0.5 or 1 parasite per 0.1ml inoculum. This was given intravenously.
2.2.6. Cryopreservation of Parasites

Blood from an infected mouse was mixed with heparinised balanced salt solution, pH8 and glycerol by the method of Lumsden et al (1966). The mixture was sealed in glass capillaries and stored in liquid nitrogen.

Infections were re-established by rapid thawing of capillaries in water at room temperature. The capillaries were cut open and the infected blood was mixed with citrate saline before intraperitoneal injection into mice. Infections became patent in mice within 4 to 7 days and were passaged at least once before being used in experiments.

2.3. Conducting a Cross

The method for crossing *P. chabaudi* clones was based on that of Walliker, Carter and Sanderson (1975), and is illustrated in figure 2. Blood forms of the two parasite lines were mixed in proportions estimated to result in an equal number of gametocytes from each parent line. The mixture was inoculated intravenously into a 4-6 week old splenectomised white rat, which enhances gametocyte development of *P. chabaudi* (McLeod & Brown, 1976). Maximum numbers of gametocytes were found 4 days after inoculation. The animal was restrained and placed in a cage containing 7-10 day-old *A. stephensi*. Feeding was encouraged by depriving the mosquitoes of glucose 24 hours prior to the blood meal.
Figure 2. Procedure used in making a cross

Parent line A

2 mice

No1 No2 → Blood forms mixed ← No1 No2

Rat

Mosquitoes

Mouse

Control line A

Progeny of the cross

Parent line B

2 mice

Rat

Mosquitoes

Mouse

Control line B
The course of the resulting infection was followed by examining midgut preparations of mosquitoes 7 days later. The number of oocysts gave an estimation of the relative success of the parental lines and mixture in establishing infections. When large numbers of sporozoites were detected in the salivary glands, usually 14 days after the initial infection, the mosquitoes were allowed to feed on uninfected mice. Infections in mice became patent within 7 days.

2.4 Studies on Blood Form Antigens

Antigens were studied by immunofluorescence and also SDS-PAGE (see section 2.6.4) after metabolic labelling of parasite proteins.

2.4.1 Antigen Preparations for IFA

Blood from mice with 10% parasitaemia containing trophozoites and schizonts was collected into heparinised RPMI. The blood cells were passed over a prewetted column of Whatmans CF11 powdered cellulose to remove white blood cells (Homewood and Neame, 1976). The blood cells were pelleted by centrifugation and washed a further two times in RPMI. The cell pellet was diluted in RPMI to 1% by volume. A 20\textmu l sample was placed on each of the wells of PTFE coated slides and allowed to dry, before being stored at -20°C.

The preparations were used in IFA by the method described in section 2.5.4.
2.4.2. Metabolic Labelling of Parasite Proteins

2.4.2.1 Labelling Parasites In Erythrocytes

Parasites were labelled with $^{35}$S-methionine by modifications of the method of Newbold et al (1982). Blood from a mouse with a 20-30% parasitaemia was collected into heparinised RPMI. This was run through a prewetted CF11 column to remove white blood cells and washed twice with RPMI. The infected red blood cells were washed once in Eagles MEM without methionine (MFM). The pellet was resuspended in MFM with 10% FCS to give 10% haematocrit. 100μCi $^{35}$S methionine was added to the culture which was kept in an atmosphere enriched in carbon dioxide at 37°C for two hours.

The free methionine was removed by two washes in RPMI. Radiolabelled parasite proteins were extracted with 1ml 1% Triton X-100 in 50mM Tris-HCl buffer, pH 8.0, containing 5mM PMSF and 5mM EDTA for 1hr at 4°C with intermittent vortexing. The mixture was centrifuged at 11,000g for 30 mins to remove insoluble material. Extracts were stored at -70°C.

2.4.2.2 Labelling Parasites Freed From Erythrocytes

Parasites freed from their host cells were labelled by the method of Tait (1981). After removal of the white blood cells and washing the erythrocytes twice packed (as above) the erythrocytes were lysed with an equal volume of 0.15% saponin in RPMI at 37°C for 10mins. 10ml of RPMI was added and the mixture centrifuged at 2,500g for 7mins. The supernatant and layer of red cell
ghosts were discarded and the parasites were washed a further two times in RPMI then once in MFM. Parasites were resuspended in 1ml of MFM with 10% FCS. 100μCi $^{35}$S methionine was added and the culture incubated for 2hrs at 37°C.

Parasites were centrifuged and washed twice with RPMI before being solubilised in extraction buffer (1% Triton X-100 in 50mM Tris-HCl pH8 with 5mM EDTA, 5mM EGTA, 2mM PMSF). This was incubated at 4°C for 1hr with intermittent mixing. Insoluble material was removed by centrifugation at 10000g for 30mins. Extracts were stored at -70°C.

2.5. Monoclonal Antibodies

Mcabs were produced in this study by fusing spleen cells from mice which had been infected with the blood stages of *P.chabaudi* to a mouse plasmacytoma line. The resulting cell lines were tested for their production of antibody to parasites by an Indirect Fluorescent Antibody test (IFA). Mcabs against *P.chabaudi* from other laboratories were also used in this study. Details are given below.

2.5.1. Immunization Protocol

Six week old female Balb/c mice were inoculated with $1\times10^6$ parasites intraperitoneally. Five days later, smears of tail blood were examined to show that the mice were infected. Eight weeks after inoculation, $10^6$ parasites were again injected intraperitoneally.
Three days later mice were bled out and the spleen was removed for the fusion process.

2.5.2 Fusion Protocols

Two slightly different modifications of the method described by Galfre et al (1977) were used in this study.

2.5.2.1. Fusion 3 Protocol

5x10^7 P3-X63-NS/1 cells, a mouse plasmacytoma cell line (Kohler et al, 1976), were mixed with 1x10^8 spleen cells from a mouse immunised with *P.chabaudi* line AS. The mixture was centrifuged at 500g for 5 mins and the supernatant was discarded. 1ml of 50% polyethylene glycol 1500 (PEG) in RPMI was added over 1 min. 1ml of RPMI was added over the next minute. 9mls of RPMI were added over 5 mins. The cells were centrifuged at 500g for 5 mins. The pellet was resuspended in RPMI with 20% FCS and the uninfected spleen cells were added. 0.5ml aliquots were plated out in each well of five 24-well plates.

24 hours later, 0.5 ml double strength HAT solution (1x10^{-4}M hypoxanthine, 4x10^{-7}M aminopterin and 3x10^{-5}M thymidine) was added to each well. 1 week later, 0.5ml culture supernatant was replaced with single strength HAT solution.

2.5.2.2. Fusion 12 Protocol

1x10^7 P3-X63-Ag8.653 cells, another mouse plasmacytoma cell line (Kearney et al, 1979), were
mixed with $1 \times 10^8$ spleen cells from a mouse immunised with *P. chabaudi* line CB. The mixture was centrifuged at 500 g for 5 mins. and the supernatant was discarded. 0.5 ml 41.6% PEG, 15% DMSO in RPMI was added dropwise over 1 min. 0.5 ml 25% PEG was similarly added. 30 ml of RPMI with 15% FCS (RP15) containing uninfected spleen cells was added slowly to the tube. 0.1 ml aliquots were plated out into each well of 5 96-well microtitre plates.

24 hours later, 0.1 ml of double strength HAT medium was added to each well. 7 days later, 0.1 ml of culture medium was replaced with single strength HAT medium.

2.5.3. Preparation of Feeder Cells

Cells growing in cultures grow best at known densities. Cells secrete factors into the culture medium. When the cell density is high, cells go into stationary phase. If the cell density is low, the cells are not receiving enough growth stimulating factor and do not grow very well. To overcome this problem when low numbers of cells are plated such as after fusion or during cloning, supplementary cells or feeders are added to the cultures. Three types of cells were used in this study. Spleen cells from a mouse which had not been infected were used during the plating out of newly fused cells. When cloning antibody producing cells, thymocytes or peritoneal exudate cells were used.
2.5.3.1. Uninfected Spleen Cells

The spleen was placed in a moist sieve and cut into small pieces. The piston of a syringe was used to break up the pieces. Cells were washed through the sieve with RPMI. The cells were centrifuged at 500g for 5 mins and resuspended in 10mls RPMI for counting.

2.5.3.2. Thymocytes

The thymus was removed from 4-6 week old Balb/c mice. It was placed in a moist sieve and teased with the piston of a syringe. Cells were washed with RPMI before being centrifuged at 500g for 5 mins and resuspended in RPMI.

2.5.3.3. Peritoneal Exudate Cells

A window of skin was removed from the peritoneal area of 6-12 week old Balb/c mice. 5ml RPMI was injected into the peritoneal cavity. The cavity was palpitated for 5 mins before withdrawing the peritoneal fluid. This was centrifuged at 500g for 5 mins, resuspended in 10mls RPMI and plated out in 100μl aliquots per well of 96 well microtitre plate.

2.5.4. Indirect Fluorescence Antibody Test (IFA)

IFA was used to screen the supernatants of growing cell cultures. The initial screen was carried out two weeks after the fusion and repeated twice before cloning. The supernatants of cloned cells were screened twelve days after the cloning and every few days as the
cells were expanded into culture flasks. IFA was also used later for screening diversity between different parasite lines.

Multispot slides of antigen preparations (2.4.1) were removed from the deep freeze and fixed immediately in acetone for 5 mins. Slides were air dried, and 25 μl of culture supernatant or serum dilution was placed on each spot. Serum from the mouse used in the fusion or supernatant known to be positive were used as positive controls. The negative control was normal mouse serum. The slides were incubated at room temperature for 30 mins in a humid box. Supernatants were removed and the slides were given 3 washes in phosphate buffered saline pH 7.2 (PBS) for 1, 2 and 5 mins before being dried gently on a hotplate. 10 μl of FITC conjugated rabbit anti-mouse (Sigma) whole IgG was placed on each spot and incubated in the humid box for 30 mins. The slides were washed 3 times in PBS before counterstaining with 0.1% Evans blue in PBS.

Preparations were examined using a Leitz Dialux 20 microscope. The FITC fluorescence was visualised using a Leitz filter N2-1.

2.5.5. Limiting Dilution Cloning of Hybridoma Cells

Once a colony of cells secreting a desired antibody covered more than half of the bottom of well, the cells were suspended and a sample was counted. Cultures were diluted to 40 cells per ml. The dilution was made into RP15 with either thymocytes or peritoneal exudate cells as feeders. Further dilution steps were
made to obtain 20, 10 and 5 cells per ml. Each quarter of a microtitre plate was plated with 0.1ml aliquots of the dilutions. One week later, each well was fed with 0.1ml of RP15. Colonies of cells were screened for antibody production 5 days later.

2.5.6. Cryopreservation of Cells

Cloned cell lines were grown up in 25cm² flasks holding 10mls of culture at 1-3x10⁶ cells per ml. The cells were centrifuged at 1500g for 5mins. The supernatant was collected and stored with the addition of 10mM sodium azide. The cell pellet was resuspended in a small volume of supernatant before the addition of 1ml of 20% DMSO in RPMI with 50% FCS. The suspension was placed in a 5ml screw cap freezing vial. The vials were put in a polystyrene box and frozen for 1hr at -20°C, overnight at -70°C before being placed in liquid nitrogen.

