INVESTIGATION OF POTENTIALLY PROTECTIVE ANTIGENS
OF THE HUMAN MALARIA PARASITE,
PLASMODIUM FALCIPARUM

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ABSTRACT

There are approximately 2,000 million people still living under the threat of malaria and present malaria control measures are becoming ineffective. Molecular biology offers a new solution to the world malaria problem. A protective antigen, which would form the basis of a malaria vaccine, could be synthesised economically and on a large scale under the direction of a gene isolated from the malaria parasite. But first the protective antigens which will induce a protective immune response must be identified.

A bank of monoclonal antibodies had been raised against the erythrocytic stages of the human malaria parasite, Plasmodium falciparum. This thesis reports the screening of these monoclonal antibodies for an ability to inhibit the in vitro, erythrocytic growth of the parasite. One monoclonal antibody, McAb 2.13, caused a strong inhibition of growth, while five others, McAbs 2.9, 6.3, 7.5, 7.6 and 7.12 may have weak inhibitory activity. The possibility that these monoclonal antibodies could be recognising protective antigens requires further investigation.

The same bank of monoclonal antibodies was also screened for cross-reaction to the surface of the sporozoite stage of the malaria parasite. Such cross-reaction by one of the monoclonal antibodies, McAb 5.1, was the first evidence to suggest that a stage-common antigen is exposed at the sporozoite surface. This monoclonal antibody could be recognising an antigen which would induce an immune response directed against two parasite stages.

The 5.1 Ag, the erythrocytic-stage protein recognised by the cross-reactive monoclonal antibody was identified and thoroughly characterised. High levels of antibody to the 5.1 Ag were found in the sera of individuals living in areas endemic for malaria, even
though this antigen is only a minor component of the erythrocytic-stage parasite. Antibody to the 5.1 Ag was affinity purified from the human endemic serum and used to probe a *P. falciparum* erythrocytic stage cDNA expression library. Two recombinant clones were isolated, one of which encoded half of the 5.1 Ag while the other contained the entire coding region of the 5.1 Ag gene. The cloned cDNAs were sequenced and the recombinant proteins that they encode were identified. Bacterial expression of the 5.1 Ag gene will generate sufficient 5.1 Ag for the protective potential of this protein to be evaluated.
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ABBREVIATIONS

Ag  antigen
bp  base pairs
A +/or T  adenosine and/or thymidine
C +/or G  cytidine and/or guanosine
CPM  counts per minute
dNTP  deoxynucleotide triphosphate
ddNTP  dideoxynucleotide triphosphate
IIF  indirect immunofluorescence
K  x1000 relative molecular weight
kb  kilobase pairs
McAb  monoclonal antibody
MWtM  molecular weight markers
OD_{650}  optical density at 650 nm
r.b.c.  red blood cell
spp  species
TCA  trichloroacetic acid
THIg  total human immunoglobulin
DHIg  THIg depleted for antibody to the 5.1 Ag
Ha 5.1  human antibody to the 5.1 Ag
Some of this work has already been published.

Introduction
Molecular biology offers a new approach to the control of malaria. One aim is to use a gene from the malaria parasite to direct economic, large-scale synthesis in bacteria of a protective antigen, an antigen which when used in a vaccine would stimulate a protective response against human malaria. First, protective antigens must be identified from amongst the many components of the malaria parasite. Second, the genes for the protective antigens must be isolated and expressed in bacteria. Third, the value of such bacterially synthesised products for protection against human malaria must be determined. Before the feasibility of this approach can be discussed, information on the biology of the parasite which causes malaria is necessary.

1. The life-cycle of malaria parasites

It is the highly synchronous development of the malaria parasite within the red blood cells of vertebrate hosts which generates the most characteristic symptom of human malaria, rhythmic bouts of fever. The unicellular malaria parasite develops within a red blood cell through ring, trophozoite and schizont stages. During this development asexual multiplication occurs and rupture of a mature schizont liberates 10 to 30 extracellular merozoites, each of which can infect another red blood cell. The bouts of fever or paroxysms coincide with each schizont rupture and involve a cold stage, a hot stage and a sweating stage. Successive paroxysms can be separated by asymptomatic periods. Unless limited, artificially with drugs or by the hosts immune system, the asexual multiplication of the parasite through the erythrocytic cycle can result in more than 50 per cent of a host's red blood cells becoming infected and cause host death.

After invading a red blood cell a merozoite may enter gametocyto-
genesis. This is an alternative route of development from the asexual multiplication of the erythrocytic cycle and leads into the rest of the parasite life-cycle.1-3 (Fig. 1). Gametocytogenesis generates macro- and microgametocytes which are still retained by the hosts erythrocytic membrane and are infective to mosquitoes.

The invertebrate hosts, responsible for transmission of the malaria parasite between vertebrate hosts, are mosquitoes, usually Anopheles species. A blood meal is required for egg laying and so is only taken by the female mosquito. Gametocytes transform into gametes upon ingestion by the arthropod vector. Macrogametocytes shed the erythrocytic membrane and microgametocytes undergo nuclear division and exflagellate to give 8 motile microgametes. Fusion of gametes generates a zygote which changes morphologically into an active ookinete. The ookinete migrates through the intestinal epithelium of the mosquito and forms an oocyst beneath the basal lamina. Rapid nuclear division of the oocyst is followed by cytoplasmic division to generate sporozoites. Sporozoites are highly motile and migrate to the lumen of the salivary ducts from which, during another blood meal, the malaria parasite is introduced into the blood stream of another vertebrate host.

Back in the vertebrate host the parasite enters a further round of asexual multiplication, exo-erythrocytic schizogony, which may be cyclical. Sporozoites invade cells of a particular host tissue (though not red blood cells) and develop through ring, trophozoite and schizont stages. At schizont rupture, 100 to 30,000 merozoites are liberated. The exo-erythrocytic merozoites may then invade red blood cells and so complete the malaria parasite's life-cycle.
Fig. 1. Life-cycle of malaria parasites. The pathways of development which occur in all species, (—), or only in some species, (-----) are indicated.
2. Species of malaria parasites

As a parasitic unicellular organism with a complex life-cycle including a sporozoite stage, the malaria parasite has been classified in the phylum Protozoa, subphylum Sporozoa. The malaria parasites have been grouped in the family Plasmodiidae, with the single genus Plasmodium, because they demonstrate two types of asexual multiplication in a vertebrate host and sexual multiplication in a mosquito host. More than 100 species of Plasmodium have been described. The characteristics of the different species are important because of the use of animal malaria parasites as models of human malaria parasites.

There are four species of Plasmodia for which man is the natural host, P. falciparum, P. malariae, P. vivax and P. ovale (Table 1). An important characteristic of P. falciparum is the sequestration of its mature erythrocytic stages in post-capillary venules of internal organs. This leads to major complications if P. falciparum infections are not controlled. Clumps of infected erythrocytes blocking blood vessels in the brain (cerebral malaria) or adrenals (algid malaria) are usually fatal. Of the four human malaria parasites, research has centred mainly on P. falciparum because of the severity of the disease it causes.

A major limitation to the study of human malaria is the specificity of the Plasmodium spp. for their vertebrate hosts. P. malariae mainly infects man, but also naturally infects Pan satyrus, the chimpanzee. For P. falciparum, P. vivax and P. ovale man is the only natural host, although abnormal simian hosts have been infected with blood inoculations of these parasites. The most widely used experimental hosts for P. falciparum are Aotus trivirgatus, the owl monkey, and Saimiri sciureus, the squirrel monkey. However, both
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Table 1. Characteristics of the human malaria parasites
monkeys are rare, expensive, difficult to keep and the course of infection is not the same as in man.

The alternative to studying *P. falciparum* directly is to study other *Plasmodium* spp. and extrapolate any results obtained to the human parasite. A few animal malaria parasites, which can be maintained in the laboratory, have been particularly well studied and are conveniently described within the framework of subgeneric classification. The criteria of this classification include host range and other biological characteristics.

Two avian *Plasmodia*, *P. gallinaceum* and *P. lophurae* have been adapted to infect ducks for study in the laboratory. These two species belong to the subgenera *P. (Haemamoeba)* and *P. (Giovannolaia)* respectively. For avian malaria parasites the cyclical exo-erythrocytic stage occurs in reticuloendothelial cells (not liver parenchymal cells) and can arise from the erythrocytic stage, characteristics distinguishing these species from human malaria parasites.

The subgenus *P. (Vinckeia)* includes the rodent malaria parasites, *P. berghei*, *P. chabaudi*, *P. yoelii* and *P. vinckei*. These species have been adapted to infect laboratory mice and the development of cyclic transmission through *Anopheles stephensi* has made them important subjects of malaria research. The main differences between members of this subgenus and human malaria parasites are small erythrocytic schizonts and, possibly, some biochemical characteristics.

Three of the human malaria species, *P. malariae*, *P. vivax* and *P. ovale* belong in the subgenus *P. (Plasmodium)* along with the two simian parasites *P. knowlesi* and *P. cynomolgi*, which have both been well studied in the laboratory. *P. vivax* and *P. cynomolgi* are morphologically very similar and closely related. *P. knowlesi* is not as closely
related to any of the human parasites and has only a 24 hour asexual cycle.

There are no good animal models for *P. falciparum* infections. This parasite is classified in the subgenus *P. (Laverania)*. The only other species of this subgenus is *P. reichenowi* which infects *Pan satyrus*, the chimpanzee, and has a very restricted geographical location in equatorial Africa. The main characteristics of this subgenus are so striking that it has led some to propose that these two species should be placed in a genus of their own, distinct from the other malaria parasites.

Apart from the differences in the parasites themselves the differences in the hosts of these model systems are important for extrapolation to the human disease. Different vertebrates may produce a different immune response upon challenge even with the same *Plasmodium* species. This restricts interpretation of results from animal models to human malaria. However, the exo-erythrocytic stage was originally demonstrated for avian malaria parasites and is now known to be a stage in the life-cycle of all *Plasmodium* species. Also, molecular characterisation of the parasites has revealed cross-species similarities of antigens e.g. the circumsporozoite protein and an abundant, high molecular weight protein of mature erythrocytic stages. So cross-species comparisons can be valid.

One final system on which *P. falciparum* study has been based was developed recently with the establishment of the erythrocytic stages of this parasite in continuous culture. This achievement has stimulated research on malaria by making parasite material available to more laboratories. Quantities sufficient for research can be obtained routinely but production of larger amounts is limited for technical reasons. A major reservation of this system is that some
characteristics of a parasite line change with prolonged culturing, e.g. presence of "knobs" on the surface of infected erythrocytes and capacity to develop into gametocytes. The same phenomenon has been observed with cryopreservation which is used routinely in parasite culturing and occasionally with passage in vivo. The changes in parasite characteristics which occur with these techniques could affect the validity of the parasite grown in vitro as a model of the parasite as it exists in vivo.

There are advantages and disadvantages to all of these systems upon which malaria research has been based. Studies of human malaria parasites, grown in vivo in abnormal hosts and in vitro in continuous culture, and of animal malaria parasites all provide complementary information from which progress towards prevention or cure of malaria, the disease, may be possible.

3. The world malaria situation

In 1981, the most recent year for which statistics are available, 7.6 million cases of malaria were reported to the World Health Organisation (WHO). This figure does not include cases in Africa, south of the Sahara, for which the most recent data, for 1979, records another 7.6 million cases, although an annual average of 5.5 million cases has been quoted for this region. However, these numbers are probably a severe under-estimate, because some affected countries do not report on all their malarious areas and surveillance is expensive and so not always thorough. Mortality figures are not readily available, but the most recent estimate, reported in 1974, was of 1 million deaths annually amongst children in Africa alone. Of the total world population of $4.5 \times 10^9$ in 1981, $2.2 \times 10^9$, or nearly 50 per cent, lived in malarious areas. Despite these figures the present malaria situation is still better than it has been.
3.1 History of malaria. Apart from the spread of falciparum malaria throughout the New World after its introduction by transatlantic explorers in the 17th century, the geographical distribution of malaria has probably always been quite constant. P.vivax and P.falciparum were mainly restricted by the 16°C and 20°C summer isotherms respectively, although certain areas within these limits such as deserts and mountain ranges remained non-malarious because transmission was not favoured for other ecological reasons. Vivax malaria extended from south-east England, areas around the Baltic Sea and Archangelsk (now in the USSR) near the arctic circle to South Africa and northern Australia in the Old World and from south-east Canada to northern Argentina in the New World. Towards the end of the 19th century, changes in pattern of land use in the USA, western Europe and in the northern Baltic region destroyed the breeding places for mosquitoes and malaria transmission, unstable anyway in these areas at the extremes of the malaria parasites' distribution, was interrupted. The distribution of malaria then continued unchanged until the 1950s.

3.2 Methods of controlling malaria. Two major approaches to combat malaria have been developed: vector control and anti-malarial drugs. Relief from malaria was first achieved in the 17th century by treatment with bark of the Cinchona, a Peruvian tree. Use of this remedy spready rapidly throughout Europe, but it wasn't until 1820 that the active principle, quinine, was isolated. From 1924, several species of synthetic anti-malarial drugs were developed. The range of drugs available however is still limited and includes the dihydrofolate reductase (DHFR) inhibitors, the 4-aminoquinolines and 8-aminoquinolines, both derivatives of quinine, and the combination drugs of a DHFR inhibitor with a dihydropteroate synthetase (DHPS) inhibitor (see Table 2). One of the uses of these drugs is for protection to
Table 2. Anti-malarial drugs

<table>
<thead>
<tr>
<th>Type of Drug</th>
<th>Drug</th>
<th>Stages acted upon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine and derivatives</td>
<td>Quinine</td>
<td>Asexual erythrocytic stages.</td>
</tr>
<tr>
<td>4-aminoquinolines</td>
<td>Chloroquine</td>
<td>Asexual erythrocytic stages.</td>
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<tr>
<td></td>
<td>Amodiaquine</td>
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<tr>
<td>8-aminoquinolines</td>
<td>Primaquine</td>
<td>Exo-erythrocytic and gametocytes.</td>
</tr>
<tr>
<td>DHFR inhibitors</td>
<td>Proguanil</td>
<td>Exo-erythrocytic and Gametes.</td>
</tr>
<tr>
<td></td>
<td>Pyrimethamine</td>
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<tr>
<td></td>
<td>Chlorproguanil</td>
<td>Weak action on asexual erythrocytic</td>
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<tr>
<td></td>
<td>Cycloguanil embonate</td>
<td>stages.</td>
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<tr>
<td></td>
<td>Trimethoprim</td>
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<tr>
<td>DHPS inhibitors</td>
<td>Sulphonamide</td>
<td>Weak but long lasting action upon</td>
</tr>
<tr>
<td></td>
<td>Sulphones</td>
<td>Asexual erythrocytic stages.</td>
</tr>
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</table>
malaria either in causal prophylaxis, aimed at exo-erythrocytic stages to prevent any erythrocytic development, or in suppressive prophylaxis aimed at erythrocytic stages to prevent infection reaching levels which can cause clinical symptoms. These drugs are also used in therapy either to treat an acute attack of malaria or to cure an individual of exo-erythrocytic stages to prevent the relapses of vivax or ovale malaria. Finally, the gametocidal and sporozontocidal activities of some of these drugs have been employed to block malaria transmission.

Vector control was initially aimed only at the aquatic stages of the mosquitoes life-cycle. Larvicidal measures include the oiling of water surfaces and the application of Paris green (copper aceto-arsenite) to known breeding places. If a breeding place is stable, larvivorous fish (e.g. Gambusia spp.) may be introduced. Alternatively, the breeding sites may be destroyed either by the installation of drainage systems or by careful water management of irrigation systems. In the 1930s, Pyrethrum extracts were found to be toxic to adult mosquitoes and were successfully used in spray form in South Africa and parts of India.

A major breakthrough came in 1939, with the discovery of the residual insecticide dichloro-diphenyl-trichloroethane (DDT) which was in widespread use by the mid-1940s. This synthetic compound combines high toxicity to insects and low toxicity to man with a long term stability, its residual activity lasting up to six months. It is also inexpensive. DDT is now mainly used to spray indoor surfaces because the cumulative toxicity of DDT on some animals, such as predatory birds, has led to its outdoor use being discouraged for environmental reasons.

Other chlorinated hydrocarbons have been developed as insecticides
13. such as Dieldrin and hexachlorocyclohexane (HCH).\textsuperscript{1,3} However, in comparison to DDT, the former is more toxic to man, the latter is more volatile (and so must be applied more often) and both are less effective. Alternatives to the chlorinated hydrocarbons are the organo-phosphates (such as Malathion) and the carbamates (such as Propoxur) which are both cholinesterase inhibitors,\textsuperscript{1,3} but these compounds are expensive and most are highly toxic to man. So, DDT still remains the insecticide of choice, where vector resistance to the chemical has not developed.

3.3 Global campaign to eradicate malaria. The power of these antimalarial developments led to the possibility of global malaria eradication, a policy officially adopted by the Eighth World Health Assembly in 1955.\textsuperscript{1,3} The World Health Organisation (WHO) was responsible for the world-wide coordination of the campaign which was to be organised at a national level the funding of individual governments being supplemented where necessary by WHO, United Nations International Childrens Emergency Fund (UNICEF), United Nations Development Programme (UNDP) and the USA Agency for International Development (USAID). The immediate plan was of malaria eradication from all countries except those in tropical Africa where it was thought present technologies would only be adequate for malaria control. It was hoped that malaria eradication would also become the eventual target in tropical Africa.

The malaria eradication campaign initially met with considerable success. Malaria eradication has been achieved and maintained by 37 of the world's 142 initially malarious countries,\textsuperscript{3} including Australia, Japan, Singapore, Korea, Taiwan and most of Europe, North America and the Caribbean.\textsuperscript{1} A considerable reduction in morbidity and mortality was also obtained in all other malarious countries.
However, as a result partly of the poor world-wide economic situation and partly of complacency stemming from the initial success, funds available for the anti-malaria campaign declined. In some areas where malaria eradication had seemed close at hand, or even achieved, malaria once again reached epidemic proportions. In India malaria cases, reduced from over 100 million cases per year to 50,000 by 1961,\textsuperscript{18} resurgued to an estimated 30 million in 1977.\textsuperscript{3} The initial improvement and subsequent deterioration in the world malaria situation from 1957 to 1977 is depicted in Fig. 2.

The initial concern about malaria resurgence is now approaching panic as the means to obtain control over malaria are gradually becoming ineffective. Resistance of many Anopheles species to chlorinated hydrocarbons has been reported in most of the malarious countries of the world and resistance to organo-phosphorous compounds and carbamates has also been detected in central America and south-east Asia.\textsuperscript{1,18}

Even more alarming is the emergence of drug resistant strains of \textit{P.falciparum}.\textsuperscript{1,19} Resistance to chloroquine, the main drug for the treatment of acute malaria, has appeared throughout south-east Asia, most of South America and in several countries of eastern Africa. The sulphonamide/pyrimethamine combination is the second line of defence for chloroquine-resistant malaria but cannot be used for treatment of pregnant women because of toxic side effects. Besides, resistance to these drugs is now spreading rapidly through South America and Asia. Quinine is the only, presently-available alternative for treatment of pregnant women and may not be completely safe for the foetus. In some places even quinine resistance has been reported. There are two drugs, mefloquine and Qinghaosu, still under
Fig. 2. World malaria situation. (Taken from Wernsdorfer, W.H.\textsuperscript{3}). Status of the antimalaria program, 1957-77, based on the total world population under malaria risk in 1960 (excluding China). Open area, population in areas freed from malaria; dotted area, population under malaria risk, protected; shaded area, population under malaria risk, not protected.
trial but acquisition of resistance by malaria parasites now appears to be matching the rate of development of new drugs.

3.4 **A possible solution to the present world malaria situation.**

Mass-immunisation could be an alternative to the application of drugs and insecticides in the campaign against malaria. Natural immunity to malaria is only acquired upon exposure to malaria infection over many years but this is at least partly due to an effect of the live parasite on the vertebrate host's immune system. Thus, immunisation with non-live parasite material may induce protection more rapidly.

Non-malaria vaccines generally consist of either a toxoid that is responsible for the clinical symptoms of the disease (e.g. tetanus, diptheria) or an attenuated form or avirulent strain of an infective organism (e.g. polio, cholera). For malaria, the clinical symptoms are caused by the infection and not a toxoid, but if the molecule(s) responsible for suppression of the anti-malaria immune response could be identified, immunisation with such a preparation might allow naturally acquired immunity to develop more rapidly. Also, protective immunisation with attenuated forms of malaria parasites has already been obtained experimentally. However, the main limitation to the vaccine approach is the generation of sufficient material for mass-immunisation.

It is generally believed that immunity to malaria is species-specific. So, vaccine material must be generated from each of the four human *Plasmodium* spp. It has only recently become possible to grow one of these species (*P. falciparum*) in the laboratory and it would be technically impossible to produce sufficient parasite material in this way for mass-immunisation. However, in vitro culturing of *P. falciparum* does provide enough material for experimental analysis.

A possible solution to this problem is the large-scale synthesis
of parasite protein under the direction of parasite genes introduced into bacteria by recombinant DNA techniques. Before this can occur, attention must be focussed on particular parasite proteins, so it is necessary to identify which of the many malaria parasite components can induce protection.

4. Identification of protective antigens of malaria parasites

Progress has already been made towards the identification of protective antigens of malaria parasites. Of the large number of antigens suggested as important for induction of protection, some have been demonstrated as protective antigens, others only implicated as potentially protective antigens, antigens which if used in a vaccine may stimulate a protective response against malaria. Different antigens may induce different levels of protection and it may be best to vaccinate with a number of antigens to generate high levels of protection.

4.1 Correlation of antigen recognition with immune status. Several approaches have been used in the search for protective antigens. In one approach, antigens of the erythrocytic stages with a range of molecular weights have been identified from correlations between the recognition of those antigens by serum samples and the immune status of the donors of those serum samples. Antigens recognised by the serum samples were identified by immunoprecipitation of radiolabelled parasite proteins and analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. This approach is based on the premise that although antibody of immune individuals is important in defence against malaria most of the antibody to the malaria parasite is not protective. Perrin et al compared the erythrocytic stage antigens of
P. falciparum, isolate SGE1 (from Senegal) recognised by pools of immune and non-immune human sera. The pooled immune sera were from Africans living in areas endemic for falciparum malaria (in Gambia and Tanzania) and the pooled non-immune sera were from Europeans recovering from the first attack of a P. falciparum infection. They identified three schizont-specific proteins (> 200 K, 137 K and 115 K) as potentially important for inducing protection. In a subsequent report, 27 antigens recognised by individual serum samples from the above pools of sera were compared and four, potentially protective, schizont-specific proteins (> 200 K, 140 K, 105 K and 82 K) were mentioned, three of which may be the same as the three reported previously.

In a series of studies, Brown et al. 28-30 searched for correlations of antigen recognition with immune status, for P. falciparum in Papua New Guinea. First, antigens recognised by pooled human sera (from individuals living in an area endemic for malaria) were compared with those recognised by the serum of a child with a high parasitaemia which was known not to inhibit P. falciparum growth in vitro. Second, the spectrum of antigens recognised by individual serum samples was compared for correlation to the ability of those serum samples to inhibit erythrocytic stage parasite growth in vitro. Both studies identified a pair of proteins (95/96 K) as potentially protective. In the final study, proteins from three different isolates of P. falciparum recognised by a pool of immune human sera were compared. The ability of the pooled immune sera to inhibit the in vitro growth of three isolates but not the other two, was attributed to the differences in the proteins recognised (three proteins of 200 K, 150 K and 65 K were identified).

A study by Gysin et al. 31 compared P. falciparum antigens detected
by immune serum and non-immune serum (obtained during a chronic infection) from the squirrel monkey. The disadvantage of using an abnormal host for the parasite is balanced by the greater control over experimental conditions. Immune and non-immune serum were obtained from the same animal and immune status could be assayed either directly by challenge with virulent parasites or indirectly by passive serum transfer. From the results obtained a 75 K protein was claimed to be more strongly recognised by the immune serum, but apart from this there is little difference in the spectrum of proteins detected.

The correlation of antigen recognition with immune status as a means to identify potentially protective antigens suffers several limitations. The antigens are usually biosynthetically labelled for detection using $^{35}$S-methionine, so only protective antigens which contain methionine will be detected. Also, protective antigens will only be detected if they are solubilised by the detergent used to generate the parasite protein extract from which antigens are immuno-precipitated. Protective antigens present at low levels may not be detected because abundant proteins dominate the spectrum of immuno-precipitated antigens. Protective antigens could be precipitated by the non-immune sera, if they can be recognised by non-protective, as well as protective, antibodies. Finally, antibodies which recognise proteins specifically precipitated by immune sera are not necessarily protective. Therefore, these studies identify a subset of potentially protective antigens which must be proved to be truly protective by further investigation.

4.2 Inhibition of parasite growth in vitro with monoclonal antibodies. A second approach used to identify potentially protective antigens exploited the specificity of monoclonal antibodies. Panels of mono-
clonal antibodies raised to the malaria parasite have been screened for the ability to inhibit parasite growth in vitro. Antigens recognised by inhibitory monoclonal antibodies have then been characterised by immunoprecipitation. There are two limitations to this approach. First, monoclonal antibodies to primate malaria parasites have been usually generated in rodents; rodent and primate immune responses to the parasite may differ. Second, very high levels of monoclonal antibodies (in the range of mg/ml) are often necessary to strongly inhibit parasite growth and vaccination may not stimulate a strong enough response to provide protection. However, it would be expected that neutralisation of a functionally important parasite antigen would be more readily achieved by a polyclonal antibody, recognising many epitopes with a range of affinities, than by a monoclonal antibody, which binds a single epitope with a single affinity. Therefore, this approach also identifies potentially protective antigens which must be investigated further by other techniques. It is important to note that protective antigens of low abundance or poor immunogenicity may not be revealed by this approach unless large numbers of monoclonal antibodies are examined.

Rat monoclonal antibodies have been raised against merozoites of P. knowlesi by Deans et al. and 8 of a total of 28 were screened for an ability to inhibit erythrocytic growth in vitro. When purified monoclonal antibodies were used, two were found to be inhibitory and examination of inhibited cultures suggested that reinvasion by merozoites, rather than intra-erythrocytic development, was affected. The two monoclonal antibodies (at 2 mg/ml) inhibited parasite multiplication by 86 and 88 per cent. Indirect immunofluorescence microscopy revealed that the antigen recognised by these monoclonal antibodies accumulated in mature schizonts and was also present on
isolated merozoites. Immunoprecipitation showed that both monoclonal antibodies recognised a minor parasite component of 66 K. This potentially protective antigen has now been more thoroughly characterised using the monoclonal antibodies.\textsuperscript{33} It is synthesised by very mature schizonts during the last 1½-2 hrs of the 24 hr erythrocytic cycle as the 66 K protein. At merozoite release, it is processed to two smaller polypeptides of 44 K and 42 K which are lost or structurally altered at red blood cell reinvasion.

Schofield et al\textsuperscript{34} generated mouse monoclonal antibodies to erythrocytic stages of two Papua New Guinea (PNG) isolates of \textit{P.falciparum}. By indirect immunofluorescence or radioimmunoassay, all 36 monoclonal antibodies reacted to 7 of 7 parasite isolates from PNG and 0 of 4 non-PNG isolates. Such extreme antigenic diversity is not usually observed.\textsuperscript{35} Nevertheless, the observation of isolate specific inhibition of \textit{in vitro} erythrocytic growth by 5 monoclonal antibodies suggests a possible importance for isolate specific protective antibodies. The antigens recognised by the inhibitory monoclonal antibodies were not characterised although it is stated that most of the 36 monoclonal antibodies reacted to high molecular weight proteins (> 200 K).

Perrin et al\textsuperscript{27,36} reported on inhibitory monoclonal antibodies which recognise \textit{P.falciparum} isolates from different geographical locations. Mouse monoclonal antibodies were raised to merozoites and schizonts prepared from \textit{P.falciparum}, isolate SGE1, from Senegal. \textit{In vitro} growth of the homologous \textit{P.falciparum} isolate was inhibited to more than 80 per cent when cultures were supplemented to 10 per cent with 6 of 20 tested ascitic fluids. Each inhibitory monoclonal antibody was derived from a different hybridoma fusion but immunoprecipitation showed that they all recognised schizont-specific
polypeptides. Three recognised a 41 K protein occasionally seen as a doublet, two immunoprecipitated 82 K and 41 K proteins and the last reacted with a 140 K protein (which may be cleaved during isolation to 96 K and 36 K polypeptides). Immunofluorescence shows the antigens to be present on the surfaces of merozoites and schizont-infected erythrocytes. Three of the inhibitory monoclonal antibodies were tested, for inhibitory activity and by immunoprecipitation, against 3 other *P. falciparum* isolates from East Africa, Vietnam and China. Identical results were obtained to those for the original isolate from West Africa (Senegal).

Clearly antibodies to antigens identified by this approach can affect parasite multiplication. The next step is immunisation with these antigens, purified by affinity chromatography using the monoclonal antibodies, for evaluation of the protective response which can be generated.

4.3 **Antigens probably susceptible to immunological attack because of location or function.** A third approach to the identification of potentially protective antigens seeks to characterise proteins with a particular location or function which would mean that they must be exposed to the host immune system. Attention can then be directed to these particular proteins to determine whether an immune response to them is protective.

A series of studies has been aimed at proteins on the surface of gametes. The intra-cellular gametocytes are transformed to extra-cellular gametes upon ingestion by a mosquito prior to fusion to form a zygote. It has been demonstrated that immunisation of vertebrate hosts with gamete preparations of *P. gallinaceum*, *P. knowlesi* and *P. yoelii* prevents zygote formation in the mosquito and so blocks malaria transmission. It is thought that antibody
ingested with the blood meal neutralises the gametes as they are formed. Surface radio-iodination of gamete and zygote preparations has been used to identify proteins exposed to the neutralising antibodies.\textsuperscript{41,42} A large number of proteins have been identified in this way and minor components, perhaps protective antigens, may have yet to be revealed. Monoclonal antibodies have been generated to gamete preparations of \textit{P.gallinaceum}\textsuperscript{43,44} and \textit{P.falciparum}\textsuperscript{45} to facilitate study of particular proteins. Two monoclonal antibodies were found which acted synergistically to prevent fertilisation probably by agglutinating male gametes.\textsuperscript{43} The antigens recognised by monoclonal antibodies which prevent infection of mosquitoes have been reported as 250 K, 59 K and 53 K.\textsuperscript{45} For \textit{P.gallinaceum} an analogous set of proteins has been reported plus an extra protein of 25 K.\textsuperscript{45}

Another extracellular stage of the malaria parasite is the merozoite and the proteins exposed at its surface have also been investigated. Viable \textit{P.knowlesi} merozoites have been isolated and surface-radioiodinated by the lactoperoxidase technique.\textsuperscript{46} SDS-PAGE and autoradiography revealed eight, strongly labelled, protein bands (150 K, 105 K, 75 K, 55 K, 53 K, 45 K, 34 K and 22 K) plus fifteen weakly labelled protein bands. Trypsin treatment, which also only affects externally exposed proteins, reduced merozoite viability from 70 per cent to 0.4 per cent and cleaved only two of the heavily labelled protein bands (150 K and 105 K to 70 K and 62 K). These two proteins could be protective antigens.

A similar study on \textit{P.falciparum} merozoites\textsuperscript{47} identified proteins with sizes apparently unrelated to those observed with \textit{P.knowlesi}. Merozoite proteins were metabolically labelled, during schizogony, with radioactive amino acids and merozoites, isolated subsequently, were treated with trypsin or chymotrypsin, which markedly reduced
viability. Total labelled merozoite proteins before and after protease treatment were compared by SDS-PAGE and autoradiography. Cleavage of five proteins was observed (3 were >200 K and the others were 130 K and 94 K) which were therefore classified as surface components and potentially protective antigens. It should be noted however that the viability of these *P. falciparum* merozoite preparations before protease treatment was only 10 per cent.

The alternative to investigation of all proteins at exposed parasite surfaces is to characterise particular parasite proteins which must be exposed because of their function. This has the advantage over the total surface protein approach that protective antigens present as minor parasite components could be identified.

One function of a merozoite surface protein which has been exploited in this way is the receptor which recognises and binds to erythrocytes prior to invasion. Major red blood cell sialoglycoproteins probably carry the determinants which the merozoite receptor interacts with. So, Jungery *et al* coupled sialoglycoproteins or N-acetyl glucosamine (a carbohydrate residue of sialoglycoproteins) to Sepharose 4B beads and purified, by affinity chromatography, 3 minor parasite proteins (140 K, 70 K and 35 K) which may include the receptor. Immunisation with the purified receptor could generate antibodies which would interfere with merozoite invasion of erythrocytes.

4.4 **Identification of protective antigens by protective immunisation.** The above procedures only identify antigens which are potentially protective. To prove a parasite protein to be a protective antigen requires demonstration of protection to malaria upon immunisation with the purified protein. Dubois *et al* achieved protection to *P. falciparum* in the squirrel monkey by immunisation with two partially purified parasite preparations. Schizont stage erythrocytic parasites
were purified from red blood cell material and total proteins were separated by SDS-PAGE. Protein was prepared from two regions of the gel (70 K to 85 K and 90 K to 120 K) and squirrel monkeys immunised with these preparations were protected to challenge with erythrocytic stage parasites. In this study, protective antigens have been partially purified, but each fraction still contains a large number of proteins and these must be separated by another technique before the protective antigens can be identified.

Other protective antigens have been identified by immunisation experiments and these are discussed individually below because of the large amount of attention they have received.

