Human immune responses to the C-terminus of the malaria vaccine candidate antigen, the major merozoite surface protein of *Plasmodium falciparum* (PfMSP1).

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Abbreviations

ADCI	Antibody-dependent cellular-inhibition.
Ag	Antigen.
AMA	Apical merozoite antigen.
AP	Affinity purified.
APC	Antigen presenting cell.
APS	Ammonium persulphate.
βME	Beta mercapthoethanol.
bp	Base pairs.
CIP	Calf intestinal phosphatase.
cpm	Counts per minute.
CSP	Circumsporozoite protein.
CTL	Cytotoxic lymphocyte.
CV	Coefficient of variation.
Da	Dalton.
DNase	Deoxyribonuclease.
DTT	Dithiothreitol.
dz	Dizygous.
EBA	Erythrocyte binding antigen.
EDTA	Ethylenediaminetetraacetic acid.
EGF	Epidermal growth factor.
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid.
ELISA	Enzyme-linked immunosorbent assay.
EMP-1	Erythrocyte membrane protein-1.
Eur	European.
GPI	Glycosylphosphatidylinositol.
GST	Glutathione S-transferase.
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid).
HLA	Human leucocyte antigen.
HRP	Horse-radish peroxidase.
HRP-2	Histidine-rich protein-2.
IFA	Indirect immunofluorescence assay.
IFNγ	Interferon-gamma.
Ig	Immunoglobulin.
IL	Interleukin.
IPTG	Isopropyl thio-ß-D-galactopyranoside.
kb	Kilobase.
kDa	Kilodalton.
LSA-1	Liver stage antigen-1.
mAb	Monoclonal antibody.
MHC	Major histocompatibility complex.
MNC	Mononuclear cell.
MRC	Medical Research Council.
MSP	Merozoite surface protein.
mz	Monozygous.
NCP	Nitrocellulose membrane.
Ni-NTA	Nickel-nitrilo-triacetic acid-agarose.

NIMR	National Institute for Medical Research.
NO	Nitric oxide.
NP-40	Nonidet P40.
OD	Optical density.
OPD	O-phenylenediamine.
OR	Odds ratio.
OVA	Ovalbumin.
PAGE	Polyacrylamide gel electrophoresis.
Pb	*Plasmodium berghei.*
PBS	Phosphate buffered saline.
Pc	*Plasmodium chabaudi.*
Pca	*Plasmodium chabaudi adami.*
Pcc	*Plasmodium chabaudi chabaudi.*
PCR	Polymerase chain reaction.
Pf	*Plasmodium falciparum.*
PHA	Phytohemagglutinin.
Pk	*Plasmodium knowlesi.*
PMSF	Phenylmethylsulphonyl fluoride.
PPD	Tuberculin purified protein derivative.
Pv	*Plasmodium vivax.*
Py	*Plasmodium yoelii.*
RAP	Rhoptry associated protein.
RBC	Red blood cell.
RESA	Ring-infected erythrocyte surface antigen.
RIA	Radio immunoassay.
RNI	Reactive nitrogen intermediates.
SALSA	Sporozoite and liver stage antigen.
SD	Standard deviation.
SDS	Sodium dodecyl sulphate.
SERA	Soluble serine repeat antigen.
SI	Stimulation index.
SICA	Schizont-infected cell agglutinin.
sIg	Surface immunoglobulin.
SSP-2	Sporozoite surface protein-2.
T20	Tween-20.
Taq	*Thermus aquaticus* DNA polymerase.
TCR	T cell receptor.
TEMED	N,N,N',N'-Tetramethylethlethylendineiamine.
Th	T helper cell.
TNFα	Tumour necrosis factor-alpha.
TRAP	Thrombospondin-related anonymous protein.
TT	Tetanus toxoid.
v/v	Volume per unit volume.
vs	Versus.
w/v	Weight per unit volume.
w/w	Weight per unit weight.

Declaration

I hereby declare that this thesis has been composed by myself, and all work described within is my own, except where otherwise stated.

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Appendix 1

Appendix 2

Human immune responses to the C-terminus of the malaria vaccine candidate antigen, the major merozoite surface protein of *Plasmodium falciparum* (PfMSP1).

Abstract

The major merozoite surface protein of *Plasmodium falciparum* (PfMSP1) is a malaria vaccine candidate antigen. The C-terminal processing product of PfMSP1 ($PfMSP1_{19}$) has a highly conserved amino acid sequence and is composed of two epidermal growth factor (EGF)-like motifs due to the characteristic spacing of cysteine residues. $PfMSP1_{19}$ is the only part of the molecule to be taken into the newly invaded erythrocyte.

Monoclonal antibodies (mAb) which bind to disulphide-constrained epitopes of $PfMSP1_{19}$ inhibit parasite growth *in vitro* and immunisation of animals with recombinant proteins representing this region of the molecule protects animals against challenge infection. Epidemiological data demonstrate that antibody responses to the C-terminus of PfMSP1 are associated with protection. To further evaluate the potential of this antigen as a vaccine candidate, I have measured human immune responses to recombinant proteins representing the C-terminus of PfMSP1. I have found that $PfMSP1_{19}$ is naturally antigenic in individuals living in a malaria endemic area, and that antibody is cross-reactive between the two major allelic prototype sequences of $PfMSP1_{19}$. However, some individuals consistently remain non responsive to $PfMSP1_{19}$ despite life long exposure. To determine whether this is due to a lack of T cell help, I have evaluated T cell responses to $PfMSP1_{19}$ in malaria exposed adults. T cell responsiveness to $PfMSP1_{19}$ is low, I have investigated whether this is due to the disulphide structure of $PfMSP1_{19}$ which may hinder its presentation to, and therefore stimulation of, T helper cells for the production of protective antibodies.

I have found that antibody responses to $PfMSP1_{19}$ are associated with resistance to clinical malaria in two populations of children actively acquiring immunity to malaria. However, not all antibody positive children were protected from malaria, suggesting that the fine-specificity of the antibody response may be important in determining its ability to provide protection against clinical malaria. I was able to demonstrate that human $PfMSP1_{19}$-specific antibody can be protective as malaria immune IgG affinity purified to $PfMSP1_{19}$ inhibits

parasite growth *in vitro*, and is equally effective against parasites from either PfMSP1 family. This suggests that if protective antibodies could be induced with vaccination with one or the other allelic form of the protein, all strains of *P. falciparum* can be controlled.

CHAPTER ONE
Introduction:

1. Malaria: a problem not solved

Although malaria has been eradicated in many regions of the world, primarily in temperate and subtropical areas rather than the African tropical heartland, there has been a recent resurgence in the disease due to the failure or premature termination of control measures against the mosquito vector, and the increasing prevalence of drug resistance. As residents in malarious areas do develop some form of protective immunity it should be possible to produce a vaccine against malaria. The aim would be to speed up the development of immunity and protect against the pathological effects of the disease. A safe, cheap vaccine would provide an attractive alternative to drug treatment for the hundreds of millions of people at risk of malaria, especially as these people live predominantly in rural areas in countries with poor infrastructure and limited health care facilities. This thesis concentrates on attempts to produce a synthetic, recombinant vaccine which may fulfil these criteria.

2. What is malaria?

Malaria is a disease caused by parasites of the genus *Plasmodium*. Malaria occurs in a wide belt around the world including tropical, subtropical and temperate zones, wherever conditions are suitable for the maintenance of the anopheline mosquito vector. The pathology of the disease is associated with the repeated cycles of parasite growth in the RBC. The disease has a complex of symptoms making it difficult to estimate how much morbidity and mortality malaria causes. In naive individuals, parasitization results in clinical disease, but in endemic areas the outcome of infection is more complicated, with various disease resistance factors (immunity) coming into effect during the individuals' life.

2a. Malaria life cycle

The infection is begun by the injection of sporozoites into the host while the female *Anopheles* mosquito takes a blood meal (see figure 1.1. for life cycle). Sporozoites migrate to the liver and invade hepatocytes where they differentiate and multiply by asexual nuclear division (schizogony). During this exo-erythrocytic stage, each intracellular parasite develops into a multinucleated schizont containing several thousands of merozoites.

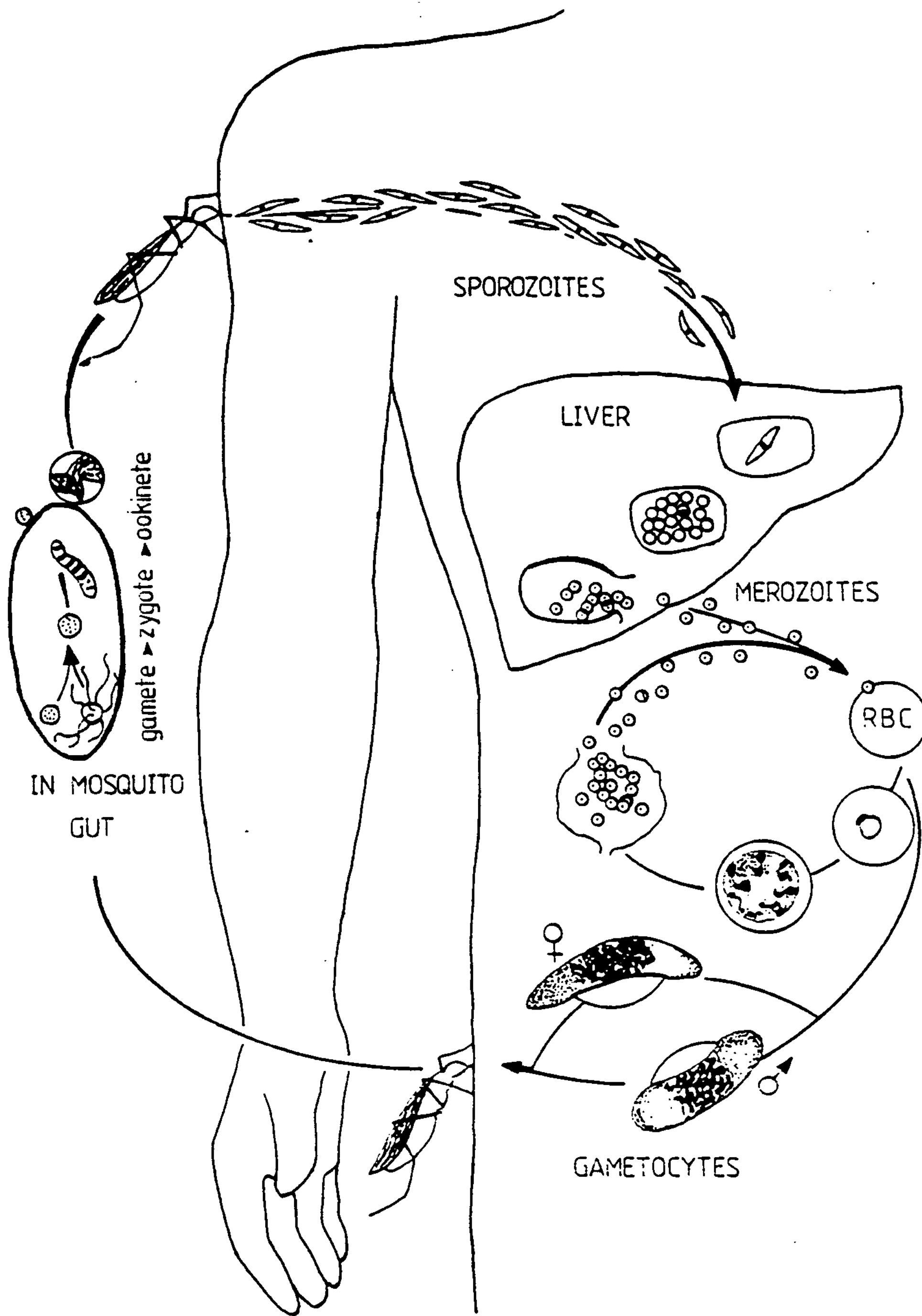


Figure 1.1. Life cycle of *Plasmodium falciparum*.

from Good *et al* (1988d) Ann. Rev. Immunol. **6**, 633-688.

Approximately eight days after the parasite first enters the human host, the schizonts burst releasing merozoites into the blood stream. It has been estimated that most infective bites inject 20 or less sporozoites, which produce approximately 800,000 merozoites (Lines and Armstrong 1992). These merozoites rapidly invade RBCs, probably within minutes after release from the liver, to begin the blood stage infection. (The invasion process is reviewed in Hadley *et al* 1986.) Merozoites interact with the RBC at any point on the merozoite surface. The parasite reorientates to bring a pair of tear-shaped organelles, called rhoptries (located at the apical end of the merozoite and associated with the electron dense micronemes), into contact with the RBC membrane. The rhoptries release their contents on to the RBC surface. The RBC invaginates and the merozoite enters the RBC. The internalised parasite is encapsulated within a parasitophorous vacuole. The intraerythrocytic parasite forms a 'ring' shape and gradually, over 24 hours, develops to form a trophozoite. Within the next 24 hours, the parasite nucleus divides to form the mature multinucleate 'segmented' schizont which contains 8-32 (on average 16) merozoites.

Only ring stage parasites and early trophozoites are present in the peripheral blood, mature parasites are sequestered in the deep capillary beds of the spleen, liver, heart, brain and other organs, where they cause most of the pathology of severe and cerebral malaria. Merozoites released by RBC rupture invade new RBCs. If not controlled by the immune system or by chemotherapy, each successive wave of rupturing schizonts, every 48 hours in the case of *P. falciparum*, causes the parasitaemia to rise and coincides with paroxysmal fever.

Some parasites, which have invaded the RBC, do not undergo asexual division but differentiate into male and female crescent-shaped gametocytes and remain in the peripheral blood. These, when taken up by the mosquito, mature into gametes and undergo fertilisation in the gut of the insect. Sexual reproduction results in zygote (ookinete) formation. The ookinetes migrate through the gut wall and develop into an oocyst on the outside of the gut wall. Further division occurs, resulting in the production of sporozoites which migrate to the salivary glands of the mosquito and await inoculation into the vertebrate host to repeat the cycle.

2b. Malaria pathology

The major clinical manifestations of malaria are chills, fever (associated with schizont rupture of the RBC), headaches, anorexia and nausea. Severity of the disease differs with each species. Falciparum malaria is the most severe

and ovale the least dangerous, though the impact of all the malarial species plays an important role in the socio-economic development of the country and drains health resources. It has been estimated that a single episode of malaria causes a two week loss of work and results in one-quarter of hospital admissions in Sri Lanka (Mendis and Carter 1995).

Severe and complicated malaria are caused almost exclusively by erythrocytic stages of *Plasmodium falciparum*. Pre-erythrocytic and gametocyte stages do not cause any pathology. The majority of *P. falciparum* infections do not result in life threatening disease, but those that do cause a whole spectrum of symptoms such as severe anaemia, acidosis, respiratory distress, renal failure, pulmonary oedema and cerebral pathology with convulsions and coma. The spectrum of symptoms can vary geographically and with age. Cerebral malaria and anaemia are the main contributors to malarial death in Africa, while in Asia and South America organ failure (other than the brain) is the main feature of severe disease (Mendis and Carter 1995). In endemic parts of Africa, younger children suffer predominantly with severe malarial anaemia (average age 27 months in The Gambia), whereas older children suffer more from cerebral malaria (average age 45 months in The Gambia) (Brewster *et al* 1990).

Malaria fever coincides with the rupture of schizonts. At this time, the parasite releases a wide range of antigens which induce cytokine production by the host. Cytokines may play a direct pathogenic role in severe malaria. In rodents, the cytokine tumour necrosis factor (TNFα) can stimulate clinical symptoms, and can be blocked with TNF-specific mAbs and other cytokines, and the level of TNF, and other cytokines including interleukin -1 (IL-1) and IL-6, increases in acute infection in humans (reviewed in Kwiatkowski 1995). There is a clear relationship between clinical severity of the disease and the level of circulating cytokines. It has been suggested that while cytokines result in the characteristic fever and associated pathology, this fever controls the density of the infection by inhibiting parasite growth (Kwiatkowski and Greenwood 1989).

Pathology caused by malaria is reviewed in Miller *et al* (1994); the main symptoms include;

1) Hypoglycaemia (caused by increased insulin secretion) can result in coma. This condition is probably exacerbated by increased demands for glucose by the parasite.

2) Lactic acidosis resulting from a restriction of oxygen supply to the tissues.

3) Renal failure due to the damage of the kidneys by renal anoxia which reduces the efficiency of glomerular filtration and tubular resorption, and usually results in death.
4) Raised intracranial pressure during a malarial attack results in headache, drowsiness and vomiting. Pressure within the intracranial space leads to a reduction of cerebral blood flow leading to respiratory arrest and encephalopathy.
5) Severe malarial anaemia which is caused by the destruction of both parasitised and non-parasitised RBCs and the inability of the body to recycle iron bound in the insoluble parasite pigment haemazoin, and also an inadequate erythropoietic response of the bone marrow. Severe anaemia is the predominant symptom of severe malaria in areas of high endemicity and is one of the main causes of malarial death of African children, affecting mainly infants to 3 year olds. After 3 years of age, the prevalence of severe anaemia decreases, at the time when the prevalence of cerebral malaria is increasing in African children.
6) Cerebral malaria causes death but occurs infrequently. In African children its incidence peaks in 3 to 4 year old children. It is paradoxical that as the child is beginning to build up its own natural immunity and control its parasitaemia, its susceptibility to cerebral malaria increases. Cerebral malaria is associated with the accumulation of mature parasitised RBCs in the cerebral microvasculature. The direct connection between sequestration of parasites within the brain and cerebral malaria has not been demonstrated. It is not known whether clinical features of cerebral malaria stem directly from histopathological ones, i.e. the physiological blocking of blood vessels. Cytoadherence of infected RBCs may lead to cerebral anoxia. The parasite modifies the surface of the infected RBC, which cytoadheres to endothelium and to uninfected RBCs (the latter process is known as rosetting, see Wahlgren *et al* 1994). Knob protrusions occur on the parasitised RBC surface as the parasite matures and are areas of cytoadherence. It is thought this allows the parasite to avoid clearance by the spleen. Cytokines, such as TNF, may up regulate cell adhesion molecules on cerebral vessels and children with cerebral malaria have high concentrations of TNF (reviewed in Miller *et al* 1994). Malarial molecules may induce the release of TNFα from macrophages; cytokines are inducers of NO and superoxides from host cells. It has been suggested that NO could be one of the final mediators in cytokine-induced pathology and coma (reviewed in Clark *et al* 1991, 1994).

3. What type of malaria vaccine?

Of the four malaria species that infect humans, *P. falciparum* and *P. vivax* are the most prevalent. However, *P. falciparum* is the only species that can directly result in death and was estimated to cause half of the 500 million cases of malaria world-wide in 1986 (Sturchler 1989). For this reason, most of the vaccine research has been directed to controlling falciparum malaria. However, it is hoped that antigens identified to be protective against *P. falciparum* will have analogues in *P. vivax* which will prove to be protective against vivax malaria. This seems highly plausible, as analogues of *P. falciparum* antigens, which appear to be associated with protection in humans, experimentally protect mice against rodent malarias (Daly and Long 1993, 1995, Ling *et al* 1994).

Most research into malaria vaccines has aimed to control the parasite, to limit its numbers and therefore reduce the clinical symptoms of the disease. However, interest has now grown in developing an anti-disease vaccine as well as an anti-parasite vaccine (reviewed in Playfair *et al* 1990). Clinical symptoms of malaria in children decline before parasite densities do, suggesting that immunity develops to control the disease as well as the parasite. Toxins released by the parasite, especially at schizont rupture, are thought to induce the pathology caused by malaria. A vaccine based on controlling these toxins may prove protective. However, the induced cytokines are both mediators of protection and pathology in malaria, and much more needs to be known about the effector mechanisms of immunity before an anti-disease vaccine can be used in humans. On the other hand, an anti-disease vaccine would not affect parasites, so severe malaria may still occur, and the population will still be infective to the mosquito vector, allowing continued transmission of the parasite. Therefore an anti-disease vaccine cannot be used on its own.