2.5.7. Production of Ascites

Balb/c mice were injected with 0.5ml 2,6,10,14-tetramethylpentadecane (Pristane) intraperitoneally. At least 4 days later 2x10⁶ cells were centrifuged at 1500g for 5mins. The supernatant was removed and the pellet was resuspended in 0.3ml of supernatant. This was injected into the peritoneum.

When tumours arose, the fluid was tapped using an 18g needle. The mouse was tapped again 2, and 4 days after the first collection. The ascitic fluid was
centrifuged at 11000g for 7 mins to pellet any cells and stored at -20°C with the addition of 10mM sodium azide.

2.5.8. Analysis of Immunoglobulin Subclass of Antibody

Culture supernatants were concentrated 10x using centicon 30 microconcentrators (Amicon). These were used in a double diffusion test (Ouchterlony 1978) with goat antiserum to the mouse immunoglobulins IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM (Serotec). The reactions were carried out overnight at 4°C.

2.5.9. Monoclonal Antibodies from other laboratories

Monoclonal antibodies which were known to react with a high molecular weight antigen (Mr 250000) associated with schizonts of *P.chabaudi* were kindly supplied by two other laboratories, as follows:-

Dr D. Boyle (NIMR, Mill Hill, London) supplied 11 mcabs. They had been raised against the AS line of *P.chabaudi* (Boyle et al., 1982) which came originally from this laboratory. They were referred to by their given number prefixed with a B eg B1.

Dr C. Hamers-Casterman (Vrije Universiteit Brussel, Belgium) raised mcabs against the 56L isolate of *P.chabaudi*. 7 of these mcabs reacted with the high molecular weight schizont antigen. Their given number was prefixed by H.
2.6 Antigen Characterisation

The antigens with which the mcabs reacted were characterised by several techniques.

2.6.1 IFA

IFA was used to characterise \textit{P.chabaudi} antigens cytologically and to screen for diversity of antigens between different parasite lines.

Most parasite preparations were examined with individual mcabs (2.5.4). In some instances, the uncloned progeny of crosses were examined by a double fluorescent antibody test, in order to show the presence of two antigenic determinants in the same parasite preparation. For these tests, preparations were incubated with two mcabs, of different isotypes. The isotypes of the mcabs examined in this procedure were either IgG2a or IgG2b. The preparations were then stained with fluorescein (FITC) and rhodamine (TRITC) conjugated reagents specific for each mcab isotype. The procedure was as follows:

\textbf{Multispot preparations of antigen} (2.4.1) were removed from the deep freeze and fixed in acetone for 5mins and then air dried. 25μl of a mixture of two mcabs of different isotypes was placed on the antigen spots. The slides were incubated in a humid box for 30mins at room temperature. The supernatants were removed and the slides were washed three times in PBS before being dried on a hotplate. 10μl of either fluorescein conjugated rabbit anti-mouse IgG2a, rhodamine conjugated rabbit
anti-mouse IgG2b or a mixture of the two was placed on the spots and incubated in the humid box for 30mins. Slides were washed in PBS three times before a coverslip was mounted with 50% glycerol in PBS.

Preparations were examined using a Leitz Dialux 20 microscope. The rhodamine fluorescence was visualised using a Leitz N2 filter and an N2-1 filter was used for the fluorescein fluorescence.

2.6.2 Immunoprecipitation of Parasite Proteins

To identify the size of the antigen with which the mcabs reacted the antibody was allowed to bind to parasite proteins. The complex was collected onto protein A bound to sepharose and electrophoresed on a sodium dodecyl polyacrylamide gel (SDS-PAGE). The parasite proteins had been labelled with $^{38}$S methionine so they could be detected by autoradiography.

Protein A-sepharose was swollen in 50mM Tris-HCl pH8.0, 1mM EDTA, and 0.5% Triton X-100 (TET) and resuspended at 50% swollen gel volume in the same buffer.

Extracts of parasite proteins labelled with $^{38}$S (2.4.2) containing $5\times10^5$ cpm were preabsorbed with 50ul of protein A-sepharose for 30mins at 4°C. The protein A-sepharose was removed by centrifugation at $11000g_{av}$ for 3mins. The extract was then mixed with 10ul of ascitic fluid and rotated for 2hrs. 100ul of protein A-sepharose in TET+6%BSA was added and rotated for a further hour.
The immunoprecipitates were washed 3 times in TET with 0.5M NaCl and 1% BSA then twice in TET. 1ml of buffer was used for each wash and the protein A-sepharose was spun down at 11,000g for 3mins. The addition of the new wash resuspended it. The washed complexes were dissolved in 50µl of 2x sample buffer, containing 2% SDS, 0.1M Tris-HCl pH7.5, 10% mercaptoethanol, 4mM PMSF, 2mM EDTA and 10% glycerol, and boiled for 3mins. The protein A-sepharose was removed by centrifugation. Samples were loaded and run on 10% SDS-PAGE. (2.6.4).

2.6.3. Peptide Mapping

Some of the mcabs reacted with different parasites to give a large antigen as the main product of the immunoprecipitate. To study this antigen further, after immunoprecipitation it was digested by a protease and the products electrophoresed on SDS-PAGE.

Aliquots of 35S-labelled parasite extracts containing 1x10^4 cpm were immunoprecipitated as 2.6.1. After the washing steps the immune complexes were eluted by the addition of 200µl of 0.125M Tris-HCl pH6.8, 0.5% SDS, 10% glycerol and boiling for 3mins. The protein A-sepharose was removed by centrifugation. 60µg/ml protease from Staphylococcus aureus strain VB was added to the immunoprecipitates and incubated at 37°C. Samples were removed at time points 0, 1, 5 and 30 mins after the addition of the protease, before being added to 50µl of 2x sample buffer and boiled for 3mins. The samples were loaded onto 12.5% SDS-PAGE (2.6.4).
2.6.4. Western Blotting

This technique involves the separation of parasite proteins by electrophoresis and the transfer of the separated proteins to nitrocellulose. Mcabs can bind to the antigens with which they react and this is identified by an antiserum to mouse IgG which is conjugated to horseradish peroxidase. A chemical reaction identifies the site of mcab binding.

Parasitised blood was collected from an infected mouse and passed down a prewetted CF11 column to remove white blood cells. The red blood cells were washed twice with RPMI before being lysed with an equal volume of 0.15% saponin in RPMI. 10ml of RPMI was added to the suspension and it was centrifuged at 4000rpm for 7mins. The parasite pellet was washed in RPMI a further two times before being solubilised in 2x sample buffer and run on a 7.5 or 10% SDS-PAG (2.6.4). Proteins were transferred electrophoretically to nitrocellulose filters by 200mA for 5hrs (Towbin et al., 1978).

Non-specific binding sites on the nitrocellulose were blocked by incubating the filter for 1hr in BLOTTO (5% non-fat milk powder in PBS, 0.02% NaN₃, 0.05% Tween 20) at room temperature. It was then incubated overnight with 1/50 dilution of ascites in BLOTTO. The first antibody was rinsed off with PBS then two washes in PBS with 0.1% Tween 20.

The blots were next incubated in 1/400 dilution of horseradish peroxidase conjugated anti-mouse IgG in PBS with 0.05% Tween 20 for 4hrs. This second antibody was
rinsed off with PBS followed by two washes in PBS with 0.1% Tween 20 then one wash in Tris-saline (10mM Tris, pH 7.4, 0.9% NaCl). They were then developed in 0.5mg/ml 4-chloronaphthol in Tris-saline with 0.01% hydrogen peroxide for 5-10 mins and washed in distilled water.

2.6.5. Polyacrylamide Gel Electrophoresis (PAGE)

This was carried out with slight modification to the method of Laemmli (1970) using discontinuous buffer system. The ratio of acrylamide to bisacrylamide was 29:2:0.8. The resolving gel was made with 0.37M Tris-HCl pH 8.7, 7.5, 10 or 12.5% acrylamide, 0.1% SDS, 0.032% Ammonium Persulphate and 0.0002% TEMED. Once the resolving gel was poured it was overlaid with 0.1% SDS.

When the gel had set, the overlay was removed and the top of the gel was washed with water and dried. The stacking gel was poured on top. It comprised of 0.11M Tris-HCl pH 6.75, 4.3% acrylamide, 0.1% SDS, 0.036% Ammonium Persulphate and 0.0005% TEMED. A 10 or 15 slot former was placed in position when the stack was poured. When the stacking gel had set the former was removed and the wells were rinsed with electrode buffer. The electrode buffer contained 0.025M Tris, 0.194M glycine and 0.1% SDS.

Samples were loaded into wells and the apparatus was assembled. The tanks were filled with electrode buffer and air bubbles removed from the bottom of the resolving gel. Tracking solution of 10% glycerol, 0.002% bromophenol blue was added to the top of the wells.
Electrophoresis was carried out at constant current of 6mA per gel for 15-18hrs.

2.6.6 Processing of Gels

Gels were fixed and stained by immersion in 0.3% Coomassie Brilliant Blue, 50% methanol and 7.5% acetic acid for at least 6hrs. The gels were then destained with several changes of 40% methanol and 10% acetic acid. After destaining they were reswollen in 5% methanol and 7.5% acetic acid.

Swollen gels were fluorographed using the method of Laskey & Mills (1975). Gels were shaken in three changes of DMSO for 30mins each and then in 22.2% PPO in DMSO for 3hrs. They were washed in several changes of distilled water for at least 1hr.

Fluorographed gels were dried onto Whatman 3MM paper using an LKB gel drier for 1hr. The dried gels were exposed to pre-flashed Kodak X-Omat AR film (Laskey & Mills, 1975) or Kodak X-Omat S film. Film and gel were exposed at -70°C.
3 RESULTS

3.1 Growth of *P. chabaudi* in Reversed Light

*P. chabaudi* grown in mice kept in normal daylight shows a synchronous infection with peak schizogony occurring at about 1 am. The antigens studied in this project were synthesised late in the erythrocytic cycle (Boyle et al., 1983). Mice were kept in reversed light with illumination from 5.30 pm to 8.30 am for at least one week before infection with parasites. Under these conditions parasites underwent schizogony during the day.

Ratios of the different developmental stages were obtained by counting over 200 parasitised cells. After schizogony the distribution of parasite stages changed from 90-100% trophozoites to 90-100% rings. This took place over 6 hours. Cloned isolates were found to go through schizogony at slightly different times. Figure 3 shows the increase in the proportion of ring stage parasites in infections of *P. chabaudi* isolates AS, CB and ER. Counts were done on three separate occasions and the graph shows the average of these results. It shows ER undergoing schizogony between 7.30 am and 1.30 pm, CB between 9.30 am and 3.30 pm and AS between 11 am and 4.30 pm.
Figure 3. Graph of % of ring stage parasites in infection of AS, CB and ER during the day when kept in reversed light cycle.
3.2 Monoclonal Antibodies

3.2.1 Production of Monoclonal Antibodies

A total of twelve fusions was carried out. Several fusions were lost very rapidly due to contamination. Other fusions produced cell lines secreting antibody but these were lost in the overgrowth of non-secreting cell lines. Two fusions, numbers 3 and 12, were successful in producing lasting hybrid cell lines. Fusion 3 was with spleen cells from a mouse immunised with the *P. chabaudi* line AS. 70% of the wells plated out produced HAT resistant cell lines with 60% of these secreting antibodies. Fusion 12 was raised against the CB line. 86% of wells plated out had HAT resistant cell lines with 73% of them secreting antibodies. From these two fusions, 17 hybrid cell lines were grown up and cloned. The mcabs were given a numerical label consisting of the number of the fusion where their cell line was produced followed by a number given to an independent cell line derived in that fusion eg 12.1 was the first line obtained from fusion 12. IFA (2.5.4) was used to screen the hybridoma culture supernatants.