4.5 Histidine-rich proteins and proteins of the plasma membrane of infected erythrocytes. A histidine-rich protein (HRP) has been purified from *P. lophurae* and used to immunise ducklings to *lophurae* malaria.\(^{52}\) Despite some controversy over these results,\(^ {53-55}\) the HRP and related proteins have been well studied since the original report. The HRP was purified from cytoplasmic granules in erythrocytes infected with *P. lophurae*.\(^ {56}\) Initial characterisation revealed that the HRP contains 73 per cent histidine and another 22 per cent of the protein consists exclusively of proline, alanine, glutamic acid and aspartic acid. It is insoluble at neutral pH and so had to be extracted in 0.9 N acetic acid. By SDS-PAGE, the HRP migrates with an apparent molecular weight of 53 K and the N-terminal 25 amino acids have now been sequenced.\(^ {57}\)

Histidine-rich proteins have also been detected in *P. falciparum* infected erythrocytes. Initially the knob protein (KP) of 80 K was studied because its synthesis correlated with the presence of knob-like protrusions on the surface of erythrocytes infected with different parasite strains.\(^ {11,58}\) Later, the HRP of *P. lophurae* and the KP of
P.falciparum were shown to have a similar amino acid composition and to be antigenically related. Studies have now revealed a second histidine-rich protein of 65 K which is present in both "knobless" and "knobby" P.falciparum strains and appears to be more homologous to the HRP than does the KP. Apart from the immunisation studies using P.lophurae HRP, there is further evidence which suggests that this family of related, histidine-rich proteins could be protective antigens. Sequestration of P.falciparum erythrocytic stages in post-capillary venules is mediated by the "knobs" and may improve parasite survival by preventing infected erythrocytes from circulating through the spleen. Strain-specific antibodies in immune sera from Aotus monkeys inhibit and reverse the in vitro binding of infected erythrocytes to amelanotic melanoma cells, a model of sequestration. Also, passive transfer of immune sera to Aotus monkeys inhibits P.falciparum sequestration in vivo. However, there are a large number of parasite-specific proteins exposed on the surface of infected erythrocytes as studies on both P.knowlesi and P.falciparum have revealed. So, antibodies preventing sequestration may not be binding to the histidine-rich "knob" protein.

4.6 The abundant high molecular weight antigen of mature erythrocytic stages. An antigen with many common characteristics has been identified in several Plasmodium species and there is evidence that it is protective. The antigen has a high molecular weight; 230/235 K in P.yoelii, 250 K in P.chabaudi and P.berghei, 250 K in P.knowlesi and 190/195 K in P.falciparum. Monoclonal antibodies to this antigen give the same indirect immunofluorescence pattern in all species, staining mature erythrocytic stages and in particular the surface of intra- and extra-cellular merozoites. Immuno-
precipitation studies to investigate synthesis of the antigen revealed complex post-translational processing events occurring at the time of schizont rupture and/or red blood cell reinvasion which generate numerous smaller molecular weight products.\textsuperscript{68,72-74,78,79} Investigation of this processing may be complicated by artefactual cleavage of the full size protein, during extraction, by proteases only present in mature erythrocytic stages.\textsuperscript{72} The high molecular weight antigen is a major protein synthesised by mature erythrocytic stages and has been shown to be glycosylated in several species.\textsuperscript{69,72,75}

The high molecular weight antigen also demonstrates antigenic diversity between isolates of each species and this has been investigated quite thoroughly for \textit{P.falciparum} using monoclonal antibodies.\textsuperscript{35,74,76} The antigen appears to consist of a constant and a variable region. Monoclonal antibodies to the constant region also react with young erythrocytic stages although the antigen is only synthesised by the mature stages. Processing may result in cleavage of the variable region during the merozoite stage so that only the constant region is carried into the red blood cell at reinvasion. Also, the antigenic cross-reaction of the high molecular weight protein from different species\textsuperscript{68,194} may be due to homology in this constant region.

There are several items of evidence to suggest that the high molecular weight antigen is protective. It is well accepted that antibodies to the merozoite surface interfere with red blood cell invasion.\textsuperscript{80} However, monoclonal antibodies to the high molecular weight antigen can block reinvasion by \textit{P.knowlesi} merozoites \textit{in vitro}\textsuperscript{71} and can protect rodents to challenge infection with \textit{P.yoelii}\textsuperscript{77} and \textit{P.chabaudi}\textsuperscript{81} erythrocytic stages \textit{in vivo}. Protective immunisation has now been achieved using the high molecular weight antigen in purified form, for \textit{P.yoelii} in mice\textsuperscript{82} and for \textit{P.falciparum} in \textit{Saimiri} monkeys.\textsuperscript{83}
The processing of this protective antigen generates many smaller fragments which could also induce protection. It is possible that potentially protective antigens identified in other ways could be processing products of the high molecular weight antigen. The large number of potentially protective antigens which have been labelled with different molecular weights could be generated from just a few primary translation products.

4.7 The circumsporozoite protein. Another family of related proteins identified in several Plasmodium species may be protective. The sporozoite surface is mainly composed of the circumsporozoite protein (CSP) which has been characterised using monoclonal antibodies. The CSP can be radiolabelled by surface radioiodination of the sporozoite and the apparent molecular weights of the CSPs of different species have been determined by immunoprecipitation and SDS-PAGE. Metabolic labelling prior to immunoprecipitation also reveals two, intracellular, precursor polypeptides (IS1 and IS2) with larger apparent molecular weights and CSP, IS1 and IS2 account for 5 to 20 per cent of $[^{35}S]$-methionine incorporated by sporozoites (Table 3). The processing to mature CSP appears to proceed in two steps with the sequential removal of 2 K and then approximately 8 K peptide fragments in all species. Analysis by 2-dimensional electrophoresis (iso-electric focussing plus SDS-PAGE) also reveals that in all species processing to mature CSP involves loss of a polypeptide containing relatively basic residues. The homology of CSPs and ISs within and between species has been further confirmed by high performance liquid chromatography and from interspecies cross-reaction of some anti-CSP monoclonal antibodies.
Table 3. Molecular weights of the circumsporozoite proteins and their precursors.

<table>
<thead>
<tr>
<th>Species</th>
<th>CSP (K)</th>
<th>IS1 (K)</th>
<th>IS2 (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P.berghei</em></td>
<td>44</td>
<td>52</td>
<td>54</td>
</tr>
<tr>
<td><em>P.knowlesi</em></td>
<td>42</td>
<td>50</td>
<td>52</td>
</tr>
<tr>
<td><em>P.cynomolgi</em></td>
<td>48</td>
<td>56</td>
<td>58</td>
</tr>
<tr>
<td><em>P.vivax</em></td>
<td>45</td>
<td>51 (may appear as a doublet)</td>
<td></td>
</tr>
<tr>
<td><em>P.falciparum</em></td>
<td>58</td>
<td>65</td>
<td>67</td>
</tr>
</tbody>
</table>

The evidence that the CSP is protective is not yet complete. The immunity that can be obtained by immunisation with attenuated sporozoites and can be naturally acquired appears to correlate with the antibody response to the CSP. Furthermore, some of the monoclonal antibodies to CSP can neutralise sporozoites both in vitro, for *P.berghei*, *P.knowlesi* and *P.vivax* and *P.falciparum*, and in vivo for *P.berghei*. However, the CSP has not been proven to be a protective antigen, because purified CSP has not yet been used to immunise and protect against a sporozoite challenge. For in vitro sporozoite neutralisation, monoclonal antibodies were present at high concentration (in the range of mg/ml) and an immune response to inoculated CSP may not be so effective. Immunisation studies are not yet possible because the sporozoite stages, from which the CSP is prepared, can not be isolated in sufficient quantities.

4.8 Proving potentially protective antigens to be protective. A large number of potentially protective antigens of malaria parasites have been identified and it is important to determine which of these antigens are really protective. To prove that an antigen is protective, protection to malaria upon immunisation with purified...
antigen must be demonstrated. However, antigen preparations are limited by the availability of malaria parasites and quantities sufficient for immunisation can not be readily isolated. Sufficient quantities could be provided by recombinant DNA techniques, isolated genes being used to direct antigen synthesis. This would have two further advantages. First, if the antigen is protective then the gene has already been isolated and the first step towards production of large quantities of antigen for use in a vaccine will have already been taken. Second, the complication of post-translational processing of proteins, as with the abundant, high molecular weight antigen, is avoided when working with the genes for those proteins. So, the large array of potentially protective antigens identified for *P. falciparum* may be simplified. Some antigen genes have already been cloned.

5. **Isolation of genes for malaria antigen**

Particular antigen genes must be isolated from a recombinant DNA library of total malaria parasite genes. Both genomic DNA\(^1\) and cDNA\(^2\) libraries have been constructed for this purpose.

5.1 **Plasmodium rRNA genes.** Ribosomal RNA genes have been isolated from genomic DNA libraries for *P. falciparum*,\(^1,3\) *P. berghei*\(^4-7\) and *P. lophurae*.\(^8,9\) These genes were isolated because nucleic acid probes, with which the recombinant clones containing rDNA sequences were detected, could be readily prepared. For *P. berghei*\(^4\) and *P. lophurae*\(^8\) the probe was prepared from rRNA purified from the respective parasite, where as for *P. falciparum* the conservation of rRNA sequences was capitalised on by probing with an rDNA sequence from *Drosophila melanogaster*.\(^1\) These rRNA genes provide a model system for study before the isolation and analysis of antigen genes.
Isolated rRNA genes were examined by restriction enzyme mapping and electron microscopy of RNA:DNA duplexes. All \textit{Plasmodium} rRNA genes so far isolated are probably present in the typical eukaryotic arrangement, 5'-small rRNA-spacer-5.8S rRNA-spacer-large rRNA-3' within a single transcription unit with 55 rRNA genes apparently unlinked. However, in all three \textit{Plasmodium} species a small number of rRNA genes were detected and these are not tandemly arranged or even linked, properties unexpected for eukaryotic genomes. For \textit{P.falciparum} and \textit{P.berghei} there are probably 4 and 2 copies respectively of each of 2 types of transcription unit per haploid genome. For \textit{P.lophurae}, there may be 7 or 8 transcription units per haploid genome which fall into 4 different classes.

The division of \textit{P.berghei} rRNA genes into two different transcription units may be functional as well as structural. Ribosomal RNA from the asexual erythrocytic stages is fully homologous to only one type of transcription unit. From duplex analysis, the coding regions of the two types of transcription unit have segments lacking homology between segments of homology and this observation has been taken as evidence that both types of transcription unit are functional. These data have led to the interesting theory that different rRNAs may be used at different points in the life-cycle of malaria parasites, though other explanations were also presented. This theory and evidence suggesting that \textit{P.falciparum} and \textit{P.lophurae}, but not \textit{P.berghei}, rRNA genes have introns require further investigation.

5.2 Categories of clones within cDNA libraries. The large number of recombinant clones generated in the construction of a cDNA library means that not all can be investigated at one time. This problem can be solved by concentrating on a subset of clones distinguished by a property which could mean that they are of interest. Once a
manageable number of clones have been isolated they may be individually examined and the interest of each evaluated.

In a study of a P.chabaudi cDNA library prepared using the E.coli plasmid pBR322 with messenger RNA from ring stage, erythrocytic forms, 9 clones out of 250 examined were found to cross-hybridise with P.falciparum ring stage cDNA. Hybridisation with P.chabaudi schizont-specific cDNA showed two of the clones to be ring stage specific whilst the other seven contain genes probably expressed throughout the erythrocytic cycle. The cDNA fragments were sized and the protein encoded by three of them was identified by hybrid selected translation. It was observed, while using a rabbit anti-
P.falciparum antiserum to immuno-precipitate hybrid selected translation products, that the degree of homology of the cDNA sequences did not correlate with the degree of immunological cross-reaction.

Kemp et al identified a subset of P.falciparum cDNA clones which encoded antigens recognised by sera of individuals immune to falciparum malaria. These clones would be expected to include those encoding protective antigens. An expression library was prepared by cloning cDNA from erythrocytic forms of P.falciparum into a derivative of λgt11. This vector directs the synthesis of recombinant proteins of E.coli β-galactosidase fused to a polypeptide encoded by the cloned cDNA insert.

The library was screened for those clones expressing a fusion protein which could be recognised by an anti-P.falciparum antigen preparation of IgG purified from immune human serum by affinity chromatography. A few hundred antigen positive clones were purified. Although these may include several clones for each antigen, this is still a large number of clones to be investigated before those coding for protective antigens may be identified. Potentially
protective antigen clones may be distinguished by a correlation between recognition of a fusion protein by a battery of sera and the immune status of the sera donors. Alternatively, antisera raised to the fusion protein may be evaluated for a protective effect.

Another category of cDNA clones which have been prepared encode proteins expressed specifically by late erythrocytic stages. Protective immunity against erythrocytic stages of malaria parasites is thought to be mainly directed against antigens on the surface of schizonts and merozoites which are only synthesised late in the erythrocytic cycle. So, schizont-specific genes are likely to code for protective antigens.

A cDNA library was prepared in pBR322 using mRNA isolated from unsynchronised *P. falciparum* asexual stages. The library was then screened with \([^32P]\) cDNA probes prepared from ring stage mRNA or schizont stage mRNA isolated from synchronised cultures. Out of 10,000 clones screened, approximately 150 were schizont specific and from cross-hybridisation studies using the cDNA inserts these could be assigned into 12 non-overlapping families. It was concluded that 12 schizont/merozoite specific genes had been cloned.

Investigation of these 12 genes may continue by identification of the proteins that they encode for correlation with antigens known or thought to be protective. Alternatively, the 12 cloned genes may be expressed in bacteria and antisera raised to the recombinant proteins may be examined for protective properties.

5.3 Isolated genes encoding antigens of *Plasmodium* species. Individual *Plasmodium* antigen genes have been isolated by a variety of procedures. In all cases, the potential of the encoded protein to elicit a protective response upon immunisation could now be checked because the antigen may be expressed to high level, from the isolated gene, in *E. coli*. 
Coppel et al.\textsuperscript{103} constructed a \textit{P. falciparum} erythrocytic stage cDNA expression library by cloning into the \(eta\)-lactamase gene of pBR322. However, \textit{in situ} immunological procedures failed to identify any clones expressing detectable levels of malaria parasite antigens. So, detergent extracts of 70, randomly chosen, cDNA recombinant clones were used to immunise mice and the antisera generated were assayed by indirect immunofluorescence microscopy against erythrocytic stage parasites. One positive clone was identified which expressed a protein of mature erythrocytic stages. Immunoprecipitation, using the mouse sera, showed the cloned gene to encode a protein of 120 K which was also recognised by human immune sera. The 120 K protein was shown to be common to two other \textit{P. falciparum} isolates, unrelated to that used to generate the cDNA library. Immunological cross-reaction was observed using the mouse antisera in immunoprecipitation studies and homology in the gene was detected using the cloned gene as a probe in Southern hybridisations to genomic DNA. However, there is no evidence to suggest that the 120 K protein is protective.

The \textit{P. falciparum} antigen encoded by another cloned gene was also identified by immunisation with bacterial lysates, however, in this case, the bacterial lysates were prepared from clones already known to be synthesising malaria parasite antigens. As discussed above (Introduction 5.2) a category of cDNA recombinant clones had been identified by Kemp \textit{et al.}\textsuperscript{101} which expressed antigens recognised by immune sera and Coppel \textit{et al.}\textsuperscript{104} fully characterised one of these clones, Ag16.

A rabbit antiserum prepared to the \textit{\textbeta}-galactosidase-antigen fusion protein of Ag16,\textsuperscript{104} immunoprecipitated a 220 K \textit{P. falciparum} protein with a \textit{pI} of 4.2. Indirect immunofluorescence microscopy on erythrocytic stages using this antiserum showed the antigen to be located on
the surface of intra-cellular trophozoites and schizonts and in small extra-cellular particles. This immunological reaction was isolate specific. These properties were similar to those reported for the isolate-specific S-antigen, a soluble, heat-stable antigen released to the sera of infected individuals. Proof of the identity of the S-antigen and the protein encoded by Ag16 came from a comparison of immunoprecipitates using the anti-fusion protein antiserum against two P.falciparum isolates for which the S-antigens are immunologically closely related but differ in molecular weight. Also, Southern hybridisation using the isolated gene to probe genomic DNA revealed homology only with the isolate which expressed immunologically related S-antigen and furthermore, the differences in size of detected restriction fragments would account for the differences in the S-antigen molecular weights.

The cDNA insert had an unusual sequence which encoded 23 identical repeats of 11 amino acids. The DNA sequences differed between repeats at the third base of 4 codons such that the amino acid sequence was not affected. It was suggested that this unusual structure may be important for defence to the immune system of the host. The complete absence of this repeating structure in other P.falciparum isolates argues against it having a functional role in parasite growth or metabolism, but the variation could be a mechanism of avoiding an immune response.

The high percentage of histidine residues (73 per cent) in the histidine-rich protein (HRP) of P.lophurae was exploited in the isolation of the gene for this antigen. A cDNA library, cloned in pBR322, was screened by colony hybridisation using the 15 bp mixed pentahistidine oligonucleotide probe, 5' GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT GT 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identified. The coding region contained 16 tandemly repeated 30 bp units which each encode 8 contiguous histidine residues plus two other amino acid residues. These clones were used in this study to characterise the mRNA and gene for the knob protein (KP) of *P. falciparum*, possible because of sequence homology between the HRP and KP.

There have been two studies with the general aim of cloning genes of high molecular weight proteins of *P. falciparum*, mature, erythrocytic stages. There is schizont-specific synthesis of a number of high molecular weight proteins which have been implicated as protective antigens and further investigations require larger quantities of these proteins. Odink et al. size-fractionated poly(A)⁺ mRNA by centrifugation on sucrose gradients and identified fractions containing mRNAs for high molecular weight proteins by *in vitro* translation. Complementary DNA synthesised from mRNA of selected fractions was used in Southern hybridisations to restriction enzyme digested genomic DNA. A genomic DNA library was prepared by cloning HindIII/EcoRI double digestion fragments or HindIII partial digestion fragments, of the sizes identified in the Southern hybridisation, into pAT153. Two gene fragments were isolated from these libraries by *in situ* colony hybridisation using the same cDNA probe. Hybrid selected translation demonstrated that both recombinant cDNA plasmids encoded 145 K proteins, at least one of which could be immunoprecipitated by pooled immune human sera. Unfortunately, a link between the 145 K protein(s) synthesised by *in vitro* translation and a protein of similar size synthesised *in vivo* has not yet been possible.

In a second study aimed at cloning genes of high molecular weight proteins, Odink et al. again size fractionated mRNA by centrifugation on a sucrose gradient and used cDNA, prepared to mRNA
of selected fractions, to probe \textit{P.falciparum} gene libraries by \textit{in situ} colony hybridisation. In this instance, the library was generated by cloning cDNA, prepared from mature erythrocytic stage poly(A)$^+$ RNA, into pUC8. Of 300 recombinant plasmids, 12 gave strong hybridisation signals and these were collected into 6 groups on the basis of cross-hybridisation of cDNA inserts. The cDNA insert of 3 of 6 selected clones (one from each group) hybridised, in northern blots of \textit{P.falciparum} RNA, to mRNAs long enough to encode high molecular weight proteins. Of these, one was shown to encode part of the abundant, schizont-specific, 195 K protein: The 800 bp cDNA insert was re-cloned into the expression vector \textit{pATtrp} and the fusion protein generated was specifically recognised in a western blot by a rabbit anti-195 K protein antiserum. In this way a link between an isolated gene and a high molecular weight protein was established. However, no further data concerning this cDNA clone were presented.

Simultaneously, a cDNA clone, which probably encodes the same protein, in this case called P190, was identified by a different method and the cDNA insert has been fully sequenced. Hall et al.\textsuperscript{83} cloned cDNA prepared from \textit{P.falciparum}, erythrocytic stage, poly(A)$^+$ RNA into the expression vector $\lambda$gt11::Tn5. Screening by an \textit{in situ} immunological procedure using a rabbit anti-P190 antiserum identified 4 positive, apparently identical clones in an amplified library which initially contained 7,000 independent recombinants.

The identity of one of the clones was confirmed by a technique called "antibody select". The expressed recombinant protein was used to adsorb IgG from the original rabbit anti-P190 antiserum and this reacted to P190 and its processed products in a western blot of total, \textit{P.falciparum}, erythrocytic-stage proteins. Therefore, the clone was not recognised by antibodies raised to a protein contaminant of the P190 preparation which had been used to generate the rabbit antiserum.
The cDNA insert has been used as a probe for northern blots of *P. falciparum* erythrocytic stage RNA and for Southern blots of genomic DNA of different *P. falciparum* isolates. In the former, a 6.7 kb mRNA was detected, and in the latter, a single band was always detected, though the size varied slightly between isolates, an observation perhaps important in connection with the antigenic variation of this protein. Sequencing of the cDNA insert revealed a region encoding 70 amino acids, a 3'-untranslated region and a poly(A) tail. This arrangement resulted from cloning the 3' end of the mRNA and in conjunction with the antibody select data implies that processing of P190 does not involve cleavage from the C-terminus.

The best studied *Plasmodium* gene is that encoding the circumsporozoite protein (CSP) of *P. knowlesi*. The CSP gene was also isolated from an expression library, poly(A)+ RNA of *P. knowlesi* infected mosquito thoraxes being used to prepare cDNA for cloning into pBR322. Pools of 48 recombinants were examined using monoclonal antibodies to *P. knowlesi* sporozoite surface antigens in a two-site immunoradiometric assay. The three positive clones identified were shown to cross-hybridise.

One of the cDNA clones when used as a probe in a Southern hybridisation failed to detect homology in uninfected mosquito DNA but hybridised to a large EcoRI fragment of *P. knowlesi* erythrocytic stage DNA. This result confirmed that the identified clones were of parasite origin. The same cDNA probe was used in a northern hybridisation to RNA from *P. knowlesi* erythrocytic stages and from infected and uninfected mosquitoes. A homologous mRNA transcript was only detected in the infected mosquito RNA and was approximately 2,050 nucleotides long. The cDNA inserts were only 350, 1,200 and 1,200 base pairs so none contain the entire gene.
The 350 bp insert was re-cloned into M13mp9 for sequencing and was found to consist of seven 36 bp repeats with an incomplete repeat at either end. The amino acid sequence was deduced from the nucleotide sequence and each 36 bp repeat encoded an identical amino acid sequence, the only nucleotide variations occurring in the degenerate third position of two codons. The deduced reading frame was proven by synthesis of dodeca- and tetraeicosa-peptides which contain one and two copies of the CSP amino acid repeat and contain the epitope recognised by the CSP monoclonal antibody. The 1,200 bp cDNA inserts were also sequenced and contained twelve, tandem copies of the 36 bp repeat which were flanked by sequences with no resemblance to the repeat.

The genomic copy of the *P. knowlesi* CSP gene was isolated. A library of EcoRI cleaved genomic DNA fragments cloned into a λ vector was probed with one of the CSP cDNA clones by in situ plaque hybridisation. Five clones were identified each containing an 11 kb fragment the size predicted from Southern hybridisation studies. After restriction enzyme mapping the region containing the CSP gene was sequenced.

A single long open reading frame was detected which ran through the twelve copies of the 36 bp repeat found in the cDNA insert and which was consistent with the reading frame determined in earlier studies. Analysis of the complete, deduced amino acid sequence for the *P. knowlesi* CSP reveals an N-terminal signal peptide, the twelve repeats flanked by charged residues and a C-terminal anchor sequence. The calculated molecular weight (36.8 K) differs markedly from the apparent molecular weight (52 K) as determined by SDS-PAGE but this could be due to anomalous electrophoretic behaviour caused by the repeating amino acid structure. There is no evidence for introns in the CSP gene.
An elegant paper describes the generation of an antiserum to the \emph{P.knowlesi} CSP by infection of rabbits with a live vaccinia virus into the genome of which had been inserted the CSP gene. However, the rabbit antiserum generated was not reported to neutralise \emph{P.knowlesi} sporozoites \textit{in vitro} a technique commonly used to demonstrate antibody to be protective. There have been no reports yet of immunisation with a recombinant malaria protein generating an antiserum which neutralises malaria parasites, to any degree, either \emph{in vivo} or \emph{in vitro}. Once this has been achieved the feasibility of using molecular biology to develop a malaria vaccine will have been demonstrated.

6. \textbf{Scope of this thesis}

A bank of monoclonal antibodies had been raised to the erythrocytic stages of a Thai isolate, K1, of \emph{P.falciparum} by J. McBride and G. Morgan. I screened these monoclonal antibodies by two techniques to identify those monoclonal antibodies which recognise potentially protective antigens.

The first technique was the assay for an ability of each monoclonal antibody to inhibit the erythrocytic multiplication of \emph{P.falciparum}, isolate K1, \textit{in vitro} and inhibitory monoclonal antibodies were identified. The second technique was indirect immunofluorescence microscopy which revealed the cross-reaction of one anti-erythrocytic stage monoclonal antibody to the surface of the \emph{P.falciparum} sporozoite stage. The erythrocytic stage antigen which the cross-reactive monoclonal antibody recognised was characterised and the gene for this antigen was isolated, sequenced and expressed in bacteria.
Materials and Methods. Chapter 1.

In vitro cultivation of *Plasmodium falciparum*
1.1 Summary of parasite cultivation

The continuous culture of the erythrocytic stages of \textit{P. falciparum} was reported 8 years ago by Trager and Jensen. The parasites were grown at 37°C in a thin settled layer of human red blood cells with a covering layer of culture medium supplemented with human serum under an atmosphere enriched for CO\textsubscript{2} and depleted of O\textsubscript{2} relative to air. Growth is maintained by diluting with uninfected red blood cells when necessary. The parasitised erythrocytes may be frozen for long term storage, the parasites remaining viable for recovery to culture.

1.2 Culture medium

Two types of medium were used. The first, "Jensen" medium, consists of RPMI1640 medium (GIBCO) supplemented with HEPES buffer (SIGMA) to 25 mM, gentamycin sulphate (SIGMA) to 50 mg/l, and NaHCO\textsubscript{3} (BDH) to 2g/l. The second, "Zolg" medium, consists of RPMI1640 medium (GIBCO) supplemented with D-glucose (Fisons) to 2 g/l, TES buffer (SIGMA) to 6.87 g/l, hypoxanthine (SIGMA) to 50 mg/l, gentamycin sulphate (SIGMA) to 50 mg/l, reduced glutathione (SIGMA) to 500 mg/l, methyl cellulose (SIGMA) to 1 g/l and NaHCO\textsubscript{3} (BDH) to 2.25 g/l. The pH was adjusted to pH 7.25 with 1 M NaOH. Both media were filter-sterilised using Millipore 0.22 μm filters and supplemented to 10 per cent with sterile pooled human serum obtained from Edinburgh Blood Transfusion Service.

The culture medium overlaying the settled red blood cells was replaced every 24 hrs. All manipulations with cultures were carried out in a sterile air flow hood. The culture vessels were gently tipped so as not to disturb the red blood cell layer and the spent medium removed by pipette. Fresh medium, prewarmed to 37°C, was then added and mixed with the infected red blood cells.
1.3 Culture vessels

Two sets of apparatus were used for culturing the parasites. In the "candle jar" method\textsuperscript{113,115} petri dishes (Sterilin) were used as the culture vessels. 85 mm diameter plates contained 1 ml of packed red blood cells and 10 ml of medium and 35 mm diameter plates contained 250 μl of packed red blood cells and 5 ml of medium. The correct atmosphere was obtained by placing the petri dishes in a glass desiccator with a candle. The candle was lit as the lid was sealed in place with silicone grease and the stop cock closed when the candle flame went out. This provides an atmosphere of 2-3 per cent CO\textsubscript{2}, 14-17 per cent O\textsubscript{2}.\textsuperscript{113} The desiccator was then incubated at 37°C.

Alternatively, tissue culture flasks (Falcon) were used. 50 ml flasks contained 0.5 ml of packed red blood cells and 10 ml of medium and 250 ml flasks contained 5 ml of packed red blood cells with 50 ml of medium. The correct atmosphere was obtained by flushing the flasks with a gas mixture of 95 per cent N\textsubscript{2}, 3 per cent O\textsubscript{2} and 2 per cent CO\textsubscript{2} and then closing the flasks tightly. The flasks were incubated at 37°C.

1.4 Culture dilution

Dilution of infected with uninfected red blood cells was necessary every 2-4 days to maintain the parasitaemia (percentage of infected red blood cells to total red blood cells) below 10 per cent. Human blood, from Edinburgh Blood Transfusion Service, was used up to 4 weeks after its withdrawal date. The blood type used was 0-Rh\textsuperscript{+} so that pooled human serum could be used in the culture medium. The red blood cells were washed 3x in culture medium lacking serum at 4°C with centrifugation (650 g, 10 mins, 4°C). Uninfected cells were mixed with infected cells from a culture prior to distribution to
fresh culture vessels and addition of culture medium plus serum.

The increase in parasitaemia was followed by making blood
smears on microscope slides every 24 hrs immediately after spent
medium had been removed. The blood smear was air dried, fixed in
methanol for 30 seconds and stained with 10 per cent Giemsa stain
(BDH) for 30 min. The stained blood smear was rinsed with water
and then examined by light microscopy at 1,000x magnification with
an oil immersion objective. At least 3,000 red blood cells were
counted and the percentage infected gave the parasitaemia. Typically,
the parasitaemia of a healthy asynchronous culture increased 2-3
to fold every 24 hrs.

1.5 Synchronisation

An asynchronous culture of *P.falciparum* with a high proportion
of ring stages was synchronised as required by treatment with
D-sorbitol. The red blood cells were centrifuged (850 g, 5 min,
room temperature) and the pelleted cells were resuspended in 5 volumes
of 5 per cent D-sorbitol for 5 min at room temperature. The
cultures were re-established by washing in 1 volume of culture medium
and diluting with washed uninfected red blood cells as necessary.
Sorbitol treatment destroys the trophozoite and schizont stages and
leaves intact the young ring stages with a spread in age of approx-
imately 10 hrs. This lysis is not due to osmotic shock of the more
fragile mature stages, because the osmolality of 5 per cent D-sorbitol
is close to physiological, but may be due to their increased
permeability. Synchrony was checked by examination of Giemsa
stained blood smears.

1.6 Saponin lysis of parasitised erythrocytes

Parasites were released from red blood cells by treatment with
saponin. Parasitised erythrocytes were washed once in Phosphate
Buffered Saline (PBS) and lysed in 10 volumes 0.1 per cent Saponin white (BDH) in PBS at 0°C, 5 min. Free parasites were then washed 3x in PBS at 4°C with centrifugation (1000 g, 10 min). Cytosolic red blood cell proteins were removed from the parasite preparation by thorough washing in this way. Red blood cell membrane ghosts, which formed a white layer over the harder black parasite pellet, could also be partly removed with care during the washing steps. This treatment leaves a pellet of intact parasites surrounded by collapsed red blood cell membranes. Many of the experiments involving western blotting (Materials and Methods 2.3.3) included a track labelled "total protein from a saponin-lysed parasite preparation". In each case, except where indicated, the parasite preparation was prepared by saponin lysis of 0.5 ml packed red blood cells, 5 per cent parasitaemia and the parasite proteins were solubilised directly in Laemmli SDS gel sample buffer (50 μl) (Materials and Methods 2.3) for analysis.

1.7 Freezing P.falciparum culture lines

When growing parasites were not required isolates of P.falciparum were stored in a frozen state. A freezing solution of 28 ml glycerol and 72 ml 4.2 per cent D-sorbitol, 0.9 per cent NaCl was filter sterilised. Parasitised erythrocytes from a healthy culture were pelleted (650 g, 10 min, room temperature) and mixed with an equal volume of the freezing solution. Aliquots of 0.5 ml were frozen in liquid N₂ for storage over liquid N₂.

Frozen ampoules were returned to culture by rapidly thawing at 37°C and pelleting the parasitised red blood cells (650 g, 10 min, room temperature). The pelleted cells were washed in an equal volume of sterile 3.5 per cent NaCl and then 3 times in complete culture medium. After diluting with an equal volume of washed uninfected red blood cells the parasites were returned to culture
as above, except that initially the culture medium was supplemented to 15 per cent with human serum. Dilution with an equal volume of red blood cells was repeated every other day and high parasitaemias were reached within 7-10 days.

1.8 [G-3H]-Hypoxanthine incorporation growth assay

The use of [G-3H]-hypoxanthine incorporation as a measure of growth in vitro of P.falciparum was first reported in a semi-automated technique for scanning new antimalarial drugs. It has since been used to measure inhibition of parasite growth by antibodies. The incorporation obtained is a direct measure of the parasitaemia reached.

The assays were carried out using healthy asynchronous cultures of P.falciparum, isolate K1. Parasitised erythrocytes were diluted with washed uninfected red blood cells and "Zolg" culture medium plus serum (but lacking hypoxanthine). The final cell suspension, 1 per cent haematocrit (proportion of packed red blood cell volume to total volume) and 1 per cent parasitaemia was distributed to U-shaped 96-well microtitre plates (Sterilin), 100 µl/well. In test assays, 10 µl of mouse ascitic fluid or other test solution filter sterilised through 0.22 µm (Millipore) filters was added per well with mixing. The plate was then incubated by the "candle jar" method.

Wells at the edge of the plate consistently gave reduced incorporation. This was attributed to evaporation altering the culture medium composition. As a result, these wells were never used in experiments and all unused wells of a plate were filled with 100 µl of sterile water.
After 24 hrs \([G^{-3}H]-hypoxanthine\) was added to each well. \([G^{-3}H]-hypoxanthine\) (Amersham) was supplied freeze dried with a specific activity of 1 Ci/mmol (37 GBq/mmol), (Mol wt 136). The labelled nucleotide was dissolved in 50 per cent ethanol to give 0.5 mCi/ml (18.5 MBq/ml), and stored at -20°C. Label of the required quantity was placed under vacuum for 2 hrs to remove the ethanol and then mixed with "Zolig" medium plus serum (but lacking hypoxanthine) to give 0.1 μCi/μl (3.7 KBq/μl). 10 μl of this prepared label was added to each well with mixing and the microtitre plate was incubated again by the "candle jar" method.

48 hrs after the test plate was set up the wells were harvested. The entire contents of each well were placed on 22 mm glass fibre filters (Whatman) and washed through with 10 ml each of 10 per cent Trichloroacetic acid (TCA) (3x), 5 percent TCA, Ethanol/Ether (1:1, vol:vol) and Ether. Once dry, the filters were placed in scintillation fluid (9 g 2,5-Diphenyloxazole, (PPO) (Beveridge) and 252 mg 1,4-bis[2-(5-Phenyloxazolyl)] benzene, (POPOP) (BDH) per 2.5 l Toluene) and incorporated tritium was counted using an Intertechnique SL 3000 scintillation counter.
Materials and Methods. Chapter 2

Antibody and protein techniques
2.1 Hybridoma technology

The bank of monoclonal antibodies used in this study were generated by J. McBride and G. Morgan. Female BALB/c mice were immunised intraperitoneally with *P. falciparum* isolate K1, erythrocytic stages from an asynchronous culture which had been released from host red blood cells by saponin lysis. Injections of 100 to 200 µg of total protein in incomplete Freund's adjuvant was carried out 2 or 3 times at 2 week intervals with a final intravenous challenge of parasites in saline without adjuvant. Three days later the mice were killed and the spleen cells used for fusion with NS-1/1-Ag4-1 (NS-1) myeloma cells. Two weeks after the fusion hybridoma cell lines producing antibodies to *P. falciparum* were identified by indirect immunofluorescence microscopy and cloned by limiting dilution. The 23 monoclonal antibodies used in the two screens of this study were prepared as ascitic fluids from these cloned hybridoma cell lines by J. McBride. I prepared the 5.1 and 7.7 monoclonal antibodies used subsequently.