Early vaccine research used attenuated or killed parasites, but due to the large scale culture of parasites that would be required, and because of cost, stability and safety, an attenuated parasite vaccine is a practical impossibility. The development of recombinant DNA technology, however, has made it feasible to characterise and express specific genes and produce recombinant proteins that could be used in a vaccine. Many proteins have been identified which are antigenic and appear to be involved in inducing protective immune responses, and have reached varying stages of development towards inclusion in a vaccine (reviewed in Romero 1992, Holder 1993, Pasloske and Howard 1994).

It is not clear whether a vaccine should aim to mimic naturally acquired immunity to malaria, or whether novel mechanisms should be sought. As natural immunity, when it develops, is very effective, most attention in vaccine research has been to mimic the mechanisms of protective immunity, but to speed up the process.

The only malaria vaccine to have undergone large scale testing in humans is SPf66 (reviewed in Tanner *et al* 1995). It is made up of three synthetic peptides; one being from the merozoite surface protein 1 (PfMSP1) and the other two from two poorly characterised merozoite proteins. The peptides are linked by a sequence representing part of the circumsporozoite protein (CSP). Initial studies of vaccine trials in monkeys and humans were encouraging but were unrepeatable by other groups. Large scale, independent randomised, double-blind, placebo-controlled trials in The Gambia and Tanzania indicate that the vaccine has a maximum of 30% efficacy, and that SPf66-specific antibodies are short lived (reviewed in Riley 1995). On the other hand, the introduction of bednets has proved to be effective in preventing malaria-related morbidity and mortality (Bermejo and Veeken 1992), so a vaccine which is introduced will have to better the protection provided by bed nets. An introduced vaccine should supplement, rather than replace, existing malaria control efforts.

A major worry for introduction of a vaccine is that as the vaccine will alter the epidemiology of malaria, causing a decrease in transmission, naturally acquired immunity will decrease. Naturally acquired immunity requires continual antigenic stimulus, as seen with African nationals who live in Europe for a number of years, and also in areas of low malaria transmission which are subject to malaria epidemics. So the introduction of a vaccine could potentially worsen the situation.

4. Effector mechanisms of anti-parasite immunity

People living in endemic areas do develop resistance to malaria but the protective mechanisms are complex and poorly understood. They are essentially of two types; natural innate resistance to the infection, which is inherited, and immunity which is at first non-specific but acquires specificity after exposure to the parasite.

4a. Innate resistance

Innate resistance is expressed regardless of malarial exposure. Individuals born of parents whose ancestors have undergone genetic selection, through immune pressure exerted by exposure to malaria, have certain genetic

polymorphisms which provide a degree of protection from malaria. There are many host factors which influence resistance to malaria (reviewed in Miller and Carter 1976). For example, the RBC surface Duffy antigen is the receptor for the attachment of *P. vivax* merozoites. Populations which are Duffy antigen negative (such as West Africans) are completely resistant to *P. vivax* infection.

A number of haemoglobin polymorphisms are associated with resistance to malaria;

1) Sickle haemoglobin (HbS) is a polymorphism in the molecular structure of haemoglobin, resulting from a single amino acid substitution. As the genetic defect is often fatal in homozygotes, the degree of protection in the heterozygous state must be high to maintain the gene frequency of over 20% in some parts of Africa. Malaria infection *per se* does not appear to be affected, but the protective affect against severe malaria is enormous, with over 90% protection against cerebral malaria and anaemia in Africa (Hill *et al* 1991). Possible mechanisms include merozoite failure to invade HbAS cells, inability of parasites to metabolise HbS, or rapid clearance of parasitised HbAS RBCs from the circulation (parasitised HbAS cells adhere to each other).

2) Resistance of neonates to malaria may be associated with high levels of foetal haemoglobin (HbF); 80% of the Hb is HbF at birth and declines slowly to be replaced by HbA by the age of 4 months. The mechanism is unclear, but parasites grow less well in HbF RBCs (Pasvol *et al* 1976).

3) Haemoglobin E (HbE) is a structural variant which is also linked with a defect in globin synthesis. Haemoglobin C (HbC) is another genetic variant found in West Africa which is thought to limit parasite growth.

4) Thalassaemia is a hereditary anaemia which is caused by a decrease in globin synthesis within RBCs; α and β thalassaemias result from either one of the two globin chains being affected. Mechanisms of protection include resistance of RBC to merozoite invasion and decreased intracellular parasite growth (Yuthavong and Wilairat 1993).

Glucose 6-phosphate dehydrogenase deficiency (G-6-PD) occurs in many malaria endemic areas, and is X chromosome-linked. Epidemiological studies indicate heterozygote females have some protection against malarial growth, but hemizygote males do not. Genes controlling host immune responses to infection are also a form of innate resistance. Heterogeneity in immune response genes may have evolved in response to disease, including malaria. Possible HLA associations with protection from the risk of developing severe malaria have been examined

in a large study in The Gambia, where the HLA class I antigen BW53 and the HLA class II haplotype DRBI 1302-DQBI 0501 were found to be associated with protection (Hill *et al* 1991). Other host genes coding for molecules involved in immunity against malaria may also have undergone selective pressure, for example genes encoding complement, cytokines and T cell receptors.

4b. Immune resistance

Only non-specific immune responses are stimulated on first contact with the parasite. During this time, antigenic information is being imprinted into the specific arm of the immune system, so that the next encounter with the pathogen will be more rapid as there is now a specific memory-primed immune system. Non-specific immune responses are often enhanced by this adaptive immunity.

4bi. Innate/non-specific immunity

Non-specific immune responses are antigenically unrestricted. Such mechanisms may be stimulated by parasite 'toxins' that induce a cytokine response in non-immune individuals. Cytokines may protect against an overwhelming parasitaemia but also cause many of the symptoms of malaria, such as fever. Non-specific immune responses are effective against all stages of the parasite. IFNγ kills parasites non-specifically in the liver stage (but may also be specifically induced), by stimulating hepatocytes to synthesise reactive nitrogen intermediates (RNIs) (reviewed in Kwiatkowski 1992). IL-6 can also kill intrahepatocytic parasites, but its production is down-regulated by the parasite (reviewed in Kwiatkowski 1992). IFNγ and TNFα can also stimulate neutrophils to phagocytose and kill intraerythrocytic parasites (Kumaratilake *et al* 1992), this is most effective in the presence of immune serum.

Experiments with murine malaria indicate that T cells are required for initial control of asexual, intraerythrocytic parasitaemia. This appears to be a Th1/IFNγ-dependent process. However, B cells are necessary to clear the infection; Th2 cell activation promotes B cells to produce specific IgG antibodies (reviewed in Taylor-Robinson 1995). Both types of T cell response are needed to survive initial challenge and to clear the infection. It is difficult to study the cytokine secretory phenotype of T cells in human malaria as activated T cells appear to migrate from the peripheral circulation, possibly to the spleen and other organs, during acute infection.

Gametocytes are vulnerable to attack by cytokines. Crisis serum inhibits the ability of gametocytes to infect mosquitoes. This inhibition can be blocked by a combination of antibodies against TNFα and IFNγ (Naotunne *et al* 1991). However, normal serum spiked with recombinant TNFα and IFNγ does not inhibit infectivity, suggesting there is some complementary gametocyte-killing factor in crisis serum. Sera taken from patients with vivax malaria during fever paroxysm, but not at other times, also inhibit gametocyte infectivity (Karunaweera *et al* 1992).

The spleen is very important in the immune response to malaria. Removal of this organ exacerbates malarial infection in animals and humans. The spleen is thought to act as a trap for abnormally-shaped infected RBCs. Activated macrophages may also have a cytotoxic effect on infected RBCs (reviewed in Good 1992).

4bii. Adaptive/specific immunity

It appears that exposure to relatively frequent infection is needed as immunity fails to develop where malaria in not endemic. Naturally acquired immunity fails to develop, or once developed, is lost with interrupted exposure (i.e. in children with seasonal exposure to malaria, or adults with extended travel out of an endemic area). Naturally acquired immunity does not sterilise against infection. Children in endemic areas exhibit higher asexual parasitaemias than do adults, however, parasitised adults rarely experience clinical attacks. Children progressively experience fewer and less severe attacks of malarial fevers with increasing age. This decrease in clinical symptoms occurs before parasitaemia decreases, suggesting an 'anti-disease' immunity develops first, with an 'anti-parasite' immunity developing later (Playfair *et al* 1990).

Development of naturally acquired immunity is thought to be a slow process requiring the years of life between infancy and adulthood to 'see' the local repertoire of strains (Day and Marsh 1991). This presumption has recently been challenged by Baird (1995). In his review article, he points out that with experimental infection of humans with malaria, naturally acquired immunity may develop after fewer than ten episodes of infection in adults. However, he suggests that immunity takes longer to develop in children due to their immune system being constitutionally less capable of mounting a protective response against the parasite. Measuring the incidence of infection in malaria-naive immigrants moving into an endemic area in Java, Baird and co-workers were able to demonstrate that adults become clinically immune relatively quickly compared to their children.

Immune evasion strategies of the parasite may account for the long period required to develop immunity, including mechanisms such as; immunodominant molecules acting as 'smoke screens' (Anders *et al* 1989); common repeat structures that are T-independent antigens that protect critical antigens (or regions of antigens) by 'epitopic suppression' (Schofield 1991); antigenic variation of the parasite antigens to frustrate the immune system (Mendis *et al* 1991). Individuals with high levels of antimalarial antibodies can still be susceptible to clinical infection. These sera may lack antibody specificities that are critical for resistance, or are of low affinity or the wrong IgG subclass to confer protection. It has been found that some antibodies cross-react with antigens from different stages of the parasite life-cycle; the parasite may present an extensive network of cross-reactive epitopes to the host in order to prevent affinity maturation of responses to less immunogenic but protective epitopes (Moelans and Schoenmakers 1992).

The actual mechanisms of naturally acquired immunity to malaria are poorly understood and there is no agreed way to measure it. Passive transfer of Ig from immune adults to non-immune children demonstrates that Ig is a component of naturally acquired immunity (Cohen *et al* 1961). It has been demonstrated that antibodies (polyclonal and monoclonal) can inhibit the *in vitro* growth of *P. falciparum* parasites. This could be due to a number of mechanisms; inhibition of intracellular parasite development, inhibition of schizont rupture, inhibition of RBC invasion by merozoites, agglutination of merozoites or inhibition of merozoite binding to the RBC. However, Bouharoun-Tayoun *et al* (1990) demonstrated that an Ig preparation from 'immune' adults (which could passively protect non-immune individuals; Sabchareon *et al* 1991) could only inhibit parasite growth *in vitro* in co-operation with blood monocytes. There is evidence that the protective effects of IgG may be mediated by cytophilic antibodies (Bouharoun-Tayoun and Druilhe 1992). Non-immunoglobulin components of passively transferred immune serum, such as complement, may also be responsible for the protection afforded.

There is evidence that T cell derived lymphokines are an important mechanism in immunity (reviewed in Kwiatkowski 1992, 1995);

1) Lymphokines can activate the killing of intracellular malaria parasites by macrophages *in vitro*, and can be neutralised using anti-IFNγ mAb (Ockenhouse *et al* 1984).

2) Serum containing the monocyte-derived cytokine TNFα can destroy murine (Taverne *et al* 1981) and human (Haidaris *et al* 1983) blood stage

parasites *in vitro* and inhibit the growth of *P. yoelii in vivo* (Taverne *et al* 1982), suggesting that TNFα secretion results in a cytotoxic mechanism that kills intracellular parasites.
3) Parasite antigens induce production of IFNγ *in vitro* in immune adults (Troye-Blomberg *et al* 1985, Riley *et al* 1988).

Immunity to malaria probably involves an immediate monocyte/macrophage derived TNF response to parasite 'toxins' that acts through fever and other non-specific mechanisms to constrain parasite growth. Then T cell-derived IFNγ, together with TNF, stimulate macrophages and neutrophils to attack parasites with nitric oxide toxic radicals. Then a more slowly evolving B cell response develops which is eventually capable of reducing parasitaemia to levels below those resulting in clinical symptoms (Marsh 1992).

4biii. Immunity during pregnancy

Pregnant women have an increased susceptibility to clinical malaria (reviewed in Menendez 1995). Immune depression, probably due to immune adaptations developed to prevent rejection of the immunologically different foetus, is especially associated with a first pregnancy and is strong enough to partially overcome anti-disease immunity.

Detrimental effects on the mother include anaemia, which contributes to maternal death, probably by leading to post-partum haemorrhage. Detrimental effects on the foetus/infant include;
1) Maternal hypoglycaemia and anaemia, resulting in a reduction in foetal birth weight, which may influence the infant's mortality and morbidity during the first year of life by directly inducing growth retardation and/or prematurity and causing impaired cell-mediated immunity.
2) Maternal malaria can cause foetal death by interfering with transplacental transfer of nutrients leading to anaemia.
Transmission of parasites from mother to child may occur during pregnancy. There is insufficient information on the effects of malaria during pregnancy on subsequent mortality or morbidity in the infant (reviewed in Carlier and Truyeus 1995). Conversely, passively acquired immunity from mothers to the foetus may play an important role in preventing or controlling the parasitic infection in the infant. It is still unknown whether placental infection during pregnancy is harmful or beneficial for the infant. However, there are high rates of premature deliveries, abortions and still births during a malaria

epidemic. An important question in a malaria vaccination campaign is whether immunosuppression during pregnancy will affect the effectiveness of a vaccine and whether pregnant women should be vaccinated at all. Avoiding infections from the onset of pregnancy may prevent the induction of immunological tolerance in the foetus. However, an asexual vaccine would control parasite densities to subclinical levels, so stimulating naturally acquired immunity in mother and foetus, and transplacental transfer of malaria-specific antibodies would occur.

5. Candidate vaccine antigens

The pre-erythrocytic stage of the infection appears to be asymptomatic with clinical symptoms of the disease associated with the asexual blood stage parasites. A prophylactic vaccine, to prevent clinical manifestations of the disease, must be directed against the blood stages of malaria. Immunity is thought to be stage-specific. A vaccine to control the initial infection by sporozoites would have to be 100% effective, as a single sporozoite could still initiate asexual parasitaemia. A vaccine which just controls the asexual stage may not suppress the formation of sexual stage parasites, so the disease would continue to be transmitted. A vaccine against sexual stages alone would not protect the vaccinated individual from the current malarial infection. So an ideal malaria vaccine will be a mixture of these, blocking all stages of the parasite life cycle. An added advantage of a multi-stage vaccine is that if the parasite develops resistance to one of the components of the vaccine (i.e. through antigenic variation in a protective epitope), parasite proliferation would still be inhibited by the parts of the vaccine controlling the other stages of the life cycle. The vaccine may also contain some anti-disease components too, directed against malarial toxins.

There are many potential vaccine candidate antigens (Howard and Pasloske 1993) and it would be impossible to study every one in detail, however there are enough potentially protective antigens now identified to warrant the formation of a multistage cocktail vaccine. Several studies have demonstrated that immunisation with even a single purified protein, or fragment of it, can protect against an experimental challenge infection.

The following section is a description of the currently most promising antigens identified in regards to vaccine development. The general characteristics of these antigens are summarised in table 1.1.

antigen	size in *P. falciparum*	location	general features	main review reference
circumsporozoite protein (CSP)	44kDa	sporozoite surface	central region of 37 repeats of Asn-Ala-Asn-Pro (NANP) and 4 repeats of Asn-Val-Asn-Pro (NVNP)	Aikawa *et al* 1981 Yoshida *et al* 1981
sporozoite surface protein-2 (SSP2) thrombospondin-related anonymous protein (TRAP)	140kDa	sporozoite surface	shares sequence motifs common to thrombospondin and properdin	Charoenvit *et al* 1987 Robson *et al* 1988
liver stage antigen-1 (LSA)	230kDa	sporozoite surface	86 amino acid repeats flanked by conserved regions	Zhu and Hollingdale 1991
Sporozoite and liver stage antigen (SALSA)	70kDa	sporozoite surface	87 amino acids	Druilhe and Marchand 1989
soluble antigen (S AG)	220kDa	released during schizont rupture	antigenically diverse repeats of 11 amino acids	Anders *et al* 1989
serine repeat antigen(SERA) serine rich protein (SERP)	126-140kDa	released during schizont rupture	35 serine residues	Inselburg *et al* 1991
Histidine-rich protein-2 (PfHRP-2)	65-75kDa	secreted into plasma	35% histine 40% alanine	Wellems and Howard 1986
rhoptry associated protein-1 (RAP-1)	80kDa	rhoptries	non polymorphic, serine rich, conserved repeat sequence, proteolytically processed	Ridley *et al* 1990b
rhoptry associated protein-2 (RAP-2)	42kDa	rhoptries	conserved no repeats hydrophobic	Saul *et al* 1992a
apical merozoite antigen-1 (AMA-1)	83kDa	rhoptries	16 conserved cysteines	Peterson *et al* 1989
ring infected erythrocyte surface antigen (Pf155/RESA)	155kDa	merozoite dense granules	2 blocks of tandem repeats	Culvenor *et al* 1991
erythrocyte binding antigen (EBA -175)	175kDa	merozoite micronemes	antigenically conserved, binds to sialic acid	Sim 1995
merozoite surface protein-1 (MSP1)	195kDa	merozoite surface	dimorphic and conserved regions, polymorphic repeats, conserved cysteine residues, 2 EGF-like regions	Cooper 1993
merozoite surface protein-2 (MSP2)	45kDa	merozoite surface	2 conserved regions flank central variable area with repeats	Al-Yaman *et al* 1994
merozoite surface protein-3 (MSP3)	48kDa doublet	merozoite surface	identified by antibody dependent cellular cytotoxicity assay, appears to be non polymorphic	Oeuvray *et al* 1994
erythrocyte membrane protein-1 (PfEMP1)	250-300kDa	infected RBC surface	undergoes antigenic variation	Leech *et al* 1984
Pf230kDa	230kDa	gamete surface	disulphide constrained	Rener *et al* 1983
Pf48/45kDa	48/45kDa doublet	gamete surface	disulphide constrained	Rener *et al* 1983
Pfs25	25kDa	ookinete surface	disulphide constrained 4 EGF-like regions	Kaslow *et al* 1988

Table 1.1. Summary of the main malaria vaccine candidate antigens.

5a. Pre-erythrocytic stage vaccine candidate antigens

The sporozoite has been intensively studied for vaccine development. Immunising mice, chickens and humans with attenuated sporozoites protected vaccinees against sporozoite challenge (reviewed in Nussenzweig and Nussenzweig 1985). The sporozoite is covered in a surface antigen, the circumsporozoite protein (CSP), which contains an immunodominant repeat region (Aikawa *et al* 1981, Yoshida *et al* 1981). Reasons for the sporozoite stage being promising as a vaccine candidate include;

1) MAbs specific to the CSP repeats passively protect mice from challenge infection.

2) Antibodies raised to a synthetic peptide representing the (NANP)n repeat of the *Plasmodium falciparum* CSP inhibit sporozoite invasion *in vitro*. However, immunisation of humans with a peptide vaccine induced limited protection (reviewed in Good *et al* 1988c).

It was noted that attenuated sporozoites could induce immunity, but that killed sporozoites could not. This suggested that hepatocyte invasion was important for an effective immune response. It was found, through depletion experiments, that cytotoxic CD8+ T cells are important to this immunity and that cytokines, in particular IFNγ, released from T cells can inhibit the development of intrahepatocytic stages (reviewed in Subrbier 1991). It has yet to be shown that sporozoite CTL responses are important in protective immunity in humans although human CD8+ cytotoxic T cells have been identified which are directed against the CSP (Sedegah *et al* 1992).