The slight variation in fusion protocols (2.5.2) did not make any difference to the specificity of the resulting mcabs. P3-X63-NS/1 secretes a light chain immunoglobulin intracellularly (Kohler et al., 1976) but P3-X63-Ag8.653 does not secrete any immunoglobulin (Kearney et al., 1979). Both cell lines fused with high efficiency.

96 wells were plated out with 0.5ml aliquots in
fusion 3 while in fusion 12, 480 wells were plated out with 0.1ml aliquots. The main advantage of plating out 0.1ml amounts was that nearly all the antibody secreting wells contained only one colony. However, all cell lines were cloned by limiting dilution (2.5.5) at least once.

3.2.2 Immunoglobulin Subclass of Antibodies

Using the method of double diffusion (2.5.8) the immunoglobulin subclasses of the antibodies produced were determined. 12.4 was IgG2b, 12.8 was IgG1. All other antibodies were IgG2a.

3.2.3 Patterns of IFA

Several different IFA patterns were observed with mcabs to *P. chabaudi*. Several antibodies reacted with the surface of all red blood cells, both parasitised and non-parasitised, and were discarded. One antibody reacted with the membranes of red blood cells infected with parasites. Some antibodies reacted with only some stages of the erythrocytic cycle of the parasite while others reacted with all. Within the parasite specific antibodies 6 different fluorescent patterns could be distinguished. The different fluorescent patterns are shown in figure 4.

3.3 Antigen Characterisation

The proteins with which the mcabs reacted were identified by immunoprecipitation (2.6.2) and Western blotting (2.6.4). The results are shown in figures 5, 6
Figure 4. Different patterns observed in IFA of late stage trophozoites and schizonts.

Type 1 mcabs produced very bright fluorescence with an irregular staining pattern. These antibodies reacted with late trophozoites and schizonts.

Type 2 mcab produced a bright staining pattern similar to that of type 1. In addition, many other apparently smaller parasites also reacted with this antibody. The antibody reacted with all stages of the parasite cycle.

Type 3 mcab produced fluorescence which appeared as small clusters of bright dots. This antibody reacted with late trophozoites and schizonts.

Type 4 fluorescence appeared as circles of bright dots, this mcab reacted with all stages of the parasite.

Type 5 fluorescence was a collection of bright spots with an associated dull diffuse type of fluorescence. This mcab reacted with all stages of the parasite.

Type 6 pattern was a hazy fluorescence with a few very bright spots. This mcab reacted throughout the erythrocytic cycle.

Mcab of type 7 reacted with the membrane of infected erythrocytes.
and 7. The characteristics of the antigens distinguished by their IFA patterns are as follows.

Type 1: The reactivity shown by mcabs 3.7, 3.8, 3.12, 12.3, 12.4, 12.6, 12.11, 12.12, 12.13, 12.15, 12.17 and the mcabs of D.Boyle and C.Hamers-Casterman. The IFA pattern was very bright fluorescent staining over an irregular shape. The antibodies reacted only with late stage trophozoites and schizonts. Figure 5 (Sect.24.2.2) shows an autoradiograph of metabolically labelled CB parasite extracts immunoprecipitated with mcabs 12.3, 12.4, 12.11, 12.12, 12.13, 12.15, 12.17 and B8. The major band on the gel is a protein of Mr 250 000. Figure 6 shows a Western blot of AS and CB parasite extracts with 3.7, 3.8 and 3.12. Mcabs 3.7 and 3.12 only react with AS while 3.8 reacts with AS and CB. The antigen of AS appears slightly larger than that of CB. H3 reacts only with the CB 250kDa protein in the Western blot shown on figure 7 and not with the protein of AS.

Type 2: Reactivity shown by mcab 12.16. The IFA pattern was similar to that of type 1 but also showed reaction with all stages of the erythrocytic cycle as small bright irregular staining. Figure 5 shows that 12.16 reacted with the 250kDa protein recognised by Type 1 mcabs.

Type 3: Mcab 12.10 had an IFA pattern of clusters of brightly staining dots. This antibody reacted with the late stages of the parasite in the erythrocytic cycle. The Western blot of figure 7, track e, shows 12.10 reacting with a protein of Mr 210 000 from AS and CB.
Figure 5. Autoradiograph of metabolically labelled CB parasites immunoprecipitated by mcabs and run on 7.5% SDS-PAGE. Numbers on left are molecular weight markers $\times 10^3$. a, serum from mouse used in fusion; b, 12.3; c, 12.4; d, 12.11; e, 12.12; f, 12.13; g, 12.15; h, 12.16; i, 12.17; j, BB.

Parasite extracts were also immunoprecipitated with normal mouse serum. A series of very low intensity bands similar to the minor bands on the mcab tracks was observed (results not shown).
Figure 6. Parasite extracts from AS and CB run on 7.5% SDS-PAGE and blotted with mcabs. Numbers on left are molecular weight markers ×10³. a, 3.7; b, 3.8; c, 3.12.

Blots probed with normal mouse serum identified bands of similar molecular weight to the minor bands observed with the mcabs (results not shown).
Figure 7. Parasite extracts from AS and CB run on 10% SDS-PAGE and blotted with mcabs. Numbers on left are molecular weight markers x10^3. a, 12.18; b, 12.14; c, 12.8; d, 12.1; e, 12.10; f, H3.

When normal mouse serum was used to blot a track a number of bands reacted. The pattern was similar to those minor bands observed with the mcabs (results not shown).
Type 4: Mcab 12.8 reaction on IFA showed circles of brightly staining dots. This mcab reacted throughout the erythrocytic cycle. Figure 7 track c shows 12.8 reacting with a protein of Mr 90,000 from CB only on the Western blot.

Type 5: On IFA 12.14 showed a reactivity pattern of a collection of spots with duller diffuse fluorescence associated with the spots. This mcab reacted with all of the erythrocytic stages. Figure 7 track b shows 12.14 reacting with a protein of Mr 110,000 from both AS and CB, although the AS protein appears slightly smaller than that of CB.

Type 6: Mcab 12.18's reaction in IFA appears as a dull fluorescence with a few brightly staining spots. This pattern was seen throughout the cycle. Figure 7, track a, shows the reaction of 12.18 with a protein of Mr 42,000 from AS and CB with the protein of AS appearing slightly larger than the CB protein.

Type 7: Mcab 12.1 reacted with the membranes of erythrocytes infected with all erythrocytic stages of the parasite. It reacts with a protein of Mr 55,000 on AS and CB shown on figure 7, track d.

The results are summarised in Table 2.

3.4 Antigenic Diversity of \textit{P.chabaudi}

In this section the extent of antigenic diversity between a number of cloned isolates of \textit{P.chabaudi} was examined using immunofluorescence, immunoprecipitation
<table>
<thead>
<tr>
<th>Mcab</th>
<th>Fluorescence pattern</th>
<th>Stage specificity</th>
<th>Mr of antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7, 3.8, 3.12</td>
<td>1</td>
<td>late trophozoites and schizonts</td>
<td>250kDa</td>
</tr>
<tr>
<td>12.3, 12.4,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.6, 12.11,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.12, 12.13,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.15, 12.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.16</td>
<td>2</td>
<td>all stages</td>
<td>250kDa</td>
</tr>
<tr>
<td>12.10</td>
<td>3</td>
<td>late trophozoites and schizonts</td>
<td>210kDa</td>
</tr>
<tr>
<td>12.8</td>
<td>4</td>
<td>all stages</td>
<td>90kDa</td>
</tr>
<tr>
<td>12.14</td>
<td>5</td>
<td>all stages</td>
<td>110kDa</td>
</tr>
<tr>
<td>12.18</td>
<td>6</td>
<td>all stages</td>
<td>42kDa</td>
</tr>
<tr>
<td>12.1</td>
<td>7</td>
<td>membrane of infected rbc</td>
<td>55kDa</td>
</tr>
</tbody>
</table>
and Western blotting.

3.4.1 Antigenic Diversity Identified by IFA

36 mcabs were available to screen 12 cloned isolates. At least two separate antigen preparations were made of each clone and they were tested several times. Both culture supernatants and ascitic fluids of mcabs produced in this study were used. Culture fluids were used undiluted and gave clear positive or negative reactions. Most ascitic fluids gave general fluorescence when used undiluted or 1/100 dilutions similar to that shown by normal mouse serum. Serial doubling dilutions of ascitic fluids from 1/1000 were tested on the isolates and the last dilution showing bright fluorescence was noted as the end point. At 1/1000 dilutions or less, positive reactions could be clearly differentiated from negative reactions. Serial dilutions of mcabs from D.Boyle were also tested. C.Hamers-Casterman provided serum containing mcabs and they were used at 1/500 dilution. Some of the results of the titrations are shown in table 3.

Differences in the brightness of fluorescence were observed. These were caused by several factors. Some mcabs gave much brighter staining than others. The group of mcabs reacting with the 250kDa antigen gave the brightest fluorescence. Mid stage trophozoites showed a reaction with the mcabs reacting with the 250kDa antigen but the brightness of the reaction increased as the parasites matured to schizonts. Thus antigen
Table 3. Titration of mcabs with AS and CB. Numbers indicate the reciprocal of the last dilution showing good fluorescence.

<table>
<thead>
<tr>
<th>Mcab</th>
<th>AS</th>
<th>CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>B8</td>
<td>4000</td>
<td>4000</td>
</tr>
<tr>
<td>12.15</td>
<td>64000</td>
<td>64000</td>
</tr>
<tr>
<td>B1</td>
<td>32000</td>
<td>32000</td>
</tr>
<tr>
<td>3.8</td>
<td>8000</td>
<td>8000</td>
</tr>
<tr>
<td>12.12</td>
<td>32000</td>
<td>32000</td>
</tr>
<tr>
<td>B2</td>
<td>64000</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>16000</td>
<td>-</td>
</tr>
<tr>
<td>12.3</td>
<td>-</td>
<td>32000</td>
</tr>
<tr>
<td>12.13</td>
<td>-</td>
<td>64000</td>
</tr>
<tr>
<td>12.16</td>
<td>64000</td>
<td>64000</td>
</tr>
<tr>
<td>12.18</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td>12.8</td>
<td>-</td>
<td>64000</td>
</tr>
<tr>
<td>12.14</td>
<td>8000</td>
<td>8000</td>
</tr>
<tr>
<td>12.10</td>
<td>4000</td>
<td>4000</td>
</tr>
<tr>
<td>12.1</td>
<td>&gt;128000</td>
<td>&gt;128000</td>
</tr>
</tbody>
</table>
Table 4. Antigenic Diversity of *P. chabaudi* Identified by IFA.