The hybridoma cell lines 5.1-1 and 7.7-4 were grown in tissue culture flasks (Falcon) at 37°C in an atmosphere of 5 per cent CO\textsubscript{2} in air with 85 to 95 per cent relative humidity. The culture medium used was RPMI1640 (GIBCO) supplemented with sodium pyruvate to 1 mM, glutamine to 2 mM, penicillin to 500 units/ml and streptomycin to 500 µg/ml (all GIBCO) and containing foetal calf serum (FCS) (Randox Laboratories) at 10 to 15 per cent (v/v). Sodium bicarbonate was added to 0.2 per cent (w/v) to produce a pH of 7.4. The cells were maintained in static culture at a density of 2-6 x 10\textsuperscript{5} cells/ml by dilution with fresh pre-warmed medium every 48 to 72 hrs.

Ascites fluids, which contain higher levels of antibody than culture supernatants, were generated by *in vivo* passage of these
hybridoma cell lines. BALB/C mice were primed 2 to 7 days before tumour inoculation by intra-peritoneal injection of 0.5 ml pristane (2,6,10,14-tetra-methyl pentadecane). Hybridoma cells cultured in vitro (3 x 10^6 cells in 0.5 ml culture medium lacking FCS) were injected intra-peritoneally and ascites tumours developed 7 to 14 days later. Ascites fluid was removed and stored at -70°C.

Once sufficient ascites fluid was obtained the cell lines were retained by freezing and storage over liquid nitrogen. Cells cultured in vitro were pelleted (250 g, 5 mins) and resuspended in a freezing mixture of 10 per cent (v/v) Dimethyl sulphoxide (DMSO) (SIGMA) in foetal calf serum. Aliquots of 10^6 cells in 0.5 ml were frozen by placing in an insulated box at -20°C overnight before transfer to storage over liquid nitrogen.

Cells were recovered to culture as required. They were thawed rapidly in a 37°C water bath and 0.5 ml aliquots were mixed immediately with 10 ml of culture medium pre-warmed to 37°C. The cells were pelleted (250 g, 5 mins) and taken up in 2 ml of fresh pre-warmed medium for introduction into a tissue culture flask. The cells were then cultured as above.

2.2 Indirect immunofluorescence microscopy

Two preparations of the erythrocytic stages of P.falciparum were used for indirect immunofluorescence microscopy. For photographing or to examine an immunofluorescence pattern, thin smears of parasitised red blood cells (50 per cent haematocrit, 5 per cent parasitaemia) were dried on to a microscope slide. To titre antibody preparations, a parasitised red blood cell suspension (10 per cent haematocrit, 5 per cent parasitaemia) was air dried to multispot microscope slides (Hendley-Essex Ltd), 20 µl per well. For both preparations the parasitised red blood cells were washed (3x) and resuspended in RPMI
parasite culture medium lacking serum. Slides prepared in this way were used immediately or stored at -20°C with desiccant.

The indirect immunofluorescence reaction was carried out on sporozoite or erythrocytic stage preparations by the same procedure. The slides were placed in acetone for 5 mins and then allowed to dry in air. Antibody preparations were diluted in phosphate buffered saline (PBS) containing 1 per cent (w/v) bovine serum albumin (BSA) and 0.01 per cent sodium azide and 25 μl amounts were applied directly to the slide. PBS is 0.15 M NaCl, 10 mM sodium phosphate, pH 7.2. After incubation for 30 min at room temperature in a humid atmosphere, the slides were washed (3x) in PBS and dried at 37°C for 5 min. Then the second antibody, fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin (Miles-Yeda Ltd) or FITC-conjugated goat anti-human immunoglobulin (SIGMA) diluted (100x) in the same PBS/BSA/azide buffer was applied to the slide in 10 μl amounts. This was again incubated 30 min at room temperature in a humid atmosphere before washing in PBS (3x).

Before examination, the slides were counterstained in Evans blue (BDH) (0.1 per cent in PBS) for 5 min, washed once in PBS and a coverslip was fixed in place with 50 per cent glycerol in PBS. The slides were then examined using an epifluorescence microscope (American Optical-Reichert Jung - microstar 110) at 1,000x magnification. Photographs were taken on Kodak Tri-X Pan film or Kodak Ektachrome.

2.3 Polyacrylamide gel electrophoresis and western blotting

Polyacrylamide gel electrophoresis (PAGE) was carried out using the discontinuous buffer system of Laemmli. Slab gels of 170 mm x 150 mm x 1.5 mm were polymerised between glass plates separated by perspex spacers. Running gels (10 per cent) consisted of:
16.67 ml 30 per cent acrylamide (BDH),
16.67 ml 0.8 per cent N,N'-methylene bisacrylamide (BDH),
6.25 ml 3 M Tris HCl (Boehringer), pH 8.8,
and 10.4 ml distilled water.

The gel mould was sealed by placing it in a trough in which 10 ml of the above running gel solution was polymerised after the addition of:

100 µl 10 per cent sodium dodecyl sulphate (SDS) (SERVA),
150 µl 10 per cent ammonium persulphate (AMPS) (BDH),
and 10 µl N,N,N',N'-Tetramethylethylene diamine (TEMED) (SERVA).

The sealer set in 15 min and the remainder of the running gel was poured after the addition of 400 µl of 10 per cent SDS, 200 µl of 10 per cent AMPS and 20 µl of TEMED. The polymerising gel was overlaid with butan-1-ol until set (1 hr). The top of the gel was then washed with ethanol (2x) and distilled water (2x) and left to drain dry (2 min). The stacking gel consisted of:

1.875 ml 30 per cent acrylamide,
1.875 ml 0.8 per cent N,N'-methylene bisacrylamide,
1.875 ml 1 M Tris HCl pH 6.8,
and 9.35 ml distilled water.

This was polymerised above the running gel, with a sample slot former in position with the addition of 150 µl 10 per cent SDS, 100 µl 10 per cent AMPS and 10 µl of TEMED. The distance from the base of the slot former to the top of the running gel was 20 mm.

Protein samples were loaded onto the gel in 1x loading buffer. This was prepared as a 3x stock solution containing:

1 ml 20 per cent SDS,
1 ml glycerol (Kochlight),
0.1 ml 1 per cent Bromophenol blue (BDH),
0.625 ml 1 M Tris HCl pH 6.8
and 0.6 ml distilled water.

When samples were prepared for electrophoresis they were made
5 per cent in β-mercaptoethanol (SIGMA) and 1x in sample buffer.
Before loading they were left 5 min at room temperature and heated to
100°C for 5 min.

The electrophoresis tank buffer consisted of 0.192 M glycine
(SIGMA), 25 mM Tris base and 0.1 per cent SDS and was prepared as a
10x stock solution. Electrophoresis was carried out for 15 hrs at
75 V.

2.3.1 Protein staining. Gels were stained with Coomassie Brilliant
Blue R to reveal protein bands by gently agitating the gel for
30 min at room temperature in staining solution: 0.5 g/l of
Coomassie Brilliant Blue R (SIGMA) in 45 per cent methanol, 10 per
cent glacial acetic acid, 45 per cent distilled water (v/v). The
gel was then destained by gentle agitation at room temperature in
repeated changes of the same solution but without the dye.

Silver staining was reported by Morrissey124 as 100x more
sensitive than protein staining with Coomassie Brilliant Blue R.
The procedure involved gentle agitation of the gel in 100 ml of a
series of solutions at room temperature:
1) 30 min Prefix 1 45% methanol, 10% glacial acetic acid, 45%
distilled water,
2) 30 min Prefix 2 5% methanol, 7% glacial acetic acid, 88%
distilled water,
3) 30 min Fix 10% glutaraldehyde in distilled water,
4) Repeated changes of distilled water either overnight or for at
least one hour.
5) 30 min in 5 µg/ml Dithiothreitol (SIGMA),
6) 30 min in 0.1% silver nitrate (BDH),
7) Rapidly rinsed in distilled water and 2 changes of developer before leaving in developer; 3% sodium carbonate, 0.0185% formaldehyde (BDH),
8) Developing stopped by adding 5 ml of 2.3 M citric acid,
9) Washed thoroughly with distilled water,
10) Stored in 0.03% sodium carbonate.

2.3.2 Protein molecular weight markers. To calibrate SDS-PAGE, high and low molecular weight markers (Pharmacia) were loaded on one track of each gel. For a Coomassie Brilliant Blue R stained gel, 1/5 of a high and 1/10 of a low molecular weight marker kit vial was loaded per track. For a silver-stained gel a tenth of this was used. One high molecular weight marker kit vial contained: ferritin (220 K and 18.5 K, 50 µg), albumin (67 K, 40 µg), catalase (60 K, 36 µg) and lactate dehydrogenase (36 K, 48 µg). One low molecular weight marker kit vial contained: phosphorylase b (94 K, 64 µg), albumin (67 K, 83 µg), ovalbumin (43 K, 147 µg), carbonic anhydrase (30 K, 83 µg), trypsin inhibitor (20.1 K, 80 µg) and α-lactalbumin (14 K, 121 µg).

2.3.3 Western blotting. Proteins were electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose filters by the western blotting procedure of Towbin et al. 125 The gel and nitrocellulose paper were sandwiched together as in Fig. 3. Each layer was pre-soaked in electrophoresis buffer and care was taken to avoid any air bubbles between layers. The sandwich was assembled between two hinged perspex grids and placed, nitrocellulose towards the anode, into a western blotting apparatus (BIO-RAD) containing electrophoresis buffer; 4 volumes of 192 mM glycine, 25 mM Tris base to
Fig. 3. Assembly for western blotting procedure. The electrophoretic protein blotting sandwich consists of a polyacrylamide gel (1), a sheet of nitrocellulose (2), three layers of blotting paper (3), "scotch-brite" scouring pads (4) and perspex grids (5), all submerged in electro-blotting buffer between the electrodes (6).
1 volume methanol. Proteins were transferred at 60 V for 5 hrs at room temperature.

The position of the gel was marked on the nitrocellulose and, after blotting, the gel was silver-stained to reveal the positions of molecular weight marker proteins run in a track of the gel. (Not all the protein was transferred from the gel.) In this way the nitrocellulose blot was calibrated with the protein molecular weight markers.

2.3.4 Immunological detection of antigens bound to nitrocellulose. The procedure used to detect antigens bound to nitrocellulose, whether after western blotting or during screening of cDNA expression libraries, was based on the procedure described by Towbin et al. The nitrocellulose was first washed briefly in 0.15 M NaCl, 10 mM Tris pH 7.4, (TS), and then unoccupied protein binding sites were blocked by agitation for at least 1 hr at room temperature in 0.15 M NaCl, 10 mM Tris pH 7.4, 5 per cent ovalbumin (crude preparation, SIGMA), 0.01 per cent sodium azide (OTS).

Immunological probing was carried out by agitation at room temperature with antibody diluted in OTS. Before and after each antibody was applied the nitrocellulose was washed in 5 changes of TS over a 30 min period. Probing with murine ascites fluids (containing monoclonal antibodies) (at a 1 in 200 dilution, at least 1 hr) or with mouse antisera (at a 1 in 50 dilution, overnight) was followed with a rabbit anti-mouse immunoglobulin antisera (prepared by R. Hall) (at a 1 in 500 dilution, at least 4 hrs) and then a horse radish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin G antisera (affinity purified preparation, SIGMA) (at a 1 in 1,000 dilution, 1 hr). Rabbit anti-β-galactosidase antisera (prepared by R. Hall) (at a 1 in 200 dilution, 7 hrs or overnight) was also followed with
the HRP-conjugated goat anti-rabbit immunoglobulin G antisera (at a 1 in 1,000 dilution, 1 hr). Human antibody probes (at a 1 in 200 dilution relative to the original human sera, 7 hrs or overnight) were followed with a HRP-conjugated goat anti-human immunoglobulin G antisera (affinity purified preparation; SIGMA) (at a 1 in 1,000 dilution, 1 hr).

After incubation with the enzyme conjugated antibody and washing, position of bound antibody on the nitrocellulose was revealed as a brown band by adding enzyme substrate: 10 mM imidazole HCl, pH 7.4, 2.5 mg/ml, o-dianisidine dihydrochloride, 0.3 per cent hydrogen peroxide (all SIGMA). The reaction was stopped by washing thoroughly with water. The nitrocellulose was blotted dry and stored in the dark.

2.4 Affinity chromatography and immunoprecipitation

Immunological purification of antigen was achieved using an immunoadsorbent of antibody covalently coupled to Sepharose beads. Preparation of this immunoadsorbent first required purification of the antibody. All procedures described in this section were carried out at 4°C, except where stated.

2.4.1 Purification of monoclonal antibodies 5.1 and 7.7.

Preliminary attempts to purify McAbs 5.1 and 7.7 from ascites fluid by Protein-A Sepharose chromatography failed because of weak binding for these particular monoclonal antibodies. So the standard procedure of diethyl amino ethyl (DEAE) cellulose ion exchange chromatography was used.

The immunoglobulins were first prepared from the ascites fluid by ammonium sulphate precipitation. One volume of ascites fluid was mixed with 2 volumes of 0.15 M NaCl, followed by the gradual addition of 3 volumes of saturated \((\text{NH}_4)_2\text{SO}_4\). The pellet obtained by
centrifugation (1,000 g, 15 min) was washed in 50 per cent saturated (NH₄)₂SO₄ and then taken up in a minimal volume of PBS. The immunoglobulin preparations were then dialysed extensively against 5 mM sodium phosphate, pH 8.0. It was noted that McAbs 5.1 and 7.7 did not precipitate well in 40 per cent saturated (NH₄)₂SO₄.

A DEAE-cellulose column (Whatman DE52) was prepared (height 30 cm, diameter 2 cm) and thoroughly equilibrated by the flow of 5 l of 5 mM sodium phosphate, pH 8.0, the starting buffer. The dialysed immunoglobulin preparation was centrifuged (1,000 g, 15 min) before application to the column and was washed in to the column with starting buffer. Elution was achieved using a convex gradient of increasing ionic strength between the starting buffer and limit buffer, 0.18 M sodium phosphate, pH 8.0, obtained with a constant mixing volume of 110 ml. Fractions of 5 ml were collected and the elution profile was obtained by measuring the OD₂₈₀ (Fig. 4). Samples (50 μl) of particular fractions were examined by SDS-PAGE on 10 per cent gels and staining with Coomassie Brilliant Blue R (Fig. 5). Fractions constituting the first peak of absorbance contained the IgG and were pooled.

2.4.2 Covalent coupling of protein to Sepharose beads. Protein was coupled to CNBr-activated Sepharose-4B (Pharmacia) according to the manufacturer's recommendations. IgG preparations were dialysed against coupling buffer, 0.1 M NaHCO₃, 0.5 M NaCl. The CNBr-activated Sepharose was swollen in 1 mM HCl for 15 min, 1 g of CNBr-activated Sepharose for 10 mg of IgG. The swollen Sepharose beads were washed in a sintered glass funnel with 1 mM HCl for 15 min before mixing with the dialysed IgG preparation for 2 hrs at room temperature. The Sepharose beads were then gently pelleted (250 g, 5 min), washed in 0.2 M glycine, pH 8.0 and resuspended in
Fig. 4. Purification of mouse IgG: Ion-exchange chromatography elution profile. Protein in fractions collected during the purification of a monoclonal antibody from mouse ascites fluid was detected by measuring the optical density at 280 nm.
Fig. 5. Purification of mouse IgG: Analysis of eluted fractions from ion-exchange chromatography. Track 1, mouse ascites fluid (5 μl). Tracks 2 to 8 are eluted fractions 5, 31, 34, 37, 42, 46 and 50 (50 μl of each) as in Fig. 4. The samples were analysed by SDS-PAGE on a 10 per cent gel and staining with Coomassie brilliant blue R.
the same buffer with mixing for 2 hrs at room temperature. The prepared immunoadsorbent was washed in 3 cycles of 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0 followed by coupling buffer and finally, washed and stored at 4°C in PBS containing azide at 0.01 per cent. The efficiency of binding was determined by measuring the OD$_{280}$ of the IgG preparations before and after binding. For McAbs 5.1 and 7.7 the binding efficiencies were at least 90 per cent.

The 5.1 Ag affinity column was prepared in a similar way using CNBr-activated Sepharose 4B. The 5.1 Ag (15 µg), prepared by affinity chromatography and initially in 8 M urea, was made 0.1 per cent in Nonidet P40 (BDH) before thorough dialysis against coupling buffer. The procedure above was then followed, using 0.2 g CNBr-activated Sepharose, except that the efficiency of binding could not be determined by measuring the OD$_{280}$ because of the Nonidet P40. So samples before and after binding were examined by SDS-PAGE and silver staining, revealing a 50 per cent binding efficiency for the 5.1 Ag.

2.4.3 Detergent extraction of parasite proteins. Parasite proteins were extracted using the non-ionic detergent Nonidet P40 by the procedure described by Hall et al modified from Holder and Freeman. Total parasitised red blood cells were extracted on ice in 5 volumes of extraction buffer (EB): 10 mM Tris, 1 mM ethylene diamine tetra-acetic acid (EDTA) (BDH), 1 mM ethylene glycol bis (amino ethyl ether) tetra-acetic acid (EGTA) (SIGMA), pH 7.4, to which was added phenyl methyl sulphonyl fluoride (PMSF) (SIGMA), as a 20 mg/ml solution in methanol to give a final concentration of 0.2 mg/ml, and Nonidet P40, to 1 per cent (v/v), immediately prior to use. The lysate was mixed by rotation for 30 min at 4°C, and insoluble material was removed by centrifugation (50,000 g, 3 hrs).
Saponin lysed, iodinated parasites were extracted for immunoprecipitation in a volume of EB equal to one fifth of the original volume of parasitised red blood cells. After mixing for 30 min the extract was centrifuged as above. If the extract was for affinity chromatography, a larger extract volume was used equal to twice the original volume of parasitised erythrocytes, to ease manipulations.

If the parasite protein extracts were not to be used immediately they were stored at -70°C and recentrifuged, as above, before use.

2.4.4 Affinity chromatography. The affinity chromatography procedure, for monoclonal antibody affinity columns, was derived from methods already described. For large scale antigen purifications, the monoclonal antibody affinity columns (2 ml packed volume) were equilibrated in the extraction buffer (EB) (see above) and the detergent extract of 100 ml packed parasitised erythrocytes (at least 5 percent parasitaemia) was applied at the rate of 50 ml/hr. The McAb 5.1 affinity column with bound antigen was washed with 5x 5 ml of EB before elution. The McAb 7.7 column with bound antigen was washed more stringently with 5x 5 ml of EB, 5x 5 ml of EB, 0.5 M NaCl, 5x 5 ml of EB with the Nonidet P40 only present at 0.1 per cent and finally 5x 5 ml of EB again, to ensure removal of non-specifically bound protein. Antigen was eluted with 8 M urea and 0.5 ml fractions were collected and stored at -70°C. Immediately prior to re-use the monoclonal antibody affinity columns were washed a second time in 8 M urea.

For small scale antigen purification, e.g. using labelled material, the same procedure was followed except that all volumes were reduced to one tenth of those above, i.e. columns had a packed volume of 0.2 ml and 50 µl fractions of eluate were collected.

Affinity purification of human antibody to the 5.1 Ag was based
on methods used in preparation of antibodies for immunoprecipitation of polysomes, in particular those of Shapiro et al. 128 Human immunoglobulin was first prepared from endemic serum by ammonium sulphate precipitation. The serum was diluted with two volumes of 0.15 M NaCl. Then, the immunoglobulin was precipitated and washed in 50 per cent saturated (NH$_4$)$_2$SO$_4$, re-dissolved in PBS, re-precipitated and washed in 45 per cent saturated (NH$_4$)$_2$SO$_4$ and finally re-dissolved in 0.15 M NaCl, 10 mM Tris, pH 7.4 (TS). Before application to the 5.1 Ag affinity column the human immunoglobulin preparation was centrifuged (50,000 g, 3 hrs).

The immunoglobulin prepared from 1 ml of human serum was applied to the 5.1 Ag affinity column (0.4 ml packed volume containing 15 μg of 5.1 Ag). The column was washed with 3x 0.5 ml TS, 3x 0.5 ml 0.5 M NaCl, 10 mM Tris, pH 7.4, 3x 0.5 ml TS, 0.1 per cent Nonidet P40 and finally with 3x 0.5 ml TS. Bound antibody was eluted with 0.15 M NaCl, 0.1 M glycine, pH 2.5. The pH of the first four 500 μl fractions was measured as 7.5, 2.6, 2.5 and 2.5. It was inferred that the antibody was eluted within the first 2½ ml, so these 4 fractions and the first 2½ ml eluted in all subsequent preparations were neutralised with 50 μl 1 M Tris. HCl, pH 7.4 and retained.

2.4.5 Immunoprecipitation. The immunoadsorbent used in immunoprecipitation experiments was the monoclonal antibodies covalently coupled to Sepharose beads. Labelled parasite proteins in extraction buffer (500 μl) were mixed with the immunoadsorbent (50 μl packed volume) for 1 hr. After binding, the monoclonal antibody-Sepharose beads were washed, by resuspension and pelleting (30 sec, 12,000 rpm, Eppendorf microfuge), in extraction buffer (EB) (3x), EB containing 0.5 M NaCl (3x), EB except 0.1 per cent Nonidet P40 (3x) and EB again (3x). Bound protein was eluted from the immunoadsorbent by resuspension
into 100 μl of 8 M urea and the total supernatant, after centrifugation, was examined by SDS-PAGE and autoradiography.

2.5 Radiolabelling of parasite proteins

2.5.1 Biosynthetic labelling. *P.falciparum* erythrocytic stage proteins were labelled biosynthetically by culturing the parasites in medium containing $^{35}$S-methionine. Parasitised red blood cells (0.5 ml packed volume, at least 5 per cent parasitaemia), pelleted (850 g, 5 mins) from a parasite culture, were resuspended in labelling medium (10 ml) and returned to normal culture conditions (i.e. 37°C, atmosphere of high CO$_2$ and low O$_2$; Materials and Methods 1.3), for 4 hrs. The labelling medium consisted of:

- 20 ml Eagles medium lacking methionine (GIBCO),
- 0.2 ml glutamine, 200 mM (GIBCO),
- 0.2 ml gentamycin, 5 mg/ml (SIGMA),
- 60 μl $^{35}$S-methionine (0.8 mCi (30 MBq), 1,000 Ci/mmol (37 TBq/mmol), Amersham).

This labelling medium was filter sterilised and the pH adjusted to approximately 7.4 (from the colour of the pH indicator in the Eagles medium) with a stream of CO$_2$ gas. The medium was warmed to 37°C and 10 μl of filter sterilised p-amino benzoic acid (1 mg/ml) (BDH) and 2 ml human serum were added before use.

Labelled proteins were extracted with Nonidet P40 as described above (Materials and Methods 2.4.3). The radiolabel incorporated into trichloroacetic acid (TCA) precipitable material was assayed for each preparation. Samples (50 μl) of labelled parasite protein extracts were mixed with 100 μl 0.1 M NaOH (to hydrolyse charged tRNAs) and 50 μl 30 percent H$_2$O$_2$ (to bleach the sample) and incubated at 37°C for 15 min. To this was added 2.5 ml 10 per cent TCA and proteins were precipitated on ice for 15 min. The precipitate
was collected on a glass fibre filter (Whatman, 2.2 cm) and the incorporated $^{35}$S-methionine counted as described above for labelling parasites with $[^3H]$ hypoxanthine (Materials and Methods, 1.8).

2.5.2 Radioiodination. Surface radioiodination of erythrocytic parasite proteins exposed by saponin lysis was carried out by the lactoperoxidase technique. Parasites prepared by saponin lysis (Materials and Methods, 1.6) from 3 ml packed parasitised erythrocytes (at least 5 per cent parasitaemia) were resuspended in 300 μl PBS. To this was added 30 μl lactoperoxidase (0.2 mg/ml in PBS) (SIGMA), 30 μl glucose oxidase (2IU/ml in PBS) (SIGMA), 3 μl $^{125}$I$^-$ (0.5 μCi, 18.5 KBq) (Amersham) and 30 μl glucose (50 mM in PBS), with mixing. After incubating 10 min at room temperature, the reaction was stopped by adding 30 μl 100 mM sodium azide. The parasites were then washed in 4x 10 ml PBS (4°C) by resuspension and centrifugation (1,000 g, 10 min) and labelled parasite proteins were extracted as above (Materials and Methods, 2.4.3).

The incorporation of $^{125}$I was measured by collecting proteins, precipitated from a 5 μl sample of the labelled parasite protein extract in 10 per cent TCA (2.5 ml, on ice, 15 min), on glass fibre filters (Whatman, 2.2 cm) washing the filters with 10 per cent TCA and ether (10 ml of each) and counting the incorporated label using a LKB Wallac 1275 Minigamma Gamma Counter.

2.5.3 In vitro translation. *P. falciparum* mRNA in vitro translation products were provided by J. Hyde. *P. falciparum* RNA was prepared by the hot phenol/chloroform extraction method and poly(A$^+$) RNA was isolated by oligo-(dT)-cellulose chromatography. *P. falciparum* poly(A$^+$) RNA was used to program protein synthesis in the mRNA dependent reticulocyte lysate in vitro translation system, supplemented with calf liver tRNA (Boehringer) with incorporation of
$^{35}$S-methionine and the total in vitro translation products were diluted with extraction buffer before use in affinity chromatography.

2.5.4 Autoradiography. Parasite proteins labelled with $^{35}$S-methionine were revealed after SDS-PAGE by fluorography. The polyacrylamide gel was first stained with Coomassie Brilliant Blue R and destained (Materials and Methods, 2.3.1) to reveal the position of unlabelled molecular weight marker proteins. The gel was then impregnated with the fluor by dehydration in dimethyl sulphoxide (DMSO) (2x 30 min), then incubation in 20 per cent (w/v) 2,5-Diphenyloxazole (PPO) in DMSO (3 hrs), all at 37°C. The fluor was then precipitated in the gel by washing for 1 hr in running tap water. The gel was vacuum-dried onto Whatman 3MM paper, the position of molecular weight marker proteins being marked with radioactive ink, and exposed to preflashed Dupont-Cronex X-ray film at -70°C. For $^{125}$I labelled proteins the same procedure was followed except that the DMSO and DMSO/PPO steps were not applied.
Materials and Methods. Chapter 3

Microbiology and molecular biology
3.1 **Bacterial strains and bacteriophages**

All of the bacterial strains and bacteriophages used in this study are listed with their genotypes (Table 4). Maps of the vectors used in construction of *P. falciparum* cDNA libraries and DNA sequencing are presented (Fig. 6).

3.2 **Media and solutions**

**L-Broth:** Difco Bacto Tryptone, 10 g, Difco Bacto yeast extract, 5 g, NaCl, 5 g, per litre adjusted to pH 7.2.

**L-Agar:** Difco Bacto Tryptone, 10 g, Difco Bacto yeast extract, 5 g, NaCl, 10 g, Difco agar, 15 g, per litre adjusted to pH 7.2.

**BBL Agar:** Baltimore Biological Laboratories Trypticase, 10 g, NaCl, 5 g, Difco agar, 10 g, per litre.

**BBL Top Agar:** As for BBL agar but only 6.5 g Difco agar per litre.

**Phage buffer:** KH$_2$PO$_4$, 3 g, Na$_2$HPO$_4$ (anhydrous), 7 g, NaCl, 5 g, 1 mM MgSO$_4$, 0.1 mM, CaCl$_2$, 1 ml of 1% gelatin solution per litre.

**Xgal indicator plates:** Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was added to molten BBL top agar to 40 mg/l from a stock solution of 20 mg/ml in dimethylformamide. Isopropyl-β-D-thiogalactoside (IPTG) when added was at 1 mM supplemented from a stock of 100 mM.

**Antibiotics:** L-broth or molten L-agar was supplemented with the following antibiotics when appropriate: tetracycline to 10 μg/ml, ampicillin to 50 μg/ml and kanamycin to 40 μg/ml.

3.3 **Microbiological Techniques**

3.3.1 **Plating bacteriophages.** Bacterial cells on which phage were to be plated were grown to an OD$_{650}$ of 0.3 in L-broth supplemented to 20 ml/l with 20 per cent maltose and with antibiotics as appropriate. The bacteria, now competent to λ infection were
Table 4. Bacterial strains, bacteriophages and their genotypes

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>NM514</td>
<td>hfl, hsdM^+R^-</td>
<td>134</td>
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<tr>
<td>ED8799/NM422</td>
<td>met, hsdM^+R^-, supE, supF, lacZ_M15</td>
<td>135</td>
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<tr>
<td>NB78</td>
<td>met, hsdM^+R^-, supE, supF, lacZ_M15, tet^R</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>derived by N. Bone (unpublished) from NEM259/ED8654</td>
<td></td>
</tr>
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<td>NB79</td>
<td>lac^A_U124, lon, tet^R</td>
<td>137</td>
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<td></td>
<td>derived by N. Bone (unpublished) from ED395</td>
<td></td>
</tr>
<tr>
<td>BTA282</td>
<td>lac^A_U139, Δlon, araD139, str^R, thi, hflA</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>(chr::Tn10), hsdM^+R^-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>derived by N. Willetts (unpublished) from Y1083</td>
<td></td>
</tr>
<tr>
<td>Y1090</td>
<td>lac^A_U169, Δlon, araD139, strA, thi, supF,</td>
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<tr>
<td></td>
<td>[trpC22::Tn10], (pMC9)</td>
<td></td>
</tr>
<tr>
<td>NM522</td>
<td>Δlac pro, hsdA (probably M^-, S^-, R^-), thi, supE</td>
<td>140</td>
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</table>

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>Genotype</th>
<th>Reference</th>
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<td>λNM1149</td>
<td>λ, imm^434, (b538)</td>
<td>134</td>
</tr>
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<td>λgt11</td>
<td>λ, lac^5, cl^857, nin^5, Sam^100</td>
<td>138</td>
</tr>
<tr>
<td>λgt11::Tn5</td>
<td>λ, lac^5, cl^857, nin^5, Sam^100, kan^R</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>derived by N. Bone (unpublished) from λgt11</td>
<td></td>
</tr>
<tr>
<td>λgt11.Amp3</td>
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<td>101</td>
</tr>
<tr>
<td>λcl^857 Sam^7</td>
<td></td>
<td>141</td>
</tr>
<tr>
<td>M13mp11</td>
<td>derived by J. Messing (unpublished) from M13mp9</td>
<td>142</td>
</tr>
<tr>
<td>λcl^-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>λv</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 6. Structure of the cloning vectors used in this study. In the \( \lambda \) vectors, the positions of the restriction sites for EcoRI(E), HindIII(H), KpnI(K) and SstI(S) are marked in kb. The arrow in the \( \text{lacZ} \) gene marks the direction of transcription.
centrifuged (2000g, 5 min) and resuspended in 10 mM MgSO₄ to one tenth of the culture volume. To 0.1 ml of phage diluted in phage buffer and 0.1 ml of plating cells was added 2.5 ml molten BBL top agar (50°C) and this was poured onto a BBL agar plate. The plate was incubated at 37°C or 42°C.

3.3.2 Generation of lysogens. λgt11::Tn5 and Amp3 cDNA libraries were immunologically screened as lysogens in E.coli BTA282. These lysogens were generated at a low multiplicity of infection (m.o.i.) and selected from nonlysogenised cells by challenging with antibiotic.

E.coli BTA282 cells were made competent to phage infection as above (Materials and Methods, 3.3.1), but concentrated 25x (instead of 10x) into 10 mM MgSO₄ to give 2.5 x 10⁹ cells/ml. Phage were added to give a m.o.i. of 0.01, and after mixing left to adsorb to the bacterial cells, 1 hr, 4°C. The cells were then pelleted (2000 g, 5 min) and re-suspended in L-broth plus tetracycline and grown at 30°C for 2 hrs. After adding kanamycin or ampicillin to select for lysogens, the cells were grown for a further 3 hrs at 30°C and concentrated 10x into 10 mM MgSO₄ for plating and screening.

The λgt11::Tn5 cDNA library was also screened as lysogens in E.coli NB79. The λgt11::Tn5 cDNA library had originally been picked as phage into an array with 10 phage clones per well. These were replicated onto BBL-agar plates with E.coli NB78 in the top agar layer to modify them, before replicating to L-agar plates spread with E.coli NB79. In both cases 0.1 ml of cells made competent to λ as above (Materials and Methods, 3.3.1) was used per plate. Lysogens in E.coli NB79 were selected by replica-plating on to L-agar plates supplemented with kanamycin and tetracycline, and once grown were pooled together for screening.

Lysogens of particular phage were also prepared for DNA
preparation and cDNA expression. λgt11 Amp3 (parent phage and cDNA recombinants) and λcl057 Sam7 were prepared as lysogens in E.coli Y1090 and BTA 282 by selecting for the presence of functional repressor. Selection on the basis of antibiotic resistance was not possible with E.coli Y1090 because this strain is already ampicillin resistant.

Serial dilutions of each phage were spotted onto plates spread with competent cells. Lysogens were selected by replica plating to a plate spread with λcl-'. This phage can not produce a functional λ repressor and so will lyse all cells except λ lysogens, which already contain functional λ repressor, and λ resistant cells. After streaking for single colonies, the two alternatives were distinguished by challenging individual clones with λV. This phage lacks the binding site for the λ repressor and so will lyse lysogens even though they contain functional λ repressor. Cells resistant to λ will survive challenge with λV. The challenge with λV is achieved by spreading a line each of λcl- and λV down a L-agar plate and streaking cells from individual clones first through λcl- and then λV. Clones which are non-lysogens and λ sensitive only grow to the λcl- streak, lysogens grow through λcl- but not λV and cells resistant to λ grow through both λcl- and λV.