A second protein on the sporozoite surface has been identified in *P. yoelii* (SSP2) (Charoenvit *et al* 1987). Mice immunised with both CSP and SSP2, but not each alone, were completely protected against challenge infection (Khusmith *et al* 1991). Antibodies raised to recombinant proteins representing PfSSP2 inhibit sporozoite invasion and development in hepatocytes *in vitro* (Rogers *et al* 1992).

5b. Liver stage vaccine candidate antigens

A liver stage antigen-1 (LSA-1) has been identified in *P. falciparum* (Guerin-Marchand *et al* 1987, Zhu and Hollingdale 1991). It has been found that CTLs recognise a conserved peptide from LSA-1 adding to its attractiveness as a vaccine candidate (Hill *et al* 1992). As well as CSP and LSA-1, other sporozoite antigens are present in, and synthesised by, liver-

stage parasites. Sporozoite and liver stage antigen (SALSA) is highly antigenic in human populations and so may be a possible vaccine candidate (Druilhe and Marchand 1989).

Some blood stage antigens also occur in the liver stage (such as the merozoite surface protein 1), so a vaccine developed against blood stages may also be effective against liver stage parasites.

5c. Blood stage vaccine candidate antigens

There are a multitude of potential antigens expressed during the blood stage, identified by immune sera, that are candidates for a vaccine (table 1.1.). Merozoite surface antigens may be involved in merozoite attachment to the RBC. Secretions of proteins from internal merozoite organelles may be important in RBC invasion and/or development within the RBC. Parasite antigens are expressed on the infected RBC surface and may be involved in cytoadherence, rosette formation with other RBCs and transport functions. Finally, when the synchronised schizonts rupture, large quantities of soluble antigens, called exoantigens, are released into the plasma. As clinical symptoms of malaria, such as fever, are associated with schizont rupture, it is likely that materials released at the time elicit the host responses. These antigens/toxins are targets for an anti-disease vaccine, reducing morbidity and mortality without necessarily reducing parasitaemia (Playfair *et al* 1990).

The selection of suitable candidates has been directed by studying the biological effects of antibodies induced to the natural infection or to purified or recombinant parasite proteins. *In vitro* functional assays have been used to define protective antigens by assaying antibody effects on parasite growth, invasion of RBCs and cytoadherence and the ability to stimulate T cell proliferation and cytokine production. *In vivo* functional assays include measuring the effects of passive transfer of immune antibodies and immunisation of animals with native or recombinant parasite proteins. Antibody and cellular immune responses to candidate antigens in malaria-exposed children and adults have been measured to determine whether responses correlate with the development of clinical immunity.

5ci. Soluble antigens

Antigens released at schizont rupture include the soluble S-antigens, but due to the wide diversity of repetitive amino acid sequence between different isolates, they have not been proposed as a vaccine candidate

(Anders *et al* 1989). However, a number of soluble antigens are serious vaccine candidates:

1) SERA: the soluble serine repeat antigen. SERA-specific mAbs can inhibit parasite growth *in vitro* (Perrin *et al* 1981, 1984) and monkeys immunised with native (Perrin *et al* 1984) or recombinant (Inselburg *et al* 1991) SERA were immune to subsequent challenge infection.

2) HRP2: Histidine-rich protein 2. Immunisation with the recombinant C-terminal half of HRP2 partially protected monkeys against challenge infection (Knapp *et al* 1988), while monkeys immunised with HRP2 plus SERA and MSP1 were totally protected (Knapp *et al* 1992).

3) Apical complex proteins: proteins released from the apical secretory complex, consisting of the large paired rhoptries and the smaller micronemes, are thought to be involved in RBC invasion. Immunisation of monkeys with the rhoptry protein complex provided protection from lethal challenge infection (Ridley *et al* 1990a) and antibody specific to the complex was able to inhibit parasite growth *in vitro* (Schofield *et al* 1986). Genes from two of these rhoptry associated proteins (RAP) have been identified; RAP-1 and RAP-2. RAP-1 is highly conserved and appears to be protective.

4) AMA-1: apical merozoite surface antigen-1 is exported to the merozoite surface from the rhoptries. Pk66 is the equivalent protein in *P. knowlesi.* Pk66-specific mAbs inhibit merozoite invasion *in vitro* (Deans *et al* 1984), and monkeys immunised with Pk66 were protected from challenge infection (Deans *et al* 1988).

5) RESA: the ring-infected erythrocyte surface antigen is localised in the dense granule organelles of the merozoite and is transferred to the cytoskeleton of the newly invaded RBC. RESA-specific antibodies, especially those directed to the repeat sequences, inhibit invasion *in vitro* but immunisation of monkeys with recombinant proteins representing RESA has produced disappointing results (reviewed in Romero *et al* 1992). Epidemiological studies have also given conflicting results regarding the correlation of RESA-specific antibodies with protection from clinical malaria (Riley *et al* 1991b, Deloron and Chougnet 1992). It is possible that the *in vitro* growth inhibition and the slight protection in monkeys is due to cross-reactivity of anti-RESA antibodies with other malarial antigens (Mattei *et al* 1989).

6) EBA-175: the 175kDa erythrocyte binding antigen binds to sialic acid on the RBC surface and the level of binding appears to correlate with the efficiency of parasite invasion, and antibodies raised to a synthetic EBA

peptide prevent the binding of purified EBA-175 to RBCs and inhibit merozoite invasion *in vitro* (reviewed in Sim 1995).

5cii. Merozoite surface proteins

There are at least three vaccine candidate antigens on the merozoite surface.

1) The merozoite surface protein-1 (MSP1) is discussed in detail below (p21).

2) The merozoite surface protein-2 (MSP2) consists of two conserved regions flanking a variable repetitive region. MSP2-specific antibodies inhibit parasite growth *in vitro* (Clark *et al* 1989) and mice immunised with recombinant proteins representing conserved regions of MSP2 are protected from a lethal challenge infection (Saul *et al* 1992b). Epidemiological studies indicate that some MSP2 antibodies are correlated with decreased susceptibility to clinical malaria (Al-Yaman *et al* 1994, R. Taylor 1995 Ph.D. thesis, University of Edinburgh).

3) The 48kDa merozoite surface protein-3 (MSP3) has recently been described (Oeuvray *et al* 1994). Antibodies to this protein label the merozoite surface and co-operate with monocytes to kill parasites. The antigen appears to be relatively conserved but further characterisation is required.

5ciii. Erythrocyte surface neo-antigens

Antigens expressed on the surface of the infected RBC are involved in cytoadherence and are thought to mediate sequestration of infected RBCs. Variant erythrocyte surface antigens, denoted schizont-infected cell agglutinins (SICA), were first discovered in *P. knowlesi* (Brown and Brown 1965). The equivalent antigen in *P. falciparum*, erythrocyte membrane protein-1 (PfEMP1), is recognised by agglutinating antibodies (Leech *et al* 1984). They appear to display considerable antigenic variation (Biggs *et al* 1991, Roberts *et al* 1992) as cloned lines appear to be able to change their phenotype *in vitro*. Although interesting with regard to its role in cytoadherence, this protein appears to be involved in immune evasion (similar to the surface variant antigens of trypanosomes; reviewed in Mendis *et al* 1991) and may not be an ideal vaccine antigen. The genes for this family of related antigens have now been identified (Baruch *et al* 1995).

Antigens expressed on the surface of the intracellular asexual parasite have received little attention as they are thought to be inaccessible to antibody. However, the identification of a parasitophorous duct (Elford *et*

al 1985, Pouvelle *et al* 1991) raised the possibility that antibody may be able to access the intracellular parasite. It has been suggested that antibody can inhibit parasite growth before schizont rupture and merozoite release (Perrin *et al* 1981), so proteins on the intracellular parasite surface are beginning to receive more attention as potential antigens for vaccine development.

5d. Transmission blocking vaccine candidate antigens

The idea of a transmission blocking vaccine is to prevent the continuation of the parasite life cycle in the mosquito vector by interfering with the sexual development of the parasite in the mosquito gut and thus reduce sporozoite numbers. Immunisation of the host with sexual stage parasites (expressing stage-specific antigens) can suppress the infectivity of subsequent blood infection to the mosquito (Carter and Chen 1976), suggesting that inhibitory agents, such as antibodies, are ingested by the mosquito during the blood meal. Gametocyte surface antigens of *P. falciparum,* 230kDa and a 45/48kDa doublet, have been identified as vaccine candidates as they are targets of transmission blocking mAbs (reviewed in Kaslow *et al* 1992). It is thought that antibody activates complement-mediated lysis of gametes or directly inhibits fertilisation. However, it has been suggested that frequent reinfection would be needed to maintain transmission-blocking immunity, so an efficient immunity induced by the vaccine may limit its own usefulness (Ranawaka *et al* 1988).

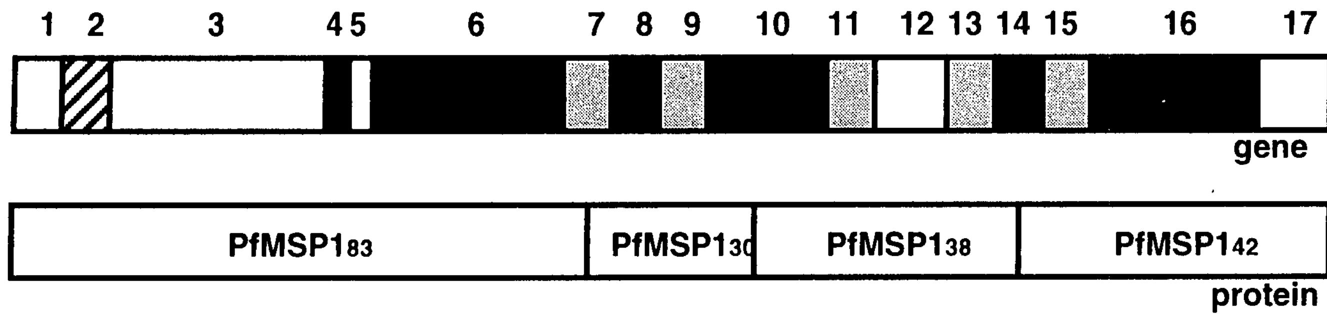
A 25kDa protein (Pfs25) was found to be expressed on the zygote and ookinete surface after fertilisation (Rener *et al* 1983). It has been suggested that Pfs25 is a ligand for an ookinete receptor on the mosquito gut wall. The deduced amino acid sequence of Pfs25 shows that it is a cysteine-rich protein consisting of four EGF-like motifs (Kaslow *et al* 1988). These EGF-like motifs have been found in Pfs25's analogue (Pgs25) in *P. gallinaceum*, an avian malaria (Kaslow *et al* 1989). The position of the cysteine residues are highly conserved between these two *Plasmodium* species. Correctly folded yeast recombinant proteins representing Pfs25 induced transmission-blocking antibodies when used to immunise mice or monkeys (Barr *et al* 1991). The adjuvant used, muramyl tripeptide, is safe to use in humans and preclinical trials of this vaccine are imminent.

6. The Merozoite Surface Protein-1 (MSP1) as a vaccine candidate

MSP1 is also known as the Precursor to the Major Merozoite Surface Antigen (PMMSA), gp195, P195, MSA-1; reviewed in Holder *et al* (1988). It is one of the most studied antigens of the parasite life cycle, and is synthesised from the beginning of schizogony in the liver and in blood stages of the parasite (Holder and Freeman 1981, 1982, Hall *et al* 1984a, Szarfman *et al* 1988).

The first complete gene sequence of *Plasmodium falciparum* MSP1 (PfMSP1) was determined in 1985 and shown to encode a protein of approximately 1700 amino acids (Holder *et al* 1985). Seven full length and at least 37 partial PfMSP1 sequences have been determined (reviewed in Miller *et al* 1993). Sequences have also been obtained for the PfMSP1 analogues in *P. yoelii* (Burns *et al* 1988, Lewis 1989), *P. chabaudi* (Lew *et al* 1989a, Deleersnijder *et al* 1990) and *P. vivax* (del Portillo *et al* 1991, Gibson *et al* 1992). Primary sequences deduced from these genes have certain features in common; a 19 amino acid N-terminal signal sequence and a putative hydrophobic membrane anchoring domain at the C-terminus of the molecule. The hydrophobic sequence is thought to be removed during biosynthesis and replaced with a glycolipid, binding MSP1 to the merozoite surface via a glycosylphosphatidylinositol (GPI) anchor (Halder *et al* 1985).

Studies with PfMSP1-specific murine mAbs suggested that the PfMSP1 molecule contained both conserved and dimorphic amino acid sequences (McBride *et al* 1982, 1985). Tanabe *et al* (1987) demonstrated that the PfMSP1 primary sequence can be divided into 17 blocks (see figure 1.2.), in which the amino acid sequence was either highly conserved (91-96% homology), semi conserved (65-75% homology) or variable (10-38% homology). The variable blocks were found to be of two versions, i.e. amino acids could be only one of two possibilities such that PfMSP1 sequences from parasite isolates fall into one of two dimorphic types. This lead Tanabe and co-workers to suggest a dimorphic allele model, where K1 and MAD20 sequences are the two allelic prototypes for all isolates of PfMSP1. They proposed that the two dimorphic forms of PfMSP1 were the result of limited intragenic recombination between two parental PfMSP1 alleles. Dimorphism at the amino acid level is strictly maintained in the nucleotide gene sequence as variation is not random; only one base differs at positions where substitutions occur and only two amino acids are permissible at any one position.



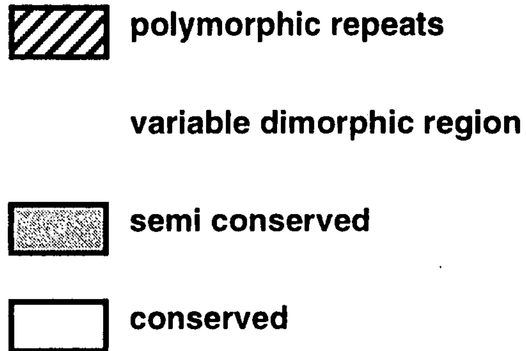


Figure 1.2. Schematic representation of the primary sequence of the PfMSP1 gene, and proteolytic processing of the PfMSP1 protein.

Comparison of the amino acid composition of PfMSP1 from different strains indicates that the polypeptide can be divided into 17 blocks with conserved, semi-conserved or variable sequence (Tanabe *et al* 1987). Variable areas are one of two dimorphic sequences. The precursor protein undergoes proteolytic processing to form four proteins on the merozoite surface (PfMSP1$_{83}$, PfMSP1$_{28\text{-}30}$, PfMSP1$_{38}$, PfMSP1$_{42}$). PfMSP1$_{42}$ undergoes secondary processing to form two peptides; PfMSP1$_{33}$ and PfMSP1$_{19}$.

There are exceptions to the dimorphic rule. Block 2 is the only truly polymorphic region of the molecule, made up of degenerate tandem repeats of amino acids, of which there are two distinct allelic prototypes. However, there is a third form of block 2 which lacks the repeats, as found in the RO33 (Certa *et al* 1987) and CSL-2 (Peterson *et al* 1988) isolates. This third form of block 2 is highly prevalent in field isolates in certain geographical regions. Kimura *et al* (1990) found that 53% of 30 Brazilian malaria patients were infected with isolates which contained the RO33 allelic form of block 2. Scherf *et al* (1991) found that 15 out of 16 (94%) blood samples from Senegal contained the RO33 allelic form, compared with 56% for K1 and 13% for MAD20 allelic forms. It seems likely that *in vitro* culturing of parasites may bias the growth rate of strains, by increasing the relative growth rate of strains which do not contain the RO33 version of block 2, suggesting that host factors, absent in *in vitro* cultures, could play a role in either limiting MAD20 and Wellcome block 2 type parasites and/or promoting RO33 block 2 type parasites. On the other hand, Jongwutiwes *et al* (1992) analysed 25 gene clones derived from 19 Thai wild isolates, and found that only 3 clones were of the RO33 block 2 type, while 16 were of the MAD20 and 6 were of the K1 type of block 2.

The reason why there are three forms of block 2, contrasting to the two forms of the rest of the molecule, is unknown. It has been suggested that the third form of block 2 may be an ancestral sequence from which the hyper variable tripeptide repeats have evolved to escape immune attack (Certa *et al* 1987). However, Kimura *et al* (1990) suggest that, due to the high conservation of the 52 amino acids of the RO33 version of block 2 among isolates from around the world, that this form of block 2 may have evolved from the repeats to form a new functional unit with as yet unknown function.

Comparison of MSP1 sequences from different *P. vivax* strains indicates there are also areas of conserved, semi-conserved and variable sequence homology within PvMSP1 (Gibson *et al* 1992). Interspecies comparisons of amino acid homology between MSP1 of different *Plasmodium* species show there is only limited sequence homology (Lewis 1989, Deleersnijder *et al* 1990, del Portillo *et al* 1991, Gibson *et al* 1992). However, there are regions of inter-species conservation which may indicate regions of MSP1 associated with essential structural or functional roles (Cooper 1993). These interspecies conserved blocks reside within the conserved regions identified by Tanabe *et al* (1987) with especially high homology in the C-terminus.

In aligned PfMSP1 amino acid sequences, the position of the 19 cysteine residues are conserved, 12 are found in the highly conserved block 17 sequence at the C-terminus of the molecule. In all *Plasmodium* MSP1 sequences (when divided into 17 blocks and aligned) the position of 14 cysteine residues are conserved, 10 of which are located within the C-terminal, block 17 (Miller *et al* 1993). In PfMSP1 this cysteine-rich region contains two EGF-like motifs (Blackman *et al* 1991a), with the characteristic spacing of cysteines and a glycine residue (Appella *et al* 1988, Engel 1989). In EGF the cysteines are linked by disulphides; C1 to C3, C2 to C4, and C5 to C6 (see figure 1.3.). In species other than *P. falciparum*, one pair of cysteines (C2 to C4) are absent from the first EGF-like motif (Blackman *et al* 1991a, Daly *et al* 1992), so not forming a true EGF-like structure.

The size of PfMSP1 has been estimated to be between 185 and 205 kDa depending on the strain of origin (McBride *et al* 1985, Schwartz *et al* 1986). Higher molecular weight homologues of PfMSP1 occur in other *Plasmodium* species; 250kDa in *P. chabaudi* (PcMSP1; Boyle *et al* 1982, Holder *et al* 1983), 230kDa in *P. yoelii* (PyMSP1; Holder *et al* 1983, Oka *et al* 1984), 230-250kDa in *P. knowlesi* (PkMSP1; Epstein *et al* 1981, David *et al* 1984)and 200kDa in *P. vivax* (PvMSP1; del Portillo *et al* 1991, Gibson *et al* 1992). MSP1 is a precursor molecule which undergoes post-translational proteolytic processing at the end of schizogony, prior to the release of merozoites from the mature schizont, to form a number of fragments on the merozoite surface (see figure 1.2.).

The exact size of the fragments depends on the parasite isolate (Lyon *et al* 1986, 1987, McBride and Heidrich 1987), but for convenience the proteins will be referred to here as PfMSP1$_{83}$, PfMSP1$_{28-30}$, PfMSP1$_{38}$ and PfMSP1$_{42}$. N-terminal amino acid sequencing of purified PfMSP1$_{83}$ (Holder *et al* 1985, Strych *et al* 1987, Cooper and Bujard 1992), PfMSP1$_{38}$ (Heidrich *et al* 1989) and PfMSP1$_{42}$ (Heidrich *et al* 1989, Blackman *et al* 1991b) confirmed that these fragments are derived from PfMSP1, and also identified the exact location of the proteolytic cleavage sites for the Wellcome and MAD20 alleles (Stafford *et al* 1994). McBride and Heidrich (1987) demonstrated that these major products of PfMSP1 proteolysis, but not uncleaved PfMSP1, are associated on the merozoite surface in the form of a non-covalently associated complex, which also includes a 22kDa and a 36kDa protein not derived from PfMSP1 (Stafford *et al* 1994). PfMSP2 is not associated with the PfMSP1 complex and PfMSP1$_{28-30}$ has not been definitively demonstrated within the complex, due to lack of a suitable antibody probe.