<table>
<thead>
<tr>
<th>Mcab</th>
<th>CAR</th>
<th>Congo</th>
<th>CAR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mr of Antigen*</td>
<td>AS</td>
<td>CB</td>
</tr>
<tr>
<td>B8, 12.4, 12.11, 12.15, 12.17</td>
<td>250,000</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12.12, H5</td>
<td>250,000</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.8</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B16</td>
<td>250,000</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B12</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B2, B4, B14, B15, B18, B20</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.7</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.12</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12.6</td>
<td>250,000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12.13</td>
<td>250,000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3.12, 3.7</td>
<td>250,000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12.15, 12.16</td>
<td>250,000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12.14, 12.15, 12.16</td>
<td>250,000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12.17, 12.18</td>
<td>250,000</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = positive
- = negative
Ca = Cameroun
* Mr varies between isolates, see section 3.4.2.
immunoprecipitate the 250kDa protein of AS, CB and DS. The precipitates were then digested with staphylococcal V8 protease. The molecular weight of the antigen recognised by the mcab was 5kDa less in CB than AS. The peptide maps obtained from the digest also showed major differences.

Figure 8 shows size polymorphisms of the 250kDa protein in seven cloned isolates of \textit{P.chabaudi}. The isolates were labelled and extracted by two methods (2.4.2). AS, AJ, DS and EF were metabolically labelled before the erythrocytes were lysed and parasite proteins extracted. This resulted in a doublet being precipitated by mcab 12.15. The other isolates were freed from host cells by saponin lysis before labelling. The precipitates had a reduced lower band. This was the opposite to that found by Epstein et al (1981). They had mcabs to a protein of \textit{P.knowlesi} synthesised in trophozoites and schizonts. Parasite proteins were metabolically labelled and extracted prior to immunoprecipitation by the mcab. If the proteins were extracted in Triton X-100, a protein of Mr 250,000 was precipitated. However, if the labeled schizont infected cells were saponin lysed before Triton X-100 extraction, a second protein of Mr 240,000 was also precipitated. This suggested that the saponin might activate a protease or glycosidase, although all samples also contained protease inhibitors.

Apart from differences in sample preparation, the immunoprecipitation shows at least three sizes of the
Figure 8. Autoradiograph of seven isolates metabolically labelled and immunoprecipitated with mcab 12.15, run on 7.5% SDS-PAGE. Numbers on left are molecular weight markers x10^3.
protein identified by the mcab 12.15 in the clones. In AS, AJ and DS, a protein of similar size is precipitated. In EF, the protein appears slightly larger while in CB, DK and ER it is slightly smaller.

Figure 7 shows other antigens which also have differences in their molecular weights between AS and CB. Mcab 12.18 reacts with a protein of Mr 42000. In AS the protein is slightly larger than in CB. The antigen of Mr 110000 recognised by mcab 12.14 is slightly smaller in AS than in CB.

3.5 Genetic Study of *P.chabaudi* Antigens

To study the genetic basis of the antigenic diversity seen in *P.chabaudi* isolates, three crosses (2.3) were made between antigenically distinct clones. Two crosses were made between clones AS and CB which in addition to the antigenic markers, differed in their response to pyrimethamine and in electrophoretic forms of three enzymes 6-phosphogluconate dehydrogenase (PGD), lactate dehydrogenase (LDH) and adenosine deaminase (ADA). The uncloned progeny of these crosses as well as 25 clones derived from the progeny were examined for their reactions with mcabs. The same clones were also examined for enzyme and drug-response characters (work of F. A. Lainson and D. Walliker). A third cross was made between clones AS and ER (work of A. Sharkey). Progeny clones of this cross were examined only for their antigen characters.
3.5.1 IFA Studies on Uncloned Cross Progeny

Antigen preparations were made of uncloned progeny from the crosses and these were used in a double IFA test (2.6.1). Figure 9 shows examples of the results.

9a and 9b show the progeny from an AS/CB cross. The progeny were stained with mcab 12.12 (isotype IgG2a) and mcab H3 (isotype IgG2b), followed by fluorescein-(FITC) conjugate specific for IgG2a and rhodamine-(TRITC) conjugate specific for IgG2b. 12.12 reacts with both AS and CB, while H3 reacts only with CB (Table 4). Thus in the double IFA, parasites stained only with FITC have AS-type antigen (1) and those stained with both reagents have CB type antigens (2). Table 5 shows the proportion of each type of antigen in a sample of the uncloned progeny. Approximately equal numbers of each type of progeny were observed. No parasites stained only with rhodamine were seen.

9c and 9d show the progeny from the AS/ER cross. These progeny were stained with mcab B14 (isotype IgG2a) and mcab H3 (isotype IgG2b). This was followed by fluorescein-conjugate specific for IgG2a and rhodamine-conjugate specific for IgG2b. Mcab B14 reacts only with AS and H3 reacts only with ER. 9c shows parasites with AS-type antigen (3) while 9d shows those with ER-type antigen (4). No parasites were found which reacted with both fluorescein and rhodamine. Table 5 shows the number of each type of progeny in a sample from this study. Two antigen preparations of AS/ER progeny were made, one being after treatment with 20mg/kg pyrimethamine for 3
Figure 9. Examples of double label IFA on uncloned cross progeny.

(a) and (b) are the uncloned progeny from the AS/CE cross. They were reacted with mcabs 12.12 (IgG2a) and H3 (IgG2b). (c) and (d) are the uncloned progeny from the AS/ER cross. They were reacted with mcabs B14 (IgG2a) and H3 (IgG2b).

Parasites were stained with antiserum to mouse IgG2a conjugated to FITC and antiserum to mouse IgG2b conjugated to TRITC.

(a) and (c) were photographed under a filter which identifies parasites stained with fluorescein. (b) and (d) were photographed under a filter which identifies parasites stained with rhodamine.

1 denotes AS/CE parasites stained only with fluorescein. 2 denotes AS/CE parasites stained with fluorescein and rhodamine.

3 denotes AS/ER parasites which stain only with fluorescein and 4 denotes AS/ER parasites stained only with rhodamine.
Table 5. Proportions of antigen types in uncloned cross progeny. AS/ER(pyr) are the cross progeny treated with 20mg/kg for 3 days.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Total Parasites Counted</th>
<th>Total FITC labelled</th>
<th>Total TRITC labelled</th>
<th>Total labelled with FITC &amp; TRITC</th>
<th>Ratio AS:CB/ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS/CB</td>
<td>245</td>
<td>0</td>
<td>113</td>
<td>132</td>
<td>54:46</td>
</tr>
<tr>
<td>AS/ER</td>
<td>134</td>
<td>13</td>
<td>121</td>
<td>0</td>
<td>10:90</td>
</tr>
<tr>
<td>AS/ER(pyr)</td>
<td>202</td>
<td>41</td>
<td>161</td>
<td>0</td>
<td>20:80</td>
</tr>
</tbody>
</table>
days and the other was without any drug. There is a great excess of ER-type progeny in both samples, with the drug treatment increasing the proportion of AS-type progeny slightly.

3.5.2 IFA Profiles of Cloned Cross Progeny

A selection of AS/CB clones shown to be recombinant for isoenzymes and pyrimethamine response were screened with the mcabs reacting with the 250kDa protein and with mcab 12.8 which reacts with a separate 90kDa protein. The results are shown in table 6.

The IFA profile of each AS/CB cross progeny clone with mcabs recognising the 250kDa protein was like that of either the AS or the CB parent. No parasites reacting with these mcabs for both parents were seen. Progeny showing recombinant combinations of 250kDa reactivity and reaction with mcab 12.8 were found. Recombinant combinations of antigens with isoenzymes and pyrimethamine response were also found.

The IFA profiles for the cloned progeny of the AS/ER cross are shown in table 7. As in the AS/CB crosses, the IFA profiles for the 250kDa protein were either AS-type or ER-type.

3.5.3 Peptide Mapping of Cloned Cross Progeny

To study the cross progeny further, the V8 protease from Staphylococcus aureus was used to digest the immunoprecipitates (2.6.3). The results are shown in figures 10 and 11.
Table 6. Characteristics of cloned progeny from AS/CB cross.

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<th>Monoclonal Antibodies</th>
<th>Enzymes</th>
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<td>AS/CB 3</td>
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| 56                    | -       | 56  | 4   | n.t.
| 57                    | -       | 57  | 4   | n.t.
| 64                    | -       | 64  | 3   | n.t.

Reaction with mcabs, + is positive, - is negative.
LDH - Lactate dehydrogenase, numbers are electrophoretic mobilities.
ADA - Adenosine deaminase, numbers are electrophoretic mobilities.
PYR - Pyrimethamine response, R is resistant, S is sensitive.
n.t. - not tested
Table 7. IFA profile of cloned progeny from AS/ER cross.

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<th>B1 3.8</th>
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<th>B3 3.7</th>
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Figure 10 shows AS, CB and the progeny of their cross. Due to loading unequal counts on the gels, they were exposed two times and the figure is a compilation from the two exposures. Differences were seen in the peptide fragments of the two parents after digestion by the protease. The peptide maps of the cross progeny appeared largely similar to the maps obtained with either AS or CB. However, AS/CB 8 shows some unique minor bands, indicated by *.

Figure 11 shows AS/ER cross progeny and the parents of the cross. The peptide maps of the cross progeny after digestion were exactly the same as that of AS or ER.

The similarity of a peptide map of the cross progeny to a parental peptide pattern was related to the similarity of the IFA profiles shown in tables 6 and 7.
Figure 10. Metabolically labelled AS, CB and cloned AS/CB progeny immunoprecipitated with mcab 12.15 and digested with protease. Samples were run on 12.5% SDS-PAGE. Numbers on left are molecular weight markers x10^3.
Before protease, 0; after 1min, 1; after 5mins, 5; after 30mins, 30. Tracks are in the same order on all gels. a, AS; b, AS/CB 10; c, AS/CB 15; d, AS/CB 34; e, AS/CB 1; f, AS/CB 8; g, CB.
Figure 11. Metabolically labelled AS, ER and cloned AS/ER progeny immunoprecipitated with mcab 12.15 and digested with protease. Samples were run on 12.5% SDS-PAGE. Numbers on left are molecular weight markers x10^3.
Before protease, 0; after 1min, 1; after 5mins, 5; after 30mins, 30. Tracks are in the same order in all gels. a, AS; b, AS/ER 7; c, AS/ER 19; d, AS/ER 8; e, AS/ER 20; f, AS/ER 22; g, ER.
4 DISCUSSION

The topics discussed in this chapter have been divided into sections and follow a similar order to the results chapter. The first section deals with the production of mcabs including the choice of myeloma, feeder cells and also discusses the choice of B-cells. In the second section the characterisation of the antigens with which the mcabs react is discussed, in relation to possible equivalent antigens in other species of *Plasmodium*. Section three surveys antigens which have been identified by mcabs and implicated in the protective response of the host. The fourth section discusses diversity of some of the antigens mentioned in section three which induce a protective response. The fifth section discusses the genetics of the antigens of malaria parasites. The final section includes general conclusions with regard to this study.

4.1 Production of Monoclonal Antibodies

Since the first generation of mcabs by Kohler & Milstein (1975), the technique has been used widely with many subsequent modifications. The basis of the technique is the fusion of spleen cells from immunised mice with murine plasmacytoma cells. Although the original fusing agent was Sendai virus, many groups now use polyethylene glycol (PEG). This procedure results in a mixture of fused and unfused cells. It is necessary to eliminate unfused tumour cells and tumour-tumour hybrids leaving the tumour-spleen cell hybrid which will secrete
antibody of the desired specificity. Unfused spleen cells cannot survive in culture so fusion with the tumour cell line confers immortality on the antibody secreting cell.