3.3.3 Generation of antisera to recombinant proteins. Antibodies were raised to recombinant proteins by a procedure based on that of MacKay et al.103,143 Lysogen cultures were grown at 30°C to OD650 of 0.4 and induced for 1½ hrs at 42°C. The cells were then pelleted and resuspended in a lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, 1 per cent Nonidet P40, 0.4 per cent sodium deoxycholate) to 4 per cent of the original volume. Aliquots of the lysate (200 μl) were stored at -70°C. Each aliquot was thawed and homogenised with
200 µl of adjuvant prior to inoculation. Mice were injected intraperitoneally with one aliquot in complete Freund's adjuvant followed by two subsequent injections at 3 week intervals using incomplete Freund's adjuvant. The mice were bled 8 days after the third injection and the sera were retained.

3.4 Characterisation of DNA

3.4.1 Preparation of λDNA. Cultures (750 ml) of *E. coli* Y1090 lysogenised with λgt11, Amp3 (parent phage and cDNA recombinants) and λCl857 Sam7 were grown at 30°C to an OD650 of 0.55. The prophage were induced into the lytic cycle by heating in a 42°C waterbath with shaking for 25 min before incubating at 37°C until complete lysis of the culture occurred (90 min after induction). Chloroform (1.5 ml) was added to lyse remaining cells.

The cultures were centrifuged (8,500 g, 10 min) to remove cell debris before pelleting the phage (43,000 g, 3 hrs). The phage were resuspended in phage buffer (10 ml) by shaking at 4°C overnight. To 10 ml of resuspended phage was added 7.3 g CsCl and, after banding by centrifugation (100,000g, 26 hrs), the phage, in a volume of 2.5 ml, were dialysed against 1 l of 10 mM Tris pH 8.0, 1 mM EDTA (TE) for 8 hrs.

DNA was then prepared by equal volume extractions of the phage with phenol/chloroform (1:1, v/v) (4x) and ether (2x). The DNA preparation was then treated with proteinase K. To 0.5 ml of λ DNA in TE was added 20 µl 1 M Tris HCl pH 8.0, 12.5 µl 20 per cent SDS and 5 µl 50 mg/ml proteinase K. This was incubated 1 hr at 37°C before re-extraction with equal volumes of phenol/chloroform (1:1, v/v) (1x) and ether (2x). All organic solvents used in these extractions were pre-equilibrated against TE. The DNA was then precipitated by adding 50 µl 3 M sodium acetate, pH 5.5 and 1½ volumes
of ethanol and leaving overnight at -20°C. The precipitated DNA was pelleted (10 min, Eppendorf microfuge), washed with 70 per cent ethanol, dried under vacuum (1 hr) and redissolved in 60 µl TE.

3.4.2 Restriction enzyme digests of λDNA. Restriction enzyme digests were carried out in the appropriate restriction enzyme buffer for 5 hrs and 1U of each enzyme was used per µg of λDNA. Restriction enzyme buffers used were:

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Tris HCl</th>
<th>MgCl₂</th>
<th>NaCl</th>
<th>Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>50 mM pH 7.55</td>
<td>10 mM</td>
<td>50 mM</td>
<td>1 mM DTT</td>
</tr>
<tr>
<td>KpnI</td>
<td>6 mM pH 8.0</td>
<td>6 mM</td>
<td>6 mM</td>
<td>6 mM βME</td>
</tr>
<tr>
<td>SatI and HindIII</td>
<td>50 mM pH 8.0</td>
<td>10 mM</td>
<td>50 mM</td>
<td>-</td>
</tr>
</tbody>
</table>

DDT, Dithiothreitol, βME, β-mercaptoethanol.

For KpnI/SatI double digests, KpnI digestion was carried out first and SatI restriction enzyme buffer was added as usual.

Restriction enzymes were obtained from Bethesda Research Laboratories and NBL Enzymes Ltd.

3.4.3 Agarose gel electrophoresis. Horizontal agarose slab gel electrophoresis was used to examine products of restriction enzyme digestion of λDNA. Agarose was dissolved by boiling in 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA pH 8.2 (TAE, prepared as a 10x stock solution) and poured into a gel mould 28 cm x 14 cm x 5 mm, with sample slot former in place, to set. Both electrophoresis tank buffer (1x TAE) and the gel contained ethidium bromide at 0.5 µg/ml added from a 5 mg/ml stock solution. To each sample to be examined, a tenth volume of TAE and a tenth volume of loading buffer (0.025 per cent bromophenol blue, 10 per cent Ficoll) were added and the samples were heated to 65°C for 10 min before loading.
Electrophoresis was carried out at 90V overnight. DNA bands were observed and photographed upon illumination with UV light.

To isolate a DNA fragment from an agarose gel a trough was cut immediately in front of the relevant band. The rear of the trough was lined with dialysis tubing and the trough was filled, and replenished as necessary, with TAE. Electrophoresis was continued at 100V for 30 min until the band had entered the trough. After reversing the current for 3 min the TAE in the trough containing the DNA fragment was removed. The trough was washed out once with TAE to recover as much of the DNA fragment as possible. The preparation was extracted with an equal volume of butan-1-ol, phenol, phenol/chloroform (1:1, v/v), chloroform and ether (2x), all pre-equilibrated with TE. The DNA was precipitated by adding 1 μg calf liver tRNA, a tenth volume of 3M sodium acetate pH 5.5 and 2 volumes of ethanol and placing at -70°C for 20 min. The DNA was pelleted (10 min, Eppendorf microfuge), washed in 70 per cent ethanol, dried under vacuum (1 hr) and dissolved in TE.

3.5 Sequencing of DNA

DNA sequencing was carried out by the dideoxy chain-termination method\textsuperscript{146} using a bacteriophage M13 cloning vector to obtain the single stranded DNA templates.\textsuperscript{147} The DNA to be sequenced was cloned into M13mp11.\textsuperscript{142,148} This vector is closely related to M13mp9\textsuperscript{142} and consists of a fragment of the \textit{E.coli} lac operon (I', O, Z') inserted into one of the ten BvuI sites of M13 DNA.\textsuperscript{149} Unique restriction sites for cloning were introduced on an oligonucleotide at the 5' end of the coding sequence of the \textit{lacZ} gene.\textsuperscript{150,151} The M13 phages replicate without lysing the host cell and viable phage containing single stranded DNA are secreted by infected bacteria.

The single stranded DNA is used as a template for primed DNA
synthesis using the Klenow fragment of DNA polymerase I\textsuperscript{152} and strand synthesis is terminated by 2',3' dideoxy nucleoside triphosphates.\textsuperscript{146} The strands are labelled by including deoxyadenosine 5'-(α-[\textsuperscript{35}S] thio) triphosphate\textsuperscript{153} and analysed by thin acrylamide gel electrophoresis and autoradiography.\textsuperscript{154}

3.5.1 Cloning of cDNA inserts into M13mp11. Ligation of cDNA inserts into M13mp11, transfection of recombinant phage into \textit{E.coli} NM522 and their plaque purification was carried out by M. Mackay. The cDNA inserts prepared as \textit{EcoRI} fragments (Materials and Methods, 3.4.3) were ligated directly into the \textit{EcoRI} cleaved, double-stranded replicative form (RF) of M13mp11, prepared by the alkaline extraction method,\textsuperscript{155} in the ratio of 3 molecules of cDNA to 1 molecule of vector. (40 ng of λIH8 cDNA insert and 75 ng of λIH9 cDNA insert were ligated to 300 ng of M13mp11 DNA in a volume of 10 μl). The ligated DNA was transfected by a method derived from that of Hanahan\textsuperscript{156} into competent \textit{E.coli} NM522 cells. Recombinant phage, with a lac\textsuperscript{-} phenotype due to insertion of the cDNA in the 5' end of lacZ, were selected as white plaques after plating on Xgal/IPTG indicator BBL plates in a lawn of \textit{E.coli} NM522 and were plaque purified. (Recombinants appeared at the rates of 7.2 per cent for IH8 and 6.1 per cent for IH9.)

3.5.2 Preparation of single-stranded M13 DNA\textsuperscript{142} A culture of \textit{E.coli} NM522 was grown to an OD\textsubscript{650} of 0.3. Into 1 ml aliquots of this culture was placed phage from single plaques of M13mp11 cDNA recombinants and these were grown with shaking (37°C, 4½ hrs). The cultures were then centrifuged (5 min, Eppendorf microfuge) and to 0.8 ml of the supernatant was added 200 μl of 2.5 M NaCl, 20 per cent polyethylene glycol (PEG) 6000. After 30 min at room temperature the phage were pelleted (5 min, Eppendorf microfuge) and care was taken to remove all of the supernatant.
DNA was then prepared from the phage. To the pellet was added 100 μl of 10 mM Tris, pH 8.0, 0.1 mM EDTA and 50 μl pre-equilibrated phenol. This was vortexed, left to stand at room temperature for 10 min, vortexed again and the phases separated (1 min, Eppendorf microfuge). The aqueous layer was collected and the DNA precipitated by adding 10 μl of 3 M sodium acetate and 250 μl ethanol. After 5 min at -70°C the DNA was pelleted (10 min, Eppendorf microfuge). The DNA was then re-dissolved in 100 μl of 10 mM Tris, pH 8.0, 0.1 mM EDTA and re-precipitated by adding 10 μl of 3 M sodium acetate and 250 μl ethanol. After a further 5 min at -70°C the DNA was pelleted again (10 min, Eppendorf microfuge), washed with 1 ml ethanol (absolute), dried under vacuum (20 min) and re-dissolved in 50 μl of 10 mM Tris, pH 8.0, 0.1 mM EDTA. The single-stranded DNA template preparations were stored at -20°C until required.

3.5.3 Sequencing reactions. First, the primer was annealed to the single-stranded DNA templates. To 5 μl of the M13mp11 recombinant DNA preparations (Materials and Methods, 3.5.2) was added 5 μl of primer mix; 0.04 pmol/μl of M13 primer pentadecamer (New England Biolabs) in 20 mM Tris, HCl, pH 8.5, 10 mM MgCl₂, with subsequent incubation at 60°C for 1 hr. For the sequencing reaction, to 2 μl of the annealed template/primer in each of 4 tubes was added 2 μl of one of the following 4 mixes of nucleotide triphosphates (Boehringer) prepared in 10 mM Tris, pH 8.0, 0.1 mM EDTA.
Nucleotide triphosphate mixes.

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>C</th>
<th>G</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>6.25 μM</td>
<td>125 μM</td>
<td>125 μM</td>
<td>125 μM</td>
</tr>
<tr>
<td>dCTP</td>
<td>125 μM</td>
<td>6.25 μM</td>
<td>125 μM</td>
<td>125 μM</td>
</tr>
<tr>
<td>dGTP</td>
<td>125 μM</td>
<td>125 μM</td>
<td>6.25 μM</td>
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<tr>
<td>ddTTP</td>
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<tr>
<td>ddCTP</td>
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<tr>
<td>ddGTP</td>
<td>-</td>
<td>-</td>
<td>320 μM</td>
<td>-</td>
</tr>
<tr>
<td>ddATP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

The proportions of each ddNTP to the dNTPs were determined empirically for *P. falciparum* DNA by M. Mackay.

Reactions were begun by adding to each tube 2 μl of Klenow mix, 0.2 U/μl DNA polymerase I, large (Klenow) fragment (P and S Biochemicals Ltd), 0.5 μCi/μl deoxyadenosine 5'-(α-[35S] thio) triphosphate (650 Ci/mmol; dried down, Amersham), 10 mM Tris, pH 8.5, 10 mM dithiothreitol. After incubation at room temperature for 25 min, 2 μl was added of a chase mix; 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM dTTP in 10 mM Tris, pH 8.0, 0.1 mM EDTA and incubation was continued for a further 20 min at room temperature. The reactions were then stopped by adding 2 μl of 0.03 per cent xylene cyanol FF, 0.03 per cent bromophenol blue, 20 mM EDTA in deionised formamide. The samples were boiled (3½ min) before loading onto a polyacrylamide gel.

3.5.5 Electrophoresis of sequencing reaction products. A gradient polyacrylamide gel system was used. The following solutions were used: 1) A 10x stock of TBE; 108 g Tris base, 55 g boric acid, 9.3 g EDTA made up to a volume of 1 l with distilled water. 2) A 0.5 TBE gel mix; 50 ml 10x TBE, 460 g urea, 57 g acrylamide,
3 g bisacrylamide, made up to 1 l and filtered. 3) A 2.5 TBE gel mix; 125 ml 10x TBE, 230 g urea, 28.5 g acrylamide, 1.5 g bisacrylamide, 25 g sucrose, 25 mg bromophenol blue, made up to 500 ml and filtered. These solutions were stored at 4°C.

A gel mould was prepared to generate a gel with dimensions 38 cm x 17 cm x 0.3 mm using two, thoroughly cleaned, glass plates, one notched and siliconised, separated by plastic spacers. To polymerise the gel, 25 per cent ammonium persulphate and N,N,N',N'-tetramethyl ethylene diamine (TEMED) were added at the rate of 2 μl/ml for each to both a 0.5 TBE gel mix and a 2.5 TBE gel mix. A gradient gel was formed by taking into a 10 ml pipette 4 ml 0.5 TBE gel mix and 6 ml 2.5 TBE gel mix and gently mixing together by introducing five air bubbles. This mixture was then poured carefully down the edge of the gel plates and the mould was filled with approximately 30 ml of the 0.5 TBE gel mix. A plastic sample well former (with 32 teeth, 5 mm deep, 2.5 mm wide, 1.5 mm apart) was put in place and the gel was left to set for 30 min.

The polymerised gel was clamped into an electrophoresis apparatus. The top and bottom tank buffers were 1.0x TBE. Prior to loading the samples, the wells were repeatedly washed out with the top tank electrophoresis buffer.

Electrophoresis was carried out for 1½ hrs at a constant 38 Watts. The gel was then removed from the electrophoresis apparatus and the siliconised glass plate was separated from the gel. The gel was fixed in 10 per cent acetic acid, 10 per cent methanol for 15 min before transfer to blotting paper. The gel was covered with Saran wrap, dried under vacuum, 80°C, 30 min and exposed to pre-flashed Dupont Cronex X-ray film.
Results. Chapter 1.

In vitro inhibition of *P. falciparum* erythrocytic growth by monoclonal antibodies.
Demonstration of a specific reduction in multiplication of a malaria parasite by a monoclonal antibody, labels the parasite component recognised by that monoclonal antibody as sensitive to immunological attack. The monoclonal antibody binds to only one epitope, possibly with low affinity. A polyspecific antisera raised to the antigen would contain many antibodies recognising many epitopes with a range of affinities and would be expected to cause a greater inhibition of parasite multiplication than the monoclonal antibody alone. Therefore, any specific inhibition of parasite growth by a monoclonal antibody, even at high concentrations, implies that that monoclonal antibody can be used to investigate a potentially protective antigen.

Antibodies can inhibit the erythrocytic growth of the malaria parasite both in vivo and in vitro. Passive transfer of gamma-globulin purified from sera of apparently immune Gambian adults markedly reduced and eventually eliminated the parasitaemia in Gambian children with severe infections of either *P. falciparum* or *P. malariae*. The dose necessary was high, the total amount given over three days being equivalent to 10 to 20 per cent of the recipients own gamma globulin.

The effect of antibody from immune sera on parasite growth in vitro has also been studied. In vitro growth has been evaluated microscopically or through incorporation of radiolabelled metabolites. Studies of *P. knowlesi* suggested that the antibody effect was on either the mature intra-erythrocytic schizont or the extra-cellular merozoite. Agglutination of merozoites and schizont-infected red blood cells was also observed. Antibody caused a reduction in the rate of reinvasion with little effect on intra-erythrocytic development.
Studies have also been carried out for *P.falciparum* with similar conclusions and with the observation of a degree of strain specificity in the inhibition of parasite growth, a phenomenon well documented for *P.knowlesi*.

Sequestration of *P.falciparum* mature erythrocytic stages in capillaries may be a mechanism of evading the host immune system and may also be inhibited by strain specific antibodies. Binding of infected erythrocytes to amelanotic melanoma cells, a model for sequestration, is blocked by sera from immunised Aotus monkeys. Antibody from immune adults in Sudan has an effect on intra-erythrocytic development inducing "crisis forms" of cultured parasites although a recent report suggested "crisis form factor" may not be associated with immunoglobulins.

However, the polyspecificity of immune serum does not allow identification of the protective antigens. Monoclonal antibodies can also inhibit erythrocytic growth of malaria parasites. Monoclonal antibodies raised against *P.yoelii* have been observed to curb an otherwise lethal infection by this murine parasite in vivo. Two rat monoclonal antibodies raised against *P.knowlesi* merozoites inhibited *P.knowlesi* growth in vitro by 90 per cent and both monoclonal antibodies recognised a parasite encoded protein of 66 K. Monoclonal antibodies have also been obtained which inhibit in vitro growth of *P.falciparum* by up to 95 per cent and have been used to characterise three potentially protective antigens of *P.falciparum* erythrocytic stages which are probably located on the merozoite surface.

This evidence demonstrates that immunoglobulins can have a deleterious effect on the erythrocytic stages of the malaria parasite. There are several mechanisms proposed for this growth inhibition.
Firstly, the antibodies may bind to the merozoite surface and block erythrocyte reinvasion by merozoite agglutination or by interfering with the receptors involved in binding to the red blood cell. Secondly, the antibody may bind to the mature schizont either to antigens at the surface of the infected red blood cell perhaps affecting metabolite transport across the membrane or to antigens on the intra-cellular parasite possible because of increased permeability of schizont-infected red blood cells.

Recently, a set of monoclonal antibodies against \textit{P. falciparum} had been prepared by J. McBride and G. Morgan and it was important to determine whether any of these had inhibitory activity in vitro. Any inhibitory antibodies could then be used to investigate perhaps new, potentially protective antigens.

1.1 Primary screen of monoclonal antibodies for inhibitory activity

I tested 23 cloned hybridoma cell lines secreting different monoclonal antibodies which reacted in indirect immunofluorescence to \textit{P. falciparum}, isolate K1. Ascites fluids generated from these cell lines were used to determine whether any of these monoclonal antibodies could inhibit the growth of this parasite in vitro. The assay used was similar to that of Desjardins et al involving incorporation of \(^{3}H\) hypoxanthine as a direct measure of parasitaemia.

Identical asynchronous cultures were set up in the wells of microtitre plates, each well containing 100 µl of parasitized blood plus culture medium (1 per cent haematocrit, 1 per cent parasitaemia). Ascites fluid or control solutions were added (10 µl per well) and cultures were incubated by the candle jar method. After 24 hrs 1 µCi (37 KBq) of \(^{3}H\)-hypoxanthine was added per well and
incubation was continued. After a further 24 hrs the radiolabel incorporated into trichloroacetic acid precipitable material was determined for each well. Results of the initial screen are presented in Table 5.

For all sets of conditions three wells were set up so that results were obtained in triplicate. The average value for each is also presented in Table 5. In Fig. 7 these averages are presented on a linear scale to demonstrate their distribution about the mean value of the incorporation by all cultures supplemented with ascites fluid, the mean value being 4160 CPM.

The background figures were obtained for a culture to which no [G-3H]-hypoxanthine had been added. Very little label was incorporated by uninfected red blood cells, 125 CPM above background. The incorporation of label by a control culture (without antibody) (4470 CPM) is close to the mean value for all the wells to which ascites fluid was added (4160 CPM).

However, it is important to compare the values obtained upon addition of different ascites fluids. Despite the high level of variation between triplicate values for some ascites fluids, one, ascites fluid 2.13, showed a marked inhibition of parasite growth, label incorporation being only 31 per cent of the mean value, after taking into account the background incorporation of uninfected cells. Inhibition relative to the control was 71 per cent. This result has been observed repeatedly (see below). Two further observations were made in this study. Firstly, there are 5 ascites fluids, 2.9, 6.3, 7.5, 7.6 and 7.12 with average label incorporations which appear distinct from the majority of values around the mean (Fig. 7). This suggests that these ascites fluids caused an inhibition of growth of between 19 and 29 per cent. Secondly, one of the ascites fluids,
Table 5. The primary screen of ascites fluids for inhibitory activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5084</td>
</tr>
<tr>
<td>McAb 2.9</td>
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<td>3177</td>
<td>3364</td>
</tr>
<tr>
<td>McAb 2.13</td>
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<td>1214</td>
<td>1487</td>
<td>1433</td>
</tr>
<tr>
<td>McAb 2.15</td>
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<td>5633</td>
<td>5453</td>
<td>5266</td>
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<td>4316</td>
<td>3993</td>
</tr>
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<td>5121</td>
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</tr>
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<tr>
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</tr>
<tr>
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<tr>
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<td>72</td>
<td>67</td>
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<td>Uninfected r.b.c.</td>
<td>192</td>
<td>191</td>
<td>193</td>
<td>192</td>
</tr>
</tbody>
</table>

* Volume in the control wells was made up by adding culture medium instead of ascites fluid.
Fig. 7. Relative distribution of inhibitory activities of the ascites fluids. The CPM of [3H]-hypoxanthine incorporated by erythrocytic-stage, parasite cultures in the presence of each tested ascites fluid is depicted on a linear scale. 'mean' is the average value for all ascites fluids. 'control' is the incorporation in the absence of ascites fluid.
2.22 caused a marked stimulation of label incorporation, 45 per cent above the mean value. A similar observation has been made for immunoglobulin preparations from the sera of some individuals living in an area endemic for malaria\textsuperscript{29,157} and for purified monoclonal antibodies\textsuperscript{32} though a possible explanation was not given.

These conclusions from one experiment must remain tentative. The variability within the triplicate results for each ascites fluid means that this data would have to be repeated in order that the apparent inhibition of growth by ascites fluids 2.9, 6.3, 7.5, 7.6 and 7.12 could become statistically significant. The observed scatter in values for this experiment is inherent in the combination of fragility of parasite culturing with the random nature of radioactive decay. Attempts to improve accuracy during the setting up of the assay failed to improve the quality of the data. Flow cytofluorimetry may be a more precise method for the determination of parasitaemia.\textsuperscript{34} Furthermore, in this experiment only one ascites fluid was used for each monoclonal antibody and the observed effect could be a property of the ascites fluid, or even the mouse in which the ascites tumour was grown, rather than the monoclonal antibody itself. This may be controlled by assaying the purified immunoglobulin.\textsuperscript{32,36}

1.2 Confirmation of inhibition by the ascites fluid 2.13

Table 6 shows the data from an experiment designed in an attempt to increase the observed inhibition of parasite uptake of $[\text{C}^3\text{H}]$-hypoxanthine by the ascites fluid 2.13 by altering the assay schedule. Again, 100 µl cultures of \textit{P. falciparum}, isolate K1, (1 per cent haematocrit, 1 per cent parasitaemia, in wells of a microtitre plate) were supplemented with 10 µl of ascites fluid 2.13, ascites fluid 2.32 or culture medium, 9 wells for each solution. Also
### Table 6. Confirmation of inhibition by ascites fluid 2.13.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td><strong>A</strong></td>
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<td>2.13</td>
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<td>Control</td>
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<td>11548</td>
<td>10314</td>
<td>10939</td>
</tr>
<tr>
<td>Uninfected r.b.c.</td>
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<td>210</td>
<td>216</td>
<td>205</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.13</td>
<td>5849</td>
<td>5354</td>
<td>5399</td>
<td>5534</td>
</tr>
<tr>
<td>2.32</td>
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<td>14608</td>
<td>17083</td>
<td>16105</td>
</tr>
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<td>Control</td>
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<td>12599</td>
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<td>Uninfected r.b.c.</td>
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<td>179</td>
<td>177</td>
<td>169</td>
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<tr>
<td><strong>C</strong></td>
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<td>10368</td>
<td>10788</td>
<td>10743</td>
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<tr>
<td>Uninfected r.b.c.</td>
<td>174</td>
<td>172</td>
<td>171</td>
<td>172</td>
</tr>
</tbody>
</table>

Background = 46 CPM

A - label present 24-48 hrs, no change of medium, harvest 48 hrs
B - label present 48-72 hrs, no change of medium, harvest 72 hrs
C - label present 48-72 hrs, change of medium at 24 and 48 hrs, harvest 72 hrs
included were wells containing uninfected red blood cells (100 μl, 1% haematocrit). Cultures were set up at 0 hrs and then three schedules of assay were followed each with triplicate wells for each solution added: A - Label was added (1 μCi(37 KBq)/well) at 24 hrs and the wells were harvested at 48 hrs. B - Label was added (1 μCi (37KBq)/well) at 48 hrs and wells were harvested at 72 hrs. C - as for B except 75 μl of culture medium from each well was replaced with fresh culture medium supplemented to 10 per cent with the respective test solution at 24 and 48 hrs. Again harvesting involved measuring the radiolabel incorporated into TCA precipitable material.

The higher levels of radiolabel incorporation throughout this experiment were due to better growth of the parasite culture used. This may also be the reason for the incorporation from 48 to 72 hrs not being much greater than from 24 to 48 hrs. The cultures may have reached maximum parasitaemia under these conditions by 48 hrs. A Giemsa stained smear of one well culture at 48 hrs showed the parasitaemia to be 8 per cent which is very high considering that the conditions in the wells of a microtitre plate may not be favourable to parasite growth. Changing the medium every 24 hrs in schedule C also failed to improve radiolabel incorporation, perhaps because it allowed the peak parasitaemia to be reached even earlier.

Although schedules B and C did not improve upon the degree of inhibition obtained in the primary screen (Table 5) and schedule A, all of the results presented in Table 6 confirm that ascites fluid 2.13 does inhibit [G-3H]-hypoxanthine incorporation. With respect to the incorporation for the control, the inhibition of label uptake by ascites fluid 2.13 was 57 per cent by schedule A and B and 45 per cent by schedule C.
1.3 Confirmation of inhibition by the monoclonal antibody 2.13

It could be objected to the results presented above that contaminating material in the ascites fluid of 2.13 was responsible for inhibiting parasite growth rather than the monoclonal antibody. Evidence is presented here arguing strongly against that view.

The original hybridoma cell line producing monoclonal antibody 2.13 (McAb 2.13) had been sub-cloned to give two cell lines 2.13-19.1 and 2.13-19.4. In the above experiments ascites fluid 2.13-19.4 AFB, produced using cell line 2.13-19.4, was used. Two further ascites fluids, 2.13-19.1 and 2.13-19.4 AFA, which had been generated independently, were also available. Inhibition of $[G^{-3}H]$-hypoxanthine incorporation by all three ascites fluids would strongly support the hypothesis that it was the monoclonal antibody 2.13 in the ascites fluid 2.13 which was causing the effect. The only characteristic that the 3 ascites fluids share, which distinguishes them from the other ascites fluids in Table 1, is that they had been produced using a cell line secreting McAb 2.13.

*P. falciparum* cultures (100 µl, 1 per cent haematocrit, 1 per cent parasitaemia, in microtitre plates) were treated with 10 µl of ascites fluids 2.13-19.1, 2.13-19.4 AFA, or 2.13-19.4 AFB or with 10 µl culture medium, in triplicate. After 24 hrs, 1 µCi (37 KBq) of $[G^{-3}H]$-hypoxanthine was added per well and after 48 hrs the tritium incorporation into TCA precipitable material was counted (Table 7).

The incorporation of radiolabel in this experiment was generally very low, a result of the parasite culture not growing well. However, all the 2.13 ascites fluids do cause an inhibition of radiolabel uptake. The inhibition observed with respect to the incorporation of the control was 48 per cent for 2.13-19.1, 62 per cent for 2.13-19.4 AFB and 75 per cent for 2.13-19.4 AFA. These different
Table 7. Confirmation of inhibition by monoclonal antibody 2.13.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>2.13-19.1</td>
<td>1695</td>
<td>1486</td>
<td>1318</td>
<td>1500</td>
</tr>
<tr>
<td>2.13-19.4 AFB</td>
<td>595</td>
<td>760</td>
<td>1011</td>
<td>789</td>
</tr>
<tr>
<td>2.13-19.4 AFA</td>
<td>1095</td>
<td>1126</td>
<td>1151</td>
<td>1124</td>
</tr>
<tr>
<td>2.32</td>
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<tr>
<td>Control</td>
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<td>2815</td>
<td>2805</td>
<td>2744</td>
</tr>
<tr>
<td>Uninfected r.b.c.</td>
<td>148</td>
<td>146</td>
<td>149</td>
<td>148</td>
</tr>
</tbody>
</table>
levels of inhibition may be due to different concentrations of the monoclonal antibody in the different ascites fluids or partly to the variability in the assay. But these results strongly suggest that McAb 2.13 can reduce the uptake of [G-3H]-hypoxanthine by a culture of P. falciparum, in vitro.

1.4 Effect on parasitaemia by the ascites fluid 2.13

To confirm that the effect on radiolabel uptake by monoclonal antibody 2.13 was due to an effect on parasite growth, the parasitaemia was followed during the course of an assay. Cultures were set up as above (100 μl, 1% haematocrit, 1% parasitaemia, in a microtitre plate) with 10 μl addition of ascites fluid 2.13-19.4 AFB, ascites fluid 2.32 or culture medium, 3 wells for each. At 24, 48 and 72 hrs a well for each of the conditions was harvested. As much as possible of the culture medium was removed without disturbing the red blood cell layer. The red blood cells were then mixed with the remaining culture medium in each well and used to make a smear on a microscope slide for Giemsa staining. At least 3,000 red blood cells were counted and the parasitaemia, the percentage which were infected, was recorded (Table 8 and Fig. 8).

These data support the hypothesis that inhibition by McAb 2.13 of [G-3H]-hypoxanthine incorporation is due to an effect on the parasites growth. The inhibition of growth by the ascites fluid 2.13-19.4 AFB was approximately 70 per cent at 48 hrs and approximately 65 per cent at 72 hrs. However, these were preliminary results each parasitaemia being obtained from a single microtitre plate well culture. Further, all slides showed a number of pyknotic degenerating parasites,36 which were not included in the estimate of the parasitaemia although there was not a clear distinction between degenerating and healthy forms. This arbitrary distinction could
Table 8. Effect of ascites fluid 2.13 on parasitaemia.

<table>
<thead>
<tr>
<th>Parasitaemia</th>
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<th>72 hrs</th>
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<tr>
<td>2.13</td>
<td>1.0%</td>
<td>2.6%</td>
<td>1.9%</td>
<td>3.2%</td>
</tr>
<tr>
<td>2.32</td>
<td>1.0%</td>
<td>2.9%</td>
<td>3.0%</td>
<td>6.1%</td>
</tr>
<tr>
<td>Control</td>
<td>1.0%</td>
<td>2.2%</td>
<td>3.8%</td>
<td>7.0%</td>
</tr>
</tbody>
</table>
Fig. 8. Effect of ascites fluid 2.13 on parasitaemia. The increase in parasitaemia of erythrocytic-stage, parasite cultures was followed over a 72 hr period. The cultures were supplemented with ascites fluid 2.13 (Ø), ascites fluid 2.32 (+) or no ascites fluid (×).
add a further element of error to this assay. The degree of error which could be present is evidenced by the high parasitaemia measured for the 24 hr culture treated with ascites fluid 2.13-19.4 AFB.

The data presented in Tables 1 to 4 is substantial evidence that McAb 2.13 can cause a severe inhibition of *P.falciparum* growth in vitro.

1.5 Future directions from this inhibition data

All monoclonal antibodies raised to *Plasmodium* species should be checked for an ability to inhibit the growth of the parasite. Such inhibitory antibodies provide ideal tools with which to isolate and characterise the potentially protective antigens with which they bind. If the McAbs 2.13, 2.9, 6.3, 7.5, 7.6 and 7.12 are all inhibitory to *P.falciparum* growth in vitro, at least 3 different potentially protective antigens have been identified. This follows from the data presented by Hall et al. in which these monoclonal antibodies fall into 3 groups on the basis of their indirect immunofluorescence pattern on the erythrocytic stages of the parasite: McAbs 2.13 and 7.12 react strongly with a discrete area of merozoites, McAbs 7.5 and 7.6 stain the surface of merozoites, and McAbs 2.9 and 6.3 give a generalised staining of all erythrocytic stages which suggests that they recognise a cytoplasmic antigen. It would be particularly valuable to extend the growth studies to confirm the weak inhibition observed with McAbs 2.9, 6.3, 7.5, 7.6 and 7.12.

Another phenomenon which could be sought is synergy between different monoclonal antibodies in parasite inhibition. This has been found before and could improve the level of inhibition observed for some of the weakly inhibitory monoclonal antibodies identified above or even reveal inhibitory potential for those that have so far appeared negative.
In contrast the inhibition observed with McAb 2.13 is strong. There is at least 50 per cent inhibition when the culture medium is supplemented to 10 per cent with 2.13 ascites fluids. This observation could be extended in several ways. First, the direct estimation of effect on the increase in parasitaemia by observation of Giemsa stained slides should be repeated to strengthen the data in Table 8. Second, McAb 2.13 could be purified from the ascites fluid and used in the growth assay to prove directly that it is the monoclonal antibody which is causing the inhibition. This would also permit studies on dose dependence, the amount of antibody necessary to give a particular level of inhibition. Third, the stage at which the monoclonal antibody acts within the erythrocytic cycle could be ascertained. At present it is thought that antibody can act either by blocking merozoite invasion or by interfering with the intra-erythrocytic development of the parasite. This could be examined using synchronised P.falciparum cultures and following a cycle of growth, with and without the monoclonal antibody, microscopically. Finally, the antigen that McAb 2.13 recognises could be identified and isolated by affinity chromatography for characterisation. Only in this way can its potential as a protective antigen be further investigated.
Results. Chapter 2

Monoclonal antibody 5.1 and 5.1 antigen characterisation
The three stages of *Plasmodium* development at which most investigations towards a vaccine have been aimed are sporozoites, merozoites and gametes. All of these are extra-cellular, the sporozoite until it invades a hepatocyte after inoculation by the mosquito, the merozoite between release from the mature schizont and invasion of an erythrocyte and the gamete after ingestion of the gametocyte by a mosquito. Immunisation to the gametes confers no protection to the disease for the vaccinated individual, but could be very important in a malaria eradication program by blocking transmission of the parasite. Of all three stages, immunisation studies aimed at the sporozoite stage have yielded the greatest success.

Sterile immunity has been obtained using attenuated sporozoites of *P. gallinaceum* in fowls, *P. berghei* in mice, *P. knowlesi* in Rhesus monkeys and *P. vivax* and *P. falciparum* in man. Sporozoites attenuated by irradiation with UV light, X-rays or γ-rays have been delivered by intravenous injection or by mosquito bite. The protective response is probably induced by the circumsporozoite protein (CSP) which occupies most, if not all, the sporozoite surface. The CSP has been studied by surface labelling and using monoclonal antibodies. Some of these monoclonal antibodies neutralise sporozoites of *P. knowlesi*, *P. falciparum* or *P. vivax* in vitro and of *P. berghei* both in vitro and in vivo. It is now clear that the CSPs of these species are all related.