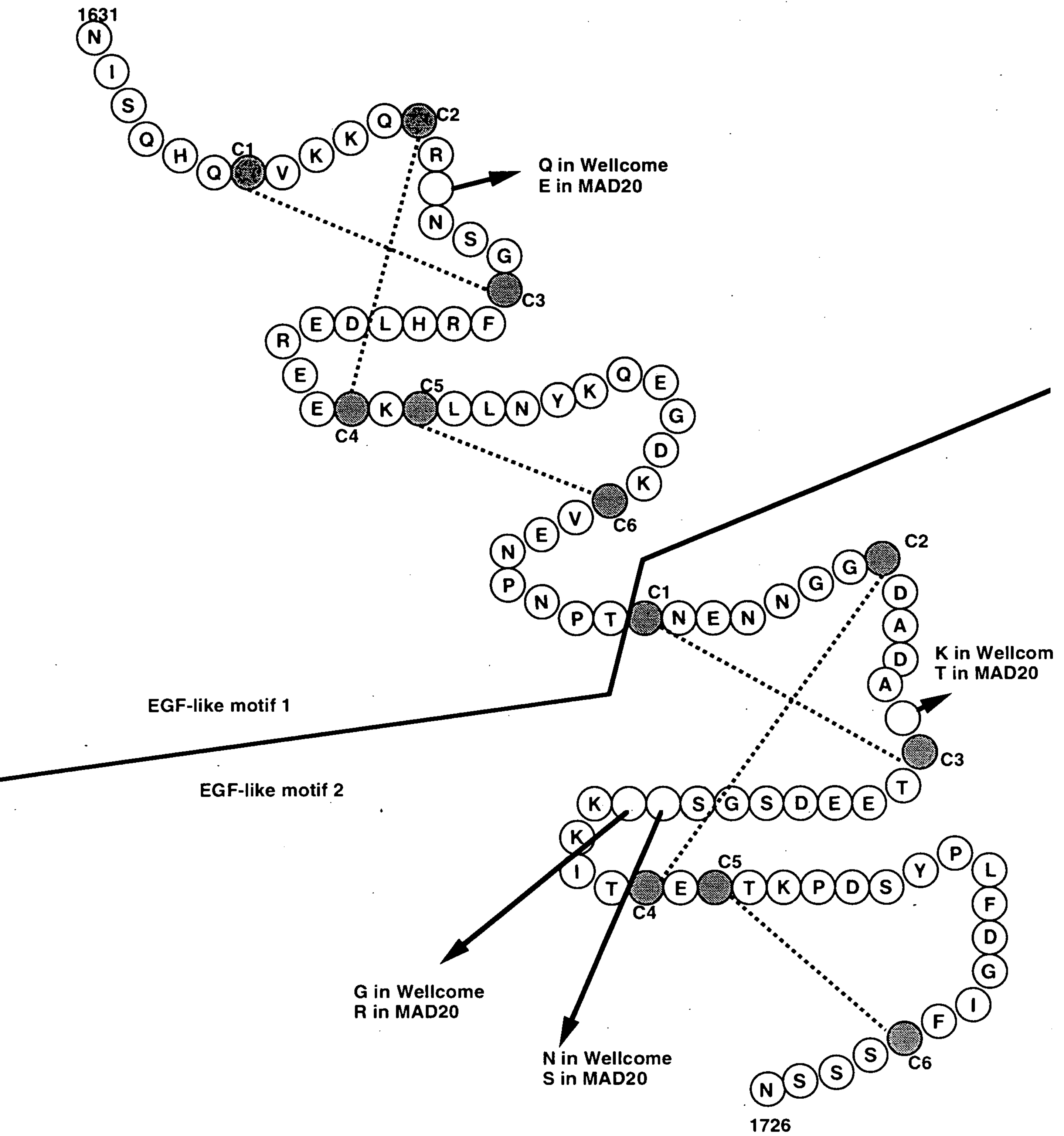


Figure 1.3. Schematic representation of the postulated EGF-like structure of PfMSP1$_{19}$

Expected disulphide-bonding is indicated by dashed line.
Dimorphic amino acid residues are indicated.

It was found that mAbs which recognise $PfMSP1_{42}$ on a Western blot also recognise a 19kDa protein, and that unlike mAbs to other parts of PfMSP1, $PfMSP1_{42}$-specific mAbs did not recognise the reduced form of the protein, suggesting that epitopes bound by these mAbs were disulphide bond-dependent (McBride and Heidrich 1987, Blackman *et al* 1991b). Metabolic labelling of $PfMSP1_{42}$ demonstrated that the 19kDa protein (henceforth referred to as $PfMSP1_{19}$) is derived from $PfMSP1_{42}$ (Holder *et al* 1987, McBride and Heidrich 1987); this was later confirmed by N-terminal amino acid sequencing (Blackman *et al* 1991b). The $PfMSP1_{19}$ protein consists of 96 amino acids, which gives a predicted size of 12kDa, which is smaller than its apparent molecular weight of 19kDa on SDS PAGE. It is difficult to predict the size of $PfMSP1_{19}$ as the protein is modified by a GPI anchor and is disulphide-constrained. After reduction and alkylation of recombinant proteins representing $PfMSP1_{19}$, its mobility on SDS PAGE increases from 19kDa to 17kDa; this is unusual as reduction of disulphide bonds usually decreases protein mobility (Burghaus and Holder 1994).

Blackman *et al* (1990) demonstrated that $PfMSP1_{19}$ is the only fragment of PfMSP1 which remains on the merozoite surface during RBC invasion. $PfMSP1_{19}$-specific mAbs bind to the parasite membrane of newly formed ring stages whereas a human mAb (X509) specific for the N-terminus of $PfMSP1_{42}$ did not (Blackman *et al* 1990). MAb reactivity with ring stages decreases with the age of the rings, with no antibody reaction with rings older than 2 hours, suggesting that the antigen is degraded. Immunoprecipitation of culture supernatants with mAb X509 demonstrated the presence of a 33kDa fragment. Together with the location of the epitope bound by mAb X509, this suggested that $PfMSP1_{33}$ and $PfMSP1_{19}$ are produced by a single proteolytic event at a site within $PfMSP1_{42}$. Henceforth this 33kDa protein will be referred to as $PfMSP1_{33}$. The absence of $PfMSP1_{33}$ in mature schizont preparations suggests that the proteolysis of $PfMSP1_{42}$ to $PfMSP1_{33}$ and $PfMSP1_{19}$ is the result of a secondary processing event, distinct from the primary processing of the PfMSP1 in schizonts.

Extracellular calcium is essential for RBC invasion by merozoites; the presence of the chelating agent EGTA inhibits RBC invasion (McCallum-Deighton and Holder 1992). Secondary processing of $PfMSP1_{42}$ was also found to be inhibited by the chelating agents EGTA and EDTA, and this inhibition is reversed by the addition of calcium but not magnesium (Blackman and Holder 1992, Blackman *et al* 1993). Primary processing was found not to be affected. This calcium-dependent proteolytic activity

was inhibited by serine protease inhibitors. It has not been proven that inhibition of secondary processing of $PfMSP1_{42}$ causes the inhibition of RBC invasion but, as the only fragment of PfMSP1 taken into the RBC is $PfMSP1_{19}$, complete proteolysis of $PfMSP1_{42}$ may be a prerequisite for RBC invasion.

At the time this project began there was considerable evidence that MSP1 might play a role in clinical immunity to malaria, and some evidence that the C-terminus of MSP1 might be particularly important. Immunisation of mice with native MSP1 (reviewed in Holder 1988), or passive transfer of MSP1-specific mAbs (Burns *et al* 1988, Lew *et al* 1989a), could protect against a challenge infection. The conserved regions of the protein contain both T and B cell epitopes (Gentz *et al* 1988, Sinigaglia *et al* 1988, Crisanti *et al* 1988). Vaccination of monkeys with native or recombinant proteins representing MSP1 protects against a lethal challenge infection (Siddiqui *et al* 1987, Holder *et al* 1988, Etlinger *et al* 1991). The C-terminal region of MSP1 is the target of the majority of the MSP1-specific invasion inhibitory mAbs (Cooper *et al* 1992, Chappel and Holder 1993), and antibodies raised to recombinant proteins representing the C-terminus inhibit parasite invasion *in vitro* (Chang *et al* 1992). Seroepidemiological studies indicated that antibody responses to PfMSP1 (Riley *et al* 1992a, 1993, Tolle *et al* 1993), and especially to the C-terminal region (Riley *et al* 1992a), were associated with resistance to clinical malaria. So PfMSP1, and especially the C-terminal region, was considered to be a major candidate for a vaccine against malaria.

7. Aims of this project

The aim of this study was to investigate human humoral and cellular immune responses to the C-terminus of PfMSP1. Firstly, to investigate the epitope specificity and the age profile of acquired antibody responses that occur following natural infection. Secondly, to assess the biological function of $PfMSP1_{19}$-specific antibodies. Thirdly, to determine whether these antibody responses are associated with resistance to clinical infection in children, and finally, to begin to evaluate cellular immune responses to $PfMSP1_{19}$.

CHAPTER TWO:
Production and antigenic integrity of recombinant proteins representing the C-terminus of PfMSP1

Introduction

Production of recombinant proteins representing PfMSP1 is necessary for;
1) characterisation of the protein and identification of immunogenic and potentially protective epitopes,
2) the production of subunits of PfMSP1 for experimental vaccination of animals (to determine whether a recombinant form of the antigen can confer a degree of immunity similar or better to that obtained with the native antigen) and
3) for an eventual large scale human vaccination trial, for which the use of killed/attenuated parasites would be impractical and uneconomical.

For these purposes it is critical to produce the synthetic antigens in the same form as their native counterparts. In the case of PfMSP1 this includes producing proteins with the correct formation of disulphide bonds to ensure formation of dominant B cell epitopes. The secondary (conformational) structure of PfMSP1 is essential for recognition of recombinant proteins representing the cysteine-rich $PfMSP1_{19}$ by murine mAbs (McBride and Heidrich 1987, Cooper *et al* 1992, Chappel and Holder 1993). Also, the protective effect of a recombinant *E. coli*-derived glutathione S-transferase (GST) fusion protein, representing the equivalent region in PyMSP1, was conformation-dependent as reduction and alkylation of this recombinant protein totally inhibited its protective effect (Ling *et al* 1994). *E. coli*-derived recombinant proteins representing $PfMSP1_{19}$ and its constituent EGF-like motifs have also been shown to display conformation-dependent determinants (Chappel and Holder 1993, Burghaus and Holder 1994).

Fragments of PfMSP1 have been expressed as recombinant proteins in *E. coli* and used to immunise monkeys (Holder *et al* 1988, Herrera *et al* 1990, Etlinger *et al* 1991, Herrera *et al* 1992). These bacterial recombinant proteins provided partial protection from challenge infection. However, in the majority of experiments, despite prolonging the prepatent period or decreasing the peak parasitaemia compared to the control animals, vaccinated animals eventually required drug treatment, and their serum IFA titres compared to native PfMSP1

were often low. A high degree of protection, and higher anti-PfMSP1 IFA titres, were obtained by immunisation of animals with purified native PfMSP1 (Siddiqui *et al* 1987, Etlinger *et al* 1991), suggesting that the bacterial recombinant polypeptides may not fully represent the native molecule in terms of correct secondary structure, i.e. correct folding of disulphide bonds, and other post-translational modifications such as glycosylation, phosphorylation, acylation and amidation.

Bacterial recombinant polypeptides are generally intracellular products. Due to the internal reducing environment of bacteria, proteins with intramolecular disulphide bridges may not fold properly inside the cell. This may be why the above immunisations with *E. coli*-derived PfMSP1 did not confer as much protection from infection as did native purified PfMSP1. The *E. coli*-derived PfMSP1 recombinant protein of the Holder *et al* (1988) study was later noted to be in a denatured configuration (Holder *et al* 1992); the immunising proteins were not tested for correct conformation in the other studies.

Due to the lack of protection conferred on monkeys immunised with bacterial recombinant proteins, investigations have turned to eukaryotic expression systems. Eukaryotic expression systems such as yeast, insect and mammalian cells produce recombinant polypeptides via the secretory pathway in a microenvironment that promotes appropriate folding and post-translational modifications of foreign eukaryotic proteins. However, some yeast cells also produce cytoplasmic proteins rather than secreted proteins, resulting in abnormal disulphide bond formation in cysteine-rich polypeptides and inappropriate glycosylation (Bathurst 1994). Mammalian cells do not have these draw backs but produce rather low yields rendering this approach impractical. Baculovirus transfected insect cells, however, produce correctly folded peptides, which appear to be appropriately glycosylated. This system also promotes other post-translational modifications such as phosphorylation, acylation and amidation (Vlak and Keus 1990).

Chang *et al* (1992) have demonstrated that the antigenic integrity of the recombinant protein is determined by the expression system used for its formation. They produced the cysteine-rich $PfMSP1_{42}$ antigen in both yeast and baculovirus systems. The baculovirus, but not the yeast, $PfMSP1_{42}$ recombinant protein was recognised by anti-PfMSP1 antibodies of animals immunised with purified native PfMSP1. Immunisation of rabbits with the

baculovirus, but not the yeast, recombinant $PfMSP1_{42}$ induced high titres of antibodies that were cross-reactive with purified native PfMSP1 in ELISA, and with schizonts and merozoites in IFA. Binding of anti-PfMSP1 antibodies to native PfMSP1 was effectively blocked in the presence of native PfMSP1 or baculovirus $PfMSP1_{42}$, while similar concentrations of yeast $PfMSP1_{42}$ were much less effective in competition binding assays. Yeast $PfMSP1_{42}$ was poorly recognised by conformation-dependent mAbs specific to $PfMSP1_{42}$. Rabbit polyclonal anti-baculovirus $PfMSP1_{42}$ antibodies completely inhibited the *in vitro* growth of parasites, whereas the anti-yeast $PfMSP1_{42}$ antibodies did not. This suggests that the antigenicity of the baculovirus $PfMSP1_{42}$ was very similar to native PfMSP1, while yeast $PfMSP1_{42}$ was antigenically distinct.

Since a major difference between the intracellular yeast polypeptide and the secreted baculovirus $PfMSP1_{42}$ seems to be the result of protein folding (demonstrated by the lack of mAb reactivity to the yeast $PfMSP1_{42}$). It is likely that native conformation-dependent determinants of the $PfMSP1_{42}$ polypeptide are critical for the optimal antigenicity and stimulation of B cells which produce growth inhibitory antibodies.

It has been suggested that the conformation of a protein also influences recognition of T cell epitopes (Atassi *et al* 1989, Vidard *et al* 1992, see chapter 7). T cell epitopes are usually short, linear peptides, however, their processing can be dependent on protein conformation, particularly, the presence or absence of disulphide bonds. The ability of a protein to activate T cells will affect the quality of antibody produced, as T cell help is required for memory responses, affinity maturation of Ig and Ig class switching.

In this chapter I report on investigation into the antigenic integrity of recombinant antigens produced by different expression systems. Some of these proteins were later used in immunological assays (presented in chapters 3, 4, 5, 6 and 7). I have used *E. coli*-derived GST fusion proteins (Chappel and Holder 1993), insect cell-secreted baculovirus recombinant proteins (Murphy *et al* 1990), and yeast-derived recombinant proteins (Kaslow *et al* 1994) to represent the C-terminus of PfMSP1. I have also produced an *E. coli*-derived GST fusion protein of the $PfMSP1_{33}$ processing fragment to further analyse human immune responses to epitopes within $PfMSP1_{42}$.

Materials, methods and results

(1) Monoclonal antibodies

MAbs used in this thesis are indicated in table 2.1. MAbs were kind gifts of Dr. J. McBride (University of Edinburgh, Scotland, UK), Drs. A. Holder and M. Blackman (NIMR, London, UK), Dr. M. Perkins (Rockefeller University, New York, USA), Drs. A. Saul and J. Cooper (Queensland Institute of Medical Research, Brisbane, Australia), and Dr. R. Reese (Agouron Institute, La Jolla, California, USA).

(2) SDS-PAGE

Samples were solubilised by boiling in SDS sample buffer (0.125M Tris-HCl pH 6.8, glycerol 20%[v/v], sodium dodecyl sulphate [SDS] 4.6% [w/v], bromophenol blue 0.01% [w/v]), with or without 0.1M dithiothreitol (DTT; Sigma) as a reducing agent, then fractionated on a 10% polyacrylamide gel by the method of Laemmli (1970). Low molecular weight markers were used (Pharmacia and BioRad). Following electrophoresis, proteins were visualised by staining with Coomassie blue (Coomassie Brilliant Blue R-250; Sigma), or by Western blotting.

(3) Western (immuno-) blotting

Standard procedures were used for Western blotting (Harlow and Lane 1988). Electrophoresed proteins were transferred to nitrocellulose paper (NCP; Schleicher and Schuell, 0.2µm pore size) by the method of Towbin *et al* (1979). When non-prestained markers were used, blots were stained with Ponceau-S (Sigma) to visualise the protein positions and size (Harlow and Lane 1988). Blots were blocked with 1% milk powder in phosphate buffered saline (PBS pH7.2)/0.05% (v/v) Tween-20, and then washed three times in 0.05% (v/v) Tween-20 in PBS (PBS/T). Antibody was diluted in blocking buffer, and incubated with the blots for one hour. Blots were then washed three times in PBS/T. Bound antibody was detected by incubation with horse radish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit or anti-human IgG (Dako Ltd, High Wycombe, UK). Blots were then washed three times in PBS/T and developed with the chromogen 4-chloro-1-napthol (Sigma).

mAb	epitope	isotype	function	reference
12.8	EGF1-both	IgG	invasion inhibitory	McBride and Heidrich 1987
12.10	double EGF-both	IgG	invasion inhibitory	McBride and Heidrich 1987
2.2	EGF1-both	IgG	block invasion inhibition	McBride and Heidrich 1987
7.5	EGF1-both	IgG	block invasion inhibition	McBride and Heidrich 1987
111.2	double EGF-both	IgG	non-invasion inhibitory	Holder *et al* 1985
111.4	EGF1-Well	IgG	nd	Holder *et al* 1985
5B1	EGF1-both	IgM	invasion inhibitory	Pirson and Perkins 1985
14-1C	EGF1-both	IgG	nd	R. Reese
4H4/19	EGF2-both	IgG	invasion inhibitory	Cooper *et al* 1992
4H4/34	double EGF-MAD	IgG	non-invasion inhibitory	Cooper *et al* 1992
X509	MAD-33	IgG	non-invasion inhibitory	Blackman *et al* 1991b

Table 2.1. MAbs specific to the C-terminus of PfMSP1.

nd=not determined.

(4) Recombinant PfMSP1 proteins

Recombinant proteins representing $PfMSP1_{42}$, (yeast) $PfMSP1_{19}$ and GST cleaved (bacteria) $PfMSP1_{19}$ used in this study were kind gifts of Drs Holder, Kaslow and Burghaus respectively. Transfected *E. coli* cells expressing $PfMSP1_{19}$ and its constituent EGF-like motifs were kind gifts of Drs Holder, Burghaus and Chappel, see table 2.2. and figure 2.1. All residue numbers are derived from the sequence numbers of Miller *et al* (1993).