Selective elimination of tumour cells is achieved by using a cell line deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT) which is responsible for the incorporation of hypoxanthine into DNA. Fused cells are grown in medium containing hypoxanthine, aminopterin and thymidine (HAT) (Littlefield, 1964). Aminopterin blocks "de novo" synthesis of DNA, and to grow in this medium cells must make DNA via the "salvage" pathway using hypoxanthine and thymidine. The only cells which will grow in HAT medium are the hybrids formed when the tumour cells fuse to the spleen cells. The spleen cell provides the HPRT enzyme and the tumour cell provides immortality.

The plasmacytoma cell line which Kohler and Milstein used in their original fusions was P3-X63-Ag8 which had the disadvantage that it secreted immunoglobulin itself and so hybrid cells secreted myeloma antibody and desired antibody. The mixture of antibodies may lower the titre of the desired antibody. The myeloma cell lines used in this project were derived from this cell line. P3-X63-Ag8-NS1 was selected by Kohler et al (1976). Although it synthesises a light chain antibody, it is not secreted. P3-X63-Ag8.653 does not synthesis any immunoglobulin and was developed by Kearney et al (1979).
In initiating new culture lines, in plating out fused cells, and growing cells at very low densities it is advisable to add extra cells to provide supplementary growth factors and increase cell density. In this study three different types of feeder cells were used. Normal spleen cell were used when plating out newly fused cells, and thymocytes and peritoneal exudate cells were used for cloning. No controlled comparisons between the different cell types were carried out but all three fulfilled their function. The main disadvantage with using thymocytes is that the donor mice have to be less than six weeks old because the thymus decreases in size after that age. Spleen cells contain a large number of red blood cells which can hinder examination of the cells through the microscope. Care must be taken when obtaining peritoneal exudate cells not to pierce the intestine. Samples of cells should always be incubated for 24hrs before use to ensure they are not contaminated. Other cell lines can be used, such as rat thymocytes, which give a much higher yield than mouse thymocytes. Another alternative is conditioned medium, which is medium from cultures which contains growth factors but has been centrifuged to remove the cells.

Apart from the choice of myeloma cell line there is also the choice of cells with which to fuse them. The majority of mcabs to malaria have been made using spleen cells from mice immunised with parasites or their extracts. Deans et al (1982) immunised a rat with *P.knowlesi* and fused the rat spleen cells to produce mcabs.
Stanley and Reese (1985) took a spleen from a monkey which was immune to *P. falciparum* and fused it with the mouse myeloma cell line P3-X63-Ag8.653. The monkey-mouse system had an efficiency equal to that of the mouse-mouse system. Their reason for using the monkey spleen was that epitopes seen by a primate should be more relevant to the production of human immunity than structures seen by a rodent, which cannot experience the disease.

Several groups have recently produced human mcabs. Schmidt-Ullrich *et al* (1986) isolated peripheral blood lymphocytes (PBL) from Gambian adults who were immune to *P. falciparum* malaria. The PBLs were cultured for seven days in the presence of pokeweed mitogen and then fused with PEG to a human lymphoblastoid line. 4 mcabs were found which could inhibit parasite growth *in vitro*. 3 of these mcabs precipitated a parasite protein of Mr 195 000. A different method for producing human mcabs was used by Brown *et al* (1986). They isolated mononuclear cells from Gambian adults who had low grade *P. falciparum* infections. The mononuclear cells were infected with Epstein-Barr virus. Culture supernatants were found to be able to inhibit *in vitro* growth of *P. falciparum*. Unfortunately there were no lasting cultures due to contamination or the surviving cells stopped producing anti-malarial antibodies.
4.2 Antigen Identification

Before the advent of mcabs, immune serum was used to study antigens of malaria. These sera showed a great deal of cross reactivity between species, for example El-Nahal (1967) was unable to distinguish \textit{P.yoelii} from \textit{P.berghei} or \textit{P.vincenki} from \textit{P.chabaudi} by IFA using antisera raised against each species. Human serum raised against \textit{P.falciparum} or \textit{P.vivax} reacted with both species on IFA (Diggs and Sadun, 1965).

Immune serum used in IFA reactions gives fluorescence on all parasites. The use of mcabs in IFA shows different patterns and can also identify stage specific antigens. In this study 11 mcabs were produced which gave a similar fluorescence pattern with late stage parasites. This was the same pattern obtained by Boyle \textit{et al} (1982) for their mcabs, some of which inhibited parasite growth. A similar pattern was also found by Holder \textit{et al} (1981) for a mcab raised against \textit{P.yoelii}. When the antigen with which this mcab reacted was purified and used to immunise mice it was found to induce a protective response. Hall \textit{et al} (1984a) also used a mcab, giving a similar IFA pattern with \textit{P.falciparum}, to purify the protein. They immunised monkeys with it and found that it induced some protection.

Using immunoprecipitation and Western blotting, the \textit{P.chabaudi} mcabs were found to react with a protein of Mr 250000. Other minor bands were also seen. These are probably the result of protein processing which has been
characterised in *P. falciparum* (Holder and Freeman, 1982) and *P. yoelii* (Holder and Freeman, 1984). The proteins were metabolically labelled when most of the parasites were at the late trophozoite stage but because the infection is not precisely synchronous, some of the parasites would be undergoing schizogony. Some processing would be taking place despite the presence of protease inhibitors.

The results of the Western blots (figure 7) do not appear as clean as the immunoprecipitations (figure 5). Many bands were common to all mcabs and were thought to be non-specific. The band with which each mcab specifically reacted is arrowed next to the track (figure 7). The specific bands generally developed first with the others appearing after a few minutes.

The other mcabs produced in this study were selected because they produced different fluorescent patterns when the supernatants from their primary wells were tested. The results from the Western blotting showed that they all reacted with different antigens. The exception to this was 12.16 which also reacted with the 250kDa protein. This mcab reacted throughout the erythrocytic cycle so probably reacts with an epitope which is on the part of the protein conserved on reinvasion. Hall et al (1984b) showed that the equivalent protein of *P. falciparum* was synthesised by trophozoites and schizonts. An epitope of this protein identified by a mcab was lost during release and reinvasion of merozoites. Another mcab recognised a
different epitope on this protein, and this was found to survive reinvasion.

The antigens with which the mcabs react can be characterised by size, IFA pattern and stage specificity, but their function is unknown at present. Oko et al (1984) studied a P.yoelii mcab with a dotty fluorescent pattern by immunoelectron microscopy. They found that the mcab reacted with the rhoptries. A similar punctate double dot fluorescence was also observed by two groups studying P.falciparum (Howard et al., 1984 and Schofield et al., 1985). They found their mcabs precipitated parasite proteins of Mr 82000 and 80000 respectively. The same antigen is probably involved in these two studies, the difference in molecular weights probably being due to experimental conditions in the two laboratories. This differs considerably from the rhoptry protein of P.yoelii which has an Mr of 235 000. One of the mcabs produced in this study, which produces dotty fluorescence, could be reacting with the P.chabaudi rhoptries. However it is not possible to determine this by IFA, and immunoelectron microscopy would be needed to study the antigen’s cellular location.

The use of fixed preparations in IFA exposes many more antigens than are exposed on the intact living parasite. Mcab 12.1 appears to react with the surface of infected erythrocytes throughout the cycle, although its reactivity with intact, unfixed parasitised cells needs to be studied to confirm this. The reaction of this mcab
through the cycle indicates it is unlikely to be related to an antigen present in the membrane of ring infected erythrocytes of *P.chabaudi* described by Gabriel *et al.*, (1986).

4.3 Protective Antigens Identified By Mcabs

Mcabs can be used to identify antigens which are involved in inducing a protective response by the host in two ways. The first is to study the effect of the antibodies on the course of infection. The second is to use the mcab to purify the antigen which is then used to immunise an animal. The effect of immunization is measured by challenging the animal with a dose of viable parasites. In both cases results are compared to controls.

Freeman *et al.* (1980) tested serum from mice bearing hybridoma tumours for their ability to control a virulent line of *P.yoelii*. They found some which reduced the parasitaemia to the levels of a nonvirulent line. The mcabs were used to purify the antigens with which they reacted (Holder and Freeman, 1981). A purified antigen, Mr 235000, was found to protect mice against infection. Another mcab, which had not been effective when given to mice directly, was used to purify the protein with which it reacted. When the purified protein, Mr 230000, was used to immunize mice, they survived challenge. Playfair *et al.* (1985) correlated the protection obtained, with T-cell mediated immunity.
Of eleven mcabs reacting with a 250kDa protein of *P. chabaudi*, Boyle *et al* (1982) tested four and found two which could delay the rise in parasitaemia and reduce its peak. When given together, the effect was slightly greater indicating a synergistic effect. These mcabs, B1 and B3, have been shown in this study to react with different epitopes. If the protein of *P. chabaudi* undergoes processing to smaller fragments on the surface of the merozoite like its counterpart in *P. yoelii* (Holder and Freeman, 1984), then these epitopes involved in inducing some immunity, might be on the merozoite surface. They would then be exposed to antibody attack when the merozoites are released from the red cells.

Mcab B8 which is common to all isolates tested was used to purify the 250kDa protein from AS parasites by Brown *et al* (1985). The purified protein was used to immunize mice. The mice were challenged with AS or CB parasites. Challenge with CB parasites showed very little difference between immunized and control groups. After AS challenge the parasitaemia was delayed and reduced in peak.

Testing the effect of mcabs *in vivo* is relatively easy with rodent malarias. However, because of the shortage of primates, experiments with simian and human malarias have mainly been done *in vitro*. Epstein *et al* (1981) produced mcabs against a 250kDa antigen on the surface of *P. knowlesi* merozoites. Free merozoites and mature schizonts of *P. knowlesi* were incubated for two hours with the mcabs. Signs of
merozoite agglutination were looked for. The mcabs were also incubated with mature schizonts and uninfected erythrocytes. The mcabs which caused the greatest amount of merozoite agglutination also caused the most inhibition of invasion. With mcabs to a different P. knowlesi antigen, Mr 140kDa, Miller et al (1984) incubated mature schizonts and free merozoites prior to culture with ascitic fluid. They found that the mcabs only affected invasion when incubated with mature schizonts. This effect was caused by agglutinating merozoites as they were released from erythrocytes, not by blocking the merozoite receptors for erythrocytes.

A mcab raised against P. falciparum was used to purify the protein with which it reacted by Hall et al (1984a). The mcab reacted against an antigen, Mr 190kDa, synthesised late in the erythrocytic cycle of P. falciparum. The purified protein was then used to immunize three monkeys. Two of the monkeys were able to recover from what is normally a lethal challenge.

Culture fluids of synchronous P. falciparum cultures were supplemented with 10% ascitic fluid containing mcabs by Perrin et al (1981b). The level of parasitaemia was measured by counting Giemsa stained smears. The incorporation of 3H hypoxanthine over a 14hr period was also measured. They found the rate of incorporation of radioactivity paralleled the level of parasitaemia. The mcabs showed a range of growth inhibition. One mcab inhibited growth 100% while others were less effective. Schofield et al (1982) found
growth inhibition levels of up to 40% when they added mcab to synchronous cultures of *P. falciparum* at the schizont stage. They assessed parasitaemias by counting parasites and also by flow cytofluorometry with a dye which stains DNA. There was no inhibition of parasites which were negative with the mcab on IFA. A second batch of mcabs made by Schofield et al (1985) included two which could inhibit growth *in vitro*. The inhibition was concentration-dependent with 100% inhibition obtained with 40ug/ml for one mcab and 250ug/ml for the other.