Such studies have shown that protection to human malaria by an immunisation aimed at the sporozoite stage is possible. Furthermore, a major antigen, which may stimulate the protective response, has been thoroughly characterised including analysis of the gene for *P. knowlesi*. 
However, there are limitations to a sporozoite based vaccine. Any sporozoites which escape anti-sporozoite immunity will produce a parasitaemia at un-immunised levels due to the cyclical nature of the erythrocytic stage. To achieve sterile immunity in all individuals requires large doses administered repeatedly. Also the immunity achieved is of only short duration, lasting just a few months in man, which would be a severe restriction to an eradication campaign. Lastly, the main limitation to study towards a sporozoite vaccine has been the availability of sporozoites. This stage of the parasite can only be obtained in small numbers by dissection of salivary glands from infected mosquitoes. The erythrocytic stage is more amenable to study because it can be grown in culture.

Vaccination with erythrocytic stages has not progressed so far, possibly because of the greater antigenic complexity compared to the sporozoite. Protective immunisations improved as preparations of parasite material became more focused. Irradiated intra-erythrocytic parasites were first used to immunise rats and mice to *P. berghei* and Aotus monkeys to *P. falciparum*. Then, parasites were released from red blood cells using the French Pressure Cell and used to immunise mice and Rhesus monkeys to *P. berghei* and *P. knowlesi*, respectively. But the greatest protection has involved immunisation with the extra-cellular merozoite. Various preparations of *P. knowlesi* merozoites have been used to immunise Rhesus monkeys, and Aotus monkeys have been immunised with *P. falciparum* merozoites isolated from an infected monkey or from culture. The problem of merozoite vaccination is its requirement for Freunds Complete Adjuvant, which has side effects preventing its use in man, but this may now be solved with a new adjuvant, CP20961, which is
effective and non-toxic. Immunisation has also been achieved using purified, erythrocytic-stage antigens for \textit{P. yoelii} in mice and for \textit{P. falciparum} in Saimiri monkeys.

In all studies, immunisation to erythrocytic stages prevented lethal infections but parasitaemias, though of finite duration, were still detected. The duration of protection has been shown to be at least 18 months between erythrocytic stage challenges.

Ideally, a human malaria vaccine should be directed against both sporozoite and erythrocytic stages inducing a defence against the erythrocytic stage as support for a primary blockade against the sporozoite and ensuring no development of clinical symptoms. In the simplest case, this would involve a protective antigen, common to the two stages, which could be readily characterised following its purification from a large scale erythrocytic stage culture. However, experiments expected to reveal such an antigen have so far been negative.

Immunisation with sporozoites confers no protection to challenge with erythrocytic stages and Rhesus monkeys immunised to \textit{P. knowlesi} erythrocytic stages still developed exo-erythrocytic forms when challenged with sporozoites of a different \textit{P. knowlesi} strain. Cross-reaction of antibody has also been sought by indirect immunofluorescence microscopy. Monoclonal antibodies or antisera raised to erythrocytic stages only cross-react to internal, cytoplasmic, sporozoite antigens and not to the sporozoite surface. Monoclonal antibodies and antisera raised to the sporozoite stages fail to react at all to erythrocytic stages.

These results support the view that the sporozoite surface and erythrocytic stages are immunologically distinct, even though a common antigen which is poorly immunogenic or of low abundance may
only be detected rarely by using monoclonal antibodies. Therefore, the monoclonal antibodies raised to erythrocytic stages of *P. falciparum* by J. McBride and G. Morgan\(^ {35,76}\) were screened against sporozoites by indirect immunofluorescence microscopy. One of these monoclonal antibodies, McAb 5.1, was found to cross-react to the sporozoite surface and the antigen that it recognises in the erythrocytic stages, the 5.1 Ag, has been characterised.

2.1 Screening of monoclonal antibodies for reactivity to sporozoites

The 23 monoclonal antibodies\(^ {35,76}\) screened for the ability to inhibit *P. falciparum* growth *in vitro* (Results, Chapter 1) were screened again by indirect immunofluorescence microscopy for reactivity to *P. falciparum* sporozoites. The *P. falciparum* sporozoites, from Thailand, were provided by Dr R. Nussenzweig (New York University Medical Centre) along with a polyspecific antibody to serve as a positive control.

The monoclonal antibodies were assayed at 10 fold dilutions from $10^{-1}$ to $10^{-5}$ on erythrocytic stages of *P. falciparum*, isolate K1, to confirm that they were active and screened at $10^{-2}$ and $10^{-3}$ dilutions on the sporozoite preparations. The monoclonal antibodies all reacted to the erythrocytic stages to a dilution of at least $10^{-3}$ (Table 9), but only one, McAb 5.1, reacted positively to the sporozoites. The immunofluorescence staining pattern of McAbs 5.1 and 7.3 on the sporozoite and erythrocytic stages is shown in Fig. 9.

Two sporozoite preparations were used. Air-dried sporozoites were prepared by the same method as used for the erythrocytic stage preparations. However, air-drying exposes internal cytoplasmic antigens, and so glutaraldehyde-fixed sporozoites, for which antibody can only interact with externally exposed antigens, were also used. The reaction by McAb 5.1 was very strong at $10^{-2}$ and $10^{-3}$ dilutions on both glutaraldehyde-fixed or air-dried sporozoite preparations.
Table 9. Indirect immunofluorescence assay of the reactivity of monoclonal antibodies in ascites fluids to erythrocytic and sporozoite stages.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Titre to which positive on erythrocytic stage parasite</th>
<th>Reaction to sporozoites of 10^{-2}/10^{-3} dilution of ascites fluids</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Glutaraldehyde-fixed</td>
</tr>
<tr>
<td>2.2</td>
<td>10^{-5}</td>
<td>-</td>
</tr>
<tr>
<td>2.9</td>
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<td>2.13</td>
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<td>7.12</td>
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</tr>
<tr>
<td>8.1</td>
<td>10^{-5}</td>
<td>-</td>
</tr>
<tr>
<td>positive sporozoite control</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

ND, not determined; +, positive reaction; -, negative reaction
Fig. 9. Indirect immunofluorescence of McAbs 5.1 and 7.3 to sporozoite and erythrocytic stages. A and B are glutaraldehyde-fixed sporozoites. C and D are air-dried erythrocytic stages. A and C were stained using McAb 5.1, B and D, using McAb 7.3.
The sporozoite image had a very sharp outline in the glutaraldehyde-fixed preparations but was much more diffuse for the air-dried specimens. Despite this, a positive or negative reaction could be easily scored for both preparations. In a negative reaction the sporozoite could be seen very faintly with a red colour, whereas in a positive reaction it fluoresced a vivid green. On the erythrocytic stage slides, a positive reaction was evident with the parasites again fluorescing vivid green over the red background, though the fluorescence pattern was different to that on the sporozoites reflecting the different morphologies of these two stages.

The ascites fluid 5.1 was then assayed at 10 fold dilutions from $10^{-4}$ to $10^{-9}$ on glutaraldehyde-fixed sporozoites and air-dried erythrocytic stages and was positive to a dilution of $10^{-6}$ on both preparations.

These results were surprising for two reasons. First, the epitope recognised by McAb 5.1 must be on the sporozoite surface because glutaraldehyde-fixation prevents reaction to internal antigens in this assay and the pattern of staining was clearly surface and not cytoplasmic. This result reveals an epitope shared by erythrocytic stages and the sporozoite surface which has remained undetected so far.\textsuperscript{2,185}

Second, none of the other monoclonal antibodies reacted to the air-dried sporozoites which should have internal antigens exposed.\textsuperscript{183} Several of the monoclonal antibodies, McAbs 2.30, 2.32, 6.3, 7.2 and 7.8 are thought to react to cytoplasmic antigens of the erythrocytic stages.\textsuperscript{76} Some cytoplasmic antigens are known to be common to several stages\textsuperscript{182-183} but only stage-specific cytoplasmic antigens are recognised by the set of monoclonal antibodies screened here.

McAb 5.1 defines an epitope common to the erythrocytic stages
and the surface of the sporozoite of *P. falciparum*. A common epitope is most simply explained by the presence of an antigen common to these two parasite forms. However, the monoclonal antibody could be reacting to a homologous region of two different, but structurally related, antigens. Alternatively, the monoclonal antibody may be binding to two unrelated antigens either through a common post-translational modification, such as glycosylation, or through anomalous cross-reaction, as is well documented for haptens.

The correct explanation can only be determined by studying the antigens from the two parasite stages.

2.2 **Background information on monoclonal antibody 5.1**

McAb 5.1 reacts in indirect immunofluorescence microscopy to mature erythrocytic stages. From the immunofluorescence pattern it was inferred that the 5.1 Ag is located in the parasitophorous vacuolar membrane and parasite-derived inclusions in the erythrocyte cytoplasm. The parasitophorous vacuolar membrane immediately surrounds the intra-erythrocytic parasite and is derived from the host cell membrane during invasion by the merozoite. One other of the 23 monoclonal antibodies used above, McAb 7.7, gives an apparently identical immunofluorescence pattern, suggesting McAbs 5.1 and 7.7 may bind to the same antigen. McAb 7.7 has therefore been studied in parallel with McAb 5.1.

One difference between McAbs 5.1 and 7.7 has been detected in indirect immunofluorescence studies on different *P. falciparum* isolates. McAb 5.1 reacted with 24 of 27 isolates tested implying that the 5.1 antigen is variable whereas McAb 7.7 reacted with all 27. In spite of this difference, the 5.1 and 7.7 epitopes could still be on one protein, the 5.1 epitope being in a variable region and the 7.7 epitope in a constant region. Note that the
reaction to sporozoites of McAb 5.1 but not McAb 7.7 is another
difference which has now been detected between these monoclonal
antibodies.

An attempt has been made to identify the antigens recognised by
McAbs 5.1 and 7.7 by immunoprecipitation of radio-labelled parasite
proteins. Four proteins, of molecular weights 160 K, 93 K, 60 K
and 35 K, were immunoprecipitated by McAbs 5.1 and 7.7 from a
detergent-solubilised extract of parasites surface-labelled with
^{125}I. The 93 K protein was not always detected. Immunopre-
cipitation with McAb 7.7 of parasite proteins labelled biosynthet-
ically with ^{35}S-methionine identified a 160 K protein, but no
proteins were detected in an immunoprecipitate using McAb 5.1.
However, all of these immunoprecipitation studies using McAbs 5.1
and 7.7 gave very weakly labelled protein bands (R. Hall, personal
communication) and so the identity of the antigens remained in doubt.

2.3 Immunodetection of 5.1 and 7.7 antigens in western-blotted
total parasite proteins

The sizes of the 5.1 and 7.7 antigens were revealed as follows.
Parasites were isolated from cultures of *P.falciparum*, isolate K1,
bysaponin lysis and solubilised in Laemmli SDS gel sample buffer.
Solubilised proteins were separated by SDS-PAGE and transferred to
nitrocellulose by western blotting. Tracks of total parasite
proteins were probed with monoclonal antibodies to reveal the
position of the antigens to which they bind (Fig. 10).

McAb 7.3 (track 1) did not reveal any antigen bands. This
monoclonal antibody immunoprecipitates a 190 K protein but the 7.3
epitope must be unstable to conditions used here, presumably the
SDS used in the electrophoresis. However, the 5.1 and 7.7 epitopes
are not irreversibly denatured by SDS. The 5.1 epitope is clearly
Fig. 10. Immunodetection of 5.1 and 7.7 antigens amongst western-blotted total parasite proteins. All four tracks contain total protein from saponin-lysed parasite preparations separated by SDS-PAGE on 10 per cent gels and western blotted to nitrocellulose. Antigen band positions were revealed immunologically using McAb 7.3 (track 1), McAb 5.1 (track 2), McAb 7.7 (track 3) and McAbs 5.1 and 7.7 (track 4).
revealed on a 23 K protein (track 2) and the 7.7 epitope on a 33 K protein (track 3).

This result suggests that the 5.1 and 7.7 epitopes are on different proteins. To confirm this and to explain the discrepancy of these results with those for the immunoprecipitation studies it would be necessary to purify the 5.1 and 7.7 antigens.

2.4 Purification of 5.1 and 7.7 antigens

The 5.1 and 7.7 antigens were purified by affinity chromatography. *

P. falciparum, isolate K1, parasites were solubilised with the non-ionic detergent Nonidet P40 in the presence of a buffer and protease inhibitors. The parasite protein extract was applied to monoclonal antibody affinity columns prepared by covalently coupling McAbs 5.1 or 7.7 to cyanogen bromide activated Sepharose.

The McAb 7.7 column could be thoroughly washed to remove any non-specifically bound proteins. Although the 5.1 Ag was removed from the McAb 5.1 affinity column by this washing regime because of low affinity of the antibody for the antigen, a shorter washing regime was sufficient to yield a reasonably pure preparation of 5.1 Ag.

Elution of antigen from the columns was achieved using 8 M urea. Fractions were collected and a sample of each fraction was monitored for the presence of antigen by SDS-PAGE and silver-staining of the proteins. An example of an elution profile for a McAb 5.1 affinity column is shown in Fig. 11.

Examination of the 5.1 and 7.7 antigen preparations by SDS-PAGE and silver-staining (Fig. 12a) revealed a single major band of 5.1 Ag at 22 K but multiple bands for the 7.7 Ag at 29 K, 33 K, 39 K, 49 K and 63 K. Always present in the 7.7 Ag preparations were two other minor bands at 93 K and 160 K. The major protein contaminating the 5.1 Ag preparation, at 14.5 K, is probably human haemoglobin, the
Fig. 11. Elution profile of 5.1 antigen from the monoclonal antibody 5.1 affinity column. A detergent extract of infected red blood cells (100 ml packed volume, 5 per cent parasitaemia) was passed through a McAb 5.1 affinity column. Eluted fractions, containing 5.1 Ag, were examined by SDS-PAGE on 10 per cent gels.

a. The total proteins in each fraction revealed by silver-staining. Tracks 1 to 10 are fractions 1 to 7, 9, 12 and 16.

b. The profile of 5.1 Ag alone, revealed by western blotting and immunodetection using McAb 5.1. McAb 7.7 was also included in the probing. Track 1 contains total protein from a saponin-lysed parasite preparation. Tracks 2 to 10 are fractions 1, 3 to 7, 9, 12 and 16.
Fig. 12. Immunodetection of 5.1 and 7.7 antigens in the antigen preparations. Proteins were separated by SDS-PAGE on 10 per cent gels. In all panels, track 1 is total protein from a saponin-lysed parasite preparation, track 2 is 50 μl of the 5.1 Ag preparation, track 3 is 50 μl of the 7.7 Ag preparation and track 4 is 25 μl of the 5.1 Ag preparation plus total protein from a saponin-lysed parasite preparation (half the usual amount used). In panel a, total protein present in the preparations was revealed by silver-staining. In panel b, the position of the 7.7 Ag is revealed by western blotting and immunodetection using McAb 7.7. In panel c, the position of the 5.1 Ag is revealed by western blotting and immunodetection using McAb 5.1.
major protein in the parasitised red blood cell extract remaining on the column only because of insufficient washing.

To confirm that these preparations were of the 5.1 and 7.7 antigens, western blots of the preparations were probed with McAbs 5.1 and 7.7 (Fig. 12b and c). This confirmed that the 33 K band in the 7.7 Ag preparation was indeed the 7.7 Ag (Fig. 12b). The other proteins in the preparation do not carry the 7.7 epitope and must specifically co-purify with the 7.7 Ag either because of covalent coupling via disulphide bonds or because of non-covalent associations in a protein complex which is not dissociated by the non-ionic detergent. However, the 22 K protein in the 5.1 Ag preparation (Fig. 12c, track 2) although clearly carrying the 5.1 epitope had apparently undergone some specific proteolysis compared to the 23 K 5.1 Ag in the saponin-lysed material (Fig. 12c, track 1). This difference in molecular weight was not due to different amounts of protein in these two preparations causing anomalous migration in SDS-PAGE, because when run together in one gel track the two bands were still resolved (Fig. 12c, track 4). This specific cleavage was observed repeatedly and could be the action of an intracellular parasite protease which is released by detergent extraction.

These results are generally consistent with the published data, which show bands of 160 K, 93 K, 60 K and 35 K immunoprecipitated by McAb 7.7. Affinity purified preparations of 7.7 Ag of molecular weight 33 K (which is close to 35 K) also have proteins at 160 K, 93 K and 63 K (which is close to 60 K). The 30 K, 39 K and 49 K proteins of the affinity purified 7.7 Ag preparation would not have been detected in the immunoprecipitation studies if they cannot be radio-labelled either with $^{125}$I at the parasite surface or bio-synthetically with $^{35}$S-methionine. In this way, the data concerning
the 7.7 Ag can be reconciled. For the 5.1 Ag, the low affinity of McAb 5.1 for its antigen may explain why it was not detected in the immunoprecipitation studies, but it is not clear why bands at 160 K, 93 K, 60 K and 35 K were obtained.

The yield of 5.1 and 7.7 Ag could only be estimated from the intensity of the protein bands upon silver-staining. Chemical protein determinations could not be attempted because of the contaminating proteins in the antigen preparations. However, the intensity of silver-staining for a particular amount of protein is reported to be independent of the protein being stained. From 100 ml packed parasitised red blood cells, 5 per cent parasitaemia, 10 to 20 μg of 5.1 Ag and 20 to 30 μg of 7.7 Ag could be obtained.

Preparations of 5.1 and 7.7 Ag consistent with the profiles in Fig. 12a, tracks 2 and 3 were obtained repeatedly by the affinity chromatography procedure described here. This demonstrates conclusively that the 5.1 and 7.7 epitopes are on different proteins.

2.5 Radiolabelling of the 5.1 and 7.7 antigens

2.5.1 Biosynthetic labelling. With the development of a reproducible preparation method, the 5.1 and 7.7 Ags could be demonstrated to be parasite encoded and not parasite-modified versions of host cell proteins. Red blood cells are not biosynthetically active, so a radiolabelled amino acid added to parasites in culture will only be incorporated into parasite proteins. If the 5.1 and 7.7 Ags were labelled in this way, as could be determined after their purification, then they must be parasite encoded.

*P. falciparum*, isolate K1, cultures were labelled by incubation for 3 hrs in methionine-free Eagles medium supplemented with ³⁵S-methionine. Labelled parasite proteins were extracted, as above, with Nonidet P40 in the presence of a buffer and protease inhibitors. Non-solubilised material was removed by centrifugation.
Antigens were purified from this biosynthetically labelled parasite protein extract by affinity chromatography using monoclonal antibody affinity columns. In addition to columns coupled with McAbs 5.1 and 7.7 two other affinity columns were used for comparison. These were coupled with McAb 7.2 (provided by D.Ll. Simmons) and McAb 7.3 (provided by R. Hall) which recognise 35 K and 190 K proteins respectively. Once the radiolabelled protein extract had been applied, the columns were washed before elution of bound antigen with 8 M urea. Eluted proteins were examined by SDS-PAGE and autoradiography. Samples of all eluted fractions were checked to determine the peak fraction for each column and these were then compared side-by-side to identify specifically bound proteins (Fig. 13a).

McAb 7.2 bound the very abundant 35 K protein (track 5) which is now known to be lactate dehydrogenase (D.Ll. Simmons, personal communication). McAb 7.3 bound the 190 K protein plus some minor protein bands (track 4).

McAb 7.7 bound the 7.7 Ag clearly labelled at 33 K (track 3). This demonstrates that the 7.7 Ag is parasite encoded. But note that the other major bands normally observed in the unlabelled affinity purified 7.7 Ag preparations (Fig. 12a, track 3) are not observed on this autoradiograph, suggesting that the 160 K, 93 K, 63 K, 49 K, 39 K and 29 K proteins, which co-purify with the 7.7 Ag, are not parasite encoded and may be red blood cell proteins which the 7.7 Ag interacts with. Alternatively, they may not contain methionine.

In track 2, the eluted 5.1 Ag contains two specific bands, at 22 K and 23 K. This was often observed and may result from partial proteolysis of the 5.1 Ag. A 23 K protein is seen upon immunodetection of 5.1 Ag in western blots of total proteins from saponin-lysed
Fig. 13. Autoradiographs of biosynthetically labelled antigens.
Biosynthetically labelled antigens were purified by affinity chromatography (panel a) or immunoprecipitation (panel b) and analysed by SDS-PAGE on 10 per cent gels and autoradiography. For both autoradiographs, track 1 is total biosynthetically labelled parasite proteins (9.0 x 10^4 TCA precipitable CPM), and tracks 2 to 5 are proteins from a biosynthetically labelled parasite protein extract (9.0 x 10^6 TCA precipitable CPM) which bound to Sepharose beads coupled with McAbs 5.1, 7.7, 7.3 or 7.2, respectively. Autoradiographs a and b were obtained by exposure to X-ray film for 2 months and 1 week, respectively.
parasite preparations. A 22 K protein is seen in large scale 5.1 Ag purifications. Nevertheless, the 5.1 Ag has been clearly labelled with $^{35}$S-methionine demonstrating that the 5.1 Ag is parasite encoded.

Usually this type of experiment is carried out by immunoprecipitation rather than affinity chromatography. However, repeated attempts at immunoprecipitation using McAbs 5.1 and 7.7 failed to give clear results. Immunoprecipitation was carried out using the same four preparations of monoclonal antibodies coupled to Sepharose beads and the same biosynthetically labelled parasite protein extract as was used in the affinity chromatography experiment above. After binding antigen, the immunoadsorbents were washed and then antigen was eluted into 8 M urea for examination by SDS-PAGE and autoradiography (Fig. 13b).

Heavily labelled antigens, such as those of McAbs 7.3 and 7.2 (tracks 4 and 5) would be clearly seen after an overnight exposure. But exposure for a week, as is necessary to reveal less well labelled antigens, results in a high background. At this exposure, the only specific band in track 3, the 7.7 Ag, can be seen, but it is quite a minor band in the track. The 5.1 Ag cannot be seen at all in track 2. This comparison (Fig. 13a and b) shows the advantage of affinity chromatography over immunoprecipitation when using antibodies with low affinity for their antigens or for detection of proteins of low abundance.

The intensity of the $^{35}$S-methionine labelled 5.1 Ag, purified by affinity chromatography, varied in relation to background proteins. For one experiment the 5.1 Ag band was particularly strong. The procedure followed was as described above except that the labelled parasite extract had a much lower specific activity. After brief
Fig. 14. Elution profile of biosynthetically labelled 5.1 Ag from a McAb 5.1 affinity column. 5.1 Ag was purified from a biosynthetically labelled parasite protein extract (3.0 x 10^6 TCA precipitable CPM) by affinity chromatography. Fractions eluted from the McAb 5.1 affinity column were examined by SDS-PAGE on a 10 per cent gel followed by autoradiography, with exposure to X-ray film for 1 week. Tracks 1 to 9 are eluted fractions 1 to 9.
washing, the column was eluted with 8 M urea. All of the eluate in the first nine fractions was examined by SDS-PAGE and autoradiography (Fig. 14).

In this particular experiment, the proportion of total parasite protein synthesis constituted by 5.1 Ag synthesis was estimated. From the specific activity of the extract, it was known that $3.0 \times 10^6$ TCA precipitable CPM had been applied to the affinity column. The region of the dried down gel which contained the eluted 5.1 Ag (all fractions) was placed in scintillation fluid and the $^{35}$S-methionine incorporated into the 5.1 Ag was measured as 1,960 CPM. From this it was estimated that 5.1 Ag synthesis constituted approximately 0.06 per cent of total parasite protein synthesis.

2.5.2 Iodination of surface proteins. Enzymatic iodination, using $^{125}$I, is often used to label proteins exposed at membrane surfaces, as the enzyme used can not traverse the membrane to label internal antigens. Iodination has also been used to study *P. falciparum* antigens. Saponin lysis of infected erythrocytes (as detailed in Materials and Methods, 1.6) leaves the parasite membrane intact. The red blood cell membrane and parasitophorous vacuolar membrane appear to collapse upon the parasite, but the precise structure of these membranes may be very disorganised. Enzymatic radiiodination of saponin-lysed parasite preparations will only label proteins external to the parasite membrane.

A saponin-lysed, iodinated and Nonidet P40 extracted parasite preparation was immunoprecipitated with McAbs 5.1, 7.7, 7.3 and 7.2 coupled to Sepharose beads. The immunoprecipitation procedure was as described above. Specifically bound proteins eluted into 8 M urea were examined by SDS-PAGE and autoradiography (Fig. 15).
Fig. 15. Autoradiography of radioiodinated antigens. Radioiodinated surface antigens were immunoprecipitated from a total parasite protein extract (1.4 x 10^6 TCA precipitable CPM) using McAbs 7.7 (track 1), 5.1 (track 2), 7.3 (track 3) and 7.2 (track 4) coupled to Sepharose beads. Bound proteins were examined by SDS-PAGE on a 10 per cent gel and autoradiography with exposure to X-ray film for 1 month. Track 5 is total surface radioiodinated parasite proteins (7 x 10^4 TCA precipitable CPM).
The 35 K protein (lactate dehydrogenase), recognised by McAb 7.2 and strongly labelled with $^{35}$S-methionine, was not $^{125}$I-labelled (track 4) consistent with its proposed location in the parasite cytoplasm and controlling for labelling of intracellular parasite proteins. By contrast, the 190 K protein, recognised by McAb 7.3, was strongly labelled (track 3).

Of the proteins bound by McAb 7.7 (track 1) just two bands were labelled at 33 K and 93 K. The other major bands seen in the unlabelled 7.7 Ag preparation (Fig. 12a, track 3) are not detected here implying either that they do not contain tyrosine, the amino acid labelled by the iodination procedure, or the tyrosines that they contain are concealed within the protein complex or a membrane lipid bilayer. In the previous published study, the 160 K and 60 K proteins were also labelled, but this may depend critically on the conditions of preparation of saponin-lysed parasites. Labelling in this way is confirmation of the interpretation of the McAb 7.7 indirect immunofluorescence pattern, that the 7.7 Ag is located outside of the intracellular parasite.

No bands are seen for the elution from the McAb 5.1-Sepharose beads (track 2). However, this could be due to the low affinity of McAb 5.1 for its antigen. So, affinity chromatography was carried out using the McAb 5.1 affinity column and the iodinated parasite protein extract. Eluted fractions were examined by SDS-PAGE and autoradiography (Fig. 16).

This autoradiograph does show a band at 23 K with a different elution profile to the other bound proteins and it could be the 5.1 Ag, confirming its location outside the parasite. However, it is not well labelled perhaps because the tyrosine(s) it contains are relatively inaccessible to enzymatic iodination. It was noted
Fig. 16. Elution profile of radioiodinated 5.1 Ag from a McAb 5.1 affinity column. A surface radioiodinated parasite protein extract (3.0 x 10^6 TCA precipitable CPM) was applied to a McAb 5.1 affinity column. Eluted fractions were examined by SDS-PAGE on a 10 per cent gel and autoradiography with exposure to X-ray film for 1 week. Tracks 2 to 10 are eluted fractions 1 to 9. Track 1 is total surface radioiodinated proteins (3 x 10^3 TCA precipitable CPM).
that the size of the radioiodinated 5.1 Ag (23 K) appears to correspond to that detected amongst western-blotted total parasite proteins using McAb 5.1 (Results 2.3).

2.5.3 *In vitro* translation of mRNA for *P.falciparum* antigens.

*P.falciparum* erythrocytic stage mRNA has been isolated and translated in the rabbit reticulocyte lysate system. Products from such an *in vitro* translation, radiolabelled by the incorporation of $^{35}$S-methionine, were analysed by affinity chromatography using the same 4 monoclonal antibody affinity columns as above. The *in vitro* translation products were first dispersed into 10 volumes of extraction buffer (see Materials and Methods, 2.4.3). After application of labelled proteins, the columns were washed and eluted as above. Samples from all fractions were examined by SDS-PAGE and autoradiography. Peak fractions, which had been stored at -70°C, were electrophoresed on one SDS-polyacrylamide gel with a track of total *in vitro* translation products, for autoradiography (Fig. 17).

The autoradiograph shows clearly the 35 K band (lactate dehydrogenase) recognised by McAb 7.2 (track 5 and D.L. Simmons, unpublished observations) and the 190 K band recognised by McAb 7.3 (track 4 and ref. 74). A strong band specifically bound by the McAb 7.7 column (track 3) is also observed at 35 K. This could be the primary translation product of the 33 K, 7.7 Ag but affinity chromatography using this column and *in vitro* translation products was only carried out once. This band was not revealed by preliminary immunoprecipitation experiments. Therefore, if this result is true, then the affinity of McAb 7.7 for the primary translation product must be less than for the mature, processed 7.7 Ag. This conclusion requires further substantiation.

Amongst the proteins eluted from the McAb 5.1 column there is a
Fig. 17. Antigens purified from the in vitro translation products of *P. falciparum* mRNA. Tracks 2 to 5 show proteins eluted from affinity columns coupled with McAbs 5.1, 7.7, 7.3 and 7.2 respectively after *P. falciparum* mRNA in vitro translation products (5.5 x 10⁶ TCA precipitable CPM) had been applied. The eluted proteins were examined by SDS-PAGE on a 10 per cent gel and autoradiography. Exposure to X-ray film was for 1 week (tracks 1 and 2) or 3 weeks (tracks 3 to 5). Track 1 contains total in vitro translated *P. falciparum* proteins (5.5 x 10⁴ TCA precipitable CPM).
specific protein band at 24.5 K (track 2). Interestingly, this protein was observed, though less clearly, in the preliminary immunoprecipitation experiments. This suggests that McAb 5.1 has a greater affinity for the primary translation product than for the 22 K processed 5.1 Ag. The 5.1 Ag is not an abundantly labelled protein in the in vitro translation products and, from scanning the autoradiographs, it constitutes approximately 0.08 per cent of total methionine incorporation.

The primary translation products and the processed, native products of the 5.1 Ag were compared after purification by affinity chromatography on the McAb 5.1 column. The peak fraction from each preparation was analysed by SDS-PAGE and autoradiography (Fig. 18). The two proteins specifically bound from the biosynthetically labelled preparation by the McAb 5.1 affinity column at 22 K and 23 K (arrowed, track 2) are both smaller than the primary translation product of the 5.1 Ag at 24.5 K (arrowed, track 3), specifically bound from the in vitro translation products. Therefore, the primary translation product of the 5.1 Ag must be cleaved to give the mature 5.1 Ag. This cleavage could occur rapidly after synthesis or as a requirement for its movement from the site of production inside the parasite to its final location in the parasitophorous vacuolar membrane.

The observation that McAb 5.1 binds to the primary translation product of the 5.1 Ag is of significance to the reaction of the monoclonal antibody to the sporozoite stage. It means that McAb 5.1 binds to amino acids in the primary structure of the 5.1 Ag and not to post-translational modifications. The reaction of McAb 5.1 to erythrocytic and sporozoite stages cannot be due to binding with a common modification, such as glycosylation, of two unrelated proteins. The two proteins must be at least related, if not identical.
Fig. 18. Comparison of the biosynthetically labelled 5.1 Ag and the 5.1 Ag primary translation product. Proteins were prepared by affinity chromatography using the McAb 5.1 affinity column from biosynthetically labelled parasite proteins (2.2 x 10^6 TCA precipitable CPM) (track 2) or *P. falciparum* mRNA *in vitro* translation products (8.4 x 10^6 TCA precipitable CPM) (track 3). Specifically adsorbed bands are arrowed. Track 1 contains total biosynthetically labelled protein (2.2 x 10^4 TCA precipitable CPM) and track 4 contains total *P. falciparum* mRNA *in vitro* translation products (8.4 x 10^4 TCA precipitable CPM). The proteins were examined by SDS-PAGE on a 10 per cent gel with autoradiography, exposure to X-ray film for 2 months.
2.6 Human antibody to the 5.1 antigen

Antibody is an important part of the immune defence against the erythrocytic stages of malaria parasites. The evidence for this was discussed at length in Results, Chapter 1 and involves sera from immune individuals giving protection upon passive transfer and causing an inhibition of parasite growth in vitro. It is also clear that cellular, as well as humoral, responses are important in protection, from studies looking at the levels of protection achieved in the absence of or after the passive transfer of T-cell or B-cell populations. One of the roles of T-cells is to help stimulate B-cell production of protective antibody. Not all anti-plasmodial antibody is protective and for this reason P. falciparum antigens recognised by human IgG have been examined to identify those that correlate with high levels of immunity. These studies have depended on immunoprecipitation of radiolabelled parasite proteins which has the major disadvantage that minor parasite components, possibly important in protection, may not be detected. The only solution to this problem requires identification of minor parasite components first, so that the antibody response to them may be evaluated. The antibody to the 5.1 Ag in sera from humans living in an endemic malaria area was investigated to determine the potential of this antigen's role in inducing protection to the erythrocytic stages.

2.6.1 Recognition of 5.1 antigen by human sera. To demonstrate that antibody reacts to the 5.1 Ag, samples of purified antigen and total proteins of saponin-lysed parasite preparations were subjected to SDS-PAGE and western blotting. The transferred proteins were probed with a pool of human sera obtained from Nigeria (an area endemic for falciparum malaria) to reveal the positions of antigens which have stimulated an antibody response. The 7.7 Ag was analysed
in parallel and the same proteins were also probed with McAbs 5.1 and 7.7 and with serum from an individual never exposed to malaria (Fig. 19).

The result was remarkable. The endemic sera reacted very strongly with the 5.1 Ag (panel c, track 2). A comparison of the 5.1 Ag bands detected by McAb 5.1 amongst total parasite proteins and in the purified 5.1 Ag preparation (panel b, tracks 1 and 2) with the antigen bands detected by the pooled endemic sera in the same preparations (panel c, tracks 1 and 2) reveals that the endemic sera gives a stronger signal to the 5.1 Ag than to any other parasite protein. No reaction of the endemic sera to the 7.7 Ag was detected (panel c, track 3) and the non-immune sera gave no bands at all on the western blots of purified antigens or total parasite proteins (panel d, tracks 1 to 3).

Such a strong reaction of the endemic sera to the 5.1 Ag was completely unexpected because the 5.1 Ag is only a minor parasite protein constituting only 0.06 per cent of parasite protein synthesis (see Results 2.5.1). Not only is there antibody to the 5.1 Ag present in the pooled sera but it may be present at high levels. Also note that the parasite proteins and 5.1 Ag are from a P. falciparum isolate from Thailand where as the endemic sera are from Nigeria.