(4a) Baculovirus-infected insect cell-derived $PfMSP1_{42}$-S42DA

The purified protein, produced according to the method of Murphy *et al* (1990) was kindly provided by Dr. A. Holder, and represents most of the C-terminal 42kDa processing product of the Wellcome allele of PfMSP1. *Spodoptera frugiperda* cells were infected with the recombinant baculovirus encoding S42DA and were grown at 27°C in medium for one week. Protein is secreted by the insect cells into the culture supernatant, which was harvested for protein purification. S42DA was purified from the culture supernatant by absorption on to a mAb (111.4 specific for the Wellcome first EGF-like motif) affinity column and ion-exchange chromatography. The protein produced represents the first 34 amino acids of the N-terminus of PfMSP1 (the first 19 of which are the signal peptide) and 271 amino acids from the C-terminus, beginning at the aspartic acid residue (1433) and terminating at the serine residue (1723), which equals 178 amino acids of the C-terminus of $PfMSP1_{33}$ and 93 amino acids of $PfMSP1_{19}$. 84 amino acids from the N-terminus of $PfMSP1_{33}$ (i.e. approximately one third of $PfMSP1_{33}$) are not represented by this recombinant protein. Three amino acids from the C-terminus of $PfMSP1_{19}$ are also missing, however the protein contains all 12 cysteine residues.

Throughout this thesis, this protein will be referred to as Well-42, see table 2.2 and figure 2.1.

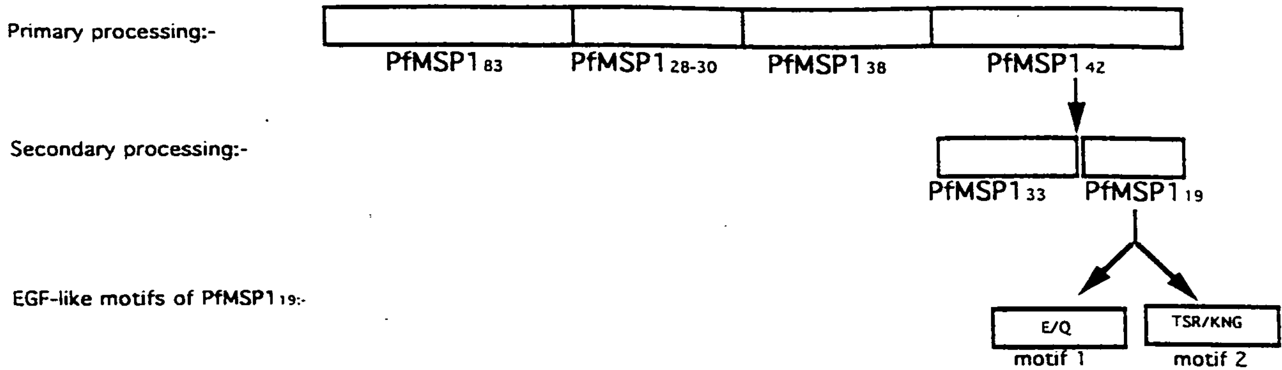
(4b) Yeast-derived recombinant proteins representing $PfMSP1_{19}$: FVO, FVO/E and 3D7

Purified yeast proteins, kindly provided by Dr. D. Kaslow, were produced as described below (Kaslow *et al* 1994). Genes encoding $PfMSP1_{19}$ (asparagine 1631 to serine 1723) were PCR-amplified from 3D7 genomic DNA and from a plasmid containing the FVO PfMSP1 gene. These were expressed in the

antigen code	domains represented	allele represented	expression system	fused to:	insert size in nucleotide base pairs	insert size in amino acids	estimated molecular weight of recombinant fusion protein (in Da)	plasmid source
MAD-EGF1 (1B)[ø]	$PfMSP1_{19}$ -1st EGF	MAD20	bacteria	GST	144	48	33,330	Chappel and Holder 1993
MAD-EGF2 (2B)	$PfMSP1_{19}$ -2nd EGF	MAD20	bacteria	GST	162	54	33,990	"
Well-EGF1 (1A)	$PfMSP1_{19}$ -1st EGF	Wellcome	bacteria	GST	144	48	33,330	"
Well-EGF2* (2A)	$PfMSP1_{19}$ -2nd EGF	Wellcome	bacteria	GST	162	54	33,990	"
Well-19/GST (Pf19III)	$PfMSP1_{19}$	Wellcome	bacteria	GST	288	96	38,610	Burghaus and Holder 1994
Well-19 (FVO)	$PfMSP1_{19}$	Wellcome	yeast	[His]6	279	93	10,890	Kaslow *et al* 1994
MAD/Well-19 (FVO/E)	$PfMSP1_{19}$	MAD20-1st EGF Wellcome-2nd EGF	yeast	[His]6	279	93	10,890	"
MAD-19 (3D7)	$PfMSP1_{19}$	MAD20	yeast	[His]6	279	93	10,890	"
MAD-33 (MAD-33)	$PfMSP1_{33}$	MAD20	bacteria	GST	843	281	58,960	A. Egan
Well-42 (S42DA)	$PfMSP1_{42}$	Wellcome	baculovirus	_	915	305	33,550	Murphy *et al* 1990
GST (GST)	_	_	bacteria	_	764	255	28,050	Chappel and Holder 1993

Table 2.2. Recombinant proteins representing the C-terminus of PfMSP1.

[ø] Author's code names for the recombinant proteins are indicated in brackets.
* insoluble, not used in immunological assays.



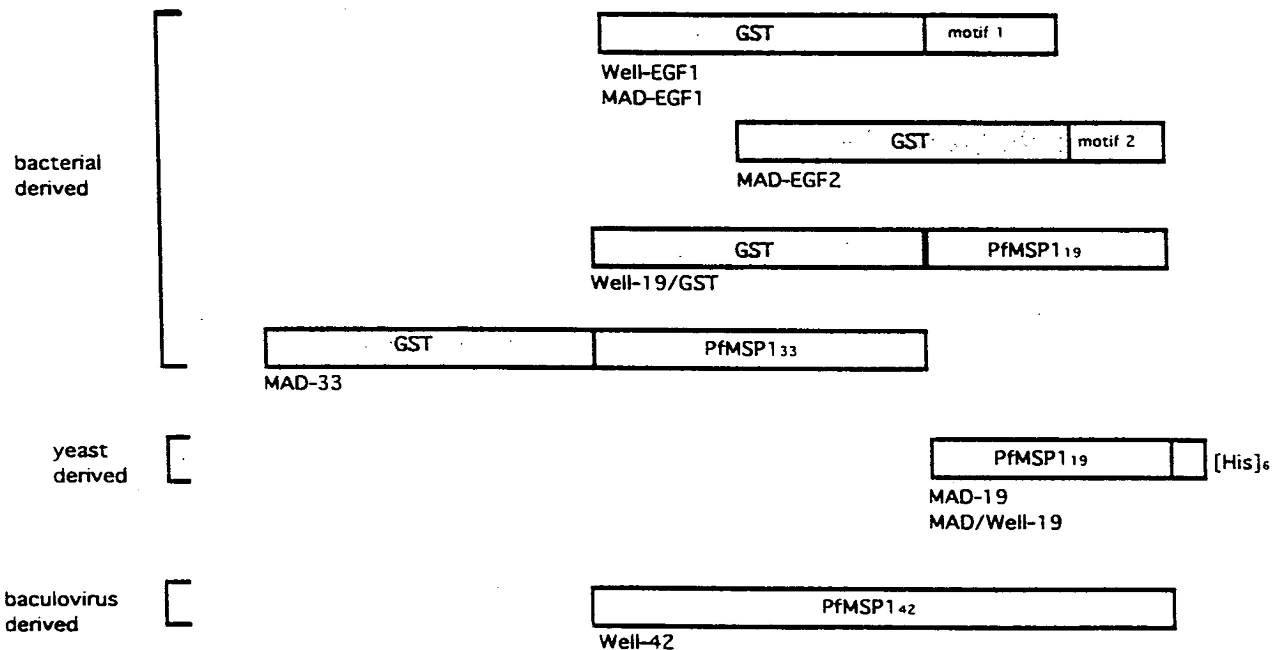


Figure 2.1. Processing of PfMSP1.

PfMSP1 is processed from a precursor molecule (gp190, PMMSA) to form four fragments ($PfMSP1_{83}$, $PfMSP1_{28\text{-}30}$, $PfMSP1_{38}$ and $PfMSP1_{42}$) which form a non-covalently linked complex on the merozoite surface. Secondary processing of $PfMSP1_{42}$ produces a membrane-bound 19kDa fragment ($PfMSP1_{19}$) which remains on the merozoite surface during RBC invasion, and a 33kDa fragment ($PfMSP1_{33}$) which is shed into the plasma. $PfMSP1_{19}$ is formed of two EGF-like motifs.

Recombinant proteins representing $PfMSP1_{42}$, $PfMSP1_{33}$, $PfMSP1_{19}$ and its constituent EGF-like motifs were expressed in *E. coli* (fused to glutathione S-transferase [GST] of *Schistosoma japonicum*), or as secreted, soluble free protein in yeast (*Saccharomyces cerevisiae*) or in baculovirus infected-insect cells.

plasmid p1XY154 to form the constructs 3D7 (the MAD20 version of $PfMSP1_{19}$ with the four dimorphic amino acids; E-TSR) and FVO (the Wellcome version, with the 4 dimorphic amino acids; Q-KNG). Through oligonucleotide-directed site-specific mutations by PCR-amplification, the hybrid construct FVO/E (representing the MAD20 first EGF-like motif, and the Wellcome second EGF-like motif; E-KNG) was formed. The proteins produced represent $PfMSP1_{19}$ minus the last 3 amino acids from the C-terminus. The addition of a 6 histidine tag to the C-terminus of these fusion proteins enabled them to be purified on nickel-nitrilo-triacetic acid-agarose (Ni-NTA).

The recombinant plasmids were electroporated into the YP47 strain of *Saccharomyces cerevisiae*. Yeast cells were grown at 30°C in medium, in a shaking incubator at 250 revs/minute for 48 hours. Recombinant protein was secreted into the culture supernatant, which was dialysed and filter-sterilised. The resultant supernatant was incubated at 4°C for one hour with Ni-NTA agarose. Centrifuged resin was loaded into a chromatography column. Recombinant protein was eluted from the resin by a low pH solution.

I was provided with purified protein. Throughout this thesis 3D7, FVO/E and FVO recombinant proteins will be referred to as MAD-19, MAD/Well-19 and Well-19 respectively, see table 2.2 and figure 2.1.

(4c) Bacteria-derived recombinant fusion proteins representing $PfMSP1_{19}$ and its constituent EGF-like motifs: Pf19III, 1A, 1B, 2A, and 2B

Recombinant *E. coli* expressing 1A, 1B, 2A and 2B were kindly provided by Dr. J. Chappel (Chappel and Holder 1993). Pf19III was kindly provided by Dr. P. Burghaus (Burghaus and Holder 1994). DNA from T9/94 (Wellcome-like) or MAD20 PfMSP1 genes were PCR-amplified with either Wellcome or MAD20 sequence-specific primers and inserted into pGEX vectors (Smith and Johnson 1988), such that they can be expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* (Smith and Johnson 1988). Recombinant proteins representing the first EGF-like motif start from asparagine 1631 ending at threonine 1678; the second-motif begins at asparagine 1674 and ends at asparagine 1726. The two constructs thus share a five amino acid overlap. The double-motif construct represents the whole of $PfMSP1_{19}$ (asparagine 1631 to asparagine 1726). I was also provided with GST cleaved (by digestion with Factor Xa; Boehringer) double-motif protein. As a control (for use in

immunological assays, see chapters 4, 5, 6, and 7), GST was produced using the pGEX plasmid without any insert.

I produced proteins from these transfected bacteria as described below. Individual colonies of ampicillin-resistant recombinant bacteria were grown overnight in 50ml of L-broth containing ampicillin at 100µg/ml, at 37°C in a shaking incubator at 180 revs/minute. Overnight cultures were diluted (1:100) into 500ml of fresh pre-warmed L-broth containing ampicillin at 100µg/ml, and grown until the absorbance of the cell suspension equalled 0.4-0.6 OD at 600nm (A_{600}), measured by a spectrophotometer. Protein synthesis was induced by the addition of iso propyl ß-D-thio-galactopyranoside (IPTG; Sigma) to equal 0.1mM final concentration. After a further three hours growth, cells were harvested by centrifugation at 5,000g for 15 minutes. Cell pellets were resuspended in 10ml ice cold PBS and underwent six x 30 second bursts of sonication. Triton X-100 (Sigma) was added to equal a final concentration of 10% (v/v), and the suspension was centrifuged for 5 minutes at 13,000g. Supernatant was incubated with 2ml of a suspension of glutathione agarose beads (Sigma) for 30 minutes on a rotator. After three washes with PBS, beads were incubated with 2ml 'elution buffer' (5mM reduced glutathione; Sigma, 50mM Tris-HCl) for 10 minutes on a rotator. After centrifugation, the supernatant, containing the eluted fusion protein, was collected (see figure 2.2. of SDS-PAGE gel of samples taken during purification).

Protein concentration of the fusion proteins was estimated visually in comparison with bovine serum albumin standards on a coomassie blue stained SDS-PAGE gel or spectrophotometrically by absorbance at 280nm (A_{280}) where an OD of 0.5 is assumed to equal 1mg/ml of protein (Dr. Holder, personal communication).

The double EGF-like motif and three of the four single EGF-like motif constructs were expressed as soluble fusion proteins. The Wellcome second EGF-like motif was insoluble in aqueous solutions and so was not used in subsequent immunological assays. (Steps taken to solubilise this protein were the same as described in 5g.)

GST-cleaved purified Pf19III was a kind gift of Dr. P. Burghaus. Purified protein had been incubated overnight with the restriction protease factor Xa (Boehringer) which cleaves the GST from the protein from the desired protein at a site-specific sequence located immediately upstream from the multicloning site on the pGEX-3X plasmid.

Figure 2.2. SDS PAGE gel of GST fusion protein purification steps.

Lane 1=sample of 2 hour growth of an overnight bacterial culture with the GST-fusion protein insert; pre IPTG induction.

Lane 2=sample of one and a half hours growth post IPTG induction.

Lane 3=sample of three hour growth post IPTG induction.

Lane 4=sonication supernatant.

Lane 5=sonication pellet.

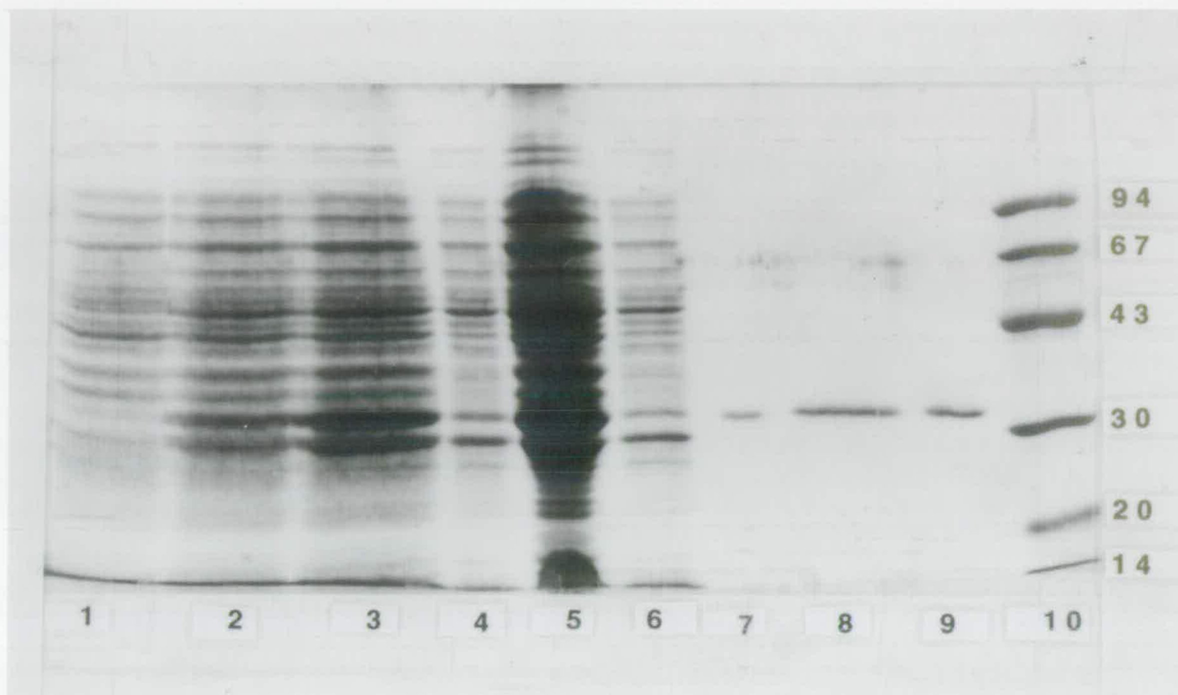
Lane 6=supernatant from incubation of agarose beads with the sonication supernatant.

Lane 7=3µl sample of purified protein (post glutathione elution).

Lane 8=15µl sample of purified protein (post glutathione elution).

Lane 9=agarose beads post glutathione elution.

Lane 10=molecular weight markers (kDa).


94
67
43
30
20
14
1
2
3
4
5
6
7
8
9
10

Throughout the thesis 1A, 1B, 2A, 2B and Pf19III recombinant proteins will be referred to as Well-EGF1, MAD-EGF1, Well-EGF2, MAD-EGF2 and Well-19/GST respectively, see table 2.2 and figure 2.1.

(5) Bacteria-derived recombinant fusion proteins representing PfMSP1$_{33}$: Construction of plasmids to express MAD20 and Wellcome versions of PfMSP1$_{33}$:

(5a) Synthetic oligonucleotides

Synthetic oligonucleotide primers were used to amplify DNA coding for PfMSP1$_{33}$. DNA was amplified using sense oligonucleotide primers (MAD20: 5' CCGCGTTGGATCCGGGCAATATCTGTCACAATGGATAAT 3', Wellcome: 5' CGGACGAGGATCCGGGCAGTAACTCCTTCCGTAATTGAT 3') and antisense oligonucleotide primers (MAD20: 5' AGAAAGGAATGATCATTATAACATATCTTGGAATTTTCCTTC 3', Wellcome: 5' GGGGGCGGGAATTCATAACATACCTTGCAAGTTTCCATC 3'). Both of the sense primers had an extension to introduce a site for the restriction enzyme *Bam* H1 adjacent to the start of the PfMSP1 coding sequences, at alanine 1349. Each of the antisense primers had an extension to introduce sites for restriction enzymes; *Eco* R1 for the Wellcome primer and *Bcl* 1 for the MAD20 primer, and a stop codon after the sequence corresponding to leucine 1630. (*Eco* R1 was not used as a restriction enzyme site for the MAD20 sequence as this stretch of DNA contains a natural *Eco* R1 site).

(5b) PCR amplification procedure

DNA encoding PfMSP1$_{33}$ was PCR-amplified from purified plasmid DNA in the form of recombinant pUC119 containing blocks 13-17 from either the MAD20 (Thai isolate 946) or Wellcome (T9/94) gene (kind gifts of Dr S. Jongwutiwes, Nagasaki University, Japan). Standard 100µl volume PCR reactions (Maniatis *et al* 1982) contained 10ng of template DNA plus the specific primer combinations (100pM of each); either MAD20 sense and antisense, or Wellcome sense and antisense. Amplification was accomplished using *Thermus aquaticus* DNA polymerase (Taq polymerase; Cetus) by denaturing DNA at 95°C for 3 minutes, followed by 30 cycles of annealing at 62°C for 2 minutes, extending at 72°C for 2 minutes and denaturing at 94°C for 1 minute. This was followed by one extension and one annealing period.