The human mcabs made by Brown *et al* (1986) and Schmidt-Ullrich *et al* (1986) both included mcabs which could inhibit *P. falciparum* growth *in vitro*.

The above are tests to study mcabs affecting the erythrocytic stages of malaria. Mcabs have also been made against sporozoites and gametes. Yoshida *et al* (1980) made mcabs against *P. berghei* sporozoites. The sporozoites were incubated with antibody for 30mins before being injected into mice. The mice did not develop parasitaemias although control mice developed lethal infections. Cochrane *et al* (1982) made mcabs against sporozoites of *P. knowlesi*. When sporozoites were incubated with mcab before injection into rhesus monkeys their infectivity was also abolished. Mcabs to the sporozoites of the human malarials *P. falciparum* and *P. vivax* were made by Nardin *et al* (1982). These mcabs were incubated with sporozoites prior to injection
into splenectomised chimpanzees. The mcabs were able to delay the onset of patency of infection.

The mcabs to *P.berghei* sporozoites (Yoshida *et al*, 1980) which were inhibitory *in vivo* were also shown to be inhibitory *in vitro*. This was shown by their ability to block entry of the sporozoites into cultured embryonic lung cells (Hollingdale *et al*, 1982). A similar *in vitro* correlation of the *in vivo* inhibitory effect of mcabs to *P.falciparum* and *P.vivax* (Nardin *et al*, 1982) was shown by Hollingdale *et al* (1984). The sporozoites were prevented from invading cultured human hepatoma cells.

This *in vitro* test was also used to study the inhibitory effect of mouse and rabbit sera raised against a synthetic peptide (Zavala *et al*, 1985 and Ballou *et al*, 1985). The peptide encoded the immunodominant epitope of the surface protein of *P.falciparum* sporozoites.

Rener *et al* (1983) mixed a suspension of *P.falciparum* gametes, erythrocytes and human serum with anti-gamete mcabs and allowed mosquitoes to feed on the mixture through membranes. 8 to 9 days after the feed, the mosquitoes were dissected and their midguts examined for oocysts. Two mcabs acted synergistically to suppress infectivity. Harte *et al* (1985) produced mcabs against the gametes of *P.yoelii*. They found antibodies which could agglutinate microgametes *in vitro*. They also infected mice and three days later injected the mice with mcab prior to allowing mosquitoes
to feed on them. One week after feeding, the mosquitoes were dissected and their midguts examined for oocysts. These authors, like Rener et al (1983), found two mcabs which reacted synergistically to block transmission of the parasites to the mosquitoes.

It is difficult to extrapolate the results from in vitro inhibition tests and the results with animal models to that of human malarias in endemic areas. Naturally occurring infections of P.falciparum have been shown to consist of mixtures of parasites (Rosario, 1981 and Fenton et al., 1985). Although no deliberate selection was employed, Fenton et al (1985) showed that the frequencies of the different parasite genotypes in the mixture altered over a period of culture. In the laboratory it is advisable to use cloned material so that the effects on the parasites are clearly seen instead of selecting a sub-population. The identification of antigens as potential vaccines is the beginning of a number of phases, including preclinical studies and vaccine trials, which must be passed before a vaccine will be in use (WHO, 1986).

4.4 Antigenic Diversity Identified By Mcabs

The introduction of a vaccine will expose parasites to a selection pressure which could lead to the selection of antigen types against which the vaccine is not effective, or to the appearance of new antigenic types. It is thus important to examine the extent of antigenic diversity within the population before
Vaccines are used. Very few of the studies to identify antigens which induce a protective response have involved tests of antibodies with different isolates to find out if they react with variable proteins.

Using a battery of mcabs raised against two isolates of *P. falciparum*, K1 and PB1, from Thailand, McBride et al. (1982) screened 27 isolates from different parts of the world. They found considerable serological diversity. The isolates in this study had been grown in laboratory cultures for some time. However, antigenic diversity was also observed when freshly isolated samples of parasites were screened (McBride et al., 1984, Knowles et al., 1984 & Howard et al., 1986). In the study of Knowles et al. (1984), antigenic diversity of isolates in PNG was examined. They found that the population of *P. falciparum* in the region of Madang to be antigenically heterogeneous. Most of the mcabs in these studies were against a 190kDa antigen (Hall et al., 1983). This protein was shown to induce some protection against *P. falciparum* in monkeys (Hall et al., 1984a).

An extended panel of mcabs to this 190kDa antigen were used by McBride et al. (1985) to further characterise the antigen. Some mcabs reacted with every isolate tested showing that the 190kDa antigens of all the isolates are related. Other mcabs recognised restricted determinants. Using the mcabs against the restricted determinants, isolates could be characterised into one of seven serotypes. These serotypes were
thought possibly to identify alleles of the gene for this protein. The DNA sequences of this gene from a few isolates were compared and showed areas of extensive homology and areas with low homology (Mackay et al., 1985, Tanabe et al., 1986). However it is not known which regions of the gene sequence encode the epitopes recognised by the mcabs.

In this study 12 cloned isolates of *P.chabaudi* were used to screen for antigenic diversity. 8 of the isolates came from the Central African Republic, 6 of them having been isolated in 1969. 30 mcabs reacted with the 250kDa protein. These could be divided into 14 groups on the basis of common reactivity. Each isolate could be distinguished by its reaction profile with the battery of mcabs including the isolates which had been collected at the same time. If these serotypes are identifying the different alleles of the gene, there are at least twelve alleles in the *P.chabaudi* population. There did not appear to be any relationship between the origin of the isolates and the number of reactive groups they had in common. 5 of the mcabs reacting with this protein reacted with all the isolates. 4 mcabs, 12.18, 12.14, 12.10 and 12.1 which reacted with different proteins also reacted with all the isolates in the sample. Only one of each reactivity was cloned. Several would be required to determine whether the whole protein or only part of it is constant in all isolates.

One of the mcabs made by Perrin et al. (1981b) which caused inhibition of growth of the erythrocytic
stages of *P. falciparum* was raised against the rodent malaria *P. berghei* so is likely to cross react widely.

Schofield *et al* (1982), raised mcabs to two isolates FCQ-27 and FCQ-30, from Papua New Guinea (PNG). Their mcabs showed diversity between 7 isolates from PNG, K1 from Thailand, NF7 from Ghana, W/L from Nigeria and NF36 from the Netherlands. No diversity was observed within the isolates from PNG. The reason may be due to the antigens which the mcabs in these studies react with. One of the mcabs was against an S-antigen (Saul *et al*, 1985). The DNA sequences of the S-antigens from FC27 and NF7 are homologous for most of their lengths and the nonhomologous regions are comprised entirely of tandem repeating units (Cowman *et al*, 1985). It is likely that the epitope with which the mcabs reacted was in the region containing tandem repeats.

Schofield *et al* (1985) also produced four mcabs against the rhoptry protein of *P. falciparum*. Two could inhibit growth of the PNG isolate the were raised against. One of these gave positive reactions on IFA with 106 primary isolates from PNG, Thailand and Brazil. The other inhibitory mcab gave positive reactions with 32 of 46 isolates from Brazil.

The mcabs made against *P. knowlesi* sporozoites by Cochrane *et al* (1982) were not tested against other lines of *P. knowlesi*. However, three were found to react with *P. cynomolgi* and one reacted with *P. falciparum*. The immunodominant epitope of the sporozoite circumsporozoite protein, encoded by a small
synthetic peptide, is present in strains of *P. falciparum* from all over the world (Zavala et al., 1985). The inhibitory mcabs to gametes of *P. falciparum* made by Rener et al. (1983) are also likely to react with many isolates. A mixture of isolates was used to immunize the mice before the fusion. A West African isolate, not in the mixture, was used to screen the primary hybridoma supernatants and a Brazilian isolate was used in the inhibition test.

Each mcab reacts with a specific site known as an epitope. It is made up of a small number of amino acids. These need not be next to each other on the primary protein structure but may be brought together in the tertiary structure. Epitopes of this type have been shown in lysozyme (Smith-Gill et al., 1982) and myoglobin (East et al., 1982). The few epitopes that have been identified in malaria proteins have been those where the amino acids are close to each other on the primary structure. Zavala et al. (1985) showed that the epitope on the circumsporozoite protein of *P. falciparum* is contained within a sequence of 4 amino acids repeated 3 times. This sequence has homology with part of the amino acid sequence of an antigen present in the blood stages of the parasite (Hope et al., 1985). A mcab reacts with this antigen in schizonts but also reacts with sporozoites indicating that this is the epitope recognised by the mcab. Hall et al. (1984b) showed that a polymorphic epitope of the 190kDa protein is encoded in the amino acid sequence by translating
mRNA in a cell free system. The product from this reacted with the mcab. Antibodies can also recognise sugar residues on glycoproteins of malaria (Ramasamy et al, 1986). This could be important when screening recombinant DNA libraries since bacterial systems do not produce proteins with the appropriate side chains.

4.5 Genetic Study of *P. chabaudi* Antigens

Diversity within populations usually arises as mutations which spread through the population when recombination occurs. Previous genetic studies with malaria had shown how markers such as isoenzymes and pyrimethamine response were inherited in a Mendelian fashion (Walliker et al, 1975). Pyrimethamine response was inherited independently of isoenzymes. Resistance to chloroquine could also be passed on to sensitive parasites during a cross (Padua, 1981).

The importance of genetic studies on antigens is that they identify unequivocally whether variant forms are due to allelic variation of a single gene, or whether genes at different loci are involved. Alleles of a single gene are identified by the fact that they segregate at meiosis. This can only be shown by crossing experiments. Genes which segregate with high frequency from one another are on separate chromosomes and are unlinked. Genes on the same chromosome are linked and segregate from one another at very low frequency.

The purpose of this work was to see if the different forms of the 250kDa protein segregated during
post-fusion meiosis thereby identifying them as alleles. The use of several markers on the one gene, also gave the possibility of identifying crossovers occurring within the gene. A crossover between mcab epitopes would lead to new combinations of mcab reactivity.

4.5.1. Allelic Variation of the 250kDa Antigen

A large amount of naturally occurring antigenic diversity identified by mcabs was found in the twelve isolates of *P. chabaudi* examined in this study. Each isolate could have a different reaction profile with the mcabs against the 250kDa antigen. These different forms were thought to be alleles of the one gene. In order to demonstrate if these different forms of the gene would segregate after pairing, a genetic cross was performed between the AS and GB isolates. Mcabs to the 250kDa protein identified 14 epitopes. 10 of these were different for their reactivity with AS and GB.

The profile of reactions of the AS/CB progeny clones with the anti-250kDa mcabs was like that of the AS or CB parent. No new combinations appeared indicating that the epitopes are very closely linked, probably within the one gene. The two forms of the 250kDa antigen seen in AS and CB can thus be concluded to be allelic.

Similar results were also found when progeny from a cross between AS and ER were examined. There were 11 mcabs identifying differences between AS and ER. The antigen profiles of the cross progeny clones were like one or other of the parents of the cross. The forms of
the 250kDa antigen on AS and ER are thus also allelic. Although all the combinations of isolates would have to be crossed to demonstrate that there are at least twelve alleles of this gene, the results so far indicate strongly that the serotyping of isolates recognises alleles.