As it turns out, every individual endemic serum sample examined had antibody to the 5.1 Ag. Aliquots from 14 serum samples obtained in the Gambia, another area endemic for falciparum malaria, had been pooled. Total protein from saponin lysed parasite preparations were separated by SDS-PAGE and western blotted for probing with the pooled Gambian serum and 9 of the 14 individual sera. Purified 5.1 Ag was treated similarly for probing with the pooled serum only (Fig. 20). The spectra of antigen bands recognised by the individual serum samples are very similar and the 5.1 Ag is recognised strongly
Fig. 19. Reaction of pooled endemic serum to total *P. falciparum* proteins and purified 5.1 and 7.7 antigens. In all panels, total proteins from saponin-lysed parasite preparations (track 1), affinity purified 5.1 Ag (track 2) and affinity purified 7.7 Ag (track 3) were subjected to SDS-PAGE on 10 per cent gels. In panel a, the proteins were silver-stained. In panels b, c and d, the proteins were transferred to nitrocellulose by western blotting and probed with McAbs 5.1 and 7.7 (panel b), pooled Nigerian sera (panel c) or human serum of an individual never exposed to malaria (panel d).
Fig. 20. Reaction of individual endemic serum samples to total *P. falciparum* proteins. In all tracks proteins were separated by SDS-PAGE on 10 per cent gels and western-blotted. Track 1 contains affinity purified 5.1 Ag. Tracks 2 to 11 contain total proteins from saponin-lysed parasite preparations. Tracks 1 and 2 were probed using pooled Gambian serum. Tracks 3 to 11 were probed with individual serum samples. (Reference numbers are 988 (J594), 907 (K806), 910 (K582), 924 (K664), 949 (J793), 953 (J1094), 992 (J695), 995 (J584) and 1000 (J505) respectively.)
by all. Note that these sera are from Gambia and in the first experiment the sera were from Nigeria; sera from two areas of Africa have now been shown to react strongly to the 5.1 Ag of a Thai isolate of *P. falciparum*.

This reaction of endemic human sera to the 5.1 Ag is consistent with it having a role in induction of protective antibody. The reaction to 5.1 Ag of serum samples from individuals with different immune status should now be investigated. If a correlation exists between level of immunity to *P. falciparum* malaria and intensity of reaction to 5.1 Ag then this would suggest that the 5.1 Ag does induce protective antibody.

2.6.2 **Polyclonal antibody specific to the 5.1 antigen.** A polyclonal antibody preparation which would specifically interact with the 5.1 Ag would be a very valuable experimental tool. Screening of cDNA expression libraries for the 5.1 Ag gene, which may be an eventual goal as explained in the introduction, is more likely to succeed using such a preparation than using McAb 5.1. Polyclonal antibody recognition only requires some of a protein to be correctly formed in an *E. coli* clone expressing the gene for that protein, whereas correct formation of a single particular epitope is required for recognition by a monoclonal antibody. A polyclonal antibody to the 5.1 Ag could also provide more information about the biology of the 5.1 Ag. For example, further processing of the 5.1 Ag could occur destroying the 5.1 epitope and generating the mature functional protein which should still be recognised by the 5.1 polyclonal antibody. The 5.1 polyclonal antibody could provide information on the amount of variation in the 5.1 Ag between different *P. falciparum* isolates. Some isolates do not express the 5.1 epitope but presumably still contain the 5.1 Ag. Finally, the 5.1 polyclonal antibody should
bind to the sporozoite protein which carries the 5.1 epitope, if it is the same or structurally related to the 5.1 Ag of the erythrocytic stage.

The conventional means of generating such a polyclonal antibody preparation is to immunise an animal with a purified protein to generate an antisera. However, the yield of 5.1 Ag from a large *P.falciparum* culture is very small (approximately 10 µg) and even several preparations may be insufficient to generate an antiserum of good titre and sufficient quantity. Furthermore, the 5.1 Ag prepared by affinity chromatography is not pure and antibodies to other proteins would complicate interpretation of results. An antiserum to 5.1 Ag purified further by SDS-PAGE may not react well to 5.1 Ag because of irreversible denaturation of epitopes by the SDS.

Therefore, generation of an anti-5.1 Ag antiserum was avoided by utilising the endemic human serum samples. These were already known to contain antibodies at levels adequate to react strongly to the 5.1 Ag. Antibodies which bound to the 5.1 Ag were purified by affinity chromatography, using a column of 5.1 Ag covalently coupled to Sepharose beads (see Materials and Methods, 2.5.2).

Human immunoglobulin was prepared from pooled Nigerian sera by ammonium sulphate precipitation. Part was retained as the total human immunoglobulin preparation and labelled "THIg". The remainder was applied to the 5.1 Ag column (see Materials and Methods, 2.5.4), the column was washed briefly and all of the flow-through was retained. Bound antibody was then eluted with a low pH buffer and labelled "Elution 1". The flow-through was re-applied to the re-equilibrated 5.1 Ag column and the procedure was repeated twice more. The final flow-through which was total human immunoglobulin depleted of antibody to the 5.1 Ag was labelled "DHIg".
The quantity of protein in the eluted fractions was examined by measuring the absorbance at 280 nm. It was assumed that an \( \text{OD}_{280} \) of 1.0 was equivalent to an immunoglobulin concentration of 0.7 mg/ml. The results suggest that in 1.0 ml of endemic sera, 240 µg of immunoglobulin can bind to the 5.1 Ag. The three eluted fractions were then pooled as affinity purified human anti-5.1 Ag antibody and labelled "Ha 5.1".

The specificity of these preparations was examined in their reaction to parasite antigens on western blots. Total proteins of saponin-lysed parasite preparations and purified 5.1 Ag were separated by SDS-PAGE and western blotted to nitrocellulose. Duplicate filters were probed using the pooled human sera (Fig. 21a, tracks 1 and 2), "THIg" (tracks 3 and 4), "DH Ig" (tracks 5 and 6) and "Ha 5.1" (tracks 7 and 8), all at dilutions of 1 in 200 relative to the original sera.

These results show that the preparation had worked well. Ammonium sulphate precipitation did not alter the spectrum of antigen bands detected. The "DH Ig" preparation no longer reacts detectably to the 5.1 Ag and reacts to all other parasite proteins as well as the "THIg" preparation. The "Ha 5.1" preparation reacts to the 5.1 Ag as strongly as the "THIg" but unfortunately also reacts weakly to many other parasite components. This means that not all the immunoglobulin in the "Ha 5.1" preparation is against the 5.1 Ag. Nevertheless, the 5.1 Ag column does adsorb all immunoglobulins which react to the 5.1 Ag, as far as can be detected.

Encouraged by this result, an attempt was made to improve upon the specificity of the "Ha 5.1" preparation using the pooled Gambian sera. The procedure above was repeated except that after application of the immunoglobulin, the 5.1 Ag affinity column was washed more
Table 10. Yield of human immunoglobulin which binds to the 5.1 antigen 1.

<table>
<thead>
<tr>
<th>Elution No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>OD&lt;sub&gt;280&lt;/sub&gt;</td>
<td>0.054</td>
<td>0.021</td>
<td>0.013 eluted</td>
</tr>
<tr>
<td>Ig Concentration (µg/ml)</td>
<td>37.8</td>
<td>14.7</td>
<td>9.1</td>
</tr>
<tr>
<td>Total Ig (µg)</td>
<td>75.6</td>
<td>29.4</td>
<td>18.2</td>
</tr>
</tbody>
</table>

(elutions obtained from 0.5 ml of serum)
Fig. 21. Examination of the specificity of the human antibody preparations. In all tracks proteins were separated by SDS-PAGE on 10 per cent gels and western-blotted. Tracks 1, 3, 5 and 7 contain affinity-purified 5.1 Ag and all other tracks contain total proteins of saponin-lysed parasite preparations. The blots in panel a were probed to test the initial human antibody preparations, prepared with minor washing of the 5.1 Ag affinity column prior to antibody elution. Tracks 1 and 2 were probed with pooled human serum, tracks 3 and 4 with "THIg", tracks 5 and 6 with "DHIG" and tracks 7 and 8 with "Ha 5.1". The blots in panel b were probed to test the second antibody preparations, prepared with more thorough washing of the 5.1 Ag column prior to antibody elution. Track 9 was probed with "THIg", track 10 with "DHIG", track 11 with "Ha 5.1", track 12 with McAb 5.1 and track 13 with serum of an individual never exposed to malaria.
thoroughly to remove non-specifically bound antibody. This time the specificities of the preparations were examined using western blots of total parasite proteins only (Fig. 21b) which showed that the specificity of the "Ha 5.1" was vastly improved and this preparation was used in all subsequent experiments.

The immunoglobulin concentrations were again examined by measuring the OD$_{280}$ (Table 11). This "Ha 5.1" preparation reacts almost exclusively with the 5.1 Ag. Therefore, the concentration of immunoglobulin in the endemic serum, which reacts to the 5.1 Ag is 0.23 mg/ml or 1 per cent of the total immunoglobulin concentration of 23 mg/ml.

To confirm this observation, the quantity of IgG in the "Ha 5.1" preparation and in total human serum was compared by SDS-PAGE and silver-staining (Fig. 22). The result shows the IgG which binds to the 5.1 Ag makes up between 0.1 and 1.0 per cent of the total IgG in the endemic human serum.

Clearly, the antibody to the 5.1 Ag makes up a significant proportion of the anti-parasite antibody in individuals living in an area endemic for malaria. Even taking the value from the silver-stained gel, at least 0.2 per cent of the total IgG reacts to the 5.1 Ag. As only 5 per cent of the total immunoglobulin reacts with P.falciparum antigens, at least 4 per cent of anti-P.falciparum antibody reacts with the 5.1 Ag, a minor parasite component.

2.6.3 Variation of 5.1 antigen between different isolates of P.falciparum. From earlier studies on antigenic diversity, McAb 5.1 was known not to react to 3 of 27 P.falciparum isolates tested; K34 (Thailand), PB1 (Thailand) and Palo Alto (Uganda). Assuming that the 5.1 Ag has a function, then the antigen must be present in all isolates, but structurally altered in some so that it no longer
Table 11. Yield of human immunoglobulin which binds to the 5.1 antigen 2.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>&quot;Ha 5.1&quot;</th>
<th>&quot;DH Ig&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>5.5</td>
<td>6.0</td>
</tr>
<tr>
<td>OD&lt;sub&gt;280&lt;/sub&gt;</td>
<td>0.030</td>
<td>2.76*</td>
</tr>
<tr>
<td>IgG Concentration (μg/ml)</td>
<td>21.0</td>
<td>1930</td>
</tr>
<tr>
<td>Total IgG (μg)</td>
<td>116</td>
<td>11600</td>
</tr>
</tbody>
</table>

* 10 fold dilution OD<sub>280</sub> = 0.276

(preparations obtained from 0.5 ml of serum)
Fig. 22. Comparison of IgG concentrations in the "Ha 5.1" preparation and total human sera. Proteins were separated by SDS-PAGE on a 10 per cent gel and silver-stained. Track 1, 100 μl of the "Ha 5.1" preparation (isolated from 10 μl of human serum). Tracks 2, 3 and 4 contain 1 μl, 0.1 μl and 0.01 μl of pooled Gambian serum (volumes obtained by dilution).
bears the 5.1 epitope. The degree of variation occurring in the 5.1 Ag may be investigated using the "Hα 5.1" and the "DHIg" preparations. The "DHIg" preparation contains no antibodies which can bind to the 5.1 Ag purified from *P. falciparum* isolate K1, but will still contain antibodies to the 5.1 Ag of other *P. falciparum* isolates if they bear alternative epitopes. These preparations may be used to reveal any antigenic similarity between 5.1 Ags which do or do not have the 5.1 epitope and any antigenic variation amongst 5.1 Ags which do carry the 5.1 epitope.

Six *P. falciparum* culture lines were provided by Dr D. Walliker; K1, MAD20 and Tak9/96 which bear the 5.1 epitope and PB1, Palo Alto 17 and Tak9/101 which do not bear the 5.1 epitope. Tak9/96 and Tak9/101 were cloned from a single isolate Tak9. Cultures for all of these parasite lines were grown and the indirect immunofluorescence reaction with McAb 5.1 or 7.7 was confirmed for each (Table 12). Purified 5.1 Ag from isolate K1 and total proteins from saponin-lysed parasite preparations for each of the six lines were separated by SDS-PAGE and western blotted. Duplicate filters were probed with "THIg" (Fig. 23, panel a), "DHIg" (panel b), "Hα 5.1" (panel c) and McAbs 5.1 and 7.7 (panel d).

The "Hα 5.1" probe reacts very strongly to all isolates showing that the 5.1 Ag varies little antigenically between strains. There is a slight reaction of the "DHIg" probe to the 5.1 Ags which lack the 5.1 epitope suggesting that there are antibodies present in the endemic sera which react to the alternative to the 5.1 epitope. Indeed the variation in the 5.1 epitope may be the only antigenic diversity expressed by this antigen. Such minor variation could be due to a single amino acid polymorphism in the 5.1 Ag of the *P. falciparum* species.
Table 12. Characteristics of six *P. falciparum* culture lines

<table>
<thead>
<tr>
<th><em>P. falciparum</em> line</th>
<th>Origin</th>
<th>IIF reaction with</th>
<th>Parasitaemia*</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>Thailand</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MAD20</td>
<td>Papua New Guinea</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tak9/96</td>
<td>Thailand</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tak9/101</td>
<td>Thailand</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PB1</td>
<td>Thailand</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Palo Alto 17</td>
<td>Uganda</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Parasitaemia when culture harvested for parasite preparation by saponin lysis
These data also show that the general pattern of antigens recognised by the endemic sera between isolates is very similar. A few bands do appear strikingly different and this may be due to variations in size of parasite proteins e.g. a band at approximately 45 K. The weakness of the Palo Alto 17 track is due to the low parasitaemia of this isolate, a result of very poor growth. It is also noted that the pattern of bands for Tak9/101 and PB1 appear nearly identical, with one clear difference of a band at 100 K.

2.6.4 Indirect immunofluorescence microscopy using the human antibody preparations. If the proteins of the erythrocytic and sporozoite stages of *P. falciparum* which carry the 5.1 epitope are the same or structurally related, then they will also share other epitopes. These common epitopes could be detected using the "Ha 5.1" preparation and this was attempted by indirect immunofluorescence microscopy using the glutaraldehyde-fixed sporozoite preparations.

The three human antibody preparations, "THIg", "DH Ig" and "Ha 5.1" were assayed, against air-dried erythrocytic stages of *P. falciparum*, isolate K1, and glutaraldehyde-fixed sporozoites, at 2-fold serial dilutions from 1 in 20 to 1 in 640 relative to the original sera. The 1 in 20 dilution was the least which could be generated using the "DH Ig" and "Ha 5.1" as they were prepared, without a further step of concentration. The results are presented in Table 13. At the same time the indirect immunofluorescence pattern of the preparations was also examined at 1 in 20 dilutions on microscope slide smears of the erythrocytic stages (Fig. 24) and the pattern of "Ha 5.1" was very similar, if not identical, to that of MoAb 5.176 (see Results, 2.1; Fig. 9).

The "Ha 5.1" preparation had a low titre to the erythrocytic stages and was negative on the sporozoites. The titre of the "THIg"
Table 13. Assay of human antibody preparations for reactivity to sporozoites

<table>
<thead>
<tr>
<th>Antibody Preparation</th>
<th>Sporozoites</th>
<th>Erythrocytic Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;THIg&quot;</td>
<td>1 in 40</td>
<td>1 in 640</td>
</tr>
<tr>
<td>&quot;DHlg&quot;</td>
<td>1 in 40</td>
<td>1 in 640</td>
</tr>
<tr>
<td>&quot;Hx 5.1&quot;</td>
<td>Negative at 1 in 20</td>
<td>1 in 40</td>
</tr>
</tbody>
</table>
Fig. 21. Indirect immunofluorescence of human antibody preparations against *P. falciparum* erythrocytic stages. The *P. falciparum*, isolate K1, erythrocytic stages were prepared as a blood smear and fixed in acetone. Indirect immunofluorescence microscopy was then carried out using the "THIg" (a), "DHIg" (b) or "Ha 5.1" (c) preparations at a dilution of 1 in 20 relative to the original human serum.
to sporozoites was apparently unaffected by depleting it for antibody to the erythrocytic stage 5.1 Ag. These results suggest that the proteins carrying the 5.1 epitopes on the two parasite stages are not identical.

However, the glutaraldehyde fixation of the sporozoites could result in a more limited region of the cross-reactive antigen being exposed for antibody binding than was exposed in the erythrocytic stages prepared by air drying. Also, the indirect immunofluorescence assay was not particularly sensitive so that if the cross-reactive proteins were only partially, antigenically related then the cross-reaction of the "Ha 5.1" preparation may not have been detected. Therefore, strong conclusions cannot be reached from this experiment.

A far better method to check for reaction of the "Ha 5.1" preparation to sporozoite antigens would be using a western blot of total sporozoite proteins separated by SDS-PAGE. This would detect a small proportion of common structure between the two 5.1 Ags and allow estimation of how closely related they are. However, sporozoite material, sufficient to carry out this experiment, has not been available.

2.6.5 Information about 5.1 antigen derived using human antibodies.

The preliminary observation of a strong antibody response to the 5.1 Ag in human sera from an area endemic for malaria led to the development of a very useful analytical tool, the "Ha 5.1" polyclonal antibody preparation. This reagent showed that the proteins of erythrocytic and sporozoite stages of *P. falciparum*, which bear a common epitope as detected by McAb 5.1, are different. A vaccine based on the 5.1 Ag of the erythrocytic stage, therefore, may not elicit an antibody response with protection against the sporozoite stage, as was initially hoped. This conclusion depends upon
further comparison of the antigenicity of the 5.1 Ag from the two parasite stages.

However, study with the endemic sera has raised the possibility that the erythrocytic stage 5.1 Ag could elicit an antibody response protective against the erythrocytic stages. The high levels of antibody to this minor parasite component are intriguing and it is important to follow the increase of antibody to the 5.1 Ag in relation to the development of immunity to *P. falciparum*. Also note that the 5.1 Ag is very similar for different isolates, so if a vaccine based on this antigen could induce protection it could be effective for all falciparum malaria.

A vital experiment to be carried out is to evaluate the effect of "Ha 5.1" antibody on erythrocytic growth of *P. falciparum*. An effect on *in vitro* growth could be easily assayed by supplementing a culture with this antibody preparation. The possibility of the erythrocytic 5.1 Ag inducing protection against the sporozoite or erythrocytic stages of *P. falciparum in vivo* requires further investigation.

2.7 Biological aspects of the 5.1 antigen

What is the 5.1 Ag? Antigenic and chemical analysis of the protein gives it the appearance of a solid structure without function. The only clue, so far available, as to what its function might be comes from indirect immunofluorescence microscopy observations. The 5.1 Ag is only detected on pigment containing trophozoites and schizonts the erythrocytic stages which are metabolically very active. The interpretation that it is observed on the parasitophorous vacuolar membrane and in parasite derived inclusions in the red blood cell cytoplasm suggests that it is being secreted out of the infected erythrocyte perhaps to have an effect on other host cells.
To obtain more information, and perhaps further clues to its function, the rate of accumulation, time of synthesis and final destination of the 5.1 Ag were investigated.

Information on time of synthesis could also provide a method of obtaining the 5.1 Ag gene by differential probing of cDNA libraries. The general pattern of protein synthesis through the erythrocytic cycle remains quite constant and synthesis of the 5.1 Ag at a particular stage, if it occurs, could be exploited in the screening of a cDNA library. A subset of genes identified through expression at that stage would include the gene for the 5.1 Ag.

2.7.1 Accumulation of 5.1 and 7.7 antigens through the erythrocytic cycle. The presence of 5.1 and 7.7 Ags on the mature erythrocytic parasites, as detected by indirect immunofluorescence microscopy, was examined semi-quantitatively. McAbs 5.1 and 7.7 were used to probe total parasite proteins prepared at various times through the erythrocytic cycle using a synchronised culture.

A *P. falciparum*, isolate K1, culture was synchronised using sorbitol. At intervals after this, a blood smear was taken for Giemsa staining and a parasite sample was prepared by saponin lysis from 0.5 ml of packed infected red blood cells. For each blood smear the parasitaemia and the proportions of parasites as rings, trophozoites and schizonts were determined (Table 14, and Fig. 25). The total proteins from the saponin-lysed parasite samples were probed with McAbs 5.1 and 7.7, after SDS-PAGE and western blotting (Fig. 26).

The result showed despite the apparently poor synchrony of the culture that the 5.1 and 7.7 Ags both accumulate gradually through the cycle of growth but disappear rapidly around the time of schizont rupture and reinvasion by merozoites. Accumulation of 7.7 Ag
Table 14. Proportions of erythrocytic stages after sorbitol synchronisation 1.

<table>
<thead>
<tr>
<th>Hrs. after synchronisation</th>
<th>Percentage of red blood cells infected by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rings</td>
</tr>
<tr>
<td>0 (presynchrony)</td>
<td>4.5</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>21</td>
<td>1.9</td>
</tr>
<tr>
<td>23½</td>
<td>1.5</td>
</tr>
<tr>
<td>26</td>
<td>0.9</td>
</tr>
<tr>
<td>30</td>
<td>0.7</td>
</tr>
<tr>
<td>34</td>
<td>1.2</td>
</tr>
<tr>
<td>38</td>
<td>2.0</td>
</tr>
<tr>
<td>42</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Fig. 25. Proportions of the different erythrocytic stages after sorbitol synchronization. 1. The graph shows the percentages of red blood cells infected by ring (○), trophozoite (□), schizont (▲) and all parasite stages (X) at intervals after synchronisation.
Fig. 26. Accumulation of 5.1 and 7.7 antigens through the erythrocytic cycle. For all tracks total proteins of saponin-lysed parasite preparations were separated by SDS-PAGE on a 10 per cent gel and western-blotted to nitrocellulose. The blot was probed with McAbs 5.1 and 7.7. Track 1 contains proteins prepared from an asynchronous parasite culture, tracks 2 to 9 contain proteins prepared from parasite samples taken 5, 21, 23.5, 26, 30, 34, 38 and 42 hrs after sorbitol synchronisation.
occurs slightly ahead of that for the 5.1 Ag but both disappear at the same time.

2.7.2 Synthesis of 5.1 antigen through the erythrocytic cycle.

Stage-specific synthesis of the 5.1 Ag is necessary, if the gene for the 5.1 Ag is to be identified by the differential hybridisation technique. This can only be investigated by biosynthetically labelling synchronised parasites and examining 5.1 Ag synthesis as a proportion of total protein synthesis at different stages of the erythrocytic cycle.

Once again, a *P. falciparum*, isolate K1, culture was synchronised using sorbitol. Blood smears were taken every 4½ hrs and, after Giemsa staining, the parasitaemia and proportions of different stages were counted (Table 15 and Fig. 27). At the same time, 0.5 ml of packed cells was removed and cultured for 4 hrs in $^{35}$S-methionine labelling medium. Labelled parasite proteins were extracted using Nonidet P40 in preparation for affinity chromatography (as above). An asynchronous culture had been labelled by a similar regime. Total labelled proteins for each labelling period were examined by SDS-PAGE and autoradiography (Fig. 28). The profile of proteins synthesised is similar for each labelling period, but the differences that there are appear more marked than reported by Deans et al. $^{193}$

To look at 5.1 Ag synthesis, the parasite protein extracts (equal CPM of incorporated radiolabel, in an equal volume, for each labelling period) were applied to a McAb 5.1 affinity column. After brief washing, the columns were eluted with 8 M urea. The peak fractions were identified and then examined by SDS-PAGE and autoradiography (Fig. 28).

The intensity of the 5.1 Ag band is poor because of the low activity of labelled material which could be applied to the affinity
Table 15. Proportions of erythrocytic stages after sorbitol synchronisation 2.

<table>
<thead>
<tr>
<th>Hrs after synchronisation</th>
<th>Rings</th>
<th>Trophozoites</th>
<th>Schizonts</th>
<th>Total Parasites</th>
<th>CPM/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.7</td>
<td>1.1</td>
<td>0.9</td>
<td>6.7</td>
<td></td>
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<tr>
<td>10</td>
<td>4.5</td>
<td>0</td>
<td>0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>14½</td>
<td>3.5</td>
<td>1.2</td>
<td>0</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2.6</td>
<td>2.1</td>
<td>0</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>23½</td>
<td>0.5</td>
<td>4.1</td>
<td>0</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>0</td>
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<td>1.7</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>32½</td>
<td>0.6</td>
<td>1.8</td>
<td>2.4</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>37</td>
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<td>6.5</td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>0.8</td>
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<tr>
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<td>7.4</td>
<td>0</td>
<td>0.4</td>
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</tr>
<tr>
<td>50</td>
<td>8.1</td>
<td>0</td>
<td>0</td>
<td>8.1</td>
<td></td>
</tr>
</tbody>
</table>

Unsynchronised material 5 3.0 x 10^2
Fig. 27. Proportions of the different erythrocytic stages after sorbitol synchronisation. The graph shows the percentages of red blood cells infected by ring (○), trophozoite (□), schizont (△) and all parasite stages (×) at intervals after synchronisation.
Fig. 28. Biosynthesis of 5.1 antigen through the erythrocytic cycle. Samples from a synchronised *P. falciparum* culture were biosynthetically labelled for 4 hrs with $^{35}$S-methionine. For tracks 2 to 10 labelling was commenced at 10, 14.5, 19, 23.5, 28, 32.5, 37, 41.5 and 46 hrs after sorbitol synchronisation. Tracks 1 and 11 contain proteins biosynthetically labelled in an asynchronous culture. In all tracks proteins were separated by SDS-PAGE on 10 per cent gels and autoradiographed with exposure to X-ray film for 1 month. Panel a, shows total labelled proteins, $1.2 \times 10^4$ TCA precipitable CPM per track. Panel b shows the proteins in the peak fraction obtained during affinity chromatography using the McAb 5.1 affinity column and each of the biosynthetically labelled parasite preparations. For all tracks, $1.0 \times 10^6$ TCA precipitable CPM had been applied to the affinity column.
column for each labelling period. The labelled extract used to
generate the profile in track 1 from an asynchronous culture is the
same as that used in the 5.1 Ag preparation of Fig. 14. Comparison
with this and with molecular weight markers identifies the arrowed
band as the 5.1 Ag. It is clear that the 5.1 Ag is synthesised in
every labelling period as a constant proportion of total protein
synthesis.

This result in conjunction with the study on 5.1 Ag accumulation
leads to an interesting conclusion. At schizont rupture all of
the accumulated 5.1 Ag is lost and accumulation must begin again after
a merozoite invades another red blood cell. The 5.1 Ag is then
synthesised as a constant proportion of total parasite protein
synthesis throughout all intra-erythrocytic stages. The low metabolic
synthesis of ring stages compared to trophozoites means that the rate
of accumulation of 5.1 Ag increases through the cycle and the antigen
only reaches levels detectable by indirect immunofluorescence or
immunodetection on western blots at the mature stages. This suggests
that the 5.1 Ag function may be required throughout the erythrocytic
cycle. The same conclusions could also be true for the 7.7 Ag,
though its biosynthesis at different points in the erythrocytic cycle
has not been investigated.

The observed accumulation of 5.1 Ag at the parasitophorous
vacuolar membrane and red blood cell cytoplasmic inclusions could
provide for an increasing requirement of its function at these sites
or result from an increased flow of the antigen into this depot en
route for secretion. To determine the site of 5.1 Ag function a
search for the 5.1 Ag in culture supernatants was undertaken. If
found in the culture supernatants, the 5.1 Ag could have been directly
secreted or incidentally released at schizont rupture. If not
found then the 5.1 Ag must function within the parasitised erythrocyte and is destroyed at schizont rupture.

2.7.3 Investigation of culture supernatants for presence of 5.1 antigen. The search for the 5.1 Ag in culture supernatants rested on two approaches. First, the culture supernatant was examined by immunological probing after SDS-PAGE and western blotting. Second, the culture supernatant was passed over an affinity column in case the antigen was present at low levels.

The supernatant of a *P. falciparum*, isolate K1, culture was centrifuged to remove any remaining red blood cells and the supernatant proteins were separated by SDS-PAGE alongside total proteins from a saponin-lysed parasite preparation. The proteins were western blotted to nitrocellulose and then probed using McAb 5.1 and the three human antibody preparations "THIg", "DH Ig" and "Ha 5.1" (Fig. 29).

The 5.1 Ag in the total parasite proteins (track 2) was detected by McAb 5.1 but no bands were observed in the culture supernatant track (track 1). This result was obtained repeatedly. Probing with the "Ha 5.1" preparation was carried out because if during secretion modification of the 5.1 Ag occurred such that the 5.1 epitope was destroyed, some epitopes recognised by the "Ha 5.1" preparation may remain intact. However, using this probe, the 5.1 Ag in the total parasite proteins was revealed (track 4) but there are no bands for the culture supernatant (track 3).

Probing with the "DH Ig" and "THIg" preparations was included as controls in case the "Ha 5.1" preparation did reveal antigen bands in the culture supernatant. But the "DH Ig" and "THIg" preparations themselves revealed three distinct antigen bands in the culture supernatant. These were recognised by the endemic sera and not by
Fig. 29. Immunological probing of western-blotted culture supernatant proteins for the 5.1 antigen. In all tracks proteins were separated by SDS-PAGE on a 10 per cent gel and western-blotted to nitrocellulose. Tracks 1, 3, 5 and 7 each contain 50 μl of culture supernatant from a *P. falciparum*, isolate K1, culture, 10 per cent haematocrit, 5 per cent parasitaemia. Tracks 2, 4, 6 and 8 each contain total proteins from saponin-lysed parasite preparations (from 0.5 ml packed red blood cells, 5 per cent parasitaemia). Tracks 1 and 2 were probed with McAb 5.1, tracks 3 and 4 with "Ha 5.1", tracks 5 and 6 with "THIg" and tracks 7 and 8 with "DHIg".
the second enzyme conjugated antibody used in the probing because they were not observed after probing with the "Ha 5.1" preparation. Whether they are only recognised by endemic sera (and not non-immune sera) or are parasite encoded is not known. Their sizes are 155 K, 100 K and 96 K.

The major limitation in this experiment for detecting the 5.1 Ag in the culture supernatant is the quantity of culture supernatant which can be loaded for immunological probing. The volume (50 µl) of culture supernatant applied per track would contain the 5.1 Ag secreted by 5 µl of parasitised red blood cells, where as 500 µl of parasitized red blood cells are used for detection of the 5.1 Ag in western blots of total parasite proteins. If the 5.1 Ag was being actively secreted this ratio would not be excessive, but, to increase the sensitivity, affinity chromatography was used in an attempt to concentrate the 5.1 Ag from the culture supernatants.

A large culture of *P. falciparum*, isolate K1, was grown. The parasitised red blood cells were extracted with Nonidet P40 (as above) in preparation for purification of 5.1 Ag by affinity chromatography. The cleared culture supernatant (1 l) was made 1 per cent in Nonidet P40 and then divided in two. Half was applied to a McAb 5.1 column, the other half to a McAb 7.7 column. After brief washing of the columns, bound proteins were eluted with 8 M urea. After thorough washing and re-equilibration, the McAb 5.1 column was re-used for the parasitised red blood cell extract. The column was again washed briefly and eluted with 8 M urea. The 3 sets of eluted fractions were examined by SDS-PAGE and silver-staining. The peak fractions of each elution were compared firstly by SDS-PAGE and silver-staining (Fig. 30a) and then by SDS-PAGE and western blotting followed by immunodetection using McAb 5.1 (Fig. 30b).
Fig. 30. Probing of culture supernatant proteins for the 5.1 antigen by affinity chromatography. In all tracks proteins were separated by SDS-PAGE on a 10 per cent gel. Track 1 contains proteins eluted from the McAb 5.1 affinity column after application of a parasitised red blood cell extract (100 ml packed volume of red blood cells, 5 per cent parasitaemia). Tracks 2 and 3 contain proteins eluted from the McAb 5.1 and McAb 7.7 affinity columns respectively after application of a P.falciparum culture supernatant (from 550 ml cultures, 10 per cent haematocrit, 5 per cent parasitaemia). Track 4 contains total protein from a saponin-lysed parasite preparation. Panel a, proteins were silver-stained. Panel b, proteins were western-blotted to nitrocellulose and probed with McAb 5.1.
The silver-stained gel revealed the 5.1 Ag at 22 K prepared from the parasitised red blood cell extract. So the McAb 5.1 affinity column was working well. But there were no differences in the profiles of the proteins eluted from the two columns after application of the culture supernatant suggesting that there is no 5.1 Ag in the culture supernatant. This interpretation was confirmed by the western blot.

It is concluded from these two experiments, direct probing of culture supernatants and affinity chromatography of culture supernatants, that the 5.1 Ag is not secreted out of the parasitised erythrocyte. It is possible that secreted 5.1 Ag rapidly adsorbs to surrounding cells where it would function in vivo and so would not be detected in the culture supernatant. But it seems more likely, because no 5.1 Ag could be detected at all in the culture supernatant, that the 5.1 Ag is synthesised by the parasite for transportation into the surrounding, red blood cell derived membranes where it has its function.

2.8 Characterisation of the 5.1 antigen in perspective

The 5.1 Ag is a *P.falciparum* encoded protein which is transported out of the intra-erythrocytic parasite in to the parasitophorous vacuolar membrane and inclusions in the red blood cell cytoplasm. The 5.1 Ag was not detected on the surface of infected red blood cells. During or prior to translocation the 24.5 K primary translation product is processed to 23 K.

A consequence of the antigens location outwith the parasite, is its complete disappearance at schizont rupture as merozoites escape the remains of the infected red blood cell. The invading merozoite or young ring form may then begin synthesis of fresh 5.1 Ag for
insertion into the new parasitophorous vacuolar membrane. Synthesis continues as a constant proportion (0.06 per cent) of total protein synthesis throughout the erythrocytic cycle. The 5.1 Ag is not secreted beyond the infected red blood cell and so 5.1 Ag accumulation matches increasing metabolic activity as the parasite matures. For this reason and because of its location it seems likely that the 5.1 Ag is involved in transmembrane transport of a metabolic requirement or toxic product of the intracellular parasite.