(5c) Cloning PCR amplified DNA

The PCR products were cloned into a TA cloning vector according to manufacturer's instructions (Invitrogen). Through heat shock methods, TA plasmids ligated with PCR amplified DNA were used to transform either TA Cloning One Shot (provided with the TA kit) or JM110 (Stratagene) competent *E. coli* cells. The MAD20 PCR product contained the *Bcl* 1 restriction enzyme site, which can only be digested from unmethylated DNA; most bacterial strains methylate DNA, therefore a strain which does not, i.e. JM110, had to be used.

(5d) Selection of recombinant bacteria and subcloning of DNA into expression plasmids

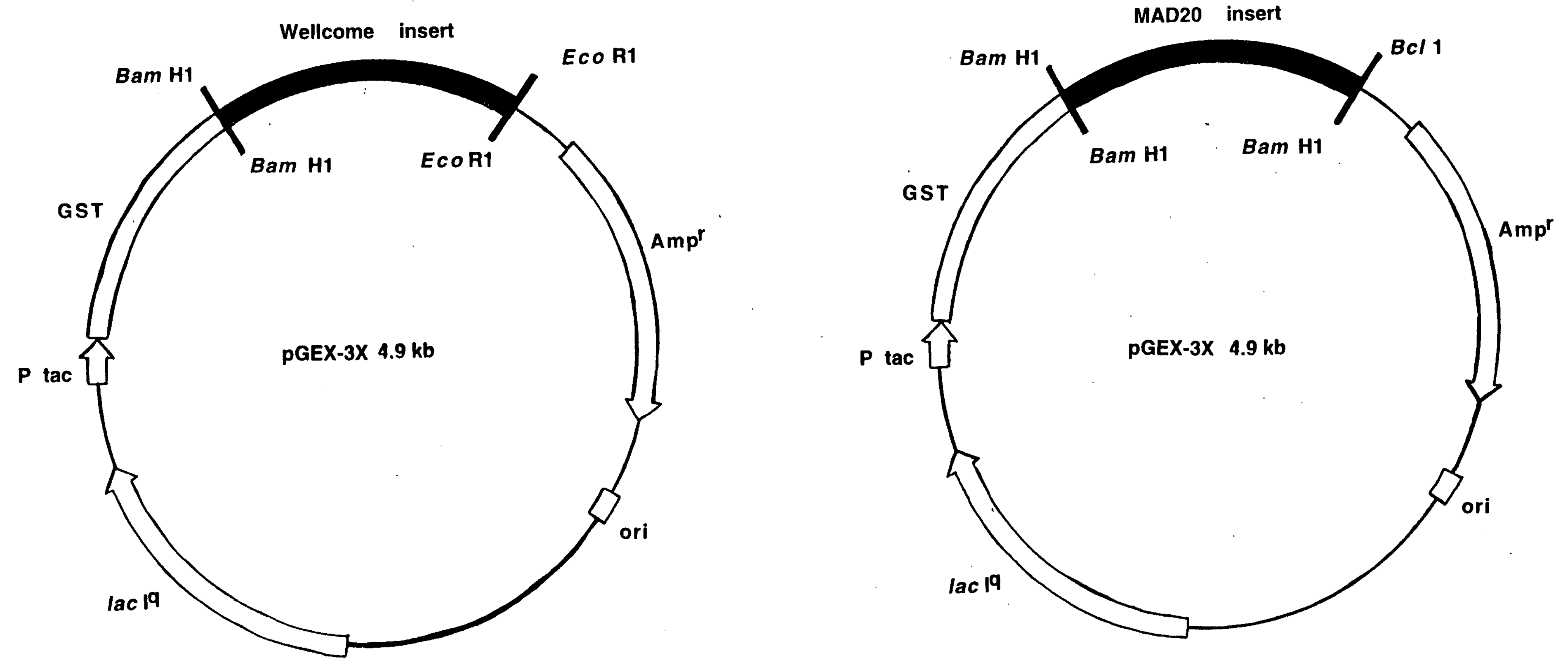
The TA plasmid contains an ampicillin resistance gene. Recombinant bacteria were selected on L-agar containing 100µg/ml ampicillin. Individual colonies on the ampicillin L-agar plates were selected, and grown up overnight in L-broth containing 100µg/ml ampicillin. Plasmid DNA was purified by a Mini Prep (Promega) purification system according to manufacturer's instructions. Purified plasmid DNA was digested with either *Eco* R1 and *Bam* H1 restriction enzymes for the Wellcome insert, or *Bcl* 1 and *Bam* H1 for the MAD20 insert. Digested DNA was separated by electrophoresis on a 1% agarose gel. A band of 4kb (i.e. linearised TA vector plasmid) plus a band of approximately 800bp (i.e. insert) indicated that the bacterial colony contained plasmid with an insert. Glycerol stocks were made of plasmids with an insert from the remainder of the overnight culture.

The 800bp insert DNA was excised from the gel, and recovered from the agarose by electroelution into dialysis tubing and then purified using an Elutip-d-column (Schleicher and Schuell) according to manufacturer's instructions. This DNA was then ligated into the bacterial expression vector pGEX-3X (Pharmacia Biotech), which had previously been digested with either *Bam* H1 restriction endonuclease and treated with calf intestinal phosphatase to prevent self-ligation (CIP; Boehringer) for the MAD20 insert, or with *Bam* H1 and *Eco* R1 restriction enzymes for the Wellcome insert (see figure 2.3.). Ligation mixes were used to transform competent bacteria (*E. coli*, strain DH5α) to ampicillin resistance and then selected on ampicillin/agar plates. Colonies containing inserts were selected as before; glycerol stocks were made.

Figure 2.3. Expression plasmid pGEX-3X with DNA insert encoding for Wellcome and MAD20 PfMSP1$_{33}$.

General features of the pGEX plasmid are shown: ampicillin resistance gene for recombinant bacteria selection, the *tac* promoter, the *lac*I^{q} gene, the origin of replication, and glutathione S-transferase [GST] for the purification of recombinant protein. The *Bam* H1 and *Eco* R1 cut Wellcome DNA was inserted into the multicloning site of pGEX-3X cut with *Bam* H1 and *Eco* R1. Due to an *Eco* R1 site in the MAD20 DNA, this insert had to be cut with *Bam* HI and *Bcl* 1 and inserted into the pGEX-3X multicloning site cut with *Bam* H1. Compatible DNA fragments for ligation are formed when DNA is cut with *Bcl* 1 and *Bam* H1: DNA cut with these two restriction enzymes will join, but neither restriction site is reformed, so this site cannot subsequently be cut with either restriction enzyme.

figure 2.3.



Wellcome = 786 base pairs
262 amino acids
56,820 Da (+GST)
28,820 Da (GST cleaved)

GST = 764 base pairs
255 amino acids
28,050 Da

MAD20 = 843 base pairs
281 amino acids
58,910 Da (+GST)
30,910 Da (GST cleaved)

(5e) Expression of DNA encoding PfMSP1$_{33}$

Bacterial colonies with the PfMSP1$_{33}$ DNA insert were tested for the production of GST fusion proteins induced in the presence of IPTG (as in section 3c).

A GST fusion protein could not be induced in any of the bacterial clones with the Wellcome insert. When this DNA was sequenced (see section 5f), I found it contained a thymidine nucleotide deletion coding for a leucine residue at position 1523 which caused a frame shift in the DNA resulting immediately in a stop codon.

The MAD20 GST fusion protein could be detected by anti-GST and anti-PfMSP1$_{33}$ antibodies (including mAb X509 which is specific for the MAD20 PfMSP1$_{33}$) in Western blot, but the protein was found to be insoluble, see figure 2.4. Throughout the thesis the MAD20 PfMSP1$_{33}$ recombinant protein will be referred to as MAD-33, see table 2.2 and figure 2.1.

(5f) Sequencing of PfMSP1$_{33}$ DNA inserts

Both inserts were sequenced in both directions across the junctions of the insert and the plasmid, to confirm the correct frame orientation and nucleotide sequence of the insert. Double stranded sequencing of purified plasmid DNA was carried out using a Sequenase kit (United States Biochemical) according to manufacturer's instructions (Chen and Seeburg 1985). Primers used were pGEX universal primers; forward primer 5' GCATGGCCTTTGCAGGG 3', reverse primer 5' ATGGCCTTTGCAGGGCT 3', plus internal primers; MAD20 forward first 5' ATCTTAAATTCACGTCTT 3', second 5' GATGATTTAGAATCAATT 3', MAD20 reverse 5' ACGAAGCTTCAGCAATCA 3', Wellcome forward first 5' ATTTTAAATTCACGATTT 3', second 5' TCAGATTTAGATTCAATT 3', reverse 5' AGGTTGCTTTAACAATAA 3'. The MAD20 DNA template used to make this recombinant protein was from a Thai field isolate, I found two amino acid differences from published MAD20 sequences (Jongwutiwes *et al* 1993); 1) glutamic acid at 1443 instead of lysine, 2) isoleucine at 1445 instead of leucine (these are not the dimorphic equivalents).

(5g) Solubilisation of MAD-33

A number of methods were used to try and solubilise MAD-33: using different cell lines; inducing protein synthesis at a lower OD of cell suspension so that more cells were in the log phase; inducing at lower temperatures, for less time,

Figure 2.4. Western blot analysis of MAD-33.

Lane 0=MW markers (in kDa).

Lane 1=sample of 2 hour growth of an overnight bacterial culture with the MAD-33 insert; pre IPTG induction.

Lane 2=sample of three hour growth post IPTG induction.

Lane 3=sonication supernatant.

Lane 4=lysis supernatant.

Lane 5=sonication and lysis pellet.

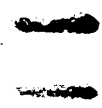
Lane 6=agarose beads incubated with pooled sonication and lysis supernatants; post glutathione elution.

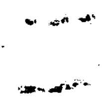
Lane 7=purified protein eluted from incubation of agarose beads with glutathione.

Lane 8=purified GST protein.

Samples were run on 10% SDS PAGE gel under reducing conditions and immunoblotted. Western blot was probed with mAb X509 which specifically recognises the MAD20 form of $PfMSP1_{33}$. A product of approximately 60kDa is recognised by the mAb, the GST protein is not. The majority of the MAD-33 product is insoluble.

Molecular weight of markers is indicated (kDa).




200
105
70
43
28
18
15
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1
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3
4
5
6
7
8

and with a lower IPTG concentration; sonicating in PBS containing 1.5% sarcosyl (Sigma), sonicating in 50mM Tris/100mM NaCl/0.1% β-mercapthoethanol (βME; Sigma), lysing cells with lysozyme (Sigma) instead of sonication and cleaving GST with Factor Xa (Boehringer) (Frangioni and Neel 1993, Smith 1993). However, none of these methods solubilised the protein. The protein became slightly soluble in 8M urea, but precipitated out after dialysis to remove the urea.

The method that worked, finally in the end, was electroelution of the lysis pellet from SDS-PAGE into dialysis bags, then precipitation with KCl to remove the SDS. Cell paste from a 500ml induced culture was resuspended on ice in 10ml 'cell lysis buffer' (25mM Tris-HCl pH8.0, 0.2% [v/v] NP-40 [nonidet P40; Boehringer], 1mM EDTA), containing 1mM PMSF (Sigma). Lysozyme (Sigma) was added to achieve a 1mg/ml final concentration, and after two hours on ice, $MgSO_4$ and DNase (deoxyribonuclease type 1; Sigma) were added to equal final concentrations of 2mM and 20μg/ml respectively. After a further two hours on ice, the cell lysate was centrifuged at 40,000g for 20 minutes. The lysis pellet was then run on SDS-PAGE with a single trough comb. After electrophoresis, thin strips at either side of the gel were cut off, stained with Coomassie blue, destained, and realigned with the rest of the gel. The band of approximately 60kDa was cut from the rest of the gel, put in dialysis bags with 5ml PBS and electroeluted in a blotting tank (Hoefer) for one hour, after which the protein solution was collected from the dialysis tubing. Addition of 500μl of 3M KCl precipitated the SDS, the solution was then centrifuged and the supernatant contained soluble protein.

(6) ELISA to determine the antigenic integrity of MAD-33

Electroelution of the MAD-33 protein resulted in slight contamination of the purified protein with co-migrating *E. coli* peptides. Since these bacterial proteins could be recognised by human serum in immunoassays, background binding had to measured. This was done by measuring antibody responses to the protein in ELISA. A few human immune sera were first tested to see if they contained antibodies to MAD-33. A high and a low titre human serum, the MAD-33 specific mAb X509 and two malaria non-exposed European sera were titrated on the MAD-33 antigen (figure 2.5.).

This titration shows that MAD-33 is not recognised by European sera, despite the bacterial peptide contamination, and is recognised by the specific

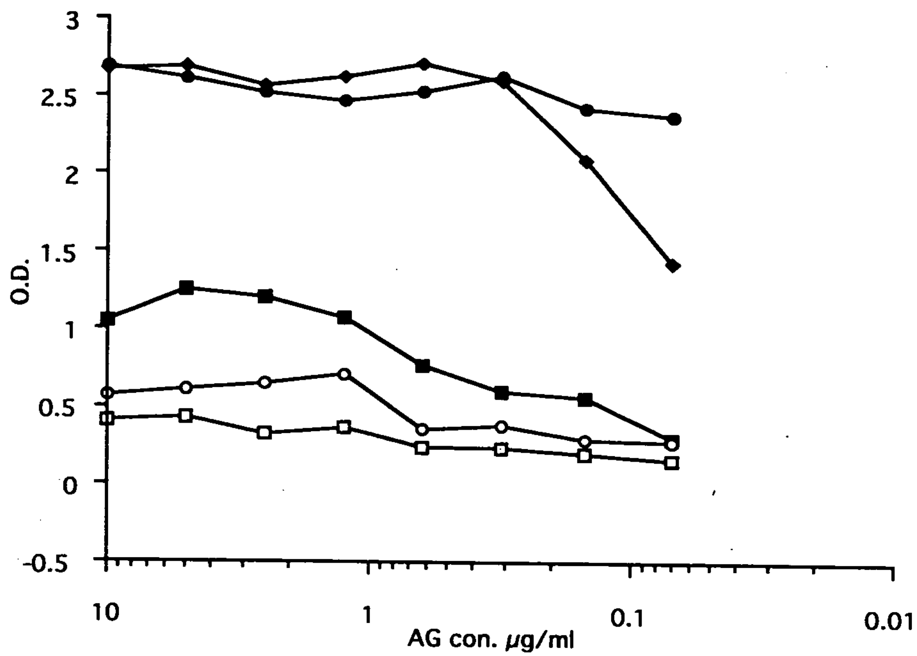


Figure 2.5. Titration of MAD-33 antigen with specific and non-specific antibodies.
High concentration of antibody (1:1000 dilution) was added to a titration of MAD-33. See chapter 3 for more details about the ELISA. ● high titre serum, ■ low titre serum, ◆ mAb X509, ○ European serum no. s28, □ European serum no. s29.

antibodies. A second titration, this time of the high affinity MAD-33 positive serum, was carried out on MAD-33 and GST saturated plates (figure 2.6.). It is clear from this titration that the malaria-exposed serum was binding specifically to MAD-33, and there was a clear difference between specific (MAD-33) and non-specific (GST) binding.

(7) ELISA to determine the antigenic integrity of PfMSP1$_{19}$ proteins

To determine whether the PfMSP1$_{19}$ recombinant proteins had assumed the correct disulphide-dependent conformation for recognition by monoclonal and human polyclonal antibody, ELISAs were performed with reduced and alkylated or non-reduced recombinant antigens.

To prepare reduced and alkylated protein, 0.5mg of antigen was incubated at 37°C for 1 hour in 0.5M Tris-HCl pH 8.1 containing 2mM EDTA and 60mM dithiothreitol (DTT; Sigma). Iodoacetic acid (Sigma), dissolved in 1M NaOH, was then added at a 2.5 molar excess over DTT. The samples were kept in the dark, pH was monitored and maintained at 8.1 for 30 minutes and then the proteins were dialysed overnight at 4°C. For ELISA, plates were coated with the appropriate saturating concentration of the reduced or non-reduced protein (for details see chapter 3).

Both human immune sera and mAbs recognised all the non-reduced GST recombinant proteins, indicating that disulphide-dependent conformational epitopes were appropriately expressed. This was confirmed by the observation that reduction of the proteins (and thus disruption of the disulphide bonds) lead to the loss of sero-reactivity (see table 2.3.). Recognition of the individual EGF-like motifs is entirely abolished after reduction though some reactivity to the double-motif proteins remains. This suggests that minor, linear epitopes are also recognised by polyclonal human antibodies. In contrast, all the mAbs tested recognised only non-reduced antigen.

(8) ELISA to examine antigenic differences between bacterial and yeast-derived recombinant proteins

Human polyclonal antibody responses to the Wellcome PfMSP1$_{19}$ double-motif protein produced in *E. coli* (Well-19/GST) and to the same sequence produced in *S. cerevisiae* (Well-19) were compared and found to be highly correlated (Spearman's rank correlation coefficient $r = 0.901$, $n=195$, $p<0.001$), indicating

that proteins produced in the two expression systems are very similar (see figure 2.7.).

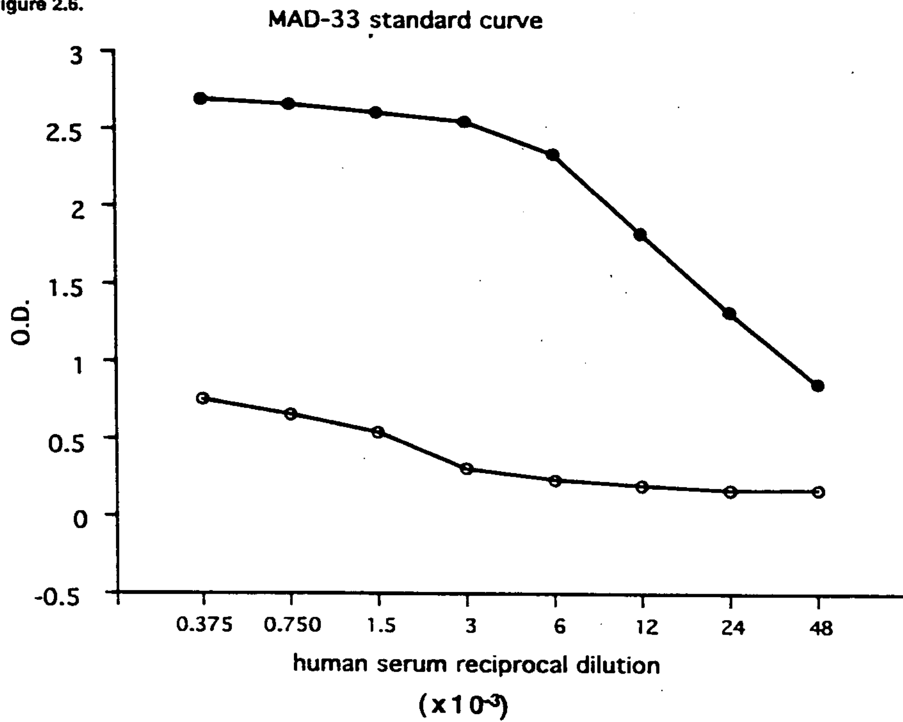


Figure 2.6. Titration of MAD-33 specific sera on specific and non-specific antigen.