The reactivity of all isolates and cross progeny with some mcabs showed that the alleles had some common regions. To study the structure of these allelic proteins further, a method for obtaining peptide fragments was used. Cleveland et al (1977) developed a method for eluting proteins from gels after electrophoresis, digesting the proteins with a protease and running the resulting peptide fragments on another gel. Newbold et al (1984), modified the technique to digest the protein immunoprecipitated by a mcab which reacts with a common epitope. The S. aureus V8 protease used in this work cleaves proteins at the carboxyl terminal side of aspartic and glutamic acid (Houmard and Drapeau, 1972).

The digestion of the proteins was stopped at various time points and the fragments obtained separated by SDS-PAGE and visualised by fluorography. The peptide maps obtained after the digest of the isolates AS, CB and ER showed some peptides with similar mobility but many bands were different. The antigen showed structural heterogeneity as well as heterogeneity of reactions with the mcabs. The peptide maps obtained from the AS/CB cloned progeny was similar to that obtained with AS or
The AS/ER progeny had maps like the AS or ER parent. The protein thus appeared to be inherited as a complete unit. The similarity of results obtained with a cross progeny to those for a parent was the same for the peptide digests and for the mcabs IFA profiles.

A minor exception to this was shown by the peptide digest of cross progeny AS/CB 8 (figure 10). A few unique minor bands were seen in the digest after 1 minute. Although the tracks had unequal amounts of radioactivity loaded these bands were seen on a repeat of the experiment. A further repeat with equal counts should be done so that a proper comparison could be carried out. The protease cuts at specific sites on the protein. A crossover within the gene could alter the number of amino acids between cutting sites causing different sized peptides. A change in glycosylation could also affect the mobility of peptide fragments.

It is not known where the epitopes recognised by the mcabs are situated on the protein. They may be spread along its length or clustered on part of the protein. There were 10 mcabs showing differences between AS and CB and 11 mcabs showing differences between AS and ER. If the markers were on one gene then recombination between these markers would be a very rare event. None was observed in the progeny from the two crosses examined. However during an infection parasites occur in the very large numbers which would be the appropriate conditions for new combinations of markers to arise to give new allelic forms of the antigen. New
alleles could also arise as a result of a mutation of a molecule in the DNA affecting the amino acid sequence of the protein. Any future vaccine must recognise a common epitope to reduce the chances of selecting a parasite population which will not respond to it. If selected, this antigen would probably spread rapidly through the parasite population.

4.5.2. Antigens at Different Loci

A different antigen, recognised by mcab 12.8 reacted with CB but not AS. The cloned progeny from the AS/CB cross were also tested for their reactivity with this mcab. Unlinked markers segregate with a frequency of 50%. Many of the progeny clones examined showed recombination between their 250kDa profile and their reaction with mcab 12.8 indicating that there was no linkage between these genes. Both genes also segregated independently of the isoenzyme markers and pyrimethamine response.

Previous work on the inheritance of antigens was by Panton et al (1984), who studied the inheritance pattern of an antigen of \( P. yoelii \) identified by crossed-immunoelectrophoresis. The quantity and electrophoretic mobility of this antigen varied between two lines. The two lines of parasites also differed in their virulence. The results of a cross between the two lines showed that the quantity of the antigen was correlated with the virulence of the parasite. The electrophoretic mobility of the antigen segregated
independently of the virulence. The antigen also segregated independently of isoenzyme and drug response.

4.5.3. Frequency of Genotypes in the Crosses

The parental lines were transmitted through mosquitoes independently at the time of the cross. When the number of oocysts in the midguts of mosquitoes infected with the parents were compared, approximately equal numbers of AS and CB oocysts were found. Many more oocyst were found on the midguts of mosquitoes infected with ER than the AS infected mosquitoes (D. Walliker, personal communication). The ability of ER to be transmitted through mosquitoes at a higher frequency than AS is reflected in the observation that many more ER type progeny were found in the uncloned progeny of the cross. Also an excess number of ER type progeny were obtained from the cloning. Almost equal numbers of AS and CB progeny were found in the uncloned progeny from that cross.

The reasons why ER is transmitted through mosquitoes more efficiently than AS is not known. In the erythrocytic stages in mice, the time of going through schizogony could affect the proportion of parasites in a mixed infection. The results of section 3.1 showed AS parasites undergoing schizogony after ER parasites. The proportions of each type of progeny in the uncloned cross progeny (section 3.5.1.) were counted one passage but several cycles of infection and reinvasion after the sporozoite induced infection. The proportions could change if counts were taken in later passages.
4.6. General Conclusions

The use of mcabs has enabled individual antigens to be studied. These mcabs have identified variant antigens between different isolates of the rodent malaria species *P. chabaudi*.

Each isolate had a distinct reactivity profile with the mcabs.

The mcabs have not established any geographical basis for the antigenic diversity.

Mcabs with similar IFA staining pattern reacted with an antigen of the same molecular weight.

Mcabs which had different IFA staining patterns were found to react with antigens of different molecular weights.

When two crosses were carried out between isolates, the antigen profiles of the cross progeny were like one or other of the parents. No recombinant profiles were found.

The peptide maps resulting from digestion of the immunoprecipitation of cloned progeny of the cross appeared like the maps obtained for one or other of the parents. The relatedness to the parent was the same as the antigen profiles.

The 250kDa antigen appears to be the product of one gene which has many alleles.

The gene for the 250kDa protein appears to be inherited independently from the gene coding for the 90kDa protein recognised by mcab 12.8.
Both antigen genes are inherited independently from the isoenzymes and pyrimethamine response used as other genetic markers.
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A POSSIBLE MOLECULAR BASIS FOR STRAIN SPECIFIC IMMUNITY TO MALARIA

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A 250 kDa antigen implicated in the induction of protective immunity to Plasmodium chabaudi was examined with a panel of 11 monoclonal antibodies in cloned parasite lines. 2 antibodies cross-reacted with the different parasite lines while 9 were specific for one line. This antigenic diversity was correlated with major differences in one dimensional peptide maps between the purified antigen from different lines of parasites. The peptide maps also revealed some apparently conserved structure which may have been responsible for the antigenic cross reactivity. Using cloned lines of P. falciparum and a second series of monoclonal antibodies, similar antigenic and structural diversity was evident in the equivalent antigen from the important human pathogen. These findings are discussed with relationship to the induction of protective immunity to malaria.

Key words: Plasmodium; Malaria; Immunity; Strain specificity; Monoclonal antibody; Peptide mapping

INTRODUCTION

Immunity to malaria is fundamentally parasite species specific [1] and at least partially strain specific [2-4]. It follows that considerable inter- and intra-species antigenic diversity can be expected in certain crucial parasite antigens. Recently, high molecular weight polypeptides synthesised late in the erythrocytic cycle of a number of Plasmodium species have been implicated in the induction of protective immunity to malaria. Boyle et al [5] showed that passive transfer of monoclonal antibodies to a 250 kDa polypeptide synthesised by the rodent malaria Plasmodium chabaudi inhibited parasite multiplication in vivo. Epstein et al [6] produced a monoclonal antibody against the simian parasite P. knowlesi which both recognised a 250 kDa polypeptide

Abbreviations: SDS, sodium dodecylsulphate; Trit, Tris (hydroxymethyl) amino ethane; bisacrylamide, N,N-methylenebisacrylamide.
and inhibited parasite multiplication in vitro. By immunising monkeys with a purified 74 kDa component from *P. knowlesi* infected erythrocytes, Schmidt-Ullrich et al. [7] were able to protect all vaccinated animals against an otherwise lethal *P. knowlesi* challenge. However, serum from the vaccinated animals precipitated parasite polypeptides up to 230 kDa which all showed extensive homology in tryptic peptide maps. It therefore appears that the immunogen in question was an in vivo processing product of a high molecular weight precursor. Most convincingly, Holder and Freeman [8] were able to confect considerable protection against challenge to mice immunised with purified antigens of 230 or 235 kDa isolated from the rodent malaria *P. yoelii*. Evidence linking the findings with the two rodent species to an equivalent antigen in the human parasite *P. falciparum* has recently been obtained. Antigenic cross reactivity has been demonstrated between both the high molecular weight polypeptides of *P. chabaudi* (250 kDa) and *P. yoelii* (230 kDa) and a 195 kDa polypeptide synthesised at the same time in the cell cycle of *P. falciparum* [9-11].

In this study we have utilised a panel of monoclonal antibodies raised against the cloned line *P. chabaudi* 2722AS to investigate antigenic diversity and cross-reactivity between this and a second cloned line of *P. chabaudi* from the same area of Africa. We have also compared structurally the 250 kDa antigen from the two parasite clones. In order to widen the relevance of our results to malaria in man, we have utilised a further series of monoclonal antibodies [12] to investigate antigenic and structural diversity in an equivalent polypeptide from the human parasite *P. falciparum*.

**MATERIALS AND METHODS**

**Parasites.** Three cloned lines of *P. chabaudi* were used, 2722AS, CB and DS. All were obtained from D. Walliker (Edinburgh). Infections were initiated in 24-26 g male CBA mice by intraperitoneal inoculation of parasitised erythrocytes from a large cryopreserved stock and used within five passages from this stablate as described previously [13]. Mice were maintained in a controlled light/dark cycle with illumination between 17.30 h and 08.30 h to enable erythrocytes containing mature trophozoites to be harvested in the morning [13]. Two cloned isolates of *P. falciparum*, C10 and D7, were maintained in in vitro culture using standard techniques [14]. C10 was derived from a Gambian isolate BW and D7 was derived from the Ugandan Palo Alto strain [14]. The two clones were antigenically distinct from each other as determined by indirect immunofluorescence with a panel of strain specific monoclonal antibodies, and the antigenic phenotypes of the clones corresponded to those of the parental isolates (12).

**Sera and monoclonal antibodies.** Normal and *P. chabaudi* AS immune mouse sera were obtained as described previously [15]. The preparation and characterisation of monoclonal antibodies to *P. chabaudi* 2722AS has been described previously [5]. The monoclonal antibodies directed against *P. falciparum* were those described by McBride et al [12]. For immunoprecipitation or indirect immunofluorescence, ascitic fluid from hybridoma bearing mice was used at appropriate dilutions.

**Metabolic labelling of parasitised erythrocytes with [35S]methionine.** *P. chabaudi* parasitised erythrocytes at the mature trophozoite stage were obtained from mice with a parasitaemia of greater than 20%. The harvesting of parasites, removal of leucocytes and the 2 h in vitro labelling with [35S]methionine was as described previously [13,15]. Identical short term in vitro labelling conditions were used for sorbitol synchronised cultures of *P. falciparum* at the mature trophozoite/young schizont stage [16].

**Indirect immunofluorescence.** Indirect immunofluorescence staining of *P. chabaudi* parasitised erythrocytes with monoclonal antibodies was as described [5].

**RESULTS**

17 monoclonal antibodies raised against the AS clone of *P. chabaudi* had previously been characterised with regard to the parasite coded polypeptides to which they were specific. 11 of these antibodies recognised an antigen of 250 kDa synthesised late in the cell cycle and gave a characteristic pattern of indirect immunofluorescence on methanol-fixed blood smears. In preliminary experiments, therefore, the reactivity of the 17 antibodies with AS or CB parasites in indirect immunofluorescence was compared. Of the 11 antibodies known to be directed against the 250 kDa antigen, 9 were found to react only with AS parasites (Nos. 2,3,4,12,14,15,16,18 and 20), but 2 cross-reacted
with CB parasites (Nos. 1 and 8). The 2 cross reacting antibodies gave typical patterns of indirect immunofluorescence with both parasite clones. Fluorescence was observed around the membrane enclosing developing merozoites and/or free merozoites. The remaining 6 monoclonal antibodies, which were directed against other parasite antigens of the AS line [5], all cross-reacted with the CB clone (data not shown).