However, thoughts on the function of the 5.1 Ag should take consideration of the 7.7 Ag. Most of the above data on the 5.1 Ag is also true of the 7.7 Ag. The molecular weight is different at 33 K and synthesis of the 7.7 Ag may represent a slightly larger proportion of total parasite protein synthesis (0.1 to 0.2 per cent). All of this would still suggest that the 7.7 Ag could have a similar function to the 5.1 Ag. However, there are more major differences. There is a large antibody response to the 5.1 Ag yet no detectable response to the 7.7 Ag suggesting that their locations may not be exactly the same (e.g. opposite sides of a membrane) or that they display considerably different antigenicities or that their final fates vary. Also, the 7.7 Ag is associated with other polypeptides in a large protein complex while no other proteins have been detected in association with the 5.1 Ag. These differences could be reconciled to related functions, but would tend to suggest that the functions of the 5.1 and 7.7 Ags are different.

Despite the large antibody response, the 5.1 Ag does not demonstrate marked antigenic variation between isolates. This may mean that the antibody response is not protective. Alternatively, the structure of the 5.1 Ag may be extremely critical for its function and the strong antibody response is protective but only develops upon long term exposure to malaria.
Initial characterisation of the 5.1 Ag was hindered by low affinity of McAb 5.1 for its antigen. This characteristic may explain the cross-reaction to a component of the sporozoite surface. Although antigens bearing the 5.1 epitope from the two *P. falciparum* stages are probably not identical it still seems likely that they are structurally and hence functionally related. The 5.1 Ag was investigated because of its potential for generating a protective response against the sporozoite yet a curiously strong antibody response to the erythrocytic stage antigen has been revealed.
Results. Chapter 3

Isolation, sequence and bacterial expression of the 5.1 antigen gene
The quantity of 5:1 Ag which can be purified from the erythrocytic parasite (10 µg per 100 ml parasitised blood) has been the main limitation to characterisation of the antigen and would make future study, to determine whether the antigen is protective, impossible. Larger cultures of *P. falciparum* erythrocytic stages can not be easily grown, so the only solution is to clone and express the 5.1 Ag gene in bacteria.

Immunisation with bacterially synthesised 5.1 Ag would generate an antiserum which could be tested for an ability to inhibit the growth of *P. falciparum* in *vitro*. Reaction of the antiserum to sporozoites may reveal how similar are the two antigens which bear the 5.1 epitope. Immunisation trials against *P. falciparum* in simian models would also require large quantities of 5.1 Ag. If the 5.1 Ag was protective, then cheap synthesis in enormous quantities, as necessary for a human vaccine, could only be possible by expression of the gene in a microbiological vector.

The isolated gene would provide structural details of the 5.1 Ag. The epitope recognised by McAb 5.1 could be defined and the cross-reaction to the sporozoite surface understood. If the sporozoite and erythrocytic 5.1 Ags are different but structurally related, the gene for the former could be obtained by probing a genomic DNA library with the gene for the latter. The sequence of the 5.1 Ag gene could be compared to known sequences of other proteins and a possible function of the 5.1 Ag might be revealed. The sequence might also show whether the antigen is membrane bound, whether there are signal sequences present and where cleavages may take place during processing.

The first step towards bacterial synthesis of the 5.1 Ag must be isolation of the gene. There are several methods which have
been used to isolate genes but most can not be applied in this instance. Screening by differential hybridisation requires the presence and absence of the 5.1 Ag mRNA in two populations of mRNA which are otherwise very similar. This approach has been ruled out because I did not detect stage specific synthesis of the 5.1 Ag through the erythrocytic cycle and I have not found a *P. falciparum* isolate in which 5.1 Ag is not synthesised (see Results, 2.6.3 and 2.7.2). A common method of gene isolation is mRNA size fractionation to select or generate a subset of cDNA clones, followed by hybrid selected translation to distinguish the clone containing the gene of interest. However for mRNAs of low abundance such as that for the 5.1 Ag, very large quantities of mRNA would be required which could not be obtained from *P. falciparum* parasites cultured in vitro. Immunoprecipitation of polysomes has been used to isolate genes with rare mRNAs and antibodies to the 5.1 Ag were available for this technique, but previous attempts to isolate polysomes from *Plasmodium* spp have been unsuccessful (R. Hall, personal communication). A powerful technique still being developed is the probing of cDNA libraries with gene specific oligonucleotides synthesised on the basis of a partial amino acid sequence of the protein of interest. Synthesis of the oligonucleotide and partial sequencing of the small amount of 5.1 Ag which can be purified would require equipment not yet widely available.

So, a recent development for the screening of cDNA libraries involving immunological probing for in situ expression of cloned genes was used to isolate the 5.1 Ag gene. This technique has already been used successfully and was the approach used in two studies on *Plasmodium* spp. Two antibody probes were available, McAb 5.1 and the polyclonal "Ha5.1" preparation, and cDNA expression
libraries constructed by J. Hyde and M. Goman were available for screening. A number of published methods were employed and one eventually proved successful.

3.1 Screening of cDNA libraries for the 5.1 Ag gene

Many cDNA libraries were screened unsuccessfully before the 5.1 Ag gene was isolated. New cDNA libraries were generated by cloning cDNA synthesised from different mRNA preparations from the erythrocytic stages of *P. falciparum* into different expression vectors. (This was all carried out by J. Hyde.) Also a number of different host bacterial strains were available in which to obtain expression of the cloned cDNA and new methods for immunological probing of cDNA libraries were tried as they appeared in the literature. Each change between successive screens attempted to maximise expression or detection of expression from the recombinant cDNA.

All of the vectors used for cloning the cDNA were derivatives of the bacteriophage λ. This vector gives a greater efficiency in cloning cDNA preparations (5 x 10^8 recombinants/μg cDNA) compared to the main alternative, plasmid vectors (10^6 recombinants/μg cDNA). Only small quantities of *P. falciparum* mRNA can be obtained so this factor becomes particularly important for the generation of the large cDNA libraries necessary to include clones for the low abundance mRNAs.

Recombinant phage were either plated on a lawn of bacterial cells for screening as plaques or used to generate lysogens for screening as colonies. Various protocols were employed to release recombinant protein from lysogens. Protein from plaques or lysed colonies was bound in situ to nitrocellulose discs which were then probed by the same immunological procedures as were being employed.
successfully to detect proteins transferred to nitrocellulose by western blotting after SDS-PAGE (see Materials and Methods, 2.4.4).

Two cDNA libraries had been independently constructed by cloning cDNA into the cI gene of λNM149²⁹,₁³⁴ and had been amplified once through E.coli NM514,₁³⁴ which does not allow growth of the parent vector. Recombinant phage (3 x 10³/plate, 10 plates) were plated for screening on a lawn of E.coli NM514 for 7 hrs or overnight. Plaque proteins were blotted to nitrocellulose for 15 min at room temperature and then probed with McAbs 5.1 and 7.7. This procedure is very similar to that used successfully by Walfield et al.²⁰⁶ However, no positive phage were detected.

The main problem with these screens was the lack of a positive control to demonstrate that the techniques were working. However, the most likely explanations for failure include use of the mono-specific antibody probes, low levels of expression from the cI gene, and cleavage of foreign protein by host cell proteases.

A third cDNA library had been constructed by cloning cDNA into the unique EcoRI site at the 3' end of the coding region of the β-galactosidase gene of λgt11::Tn5. This phage vector was derived from the Young and Davis expression vector λgt11,₁³⁸ with the introduction of the kanamycin resistance selectable marker of the bacterial transposon, Tn5, by N. Bone (unpublished), so that cells lysogenised with this phage could be selected away from non-lysogenised host cells.

The library was plated directly on lawns of E.coli ED8799 (relevant genotype hsdR⁻ M⁻, SUPF, lacZM₁₅) on Xgal indicator plates.²¹⁴ A lack of blue colouration in a plaque indicates that β-galactosidase activity has been destroyed by insertion of cDNA into the β-galactosidase gene and 7,000 white plaques were picked. This recombinant phage
stock was used to generate lysogens in *E. coli* NB79 (relevant genotype \( \text{lac}^{\text{u124, lon}} \)) after a second round of amplification, through *E. coli* NB78 (relevant genotype \( \text{hsdR}^{-}, \text{SUPF}, \text{lacZ}^{\text{M15}} \)), to modify the recombinant phage DNA. A second set of lysogens were constructed using this recombinant phage stock, directly in *E. coli* BTA282 (relevant genotype \( \text{lac}^{\text{u139}, \text{alon, hflA150, hasR}^{-}, \text{SUPF}} \)) by N. Bone.

Lysogens of \( \lambda \text{gt11} \) derivatives in these two *E. coli* strains, NB79 and BTA282, have the following characteristics: 1) The repressor of the \( \lambda \text{cl}_{\text{857}} \) gene is temperature sensitive so that the phage can be induced into the lytic cycle by heating to 42°C. This generates many copies of phage DNA from which expression of the cDNA can occur. 2) The \( \text{Sam}^{-} \) mutation of \( \lambda \text{gt11} \) means that, in the absence of \( \text{SUPF} \), phage induction does not result in cell lysis, so induced phage and recombinant proteins are retained in the cell. 3) The host cell is \( \text{lon}^{-} \) and so is deficient in a protease which could act on recombinant proteins. 4) The host cell is deleted for the \( \text{lac} \) region so that homologous recombination involving the \( \text{lacZ} \) gene of \( \lambda \text{gt11} \) cannot occur.

This \( \lambda \text{gt11::Tn5} \) cDNA library, as these two sets of lysogens, was screened by the method of Kemp *et al*. Lysogen colonies (10³ colonies per plate, 10⁴ total colonies) were replicated to nitrocellulose and growth at 30°C was continued before induction of phage into the lytic cycle by elevating the temperature to 42°C. The cells were lysed by laying the filters on blotting paper saturated with 1 per cent SDS (15 min) and then placing them in an atmosphere saturated with chloroform (15 min). The filters were blocked with ovalbumin and DNase treated before probing with McAbs 5.1, 7.7 and 2.13 and with the polyclonal "Ha 5.1" preparation. No positive lysogens were obtained.
With the λgt11 vectors the production of β-galactosidase can be immunologically detected to check that all techniques in the screen are working correctly. For this reason, a rabbit anti-β-galactosidase antibody probe was used on one filter in this and all subsequent library screens and a strong signal was always obtained.

After these screens there were two further changes. Firstly, a new cDNA library had been constructed using the vector λgt11.Amp3 of Kemp et al. This is a derivative of λgt11 similar to λgt11::Tn5 except that, instead of kanamycin, an ampicillin resistance selectable marker had been introduced and it had a slightly larger cloning capacity: λgt11::Tn5 is 48.5 kb, λgt11.Amp3 is 45.5 kb and optimal lambda packaging is obtained for lengths of 36.8 to 51.5 kb. Secondly, a new method of immunological probing was used. Stanley found that some hybrid proteins involving β-galactosidase were insoluble and had to be solubilised with high concentrations of SDS and heating before immunological detection.

Lysogens were formed using the λgt11::Tn5 and λgt11.Amp3 cDNA libraries in E.coli BTA282. The colonies were grown (10^3 colonies per plate, 10^4 colonies total for each library) replicated to nitrocellulose and induced as before, but cells were then lysed by placing the filters on blotting paper saturated with 5 per cent SDS and heating (100°C, 15 min) before exposure to an atmosphere saturated with chloroform (15 min). Remaining protein binding sites were blocked with ovalbumin and SDS was electrophoretically removed from bound proteins, to allow them to renature, in a procedure analogous to that used in western blotting from SDS-polyacrylamide gels. The filters were then blocked again with ovalbumin and DNase treated before probing with McAb 5.1 and the "Ha 5.1" preparation. This procedure was used successfully to isolate part of the gene for P190 of

168.
Table 16. Screens of cDNA libraries for the 5.1 Ag gene

<table>
<thead>
<tr>
<th>Library</th>
<th>Screened as</th>
<th>Host Cell</th>
<th>Method</th>
<th>Ref</th>
<th>Ab probe</th>
<th>Success</th>
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<tbody>
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<td>PP</td>
<td>NM514</td>
<td>Walfield et al</td>
<td>206</td>
<td>McAbs 5.1 + 7.7</td>
<td>-</td>
</tr>
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<td>PP</td>
<td>NM514</td>
<td>Walfield et al</td>
<td>206</td>
<td>McAbs 5.1 + 7.7</td>
<td>-</td>
</tr>
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<td>LC</td>
<td>NB79</td>
<td>Kemp et al</td>
<td>101</td>
<td>McAbs 5.1, 7.7</td>
<td>-</td>
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<tr>
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<td>Kemp et al</td>
<td>101</td>
<td>&quot;Ha 5.1&quot;</td>
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<tr>
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<td>Kemp et al</td>
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<td>Stanley</td>
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<td>Young &amp; Davis</td>
<td>139</td>
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<td>Y1090</td>
<td>Young &amp; Davis</td>
<td>139</td>
<td>&quot;Ha 5.1&quot;</td>
<td>++</td>
</tr>
</tbody>
</table>

PP - phage plaques; LC - lysogen colonies; Ref - Reference
Once again, the 5.1 Ag gene was not detected. However, two other clones of interest were (see Results 3.2).

It is uncertain how representative these cDNA libraries remained during their formation into lysogens. At least one round of amplification had occurred prior to lysogen generation, the efficiency of lysogen formation for both λgt11 libraries was less than 1 per cent relative to input phage and after lysogen formation, amplification of the cDNA libraries occurred in order to select against non-lysogenised cells. Each of these steps could reduce the representativity of the library.

This problem was avoided by returning to screening the cDNA libraries as plaques by a method reported by Young and Davis\textsuperscript{139} and utilised successfully by Goto and Wang.\textsuperscript{210} This procedure involves a new host strain, \textit{E.coli} Y1090, which carries the SUPF gene so that at elevated temperatures λgt11 and its derivatives will complete the lytic cycle. This bacterial strain also carries the plasmid pMC9, which contains the lacI\textsuperscript{+} gene, so that high levels of lac repressor are produced preventing any expression from lacZ, the β-galactosidase gene, in the absence of an inducer. This has the advantage that genes for proteins toxic to the host cell may also be isolated.

This strain also has deletions of the lon gene and the lac region.

Both cDNA libraries in λgt11::Tn5 and λgt11,Amp3 were plated as phage directly on \textit{E.coli} Y1090 (2 × 10\textsuperscript{4} plaques per plate, 10 plates) for 3½ hrs at 42°C. Dry nitrocellulose filters containing the gratuitous lac inducer, isopropyl-β-D-thiogalactoside (IPTG), were laid over the plaques and incubation was continued for 5 hrs at 37°C. The filters were then removed and probed with the "Ha 5.1" preparation. No positive clones were identified in the λgt11::Tn5 cDNA library.
but two 5.1 Ag gene clones were obtained from the λgt11.Amp3 cDNA library (see Results, 3.3).

By these steps the technology for screening cDNA expression libraries with antibody probes gradually improved and eventually the gene for the 5.1 Ag was obtained.

3.2 Isolation of clones IHHS 1 and 2

While probing the λgt11::Tn5 cDNA library, as lysogens in E.coli BTA282, by the method of Stanley, 216 two clones were independently identified which reacted exceedingly weakly with the "Ha 5.1" probe. The strength of signal was just strong enough to allow purification of the clones but was too weak to be photographed. However, in an attempt to prove that these clones were expressing part of the 5.1 Ag, they were probed with the "DHIg" and "THIg" antibody preparations, after the removal of antibodies to E.coli antigens, and both reacted specifically and very strongly with both probes (Fig. 31). This meant two things. Firstly, although these clones did not contain the 5.1 Ag gene, they did contain genes for other *P.falciparum* antigens which were recognised by antibodies from the endemic sera and which were contaminating the "Ha 5.1" antibody preparation at low levels. This explained the weak signal obtained when using the "Ha 5.1" probe. Secondly, all of the probing techniques were working well and expression of the 5.1 Ag gene by a clone in this library would have given a very strong signal which could not have been missed.

These two clones were labelled IHHS1 and IHHS2 and a preliminary investigation of them was carried out. Identification of the protein product synthesised in these clones which was allowing them to be detected by the human antibody probes, was attempted by a similar method to that reported by Young and Davis. 138 Cultures of
Fig. 31. Reaction of the human antibody probes to the \(\lambda\)IHHS 1 and 2 lysogens of \textit{E. coli} BTA282. Three lysogens were streaked for single colonies, \textit{E. coli} BTA282 (\(\lambda\)IHHS2) (a), \textit{E. coli} BTA282 (\(\lambda\)IHHS1) (b) and \textit{E. coli} BTA282 (\(\lambda\)gt11::Tn5) (c). The lysogen colonies were transferred to nitrocellulose and induced at 42\(^\circ\)C. The cells were then lysed and released proteins were probed with "THIg" (A), "DHlG" (B) or "H\(\alpha\) 5.1" (C).
each clone and of *E. coli* N881 (*E. coli* BTA282 lysogenised with λgt11::Tn5, the parent vector) were grown to OD$_{650}$ of 0.3 and induced by incubation with shaking at 42°C for 2 hrs. Cells were then pelleted (2000 g, 5 min) and taken up in Laemmli SDS-sample buffer to 3 per cent of the original volume. After heating to 100°C for 5 min non-solubilised proteins were pelleted (Eppendorf microfuge, 12 K, 5 min) and solubilised proteins were separated by SDS-PAGE on 10 per cent gels. After western blotting to nitrocellulose, proteins were probed using the rabbit anti-β-galactosidase antibody probe and the "THIg" preparation (Fig. 32).

The recombinant proteins responsible for reaction with the human antibody probe were not identified. The "THIg" probe reacted weakly in this experiment to total parasite proteins (Panel b, track 7) because a poor parasite preparation was used. However, there were no extra bands detected in the recombinant lysogen tracks (tracks 4 to 6) compared to those for cells lysogenised with the parent phage (tracks 2 to 3). The rabbit anti-β-galactosidase antibody probe gave strong signals (Panel a). For λIHHS1 the β-galactosidase band (tracks 3 and 4) appears the same size and intensity as the parent β-galactosidase. Therefore, if the β-galactosidase detected for λIHHS1 is a hybrid protein it can only contain a few amino acids of a parasite antigen. In contrast, for λIHHS2 there is no detectable β-galactosidase band. This suggests that the antigen sequence attached to the β-galactosidase in this case makes the hybrid protein extremely unstable in the *E. coli* cell. Failure to identify recombinant proteins in a clone reacting with a human antibody probe has been observed before (A. Osland, personal communication). One possible explanation is that the recombinant protein is degraded to levels too low to detect in this procedure. Alternatively, DNA rearrangements could result in the recombinant protein being
Fig. 32. Investigation of λIHHS 1 and 2 recombinant proteins. In all tracks proteins were separated by SDS-PAGE on a 10 per cent gel and western blotted to nitrocellulose for probing with a rabbit anti-β-galactosidase antisera (panel a) or the "THIg" preparation (panel b). Track 1, β-galactosidase (SIGMA, 1 μg). Tracks 2 and 3 contain proteins from _E. coli_ BTA282 (λgt11::Tn5), tracks 4 and 5, from _E. coli_ BTA282 (λIHHS1) and track 6, from _E. coli_ BTA282 (λIHHS2). For tracks 2 to 6, proteins were extracted from the pelleted cells of a 1 ml culture with Laemmli SDS gel sample buffer (30 μl). Track 7 contains total proteins from a saponin-lysed parasite preparation.
synthesised as a small peptide which would not be detected using a 10 per cent polyacrylamide gel. Indeed, severe rearrangements in the DNA of these recombinant clones was detected by N. Bone (personal communication) during investigation of the cDNA inserts they contained.

Parasite proteins which the cloned cDNAs code for were identified by a procedure based on that of MacKay et al. Mice were immunised with a bacterial lysate of the lysogens, E.coli BTA282 (λgt11::Tn5), E.coli BTA282 (λIHH51) or E.coli BTA282 (λIHH52), and the antisera generated, which should contain antibodies to the recombinant proteins, were used to probe total parasite proteins in a western blot after SDS-PAGE. Four tracks were probed with the "TH1g" preparation or with the mice antisera raised to one of the three cloned lysogens (Fig. 33). The result showed a weak reaction of the anti-λIHH51 lysogen sera to a parasite protein of 44 K and a stronger band for the anti-λIHH52 lysogen sera at 13 K. No bands were seen using the control mouse sera probe.

Thus, two cDNA recombinant clones have been identified which react with antibody in endemic sera. The recombinant proteins responsible for this reaction have not been identified but the parasite proteins which the cDNAs code for were shown to be 44 K and 13 K. There is no information to suggest that these parasite antigens will be of use in the development of a malaria vaccine.

3.3 Isolation of 5.1 Ag cDNA recombinant clones

Screening the λgt11.Amp3 cDNA library by the Young and Davis procedure identified two clones which reacted repeatedly and strongly with the "Ha 5.1" antibody probe. By picking and rescreening (Fig. 34) these two clones were purified and with each round of purification the strength of signal from a single plaque increased.
Fig. 33. Reaction to parasite proteins of mouse antisera raised to $\lambda$IHHS 1 and 2 lysogens of *E. coli* BTA282. All tracks contain total protein of saponin-lysed parasite preparations separated by SDS-PAGE on a 10 per cent gel and western blotted to nitrocellulose. Track 1 was probed with "THIg", track 2 with mouse anti-*E. coli* BTA282 ($\lambda$IHHS1) antisera, track 3 with mouse anti-*E. coli* BTA282 ($\lambda$IHHS2) antisera and track 4 with mouse anti-*E. coli* BTA282 ($\lambda$gt11::Tn5) antisera.
Fig. 34. Purification of λIH8 and λIH9. For each filter plaque proteins were probed using the "H5 5.1" antibody preparation.
Filters 1 to 3 show the purification of λIH8 and 4 to 6, the purification of λIH9. Filters 1 and 4 are from the primary screen; 2 x 10⁴ plaques per plate, a single positive plaque on each filter. Filters 2 and 5 are from the secondary screen; 1.0 x 10³ plaques per plate, 15 positive plaques on filter 2 and 7 on filter 5. Filters 3 and 6 are from the final screen of the purified phage; 47 plaques on filter 3 and 42 on filter 6 and all are positive.
One of the clones, λIH8, always gave a stronger and larger response than the other, λIH9.

The most likely explanation for the success of this screen is the larger number of independent clones examined compared to all previous screens. Just two positive clones were identified amongst $2 \times 10^5$ phage of the unamplified λgt11.Amp3 cDNA library. The λNM1149 cDNA libraries were not originally prepared as expression libraries and only $6 \times 10^4$ independent clones were probed. The λgt11::Tn5 cDNA library could contain $7 \times 10^3$ recombinants at most, the original number of hand picked plaques and this only if no clones had been selected against during the various amplification steps involved in some of the screening procedures. The only previous screen of the λgt11.Amp3 library was of $10^4$ clones, once again after amplification. However, important in this comparison, is that many parent phage were present in the λgt11.Amp3 cDNA library and probably only 10 per cent, the proportion of white plaques in samples plated on Xgal$^{214}$ indicator plates (M. Goman, personal communication), were recombinants.

It is inferred that only one in approximately 10,000 recombinants of the cDNA library was expressing detectable levels of 5.1 Ag. This is not a surprisingly low frequency. The 5.1 Ag only accounted for approximately 0.08 per cent of the methionine incorporated into total P.falciparum mRNA in vitro translation products (see Results 2.5.3). This percentage of methionine incorporation can be taken as an estimate of the detection frequency for a 5.1 Ag gene recombinant clone in the cDNA expression library after consideration of two factors. First, the 5.1 Ag is small (24 K) and because larger proteins incorporate more methionine per intact mRNA the frequency of a 5.1 Ag cDNA clone will be slightly higher than the percentage
of methionine incorporation into the 5.1 Ag. It is assumed that
the 5.1 Ag has an average methionine content and that efficiency of
5.1 Ag mRNA in vitro translation is also average. Second, only 1
in 6 cDNA recombinants will contain the cDNA insert in the correct
orientation and phase with respect to the host gene for maximal
expression. However, a value of 1 in 2 was found in one study suggesting that detectable expression can occur without fusion to a
host protein. After taking into account these considerations, the
frequency with which 5.1 Ag cDNA recombinant clones were detected,
1 in 10^4 recombinants, is reasonable.

Evidence that λIH8 and λIH9 were clones containing 5.1 Ag coding
sequences came from a comparison between their response and the
response of λgt11.Amp3, the parent phage, upon probing, by the Young
and Davis method, with different antibody preparations (Fig. 35).
All three phages reacted equally well with the rabbit anti-β-galactosidase
antisera (filters 1, 7 and 13). None of the human antibody
preparations reacted to λgt11.Amp3 (filters 2 to 4). However, both
the "THIg" and "Ha 5.1" probes, but not the "DHIg" probe, reacted to
λIH8 and λIH9 (filters 8 to 10 and 14 to 16). This was good evidence
that λIH8 and λIH9 were 5.1 Ag cDNA clones, but this interpretation
was proven when both clones reacted specifically with McAb 5.1
(filters 11 and 17). McAb 5.1 did not react to λgt11.Amp3 (filter 5)
and McAb 7.7 did not react to any of the three phages (filters 6, 12
and 18).

Several significant conclusions were drawn from this study.
First, the reaction of McAb 5.1 to clones λIH8 and λIH9 provides
proof that the 5.1 epitope is contained in the primary structure of
the 5.1 Ag as was concluded in earlier studies (see Results, 2.3.5)
using McAb 5.1 and P.falciparum mRNA in vitro translation products.
Fig. 35. Reaction of different antibody probes to $\lambda$IH8 and $\lambda$IH9. Each filter was prepared from plates with 150 plaques of $\lambda$gt11.Amp3 (filters 1 to 6), $\lambda$IH8 (filters 7 to 12) or $\lambda$IH9 (filters 13 to 18) plated on *E. coli* Y1090. The filters were probed with rabbit anti-\(\beta\)-galactosidase antiserum (filters 1, 7 and 13), "THIg" (filters 2, 8 and 14), "DHIg" (filters 3, 9 and 15), "H\(\lambda\) 5.1" (filters 4, 10 and 16), McAb 5.1 (filters 5, 11 and 17) or McAb 7.7 (filters 6, 12 and 18).
Second, the primary structure of the 5.1 epitope is encoded by both λIH8 and λIH9 cDNA inserts and must be generated by correct folding of the recombinant proteins. Finally, the stronger reaction to λIH8 than to λIH9 for the human antibody probe was also found for the McAb 5.1 probe. One explanation for this is that the λIH8 recombinant protein is synthesised faster or is degraded more slowly than that of λIH9. Another explanation, that λIH8 encodes a larger proportion of the 5.1 Ag only holds if the two cDNA inserts encode different numbers of a repeating 5.1 epitope. (This point will be returned to later in the chapter.)

The activity of β-galactosidase in these clones was investigated. Usually insertion of a cDNA insert into the 3' end of the β-galactosidase coding sequence inactivates the enzyme and a lack of β-galactosidase activity in these clones would support the prediction that the 5.1 Ag coding sequences have been correctly introduced into the unique EcoRI site of the λgt11.Amp3 expression vector. The test was carried out by plating λIH8, λIH9, λgt11.Amp3 (active β-galactosidase encoded) and λcI857Sam7 (no β-galactosidase encoded) on a lawn of E.coli Y1090 with Xgal214 in the top agar. The phage were grown 3½ hrs at 42°C and then induced by laying dry nitrocellulose filters containing IPTG over the plaques. After incubation at 37°C for 5 hrs, the filters were removed and photographed. The blue colour, generated by active β-galactosidase, transferred well to the filter (Fig. 36). The recombinant phage, λIH8 and λIH9, do not express an active β-galactosidase as expected.

These 5.1 Ag gene clones were pursued further by a closer analysis of the cloned DNA. Not only did this confirm the cloning events for these recombinant phage, but more importantly provided new information about the 5.1 Ag gene and the 5.1 Ag itself.
Fig. 36. Examination of $\beta$-galactosidase activity in $\lambda$IH8 and $\lambda$IH9. The phage, $\lambda$gt11.Amp3 (filter 1), $\lambda$cI$\_857$ Sam7 (filter 2), $\lambda$IH8 (filter 3) and $\lambda$IH9 (filter 4), were plated on *E. coli* Y1090 (approximately 100 plaques per filter) with Xgal, in the top agar. Induction, by laying an IPTG saturated nitrocellulose filter over the plate leads to a blue colouration in the vicinity of active $\beta$-galactosidase.
3.4 Analysis of \( \lambda \)IH8 and \( \lambda \)IH9 DNA

The \( \lambda \)IH8 and \( \lambda \)IH9 DNAs were subjected to restriction enzyme analysis in preparation for isolation of cDNA inserts for sequencing. First, lysogens of these phage and of \( \lambda \)gt11.Amp3 and \( \lambda cI_{857}Sam7 \) were prepared in E.coli Y1090 so that DNA could be rapidly prepared for each of them, whenever necessary. E.coli Y1090 was used because, \( \lambda \)IH8 and \( \lambda \)IH9 were known to be stable in this host. DNA of \( \lambda \)gt11.Amp3 was required for comparison with the 5.1 Ag recombinant phage DNA and \( \lambda cI_{857}Sam7 \) was used for molecular weight markers. The lysogens were prepared by selecting for the presence of active \( cI \) repressor. Selection through drug resistance was not possible because ampicillin resistance, the \( \lambda \)gt11.Amp3 selectable marker, was already present in E.coli Y1090.

Confirmation that the desired lysogens had been formed was obtained. Small cultures were grown at 30°C and induction by heating to 42°C caused cell lysis liberating phage. The phage for each lysogen were examined by plating on E.coli Y1090 at 42°C and probing as before with the "Ma 5.1" antibody probe. As predicted, the phage from lysogens E.coli Y1090 (\( \lambda \)IH8) and E.coli Y1090 (\( \lambda \)IH9) were positive and those from E.coli Y1090 (\( \lambda \)gt11.Amp3) and E.coli Y1090 (\( \lambda cI_{857}Sam7 \)) were negative.

To prepare DNA, large cultures of the lysogens were grown at 30°C, and induced by heating to 42°C, with lysis completed at 37°C. The phage were purified and the DNA extracted for restriction enzyme analysis.

3.4.1 EcoRI digestion of \( \lambda \)IH8 and \( \lambda \)IH9 DNA. The cDNA inserts should be bounded by the only EcoRI restriction sites in the recombinant phage DNA. The cDNA had been cloned into the unique EcoRI site of the \( \lambda \) vector using EcoRI linkers. The cDNA was not
Fig. 37. $\lambda$IH8 and $\lambda$IH9 DNA digested with EcoRI. DNA preparations (30 µg) of $\lambda$gt11.Amp3 (track 1), $\lambda$IH8 (track 2) and $\lambda$IH9 (track 4) were digested with EcoRI. Size markers (track 3) are the HindIII digestion products of $\lambda$cl $\lambda_{857}$ Sam7 DNA (30 µg). The digestion products were analysed by electrophoresis on a 2 per cent agarose gel.
modified to avoid cleavage by the EcoRI digestion immediately prior to ligation into the vector, so there should be no EcoRI sites within the cDNA insert. Therefore, cleavage of the recombinant phage DNA with EcoRI should liberate the cDNA inserts and sizes of the cloned segments can be immediately determined.

Cleavage of λIH8 and λIH9 DNA with EcoRI liberated 335 and 660 bp fragments respectively (Fig. 37, tracks 2 and 4). No fragment was liberated from λgt11.Amp3 (track 3). These cDNA inserts are quite large and the λIH9 insert probably contains most of the coding sequence. The 5.1 Ag (24.5 K) would be expected to be encoded by an mRNA of at least 740 bases. It was noted that λIH9, which gave the weaker signal upon immunological probing, contains the longer cDNA insert.

The two cDNA inserts were purified from the agarose gel and phenol extracted in preparation for sequencing.

3.4.2 KpnI/SstI digestion of λIH8 and λIH9 DNA. The clones λIHHS 1 and 2 (see Results, 3.2) and some other cDNA recombinant phage isolated from the λgt11::Tn5 library on the basis of reaction with human sera by A. Osland, had been found upon restriction enzyme analysis by N. Bone to have undergone severe rearrangements (personal communication). To check that λIH8 and λIH9 had not suffered similarly, their DNA was digested with KpnI and SstI and the restriction fragments generated were examined.

These enzymes cleave λgt11.Amp3 at a number of sites around the point of cDNA insertion. Gross rearrangements in this region should be easily revealed by this analysis. This double restriction has been used by others (N. Bone, R. Hall, J. Hyde, D. Simmons, personal communications) to obtain the size of small cDNA inserts because it leaves the inserted DNA on a 2.08 kb fragment.
Fig. 38. λIH8 and λIH9 DNA digested with KpnI and SstI. DNA preparations (4 μg) of λIH8 (track 1), λgt11Amp3 (track 2) and λIH9 (track 3) were digested with KpnI and then SstI. Size markers (track 4) are the HindIII digestion products of λcl857sam DNA (4 μg). The digestion products were analysed on a 0.7 per cent agarose gel.
The result showed no gross rearrangement of \( \lambda \text{IH8} \) or \( \lambda \text{IH9} \) DNA (Fig. 38, tracks 1 and 3) compared to \( \lambda \text{gt11.Amp3} \) (track 2). Fragments of 1.5, 6.2, 17.1 and 18.6 kb remain the same in all three phage. The 2.08 kb fragment of \( \lambda \text{gt11.Amp3} \) into which cDNA is inserted, increased in size for \( \lambda \text{IH8} \) and \( \lambda \text{IH9} \) to 2.35 and 2.64 kb as accurately as could be measured. There is one partial digestion fragment in the \( \lambda \text{IH9} \) track at 4.2 kb.

The result provided further assurance that the 5.1 Ag cDNA inserts have been cloned in the manner intended.

3.5 **Sequencing of \( \lambda \text{IH8} \) and \( \lambda \text{IH9} \) cDNA inserts**

The \( \lambda \text{IH8} \) and \( \lambda \text{IH9} \) cDNA inserts were sequenced by the dideoxy chain termination method using the single-stranded bacteriophage M13 cloning vector. The cDNA inserts obtained after EcoRI cleavage of \( \lambda \text{IH8} \) and \( \lambda \text{IH9} \) DNA were cloned into M13 mp11 by M. Mackay.