Plates were coated with 1µg/ml of antigen, high to low concentrations of a high affinity serum were added to the plate. See chapter 3 for more details about the ELISA.●MAD-33,○GST.

	mAbs						human sera					
	14-1C	7.5	111.4	6E2/53	12.10	4H4/34	1559	1572	1579	1584	1592	1599
Well-EGF1												
non-reduced	**2.531**	**2.688**	**2.658**	0.170	0.150	0.193	**1.433**	0.129	0.153	0.145	**1.856**	**1.188**
reduced	0.117	0.199	0.172	0.102	0.137	0.113	0.089	0.127	0.112	0.121	0.151	**0.403**
MAD-EGF1												
non-reduced	**2.475**	**0.479**	0.137	0.181	0.199	0.130	**1.859**	0.184	0.100	0.126	**1.878**	**1.168**
reduced	0.124	0.118	0.106	0.114	0.135	0.109	0.095	0.125	0.113	0.113	0.160	**0.353**
MAD-EGF2												
non-reduced	0.311	0.123	0.148	**1.496**	0.309	0.133	**1.491**	0.329	0.194	**1.405**	0.163	**2.392**
reduced	0.159	0.138	0.113	0.191	0.135	0.125	0.198	0.251	0.284	**0.355**	0.108	**0.410**
MAD-19												
non-reduced	**1.914**	**0.995**	0.142	**2.130**	**0.710**	**1.721**	**1.899**	**0.824**	**2.516**	**1.270**	**1.655**	**2.623**
reduced	0.157	0.114	0.086	0.099	0.104	0.093	0.295	0.220	0.282	0.222	**0.612**	**0.813**
MAD/Well-19												
non-reduced	**1.932**	0.241	0.152	0.140	0.309	0.104	**1.828**	**0.691**	**2.333**	**0.829**	**1.521**	**2.334**
reduced	0.287	0.106	0.089	0.092	0.104	0.108	**0.664**	**0.316**	**0.851**	0.132	**1.016**	**1.486**
Well-19												
non-reduced	**1.998**	**1.971**	**2.124**	0.126	**1.712**	0.105	**1.712**	**0.627**	**2.408**	**0.820**	**1.410**	**2.412**
reduced	0.211	0.158	0.249	0.094	0.132	0.087	**0.523**	0.272	**0.655**	0.121	**0.824**	**1.314**
Well-19/GST												
non-reduced	**2.549**	**2.403**	**2.449**	0.125	**2.324**	0.225	**2.176**	**0.646**	**2.553**	**1.159**	**1.747**	**2.480**
reduced	0.138	0.138	0.188	0.091	0.178	0.080	**0.686**	0.272	**0.593**	0.143	**0.660**	**0.905**
GST												
non-reduced	0.262	0.119	0.208	0.235	0.234	0.169	0.105	0.101	0.108	0.102	0.121	0.120
reduced	0.134	0.124	0.101	0.122	0.128	0.116	0.078	0.084	0.087	0.078	0.105	0.120

Table 2.3. Comparison of OD values for ELISA assay using non-reduced and reduced recombinant antigens.

Underlining is used to highlight values for positive antigen/antibody combinations.(Human sera were tested at a dilution of 1:3000, which represents the mid point of the titration curve. MAb were tested at a dilution of 1:1000 which represents a saturating concentration of antibody.)

figure 2.7.

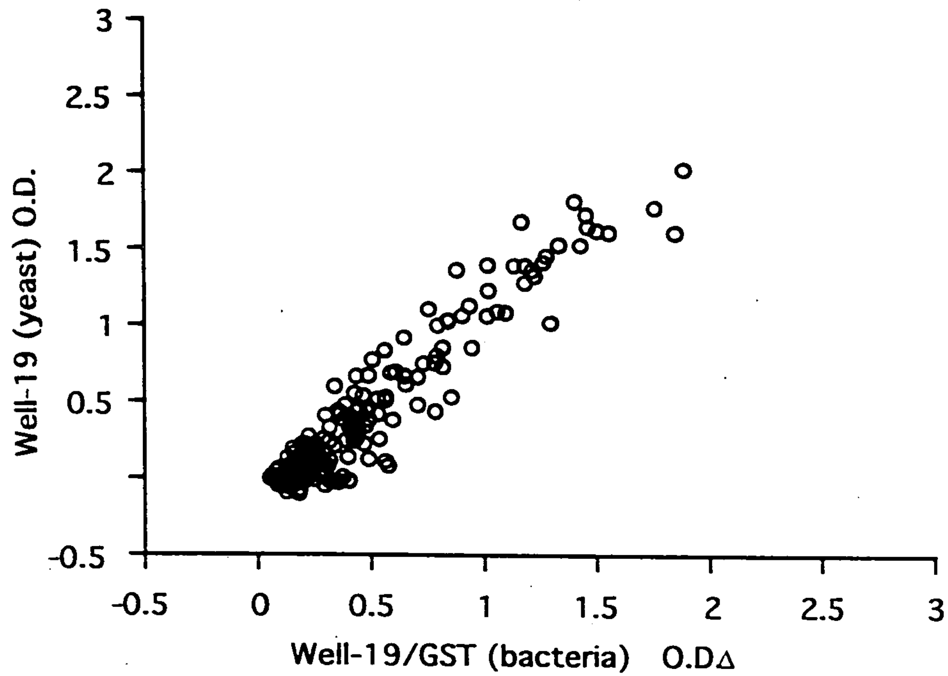


Figure 2.7. Comparison of antibody recognition of yeast derived Well-19 vs *E. coli*-derived Well-19/GST.

Each point represents reactions of a single immune human serum from one individual (dilution 1:3,000). For more details on this type of ELISA see chapter 4.

Discussion

Proteins expressed as intracellular products in *E. coli* are not generally thought to form appropriate intramolecular disulphide bonds (reviewed in Marston 1986). This is potentially a serious problem for expression of the C-terminus of PfMSP1 which contains 12 cysteine residues thought to make up 6 intramolecular disulphide bonds (Blackman *et al* 1991a). Indeed, Holder *et al* (1988) found that vaccination of monkeys with two recombinant proteins (*trp* E' and β-galactosidase hybrid fusion proteins) representing the C-terminus of PfMSP1, including the two EGF-like motifs, induced only partial protection against subsequent challenge infection. Native PfMSP1 was poorly recognised by serum from these vaccinated monkeys and the authors concluded that the recombinant proteins were not correctly folded. In contrast, Muller *et al* (1989), Chappel and Holder (1993), and Burghaus and Holder (1994) have all been able to produce *E. coli*-derived recombinant proteins representing the C-terminus of PfMSP1 (including the EGF-like motifs) which were recognised by conformation-dependent mAbs. In confirmation, the binding of all these mAbs was abolished by the prior treatment of the proteins with a reducing agent. The fact that *E. coli*-derived GST fusion proteins representing $PfMSP1_{19}$ are recognised by conformation-dependent mAbs suggests that the correct tertiary structure had been obtained by this prokaryotic expression system. Correct cysteine pairing has also been demonstrated for recombinant proteins representing the C-terminus of PfMSP1 produced in eukaryotic expression systems (Murphy *et al* 1990, Kaslow *et al* 1994). It appears therefore that, once placed in a non-reducing environment, $PfMSP1_{19}$ proteins automatically fold, and disulphide-bond, into the native conformation. Indeed, if the proteins are artificially reduced but not alkylated, they will spontaneously refold into native conformation once returned to a non-reducing environment (Dr. A. Holder and A. Egan, unpublished observations). It is fortuitous, therefore, that the native conformation of $PfMSP1_{19}$ is also the most stable conformation. This has made expression of $PfMSP1_{19}$ in bacteria much easier than might have been anticipated.

A potential draw back of eukaryotic expression systems could actually be their ability to glycosylate proteins (Bathurst 1994), as there is no direct evidence of glycosylation in PfMSP1 despite the presence of potential sites (Dieckmann-Schuppert *et al* 1992). So, despite their apparent similarity,

eukaryotic expression systems may not produce proteins with exactly the same antigenicity as native PfMSP1 (reviewed in Chang 1994)..

E. coli-derived PfMSP1$_{19}$ recombinant proteins were used to vaccinate *Aotus* monkeys against subsequent homologous and heterologous challenge infection. However, no protection (relative to control animals) was observed. Only three of the five post vaccination/prechallenge sera were able to inhibit parasite invasion of RBCs *in vitro*, but all were able to compete with the binding of conformation-dependent mAbs to the recombinant protein (Burghaus *et al*, manuscript in preparation, also see chapter 5). Kumar *et al* (1995), vaccinated *Aotus* monkeys with either an *E. coli*-derived PfMSP1$_{42}$ GST fusion protein or a PfMSP1$_{19}$ recombinant protein secreted from yeast. One of the three monkeys immunised with the bacteria-derived PfMSP1$_{42}$ GST fusion protein was able to control its parasitaemia but eventually needed drug treatment; the other two monkeys were unprotected. Two of the four monkeys immunised with the yeast PfMSP1$_{19}$ protein resolved an otherwise lethal infection without treatment, a third monkey eventually required drug treatment after a prolonged prepatent period and the fourth monkey was unprotected. Both bacterial PfMSP1$_{42}$ and yeast PfMSP1$_{19}$ were recognised in ELISA and on Western blot by reduction-sensitive mAbs. Sera from the protected monkeys were unable to inhibit parasite invasion of RBCs *in vitro*. Thus, although both proteins were conformationally correct, and although PfMSP1 proteins produced in the yeast expression system proved to be more protective than those produced in the bacteria, in neither case was complete protective immunity induced. Whether this was due to the expression system, or to the failure of the immune response to focus on critical epitopes within PfMSP1$_{19}$ is not known.

In summary, there is definitely some confusion over which is the best expression system for the production of PfMSP1 recombinant proteins for use in vaccines. Recombinant proteins from all three expression systems described here were recognised by conformation-dependent mAbs, suggesting that all three systems are capable of producing proteins with correctly formed disulphide bonds. Direct comparison of human antibody responses to the bacteria- and yeast-derived recombinant proteins, representing the same sequence of the Wellcome PfMSP1$_{19}$, indicate that recombinant protein produced in either expression system is suitable for the detection of antibody responses by ELISA. However, differences in protein structure may have more profound effects in vaccination of animals than in immunological detection of

pre-formed antibody. Correct disulphide bond formation appears to be the most easily rectified problem with PfMSP1₁₉ expression, and the mAbs provide a quick and easy way of assessing disulphide-dependent conformation. However, other post-translational modifications, which are more difficult to assess, may be crucial in determining the immunogenicity of the molecule and may explain the difference between vaccination with native PfMSP1 (which protects) and recombinant PfMSP1 (which does not always protect). Also, the stimulation of T cell help may be greatly affected by post-translational modifications of the vaccinating protein.

PfMSP1₃₃ contains no cysteine residues and recognition of the protein by the mAb X509 is not reduction-sensitive (Blackman *et al* 1991b). Therefore I decided that the bacterial expression system would be an appropriate method for the production of recombinant proteins representing PfMSP1₃₃. Only the MAD20 allelic form of the protein was expressed. DNA encoding the Wellcome allelic form was found to contain a nucleotide deletion which resulted in a stop codon terminating protein expression. This deletion probably occurred as a result of a PCR-induced mutation (PCR technology-Principle and applications of DNA amplification, Ed. Erlich, H. A., Stockton press, 1989). Successful expression of this protein would therefore require that the whole cloning process be repeated from the original pUC119 plasmid. An expression product was obtained for the MAD20 construct. Sequencing confirmed that the whole sequence was present, correct and in-frame. However, a problem was encountered with the expression of this relatively large recombinant protein, in that the product was insoluble. This is a common problem in the expression of large proteins in *E. coli* but was eventually solved. The resulting protein was slightly contaminated with *E. coli* peptides which migrate at the same molecular weight as the fusion protein. However, in ELISA assays only low ODs were obtained with serum from malaria-unexposed individuals indicating that the bacterial peptides were not recognised by human serum and therefore had little effect on antibody responses to this protein.

These recombinant proteins were used to measure human immune responses to the C-terminus of PfMSP1 (chapters 3, 4, 5, 6 and 7).

CHAPTER THREE:
Detection of malaria-specific antibody responses by Enzyme Linked Immunosorbent Assay (ELISA)

Introduction

The development of the enzyme-linked immunosorbent assay (ELISA) by Engvall and Perlmann in 1971 revolutionised the experimental detection of an analyte in a sample, and is now used to measure a wide range of antigens, antibodies, cytokines and hormones. Before the development of ELISA the main method of detection of an antibody/antigen reaction was by radioimmunoassay, which has several draw backs; the main one being that it requires the use of hazardous reagents. The ELISA technique takes advantage of the fact that proteins stick to plastic, presumably via hydrophobic forces (Kemeny 1992). The use of multiwell microtitre plates was developed as a solid phase, instead of tubes, which facilitates easy handling and can be used with automated well washers, readers and multiple well dispensers, allowing large numbers of samples to be assayed. ELISA is now a standard laboratory technique and a very useful tool in the study of parasitology.

ELISA has enabled the large scale study of serological responses to parasitic infections and has become an important device for the investigation of human immune responses to malaria. In this thesis I describe measurements of antibody responses to recombinant proteins representing the C-terminus of PfMSP1 in hundreds of individuals; this would have been a daunting task with a less automated immunological assay such as Western blot. As so many samples can be tested in one experiment, the antibody responses of a whole study population can be compared within one experiment, and if care is taken to standardise the parameters of the ELISA assay, results from different experiments can also be compared. So, ELISA has become an invaluable technique for large-scale batch testing of immune responses in seroepidemiological studies. However, ELISA can be inaccurate if its limitations are not taken into account and controlled for.

The indirect ELISA technique which has been used in this study, is the most widely used form of ELISA (see figure 3.1.). The unlabelled partner in the antibody/antigen complex is attached to a solid matrix. Either the antibody or

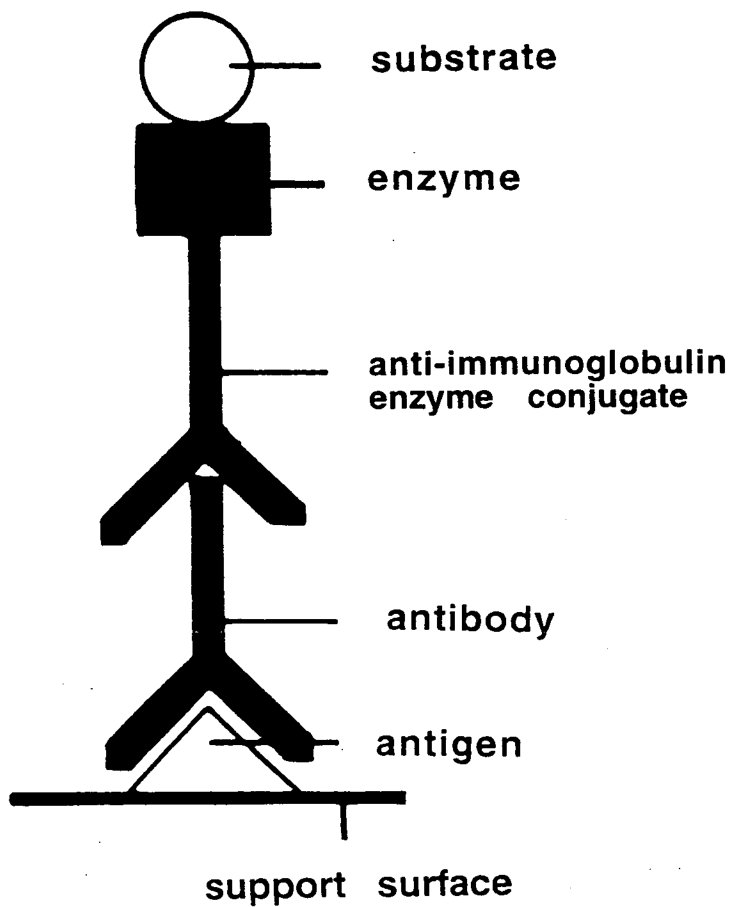


Figure 3.1. The components of a standard indirect ELISA

From Vankatesen and Wakelin (1993), Parasitology Today,9:228.

the antigen can be attached to the microtitre plate; in this study, antigen was attached to the microtitre plate to detect antibodies in the sample solution. The sample antibody is detected by a second antibody which has an enzyme label (a direct ELISA would have the enzyme label attached to the sample antibody). The concentration of the primary antibody bound to the antigen is estimated by the addition of the second antibody's enzyme substrate and an indicator that changes colour when the substrate is acted upon by the enzyme. In this study, the detecting secondary antibody is either a rabbit anti-mouse or rabbit anti-human IgG heavy chain antibody, depending on what primary antibody is to be detected. Attached to this secondary antibody is the enzyme horse radish peroxidase (HRP), for which the substrate is hydrogen peroxide (H_2O_2). The action of this enzyme on the H_2O_2 causes a colour change in the chromogen o-phenylenediamine (OPD). This colour change/enzyme activity is stopped by the addition of sulphuric acid (H_2SO_4), and the degree of colour generation, the optical density (OD), of each reaction is measured at the wavelength of 492nm on a spectrophotometer. The OD obtained is given as a log of percentage light absorbance. 100% light absorbance gives an OD of 2, so an OD of 1 is not 50% absorbance, but rather 10%, and OD readings over the value of 2 are inaccurate.

It is important to include a standard curve of a positive serum on one antigen in every assay. This will indicate how comparable the results are from separate assays. A measure of this homogeneity can be determined by working out the coefficient of variation (CV) between assays;

$$\text{coefficient of variation (\%)} = \frac{\text{SD} \times 100}{\text{mean}}$$

This needs to be worked out within batches and between batches of ELISAs to find an acceptable CV for subsequent assays. It has been suggested that variation should not be more than 20% (Kemeny 1992).

In this chapter I will discuss the importance of, and the procedure for, optimising the ELISA protocol. The solid phase system used in this study is a polystyrene 96-well microtitre plate. There are different types of microtitre plates, with different binding capacities, some are good at binding small peptides while others are better at binding larger peptides (Kemeny 1992).

Immulon 4 plates (Dynatech) are used in our laboratory as they were found to be the most efficient plates at binding small recombinant proteins.

The initial binding of the antigen to the plate is the most important stage of the ELISA to be optimised; if this stage is not properly standardised, standardisation of the of the other components of the ELISA will be meaningless. Antigen is passively adsorbed on to the surface of the plastic in a carbonate buffer, which facilities this binding. Antigen should be firmly bound to the plate and should not be damaged by adsorption. However, conformational changes can occur when an antigen binds to plastic. Also proteins can become bound to the plate in a specific orientation, i.e. hydrophobic regions of peptides bind and are not available as an epitope, so some antigenic determinants fail to be expressed. This could also result in a change in the protein's natural tertiary structure (Davey and Steward 1988, Kemeny 1992).

It is important that the concentration of the coating antigen is standardised. A saturating protein concentration is desired for all antigens, so that maximum binding of specific antibody can be achieved (Davey and Steward 1988). High epitope frequency (high antigen concentrations) encourages bivalent interactions between the two IgG heavy chains with the antigen. This is more stable, i.e. less susceptible to dissociation, compared to monovalent binding of the Fab fragment. On the other hand, bivalent interactions may be rare due to unsuitable orientation of epitopes on the solid phase. However, a plate should not be over saturated with antigen as this will cause concealment of epitopes (Kemeny 1992). Also protein-protein connections are less stable than protein-plastic, so antibody bound to an antigen bound to another antigen is more likely to dissociate during washing stages (Venkatesan and Wakelin 1993).

It has been suggested that the detecting antibody should be used in excess so as to detect all specific binding (Venkatesan and Wakelin 1993). However, a high concentration of secondary antibody can increase non-specific binding and decreases the measurable analyte range (Kemeny and Chantler 1988). A high concentration of secondary detecting antibody would cause many samples to go over the OD of 2, which would be an inaccurate reading so it would not be possible to compare these samples' OD values with one another. On the other hand, lower concentrations decrease the specific absorbance but non-specific binding is only slightly reduced (see figure 3.2.). The detecting antibody can bind non specifically to vacant plastic sites and to

Figure 3.2.