This immunofluorescence data with the anti-250 kDa antibodies was confirmed by immunoprecipitation of late stage parasite polypeptides labelled metabolically with \(^{35}\text{S}\) methionine (Fig. 1(a)). Only monoclonal antibodies 1 and 8 reacted strongly with the CB line whereas, as expected, all 11 antibodies precipitated a parasite polypeptide of 250 kDa from AS parasites. In addition, when immunoprecipitates (not shown) or total metabolically labelled parasite lysates were run side by side, the molecular weight of the antigen recognised by the cross-reactive antibodies was approximately 5 kDa less in CB than in AS parasites (Fig. 1(b)). Thus the equivalent antigen from two cloned lines of \(P. chabaudi\) showed both antigenic and molecular differences whilst at the same time conserved antigenic regions could be demonstrated. To investigate further the structural relationship between the corresponding polypeptides from the two isolates, monoclonal antibody 8 was used to precipitate \(^{35}\text{S}\) methionine-labelled antigen from both parasite lines. The washed precipitates were then digested for various times with staphylococcal V8 protease and the resultant polypeptides analysed by SDS-PAGE and fluorography (Fig. 2). All of the peptides present in addition to the 250 kDa antigen in the time 0 tracks were subsequently shown to be derived from the 250 kDa polypeptide by pulse-chase experiments (Newbold and Schryer, unpublished). Control experiments, where two different samples of antigen from the same parasite clone were mapped on one gel showed that under these conditions the digestion patterns were indistinguishable.

Examination of the one dimensional maps reveals that the majority of major digestion products of the antigen from the two parasites are not obviously related, suggesting that a high degree of structural diversity exists between this important antigen in two related parasite clones. However, some digestion products of the two antigens have the same mobility (arrowed in Fig. 2) and therefore suggest common conserved structure that may be responsible for the cross reaction observed with two of the monoclonal antibodies.

Fig. 1. Immunoprecipitation of metabolically labeled polypeptides of \(P. chabaudi\) clones CB and AS by monoclonal antibodies raised against clone AS. (a) CB or AS parasites were labelled for 2 h in vitro during the development of mature trophozoites to schizonts. Triton X-100 soluble CB or AS antigens were immunoprecipitated with monoclonal antibodies 1-4, 8, 12, 14-16, 18, 20, normal mouse serum (n) or immune serum directed against AS parasites (i). The particular ascitic fluid containing clone 2 gives high non-specific background precipitation in this experiment. Precipitates were analysed by SDS-PAGE on a 10% gel and visualised by fluorography. (b) Lysates of AS and CB parasites labelled as in (a) were analysed by SDS-PAGE on a 10% gel and the labelled bands visualised by fluorography. Molecular weight markers used throughout were human spectrin (240 and 220 kDa), \(E. coli\) RNA polymerase (165, 155 and 39 kDa), bovine serum albumin (68 kDa) and soybean trypsin inhibitor (21 kDa).
A monoclonal antibody was used to immunoprecipitate 250 kDa antigens labeled metabolically with $[^{35}]$S-methionine from CB or AS parasites as in Fig. 1. Precipitates were treated with V8 protease and analysed by SDS-PAGE on a 10% gel. Labelled digestion products were visualised by fluorography. From left to right, tracks are alternatively AS or CB antigen at 0, 30 and 90 min digestion time. Peptides of equivalent mobility derived from the antigen from the two parasite clones are arrowed on the right.

Using the same mapping technique, the 250 kDa antigen from AS and CB parasites was compared with a third, geographically distinct cloned isolate of *P. chabaudi* designated DS. Since monoclonal antibody 8 also reacted with this line of parasites, it could be used to immunoprecipitate antigen from all three parasite clones. The resulting peptide map on a 12.5% w/v acrylamide SDS gel is shown in Fig. 3. It is evident that the same structural similarities are present in the third line of *P. chabaudi*, but that in other respects the antigen from the three different parasites shows considerable diversity in structure.

It was pointed out in the introduction that similar high molecular weight antigens from asexual intraerythrocytic cycle of a number of *Plasmodium* species have been implicated in the induction of protective immunity. The equivalence of the 250 kDa antigen from *P. chabaudi*, the 230 kDa antigen from *P. yoelii* and a 195 kDa polypeptide from *P. falciparum* has recently been demonstrated by showing antigenic cross-reactivity between the molecules from the three different species [9]. McBride et al. [12] had produced a number of monoclonal antibodies to *P. falciparum* which showed strain specificity and some of which recognised a parasite polypeptide of approximately 200 kDa [19]. By this and a number of other criteria (see Discussion) it was evident that at least some of these strain specific monoclonal antibodies recognised a *P. falciparum* antigen which was equivalent to the 250 kDa component of *P. chabaudi*. It was therefore of interest to determine whether or not the same structural diversity was present in the 250 kDa component of *P. chabaudi*.
shown by the antigen from the rodent parasites was also present in different cloned isolates of the human malaria.

Clones C10 and D7 of \textit{P. falciparum} were labelled metabolically with $^{[35S]}$methionine and parasite polypeptides immunoprecipitated with three monoclonal antibodies (7.3, 9.2 and 9.8 [12]). All precipitated components of approximately 200 kDa from the labeled schizont infected cell extracts. Antibody 7.3 was specific for clone C10, 9.2 was specific for clone D7, while the third antibody (9.8) cross-reacted with both parasites isolates (data not shown). This confirmed the immunofluorescence specificities of the monoclonal antibodies described previously [12]. The cross-reactive antibody [9.8] was therefore used to repeat the immunoprecipitation and peptide mapping experiments described earlier (Fig. 4). It is evident that the antigen molecular weights from the two cloned isolates are slightly different, as had been seen in the case of the \textit{P. chabaudi} antigen. Again, the peptides in addition to the 200 kDa bands in the time 0

![Fig. 4. V8 protease digest of high molecular weight schizont antigen from \textit{P. falciparum} clones C10 and D7. \textit{P. falciparum} clones C10 and D7 were labelled metabolically for 2 h with $^{[35S]}$methionine at the late trophozoite/schizont stage. Monoclonal antibody 9.8 [12] was used to precipitate antigen from Triton X-100 extracts of the labeled cells. The immunoprecipitates were then digested for increasing times with V8 protease, the products analysed by SDS PAGE on a 12.5% gel and visualised by fluorography. Tracks from left to right are alternate antigen from clones C10 or D7 digested for 0, 30 and 90 s and 5, 15 and 30 min.](image)

tracks are cleavage products of the major antigen and correspond closely in molecular weight to the processing products described by Holder and Freeman [10]. The map itself demonstrates apparently conserved peptides between the antigen from the two clones, but very considerable diversity in the other peptides produced during the digestion.

**DISCUSSION**

The evidence equating high molecular weight polypeptides from various \textit{Plasmodium} species is manifest in the following common features. They are all synthesised late in the intraerythrocytic cycle of the parasite [6-11,13,15,16,19]. Monoclonal antibodies directed against these antigens show a characteristic pattern of indirect immunofluorescence on methanol fixed blood smears. Fluorescence is observed around developing merozoites within schizonts and on free merozoites [5,6,8,10,12,19]. Monoclonal antibody immunoprecipitates of metabolically labelled antigen either alone or in conjunction with pulse-chase experiments suggest that these antigens are cleaved post translationally in a specific fashion [5,8,9,10,19]. The antigens are lost during the process of schizont rupture and merozoite invasion and are not carried through to the subsequent cell cycle as shown by pulse-chase experiments [16,20] and/or lack of monoclonal antibody reactivity with ring stages [5,6,8,10]. In a surface-specific immunoprecipitation assay, these antigens were found to be available to bind antibody in undisturbed schizont infected erythrocyte preparations of two rodent malarial [15], in \textit{P. falciparum} [16] and in \textit{P. knowlesi} (unpublished observations, Newbold and Boyle). Finally, antigenic cross reactivity has been demonstrated between antigens from \textit{P. yoelii}, \textit{P. chabaudi} and \textit{P. falciparum} [9].

A further feature of these antigens suggested by immunofluorescence data with monoclonal antibodies to \textit{P. falciparum} [12], or here to \textit{P. chabaudi}, is considerable antigenic diversity. In this study we have extended these observations by demonstrating that both the antigenic diversity and cross reactivity in this family of high molecular weight antigens can be represented at the structural level. Not only are there small differences in molecular weights between the antigens from different cloned lines of parasites, but one dimensional peptide maps reveal that apparently conserved sequences as well as completely diverse structures are present within different parasite of a single species. Since cross-reactive antibodies were used in the mapping studies, it should be stressed that without the availability of other antibodies to the same antigen of different epitope specificity, or the data obtained by the peptide maps, it may have been concluded that the antigen from different parasite lines was identical. This may be a consideration when examining possible intraspecies diversity in other parasite antigens.

However, the most important conclusions to be drawn from these experiments is how the antigenic and structural diversity in this family of plasmodial antigens relates to the induction of protective immunity. So far, vaccination studies with purified
antigen have clearly demonstrated protection against homologous parasite challenge with the 230 kDa antigen from *P. yoelii* [8]. In vitro inhibition of parasite multiplication has also been demonstrated with a monoclonal antibody directed against the equivalent 250 kDa antigen from *P. knowlesi* [6]. By inference, the protection achieved by Schmidt-Ullrich et al [7] using a 74 kDa *P. knowlesi* polypeptide for vaccination was very likely related to these findings, since vaccinated animals produced antibodies which recognised a family of structurally related polypeptides up to 230 kDa, typical of the in vivo processing known to occur with this family of antigens. With *P. chabaudi*, passive transfer of anti 250 kDa monoclonal antibodies conferred a degree of protection to mice against challenge [5]. Taken together with the data presented here, these findings suggest that: (1) this family of high molecular weight antigens is somehow involved in the induction of protective immunity to malaria. (2) The considerable antigenic and structural diversity in these antigens from at least two species of malaria is unlikely to be fortuitous. Since they seem to be involved at least in part in the induction of immunity, natural selection may well have resulted in the generation of diversity as one mechanism of evading the hosts immune response. (3) Vaccination studies with purified 250 kDa antigen from AS and CB lines of *P. chabaudi* suggest that the protection afforded to mice against challenge is also strain specific (Schryer, Newbold, Jarra and Brown, in preparation).

Immunity to malaria involves a complex series of events and takes many years to acquire under conditions of natural transmission [1]. It is characterised by components which show strict parasite species specificity, at least partial strain specificity and, in some cases at least, variant specificity [1–4]. There are also components of the immune response, which appear to be non-specific in effect, that can confer a limited degree of protection across strain and species barriers [21,22]. Attempting to explain such a complex phenomenon in terms of an immune response to a single antigen would be simplistic in the extreme. Nevertheless, the fact that an antigen involved in the induction of immunity shows such inter- and intra-species diversity suggests that at least part of species and strain specific components of malarial immunity may be directed against this class high molecular weight parasite antigens.

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