For each cDNA insert ten M13 recombinants were purified and labelled IH8 (1 to 10) and IH9 (11 to 20). Single-stranded DNA templates were prepared for IH8 (3 and 6 to 10) and IH9 (13, 15, 16, 18, 19, and 20). The IH8 (8 to 10) and IH9 (18 to 20) DNA was sequenced using a commercial primer for template directed DNA polymerase I DNA synthesis and strand synthesis was specifically terminated using dideoxynucleotides. The products of the reaction were analysed by thin acrylamide gel electrophoresis (Fig. 39). The sequences determined (Fig. 41) were confirmed by M. Mackay using the above DNA preparations and DNA prepared from the other IH8 and IH9 M13 clones.

As the \( \lambda \text{IH8} \) and \( \lambda \text{IH9} \) DNAs both code for the 5.1 Ag, sequences shared by the two cDNA inserts was expected. The sequences for IH8 (8) and IH9 (18 and 19) were identical except for the length of A residues, 18 for IH8 and 12 for IH9. These long runs of A residues at the
Fig. 39. Sequencing gel for 1H8 (8, 9 and 10), 1H9 (18, 19 and 20). The sets of 4 tracks contain the sequencing reaction products for 1H8(8) (set 1), 1H8(9) (set 2), 1H8(10) (set 3), 1H9(18) (set 4), 1H9(19) (set 5) and 1H9(20) (set 6). G, A, T and C indicate the tracks for which the sequencing reactions were carried out in the presence of ddGTP, ddATP, ddTTP and ddCTP, respectively.
Fig. 40. Relationship of the 1H8 and 1H9 cDNA inserts to the 5.1 Ag mRNA. 

\[
\begin{align*}
5' & \quad \text{poly(A)} \quad 3' \\
\text{---} & \quad 650\text{bp.} \quad (\text{A})_{12} \quad \lambda 1H9 \text{ cDNA insert} \\
\text{---} & \quad 336\text{bp.} \quad (\text{A})_{18} \quad \lambda 1H8 \text{ cDNA insert} \\
\end{align*}
\]

\[
\begin{align*}
1H9(20) & \quad \text{---} \quad \text{240bp.} \quad \text{---} \quad 1H8(8) \text{ and} \\
\text{---} & \quad \text{---} \quad 1H8(9+10) \quad \text{---} \quad \text{---} \quad 1H9(18+19) \\
\end{align*}
\]

, mRNA. ---- , DNA. ----- , sequencing reactions.
ends of the cDNA inserts are probably derived from the poly(A) tail of the mRNA, so the two inserts correspond to different lengths of the 5.1 Ag mRNA both starting from the 3' terminus (Fig. 40).

The sequence for the λIH8 insert (Fig. 41) was completed using IH8 (9 and 10) which have the cDNA cloned in the M13mp11 cloning vector in the opposite orientation to that in IH8 (8). Sequencing in the two directions gave an overlap at the position predicted for the size of the insert. The coding frame was determined by reference to the known reading frame of the β-galactosidase gene of the λgt11 vector into which the cDNA had originally been introduced for expression. This gives a long open reading frame which almost certainly codes for part of the 5.1 Ag because there are many nonsense codons in the other two frames. This suggests that for λIH8 the antibody probes were recognising a hybrid protein of β-galactosidase and the 5.1 Ag. The 5.1 epitope must be present in this sequence of 73 amino acids but a closer description will require further analysis.

The full sequence of the λIH9 cDNA insert has not been derived yet. However, 330 bp have been obtained from the IH8 cDNA sequence and 240 bp from the 5' end were obtained from sequencing IH9 (20) (Fig. 41). Unexpectedly, immediately at the 5' end of the IH9 cDNA insert was found a nonsense codon in phase with the β-galactosidase coding sequence. Therefore, expression of a hybrid protein of β-galactosidase and the 5.1 Ag cannot occur in this case and expression of 5.1 Ag coding sequences must involve initiation of translation from a site other than that used for β-galactosidase synthesis. The weak signal obtained upon immunological probing of λIH9 is probably due to a reduced rate of translation of 5.1 Ag sequences for λIH9 compared to that for the recombinant protein of λIH8.
Fig. 41. Sequence of the $\lambda$IH8 and $\lambda$IH9 cDNA inserts and of the 5.1 antigen.

$$\text{(GAATTCC)}\text{TTT AAT TTA TTT AAT ATA TTC AAA ATG AAA}$$

EcoRI linker

$$\text{ATC TTA TCA GTA TTT TTT CTT GCT CTT TTC}$$

Ile Leu Ser Val Phe Phe Leu Ala Leu Phe

$$\text{TTT ATC ATT TTC AAT AAA GAA TCC TTA GCC}$$

Phe Ile Ile Phe Asn Lys Glu Ser Leu Ala

$$\text{GAA AAA ACA AAC AAA GAA ACT GGA AGT GGT}$$

Glu Lys Thr Asn Lys Glu Thr Gly Ser Gly

$$\text{GTT AGC AGC AAA AAA AAA AAT AAA AAA GGA}$$

Val Ser Ser Lys Lys Lys Asn Lys Lys Gly

$$\text{TCA GGT GAA CCA TTA ATA GAT GTA CAC GAT}$$

Ser Gly Glu Pro Leu Ile Asp Val His Asp

$$\text{TTA ATA TCT GAT ATG ATC AAA AAG AGA GAC}$$

Leu Ile Ser Asp Met Ile Lys Lys Arg Asp

$$\text{TGT GAG TAC AAG AAT CGA TTA ACT GC. ...}$$

Cys Glu Tyr Lys Asn Arg Leu Thr Ala ...

$$\text{approximately 80 bp ... ... ... not yet sequenced}$$

$$\text{(GAATTCC)GC AAG TTT ATA TTT GGA GGT GTT GGT TTA}$$

EcoRI linker

$$\text{GTA TTA TAC ACT GAA AAA GGA AGA CAC}$$

Val Leu Tyr Asn Thr Glu Lys Arg His

$$\text{CCA TTC AAA ATA GGA TCA AGC GAC CCA GCT}$$

Pro Phe Lys Ile Gly Ser Ser Asp Pro Ala

$$\text{GAT AAT GCT AAC CCA GAT GCT GAT TCT GAA}$$

Asp Asn Ala Asn Pro Asp Ala Asp Ser Glu

$$\text{TCC AAT GGA GAA CCA AAT GCA GAC CCA GAA}$$

Ser Asn Gly Glu Pro Asn Ala Asp Pro Glu

$$\text{GTT ACA GCT CAA GAT GTT ACA CCA GAG CAA}$$

Val Thr Ala Gln Asp Val Thr Pro Glu Gln

$$\text{CCA CAA GGT GAC GAC AAC AAC CTC GTA AGT}$$

Pro Glu Asp Asp Asn Asn Leu Val Ser

$$\text{GCC CCT GAA CAC TAA ACG CAC GCT GTA AAC TTT}$$

Gly Pro Glu His ...

$$\text{TGT GGG TTT TTT TGA AAT ATT ACG TGA}$$

AAA TAA TTT TTA TTT ATG ATT ATA TTA TAT

ATA TIG CTA TTT TAA (A) 12 or 18 (GGAATTCC) EcoRI linker
An open reading frame extends throughout the IH9 5' sequence and the other reading frames contain many nonsense codons. There is a methionine codon 25 nucleotides from the 5' terminus which may mark the start of the 5.1 Ag coding sequence. In the parasite, translation of the mRNA could begin from a site not present on this cDNA clone which is 5' to this methionine codon. However, this seems unlikely because there is a perfect signal sequence in the amino acid sequence as described (Fig. 41). There is a charged residue at the N-terminus (lys-2), then a long hydrophobic region (ile-3 to phe-16) followed by amino acids ser-20, ala-22 and glu-33 which are all expected for cleavage of the signal sequence after ala-22. Such a cleavage would remove 2.6 K, which may be part of the explanation for the difference in apparent molecular weights of the 5.1 Ag \textit{in vitro} translation product and the mature 5.1 Ag (see Results 2.5.3). It is tempting to speculate that signal sequence cleavage from the primary translation product (24.5 K) generates a 22 K protein and the mature 23 K product contains a further labile modification which may be lost during 5.1 Ag purification.

The 240 bp at the 5' end of the IH9 cDNA insert code for 71 amino acids with a molecular weight of 8.0 K. The 335 bp of the IH8 cDNA insert code for 73 amino acids with a molecular weight of 7.7 K. This leaves approximately 90 bp only of the IH9 cDNA insert unsequenced to code for 8.8 K of the 5.1 Ag \textit{in vitro} translation product of apparent molecular weight 24.5 K. The 5.1 Ag may migrate anomalously in SDS-PAGE. The remaining segment may be sequenced using the BclI restriction site at nucleotide 193 of the \textlambda IH9 cDNA insert. This restriction enzyme leaves the same 5' unpaired sequence as BamHI and so can be used for cloning directly into M13mp10 to obtain the remainder of the 5.1 Ag sequence.
3.6 Investigation of 5.1 Ag recombinant proteins

Reaction of antibody probes with λIH8 and λIH9 demonstrated that these clones are expressing 5.1 Ag coding sequences. Identification of the recombinant proteins responsible for this reaction was attempted. Recombinant cDNAs were expressed within bacterial cells and total cell proteins were probed, after SDS-PAGE and western blotting, with the polyclonal "Ha 5.1" antibody preparation.

Two strategies were used to obtain expression of the 5.1 Ag cDNA inserts. First, cultures of E.coli Y1090 lysogenised with λIH8, λIH9 or λgt11.Amp3 were grown at 30°C and expression from lacZ was induced at an OD$_{650}$ of 0.3 by adding filter sterilised IPTG to 1 mM. Culturing at 30°C was continued for a further 2 hrs. Under these conditions the prophage are not induced into the lytic cycle so this approach requires expression of sufficient recombinant protein for detection from the single copy of the recombinant gene integrated into the host chromosome.

The second approach required preparation of E.coli BTA282 lysogenised with λIH8, λIH9 and λgt11.Amp3. An attempt to prepare these by selecting against non-lysogenised cells with ampicillin (BTA282 is amp$^S$) failed for unknown reasons. But they were successfully constructed in the same way as for the E.coli Y1090 lysogens by selecting for the presence of functional cI repressor. The three E.coli BTA282 lysogens were grown at 30°C to OD$_{650}$ of 0.3 and were then induced by incubating at 42°C for a further 2 hrs. In this way the prophage is induced and large copy numbers of the recombinant genes are generated, but cell lysis does not occur because E.coli BTA282 does not carry the SUPF gene that λgt11 phage requires to complete the lytic cycle.

Both sets of lysogens were then harvested by pelleting the cells
and solubilising them in Laemmli \(^{123}\) SDS-sample buffer to 3 per cent of the original volume. Total cell proteins were examined by SDS-PAGE, western blotting and immunological probing using the rabbit anti-β-galactosidase antisera and the "Ha 5.1" preparation (Fig. 42).

Both sets of lysogens gave the same pattern of results (although higher levels of expression were observed for the \texttt{E.coli} Y1090 lysogens) confirming the interpretation of the sequence data. The \(\lambda IH8\) cDNA insert is expressed to give a fusion protein of β-galactosidase and part of the 5.1 Ag. This hybrid protein (Fig. 42, panel a, tracks 1 and 6) is larger than the parent β-galactosidase (panel a, tracks 3, 4 and 7) although the increment in size is difficult to measure on this gel of 10 per cent acrylamide. This \(\lambda IH8\) hybrid protein clearly contains 5.1 Ag epitopes (panel b, tracks 1 and 6) which are not present on the parent β-galactosidase (panel b, tracks 3 and 4).

For \(\lambda IH9\), the 5.1 Ag coding sequences are not expressed to give a hybrid protein. The \(\lambda IH9\) lysogens still generate a β-galactosidase (panel a, tracks 2 and 5) which is of apparently identical size to the parent β-galactosidase (panel a, tracks 3, 4 and 7). In panel b, the β-galactosidase of the \(\lambda IH9\) lysogens was not detected by the anti-5.1 Ag probe (tracks 2 and 5) and instead 3 new bands were detected at 23 K, 24 K and 22 K in order of increasing intensity. This is a remarkable result because the 5.1 Ag \texttt{in vitro} translation product was measured as 24.5 K, the mature 5.1 Ag is 23 K and this is degraded during purification to 22 K. This result strongly suggests that the entire 5.1 Ag coding sequence is present in and expressed from the \(\lambda IH9\) cDNA insert. Not only that but the primary translation product appears to be processed in \texttt{E.coli} in a similar manner to that which occurs in the parasite.
Fig. 42. Identification of the recombinant proteins of λIH8 and λIH9. Proteins were separated by SDS-PAGE on a 10 per cent gel, western blotted to nitrocellulose and probed with rabbit anti-β-galactosidase antiserum (panel a) or the "Ha 5.1" preparation (panel b). Tracks 1 to 3 contain the bacterial proteins from cultures (750 µl) of E.coli Y1090 lysogenised with λIH8, λIH9 and λgt11.Amp3, respectively, after induction with IPTG. Tracks 4 to 6 contain the bacterial proteins from cultures (750 µl) of E.coli BTA282 lysogenised with λgt11.Amp3, λIH9 and λIH8, respectively, after induction at 42°C. Track 7 contains β-galactosidase (SIGMA, 0.2 µg). Track 8 contains total proteins from a saponin-lysed parasite preparation.
The 5.1 Ag recombinant proteins of λIH8 and λIH9 have been identified. For one clone, part of the 5.1 Ag is synthesised in a hybrid protein with β-galactosidase and for the other the entire 5.1 Ag is synthesised without fusion to a prokaryotic protein. Both of these recombinant proteins could now be purified by affinity chromatography for further characterisation.

3.8 Comments on the cloning of the 5.1 Ag gene

The isolation of the gene for the 5.1 Ag emphasises the power of immunological screening of cDNA expression libraries to obtain the genes of proteins expressed at low levels. The 5.1 Ag mRNA is at very low levels in the *P. falciparum* erythrocytic stages, approximately 0.08 per cent of total parasite mRNA, yet once the technology had been developed, two phage containing 5.1 Ag cDNA sequences were detected amongst a library of $2 \times 10^4$ recombinant phage. Also, if the gene is required for production of the protein, as with the 5.1 Ag, then this method of gene isolation, dependent on the expression of that gene, avoids the subsequent steps necessary to obtain expression of that gene after its isolation by other methods.

Fortuitously, the two clones obtained which express 5.1 Ag sequences each have a distinct useful characteristic. With λIH8, a hybrid protein containing a part of the 5.1 Ag which includes the 5.1 epitope can be produced at high levels. With λIH9, synthesis can be obtained, admittedly at quite low levels, of the entire 5.1 Ag. It should be possible to synthesise sufficient quantities of these two recombinant proteins in *E. coli* for experimental immunisations to evaluate the protective potential of the 5.1 Ag. Subsequent genetic manipulation may be necessary to obtain higher levels of synthesis if the 5.1 Ag is proven to be protective and of value for development as a human malaria vaccine.
Discussion
The protective immune response of man to *P. falciparum* is directed to only a few of the total proteins synthesised during the complex life-cycle of this malaria parasite. The present hope of a malaria vaccine depends on the identification of these few protective antigens, isolation of their genes and their economic and large-scale synthesis in a microbial system. Although several potentially protective antigens have already been characterised, many more may need to be investigated before a protein effective in a vaccine is identified. In this study, the search for a protective antigen of the human malaria parasite, *P. falciparum* has been continued.

1. **Antigens recognised by inhibitory monoclonal antibodies.**

A bank of 23 monoclonal antibodies raised to saponin-released erythrocytic stages of *P. falciparum* were assayed for an ability to inhibit the *in vitro* erythrocytic growth of this parasite. One monoclonal antibody, McAb 2.13, caused a 50 per cent inhibition of growth and five others, McAbs 2.9, 6.3, 7.5, 7.6 and 7.12, appeared to cause an inhibition of approximately 20 per cent. Although McAbs 2.9 and 6.3 have not been investigated beyond that reported by Hall *et al.* and McBride *et al.*, the antigens recognised by the other four monoclonal antibodies have been further characterised.

The antigen, P190, recognised by McAbs 7.5 and 7.6 has been studied by Hall *et al.* using the two non-inhibitory, anti-P190 monoclonal antibodies, McAbs 2.2 and 7.3. It was demonstrated that P190 is only synthesised by mature erythrocytic stages and undergoes processing at release/reinvasion of merozoites. Saimiri monkeys have been immunised with purified P190, and protection was observed to challenge with a heterologous isolate of *P. falciparum*. The gene for P190 has now been isolated and expressed in bacteria.
Previously, McAbs 2.13 and 7.12 were reported to immunoprecipitate a biosynthetically labelled, 160 K, parasite protein. Also, both monoclonal antibodies gave the same indirect immunofluorescence pattern on erythrocytic stages. Although it is still thought that McAbs 2.13 and 7.12 recognise the same antigen, in a subsequent study, they both detected proteins of 64 K and 70 K amongst western-blotted, total, erythrocytic-stage, parasite proteins (D.Ll. Simmons and J. Hyde, personal communication). To explain this discrepancy and to evaluate its protective potential, further characterisation of this antigen is necessary.

2. Relationship between the sporozoite and erythrocytic stage antigens recognised by monoclonal antibody 5.1

Most of the work in this thesis was stimulated by the cross-reaction of McAb 5.1 to the sporozoite surface and erythrocytic stages of *P. falciparum*. A number of studies have used this type of cross-reaction as evidence of homology between proteins, although further analysis of the proteins involved is necessary to prove the relationship.

Cross-reaction of monoclonal antibodies to structurally-unrelated haptens has been demonstrated. However, haptens occupy only part of the antigen binding cleft of an antibody and so hapten binding sites of a single antibody need not necessarily overlap. Antibodies generally bind to 5 or 6 amino acid residues of a protein which would fill the antigen binding cleft.

Indirect evidence that monoclonal antibodies could cross-react to structurally-unrelated proteins comes from observations involving a polyclonal antiserum. The antiserum was generated to a peptide fragment of a protein encoded by Rous Sarcoma Virus. When used at
sufficiently high concentrations in indirect immunofluorescence microscopy, the antiserum reacted specifically with proteins in uninfected host cells. After further analysis, the conclusion reached was that a proportion of the antibodies in the antiserum recognised a fortuitous similarity in the primary sequences of two otherwise unrelated proteins. It follows that a monoclonal antibody, which is the amplification of a single antibody of an antiserum, could cross-react to proteins similarly related. The degree of homology between two proteins recognised by a monoclonal antibody can only be determined by further characterisation of the proteins involved.

This thesis reports on the characterisation of the erythrocytic stage 5.1 Ag. The sporozoite antigen recognised by McAb 5.1 has been investigated by R. Nussenzweig et al (personal communication). The sporozoite 5.1 epitope was shown to be carried on the \textit{P. falciparum} circumsporozoite protein (CSP). McAb 5.1 detected a protein of identical mobility to the CSP amongst western-blotted, total, sporozoite proteins. The CSP has an apparent molecular weight of 58 K upon SDS-PAGE and is larger than the 23 K erythrocytic-stage 5.1 Ag. Also, in radioimmunoassay, McAb 5.1 inhibited the binding to the CSP repeating epitope by other monoclonal antibodies which do not cross-react to erythrocytic stages. The affinity of McAb 5.1 for the CSP was low and a similar observation of low affinity of McAb 5.1 for the erythrocytic stage antigen was made in this study (see Results, 2.4). These data, combined with the failure to detect a reaction of the affinity-purified polyclonal human anti-5.1 Ag antibody preparation to glutaraldehyde-fixed sporozoites by indirect immunofluorescence microscopy (see Results, 2.6.4), are strong evidence that the sporozoite and erythrocytic stage 5.1 Ag
are not identical. However, determination of how closely related are these two proteins requires comparison of their amino acid sequences.

The gene for the 5.1 Ag has now been isolated and most of the antigen's amino acid sequence has been deduced from the nucleotide sequences of the cloned cDNAs (see Results 3.5). The gene for the *P.falciparum* CSP has not yet been obtained and so a comparison of the two antigens to reveal the extent of their homology or to localise the 5.1 epitope is not possible. The CSPs of different *Plasmodium* spp. are thought to be closely related and a monoclonal antibody which recognised the repeating epitope of the *P.knowlesi* CSP cross-reacted to the *P.falciparum* CSP. However, a comparison of the amino acid sequences of the *P.knowlesi* CSP and the *P.falciparum* erythrocytic stage 5.1 Ag revealed no significant homology. Determination of the extent of homology between the *P.falciparum* CSP and erythrocytic stage 5.1 Ag must await the cloning of the *P.falciparum* CSP gene.

A tandemly repeating epitope in the 5.1 Ag, as observed for the *P.knowlesi* CSP and the *P.falciparum* S-antigen, would have been a good candidate for the 5.1 epitope because McAb 5.1 competed with other monoclonal antibodies for binding to the repeating epitope of the *P.falciparum* CSP. However, a tandemly repeating epitope is not apparent in the 5.1 Ag amino acid sequence so far derived. The 5.1 epitope is known to be included in the C-terminal 73 amino acids of the 5.1 Ag encoded by λIH8 because of the reaction of McAb 5.1 to this clone (see Results 3.3) but a more precise description is not possible at present.

The site of the 5.1 epitope could be more closely defined by re-cloning and expressing progressively smaller fragments of the λIH8 cDNA insert until the ability of McAb 5.1 to recognise the
recombinant protein is lost. However, the expanse of the 5.1 Ag which contains the 5.1 epitope, as defined by the cDNA insert of \( \lambda \)IHB, may be reduced only slightly by this approach: The 5.1 epitope may be disturbed by disruption of the secondary/tertiary structure of the recombinant 5.1 Ag before the sequence encoding the 5.1 epitope itself is encroached upon. Epitope mapping by transposon insertion suffers similar limitations.

Defining the 5.1 epitope by comparison of the sporozoite and erythrocytic stage 5.1 antigens requires isolation of the CSP gene. This gene might be isolated by screening a \textit{P. falciparum} genomic DNA library by \textit{in situ} hybridisation using the cloned erythrocytic stage 5.1 Ag gene but this would depend on more homology between the two proteins than a single common epitope. Screening a genomic DNA expression library with McAb 5.1 is more likely to succeed because it only depends on formation of the 5.1 epitope in a CSP recombinant protein and this has already been observed with the recombinant erythrocytic stage 5.1 Ag. Characterisation of the erythrocytic stage 5.1 Ag, sufficient for comparison to the CSP, has been achieved in this study.

3. **Comments on the gene for the 5.1 antigen**

The proportions of the A + T nucleotides in the genome of \textit{Plasmodium} spp is very high ranging from 76 to 83 per cent. The cDNA sequence for the 5.1 Ag is also A + T rich, particularly in the 5' and 3' non-coding regions (see Table 17). This difference in proportions of A + T residues for coding and non-coding regions has previously been observed for the \textit{P. knowlesi} CSP gene (see Table 17). Also, the \textit{P. falciparum} rRNA coding regions have 60 per cent A + T and are interspersed with regions of even higher A + T content.
Table 17. Base composition of 5.1 antigen cDNA and the *P. knowlesi* CSP gene

<table>
<thead>
<tr>
<th>Region of gene</th>
<th><em>P. falciparum</em> 5.1 Ag</th>
<th></th>
<th><em>P. knowlesi</em> CSP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion A + T</td>
<td>Percentage A + T</td>
<td>Proportion A + T</td>
<td>Percentage A + T</td>
</tr>
<tr>
<td>5' non-coding</td>
<td>23/24</td>
<td>96%</td>
<td>195/269</td>
<td>72%</td>
</tr>
<tr>
<td>5' coding</td>
<td>149/212</td>
<td>70%</td>
<td>568/1092</td>
<td>52%</td>
</tr>
<tr>
<td>3' coding</td>
<td>129/224</td>
<td>58%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3' non-coding</td>
<td>71/88</td>
<td>81%</td>
<td>266/379</td>
<td>70%</td>
</tr>
<tr>
<td>Total</td>
<td>372/548</td>
<td>68%</td>
<td>1029/1740</td>
<td>59%</td>
</tr>
</tbody>
</table>
The codon usage for the 5.1 Ag gene and for all sequenced *Plasmodium* genes (see Table 18) is clearly biassed so that A + T are the preferred nucleotides in the third position of degenerate codons. In the 5.1 Ag, the codons for proline, valine, threonine, glycine and leucine end in A or T rather than G or C in 9/9, 8/8, 6/6, 11/12 and 9/10 instances, respectively. One possible explanation for this phenomenon is that in the environment, for one of the multiplication steps of the malaria parasite, a requirement for synthesis of guanosine or cytosine nucleotides is limiting.

Both 5.1 Ag cDNAs have a long stretch (12 or 18 residues) of adenosine nucleotides at one of their termini which is likely to be generated from the poly(A) tail of the 5.1 Ag mRNA. It is possible that these tracts could result from hybridisation of the oligo(dT) primer, used in cDNA synthesis, to an (A) rich section of the 3’ non-coding region of the mRNA. However, the ubiquitous polyadenylation signal, AAUAA, is observed 37 nucleotides away from the poly(A) tract. Although this distance is longer than usual it is reasonable to think that the 5.1 Ag cDNAs include the poly(A) tail and thus probably the entire 3’ non-coding region of the 5.1 Ag mRNA has been cloned.

4. Comments on bacterial expression of the 5.1 antigen

It was suggested (in Results 3.6) that bacterial translation of the 5.1 Ag coding sequences in λIH9 could initiate at the methionine codon 24 nucleotides from the start of the cDNA insert. A similar observation, of translational re-start occurring when synthesis of a hybrid protein had been expected, was made with the cloning of Hepatitis B virus DNA into the β-lactamase gene of pBR322. It is noted that there is no typical Shine-Dalgarno sequence in the
cloned 5' non-coding region of the 5.1 Ag mRNA although 3 nucleotides, TAA, at positions 12 to 14, could hybridise to the 3' terminus of the *E. coli* 16S rRNA. However, translation of the λCI gene is known to occur in the apparent absence of a Shine-Dalgarno sequence. Furthermore, the genomic *P. knowlesi* CSP gene also lacks a good ribosome binding site yet translation of this gene in *E. coli* could be detected immunologically.

A study comparing translational initiation sites used in *E. coli* has revealed a non-random use of nucleotides at several sites, other than the Shine-Dalgarno sequence, around the initiation codon. In the sequence at the 5' end of the 5.1 Ag mRNA

5'-UUUAUUUAUUUAUUAAUUCAAAAUGAAAAUCUUAUCAGUA-3'

underlined nucleotides fit and overlined nucleotides deviate from the preferred nucleotide usage. Indeed the run of 4 A residues immediately after the initiation codon is a particularly common observation and the lack of G residues in the vicinity of the AUG is also thought to be beneficial. It is possible that these nucleotides help in the initiation of translation and could compensate for the lack of a typical Shine-Dalgarno sequence. In the λIH9 lysogens there is less 5.1 Ag detected than β-galactosidase (see Results 3.6) even though they are presumably translated from the same mRNA. Also plaques of λIH8 give a stronger signal than λIH9 when probing for the 5.1 Ag. Explanations for these observations could include poor translation of the 5.1 Ag coding sequence because of the lack of the Shine-Dalgarno sequence as well as increased degradation of the non-fused recombinant 5.1 Ag. Of course, a requirement for the Shine-Dalgarno sequence for expression in the eukaryotic malaria parasite would not be expected.
Table 18. Codon usage for the 5.1 antigen gene and other sequenced
*P. falciparum* genes

<table>
<thead>
<tr>
<th>First base of codon</th>
<th>Second base of codon</th>
<th>T</th>
<th>C</th>
<th>A</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>a</td>
</tr>
<tr>
<td>Phe</td>
<td>Ser</td>
<td>5</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>Ser</td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Ser</td>
<td>7</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Ser</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Pro</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Pro</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Pro</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Leu</td>
<td>Pro</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Pro</td>
<td>8</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Pro</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>Leu</td>
<td>Pro</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>Thr</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>Thr</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>Thr</td>
<td>4</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>Thr</td>
<td>2</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Ala</td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Ala</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Ala</td>
<td>4</td>
<td>8</td>
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<tr>
<td>Val</td>
<td>Ala</td>
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</tbody>
</table>

- a - amino acid encoded, b - codon usage in 5.1 Ag,
c - codon usage in cDNA clones for 5.1 Ag, P190, Lactate Dehydrogenase (D. L. Simmons, unpublished observations) and S-antigen. The figures in column 'c' were compiled by J. Hyde.
Recombinant 5.1 Ag is detected at higher levels when synthesised in λIH8/9 lysogens of \textit{E. coli} Y1090 as compared to \textit{E. coli} BTA282 (see Results 3.6). This observation is interesting because the poor expression in \textit{E. coli} BTA282 lysogens occurs from several hundred unpressed copies of the 5.1 Ag recombinant genes, the result of prophage induction, whereas the higher 5.1 Ag levels in lysogens of \textit{E. coli} Y1090 are produced from only one copy of the gene, expressed after induction with IPTG. The different levels of recombinant 5.1 Ag could be due to the different genetic backgrounds of the two host strains. Alternatively, elevation of temperature to induce the prophage in lysogens of \textit{E. coli} BTA282 could be detrimental to synthesis or stability of the recombinant protein. Heat shock induces expression of 17 \textit{E. coli} genes and at least one of these is known to be a protease.\footnote{241}

It was proposed (in Results 3.6) that the bacterially synthesised 5.1 Ag may be processed by cleavage of the N-terminal signal peptide. Similar observations, such as those for rat insulin,\footnote{237} have led to the suggestion that the eukaryotic and prokaryotic secretion mechanisms may be very closely related. Signal peptide processing is probably important for the localisation of the 5.1 Ag to the parasitophorous vacuolar membrane of the intra-erythrocytic malaria parasite. The absence of a C-terminal anchor-sequence, as observed for the \textit{P. knowlesi} CSP,\footnote{111} suggests that the 5.1 Ag is secreted by the intracellular parasite. The 5' end of the λIH8 cDNA insert encodes a region of 10 hydrophobic residues. "Stop-transfer" signals,\footnote{238} required for the generation of integral membrane proteins, are usually 20 to 25 residues long.\footnote{239} However, this hydrophobic region in the middle of the 5.1 Ag could be important for insertion into the parasitophorous vacuolar membrane after release from the
parasite surface. Adjacent regions on the 5.1 Ag could be important for interactions which target the protein to the correct membrane.

5. **Is the 5.1 antigen protective?**

The crucial question of whether or not vaccination with purified 5.1 Ag can induce immunity to *P. falciparum* malaria can now be addressed. Expression of the 5.1 Ag gene in bacteria means that large quantities of the protein can be easily prepared without the need for the culturing of malaria parasites. The recombinant protein can be purified, by affinity chromatography, from a detergent extract of the bacterial cells in which the 5.1 Ag has been synthesised. Saimiri monkeys may then be immunised with the purified 5.1 Ag before challenge with sporozoite or erythrocytic stages of *P. falciparum*. Even a small degree of protection would be important. The final malaria vaccine may well consist of many malarial proteins that together can induce complete protection. Will the 5.1 Ag be one of the components? Is the 5.1 Ag protective?
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ADDENDUM

The gene for the *P. falciparum* circumsporozoite protein (CSP) has now been isolated and sequenced. In one study, an expression library was constructed by cloning cDNA, prepared from *P. falciparum* sporozoite poly(A)⁺ RNA, into the β-lactamase coding sequence of pBR322. In a second study, genomic DNA, prepared from *P. falciparum* erythrocytic stages, was cleaved using mung bean nuclease and the fragments generated were cloned into the expression vector λgt11. Clones containing CSP coding sequences were isolated from both libraries by their immunological detection using monoclonal antibodies to the *P. falciparum* CSP.

The cloned CSP DNAs were examined by sequencing and restriction enzyme analysis. None of the clones isolated from the λgt11 genomic DNA library expressed the cloned DNA to generate a hybrid protein with β-galactosidase. In all cases it appeared that translation of the CSP coding region began within the parasite sequences in the absence of a typical Shine-Dalgarno sequence as was observed for λIH9 (see Discussion, 4).

The amino acid sequences of the *P. falciparum* and *P. knowlesi* CSPs were compared. The overall structure of the two proteins appeared very similar each having an N-terminal signal peptide, a central region consisting of tandemly repeated sequences flanked by charged regions and a C-terminal anchor sequence. The only segments of strong sequence homology between the two proteins are two regions of 15 and 13 amino acids which flank the tandem repeats. The apparent molecular weights of both proteins, as determined by SDS-PAGE are larger than their molecular weights as deduced from the amino acid sequences.

In the screen of the genomic DNA expression library, seven
clones containing CSP coding sequences were identified along with two other clones, \( \lambda \text{mPf6} \) and \( \lambda \text{mPf9} \), with sequences sharing no detectable homology with the CSP gene. Only one of the five anti-CSP monoclonal antibodies used to screen the library reacted to the two extra clones and it was suggested that they may encode the 5.1 Ag. No further data concerning \( \lambda \text{mPf6} \) or \( \lambda \text{mPf9} \) were presented.

The \textit{P.falciparum} CSP and 5.1 Ag can now be compared. The results of a computer comparison of the amino acid and nucleotide sequences, using programs generated by the University of Wisconsin Genetics Computer Group (UWGCG), are presented (Fig. 43a and b). The striking feature of the two comparisons is a strong homology between a region of the \( \lambda \text{IH}8 \) 5.1 Ag cDNA insert and the tandemly repeating epitope of the CSP. This homology is striking in both the nucleotide and amino acid sequences (Fig. 44). Apart from this stretch of 54 nucleotides (18 amino acids) there is no homology of the 5.1 Ag to the CSP, so it seems very unlikely that the proteins evolved from a common ancestral gene. But, it is hard to believe that this segment of homology arose by chance. Whatever the explanation of this homology it is almost certain that this homologous region contains the epitope recognised by McAb 5.1.


Fig. 43a. Dot-plot comparison of the nucleotide sequences encoding the *P. falciparum* CSP and 5.1 Ag. Blocks of 21 nucleotides were compared and a dot marks the positions for which there are at least 14 matches.
Fig. 43b. Dot-plot comparison of the amino acid sequences of the *P. falciparum* CSP and 5.1 Ag. Blocks of 30 amino acids were compared and a dot marks the positions for which there are at least 8 matches.
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![Fig. 44. The regions of the P.falciparum CSP and 5.1 Ag which show homology. Due to the degeneracy of the CSP tandem repeats alternative nucleotides and amino acids occur. The most frequently used alternative is placed uppermost. Amino acids common to the two proteins are underlined.](image)