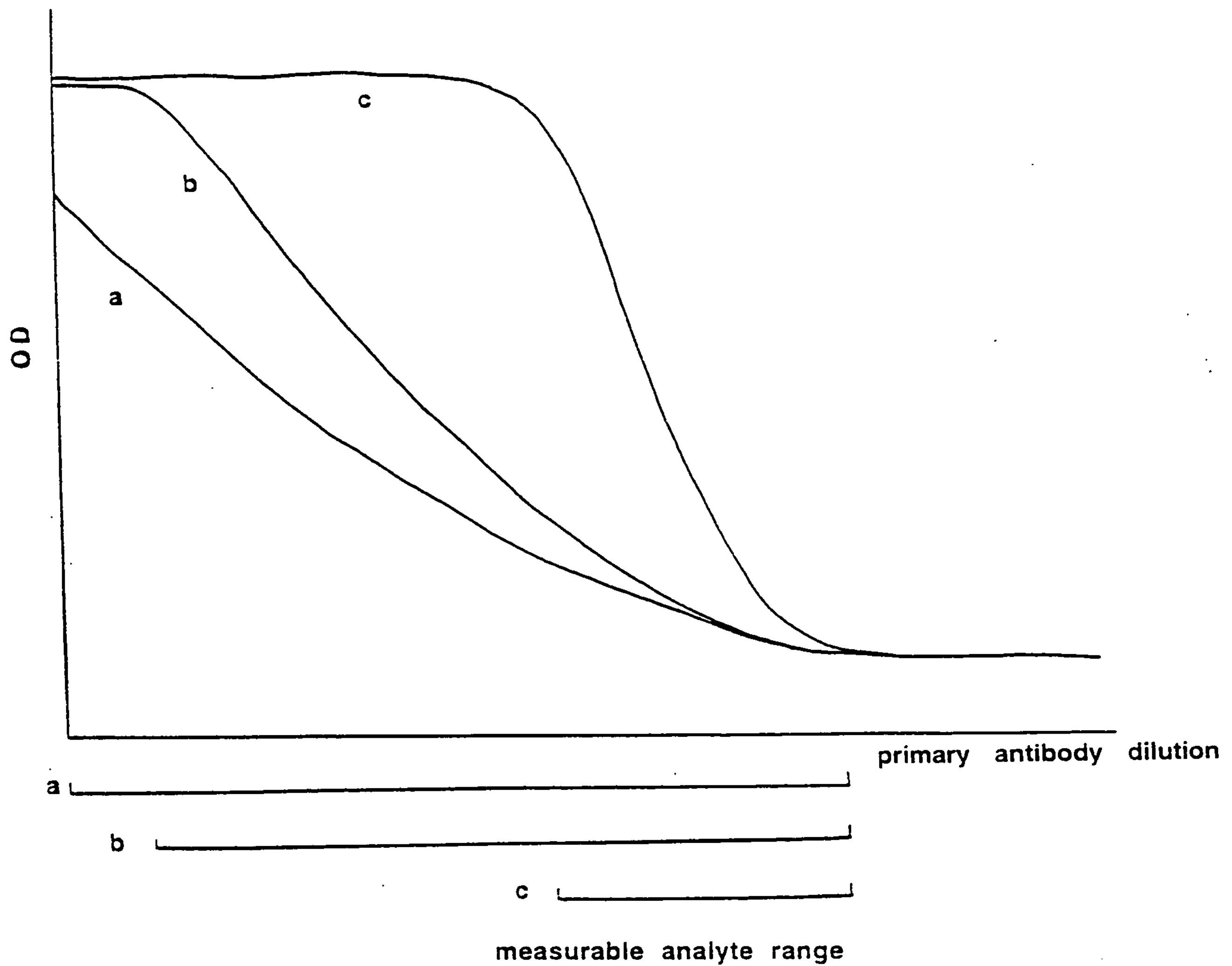


Figure 3.2. Schematic diagram to demonstrate the optimal concentration of secondary antibody. High concentrations of conjugated antibody (c) causes a steep titration curve, decreasing the measurable analyte range. Low concentrations of the secondary antibody (a) results in a shallow titration curve where large differences in primary antibody concentration results in a small difference in the OD. An optimal secondary antibody concentration (b) would result in a large measurable analyte range but which reaches the plateau of 100% absorbance (i.e. OD 2).

the antigen layer itself; diluting the detecting antibody in blocking buffer (i.e. excess non-specific protein) before hand reduces this affect.

Although there is an apparently linear relationship between antibody concentration and binding to the antigen (Koertage and Butler 1985), increase in the concentration of the secondary antibody does not result in a linear increase in OD. With an IgG antibody, there is potential variation in binding of one or both of the heavy chains. This variation becomes more evident at higher secondary antibody concentrations as less antibodies are able to bind with both arms (Davey and Steward 1988). Variation in stoichiometry may be an important variable in day to day variation.

A dilution of serum which results in a OD on the linear part of the titration curve increases the sensitivity of the assay for detecting changes in antibody binding and is thus the optimal concentration at which to compare large numbers of sera. It is not good to chose a dilution from the shallow part of the dilution curve as a large change in antibody concentration will only result in a small change in colour, making it difficult to compare individual samples within an assay (Kemeny 1992).

Most problems of non-specific binding and high backgrounds occur from this stage of the ELISA. Non-specific binding of antibodies in the serum to exposed sites of the plastic plate, and to the antigen layer itself, results in high backgrounds. This is particularly the case with sera from malaria-exposed individuals as malaria infection induces production of large amounts of antibody (hypergammaglobulinaemia) much of which is not specific for malarial antigens (Crane 1986). Non-specific binding can be reduced during the washing stages, however over vigorous washing can wash off specifically-bound antibody too, especially low affinity antibody (Davey and Steward 1988). However, when antigen/antibody complexes have formed, their dissociation is slow (Avrameas 1992). Detergents reduce background by interacting with hydrophobic sites on the plastic and so are included in the buffers (Avrameas 1992, Venkatesan and Wakelin 1993). Vacant sites on the plastic are blocked with non-specific protein (typically serum albumin or milk proteins) to reduce non-specific binding. Preincubation of sera with this same non-specific protein selects out non-specific antibody in the sample as well.

This optimised ELISA is the basis of the seroepidemiological analysis of antibody responses to PfMSP1 carried out in subsequent chapters in this thesis.

Methods and materials

The indirect enzyme linked immunosorbent assay (ELISA)

(1) General method

Antibodies reacting with recombinant PfMSP1 proteins were detected by ELISA. Microtitre plates (Immulon 4, Dynatech, Billingshurst, UK) were coated overnight at 4°C with proteins diluted in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$ pH9.5). The saturating concentration of protein was from 0.1μg/ml to 5μg/ml depending on the antigen. Plates were washed three times in phosphate buffered saline (PBS pH7.2)/0.05% (v/v) Tween 20 (PBS/T; washing buffer), blocked with a 1% (w/v) solution of non-fat powered milk in PBS/T (blocking buffer) and washed again. 100μl of serum diluted in blocking buffer, were added to duplicate wells and incubated overnight at 4°C.

After washing, horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG (100μl diluted 1:5000 in PBS/Tween 20) (Dako Ltd, High Wycombe, UK) was added to the plates, incubated for 3 hours at room temperature and developed with H_2O_2 and o-phenylenediamine (OPD; Sigma) for 10 minutes at 4°C. The reaction was stopped by the addition of 20μl of 2M H_2SO_4. Plates were read at 492nm. For GST fusion proteins the OD value of the GST control was subtracted from the OD of GST-recombinant fusion proteins, to give a specific OD (ΔOD) for the response to the PfMSP1 antigens.

(2) Optimisation of the general method

A saturating concentration of protein was determined by titration of antigen from high to low concentrations (0.005μg/ml to 10μg/ml). After the saturating concentration of each antigen was determined, the optimal concentration of the primary and secondary antibodies was determined. Plates were coated with saturating concentrations of antigen. Plates were washed and blocked as before. 100μl of high, medium and low titre serum, diluted in blocking buffer, was titrated from high to low concentrations in duplicate wells and incubated overnight at 4°C as before. After washing, high to low concentrations of secondary conjugated antibody was added to the test wells. The rest of the ELISA was carried out as before.

Results

(1) Antigen layer

To determine the optimum antigen concentration (maximum binding without over saturating), antigen was titrated from high to low concentrations, with all other parameters of the ELISA in excess so that the only limiting factor was the antigen concentration. The resulting titration resulted in a sigmoidal curve (see figure 3.3.), where the top of the curve represents optimum binding, i.e. saturation of all available binding sites. After this level has been reached, the OD does not increase with increasing antigen concentration, and may even decrease due to overcrowding or unstable protein-protein interactions.

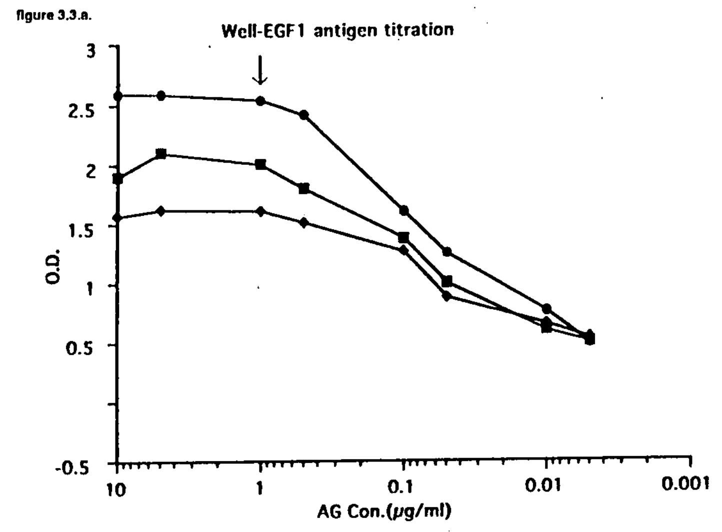
A high concentration of high, medium and low titre antibody was used for the antigen optimisation titration, as high affinity antibody will detect antigen at lower antigen concentrations than lower affinity antibodies (Davey and Steward 1988). So if an antigen concentration was chosen by only titrating with high affinity antibodies, the assay would not necessarily be capable of detecting low affinity antibodies.

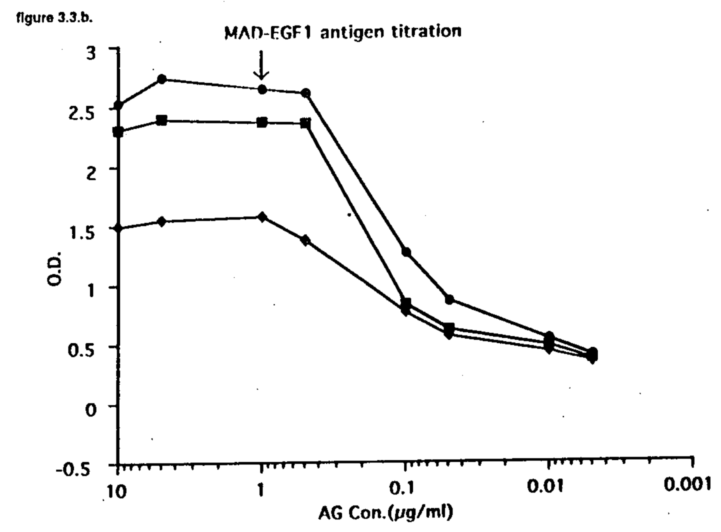
From the antigen titration graphs (figures 3.3.a-g.), the following concentrations of proteins were chosen to coat ELISA plates with; 0.1µg/ml of MAD/Well-19, Well-19 and Well-19/GST, 1µg/ml of the first EGF-like motif proteins and MAD-19, and 5µg/ml of the second EGF-like protein. 1µg/ml of MAD-33 is also used to saturate ELISA plates as determined from the antigen titration in chapter 2 (figure 2.5.).

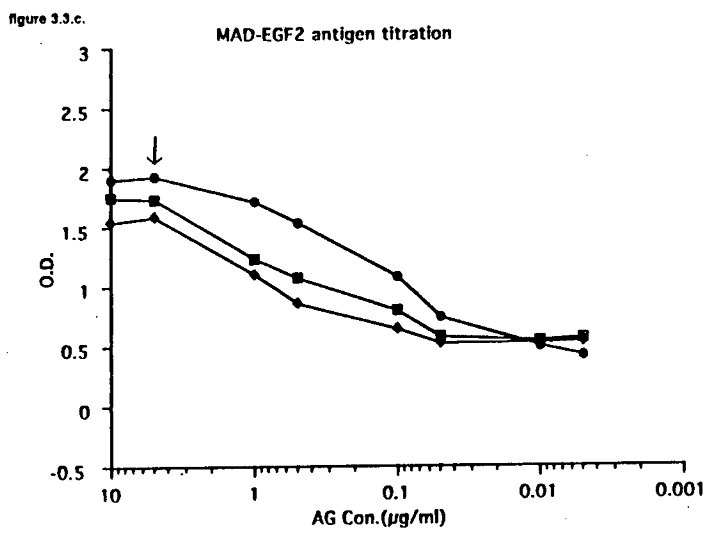
(2) Optimisation of the secondary antibody

The next component of the ELISA to be optimised was the detecting, enzyme-conjugated secondary antibody. The plate was coated with the saturating antigen concentration. Checkerboard titrations of high to low concentrations of high, medium and low titre sera were added. To this was added high to low concentrations of the secondary antibody.

I found that a dilution of 1:5000 of the conjugated antibody resulted in a titration curve which is neither too shallow nor too steep, as well as resulting in maximal difference between the binding of high, medium and low titre primary antibody on specific (Well-EGF1) and non-specific (GST) antigen (see figures 3.4.). The 1:10,000 dilution was too shallow, while the 1:500 and 1:1000







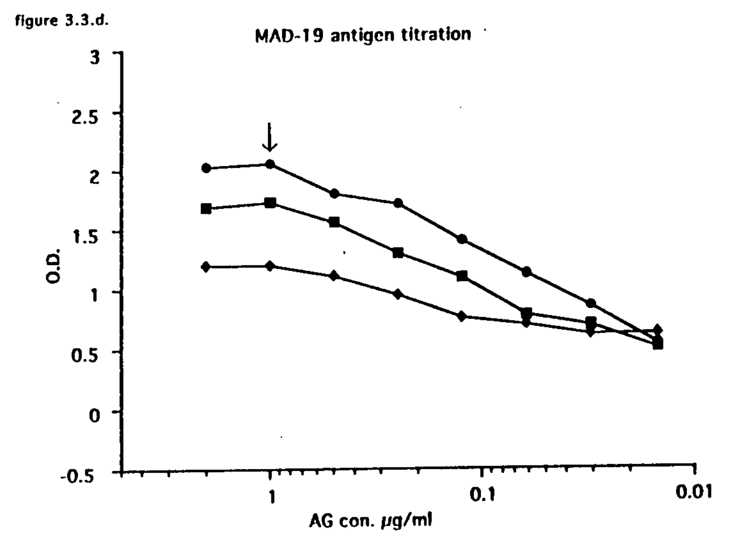


Figure 3.3. Titration of antigen to find the optimum concentration with which to coat the ELISA plate. Titrations are carried out with a 1:1000 dilution of high , medium and low titre sera, and a 1:1000 dilution of the secondary antibody. Samples were tested in duplicate, averaged well values are plotted. Arrow indicates optimum antigen concentration chosen for subsequent assays.

figure 3.3.e.

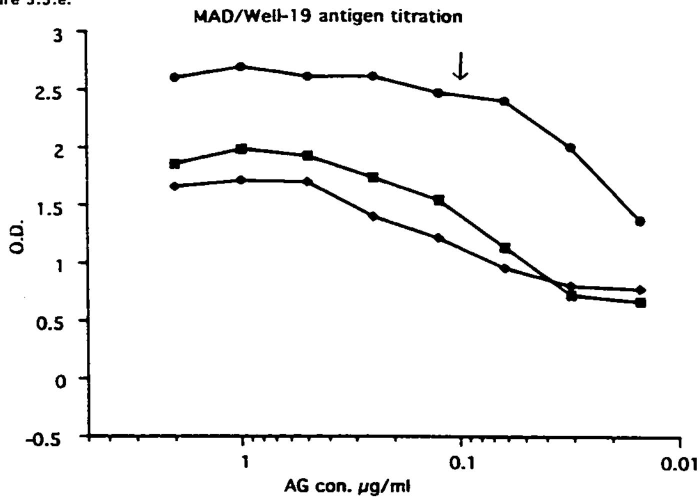


figure 3.3.f.

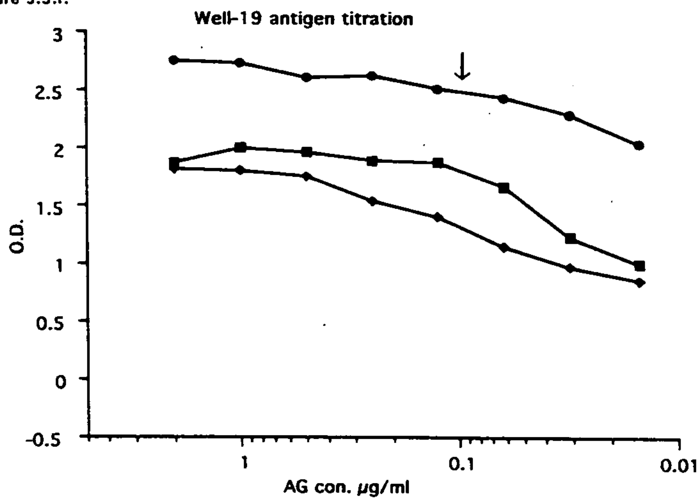


figure 3.3.g.

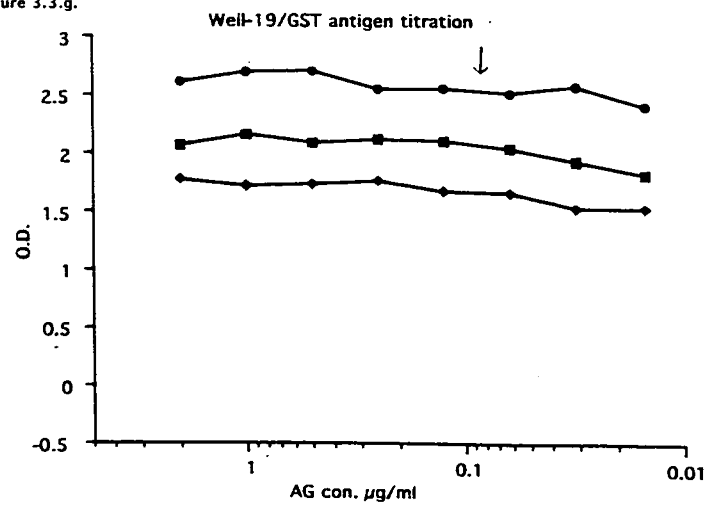


figure 3.4.a.

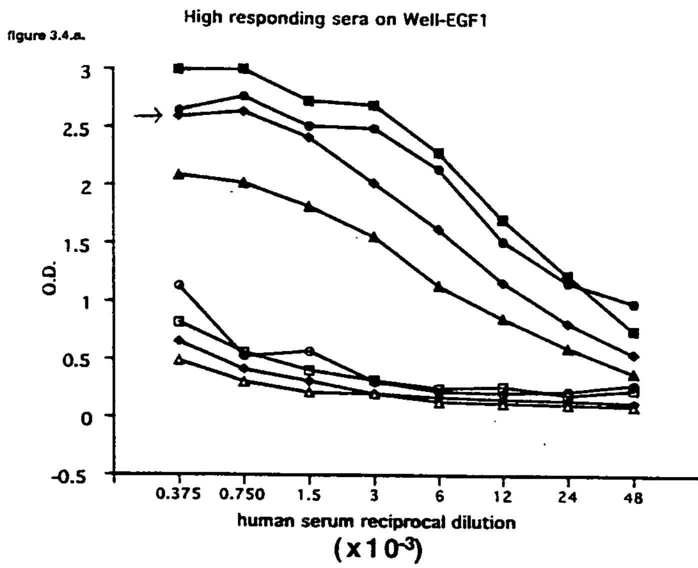


figure 3.4.b.

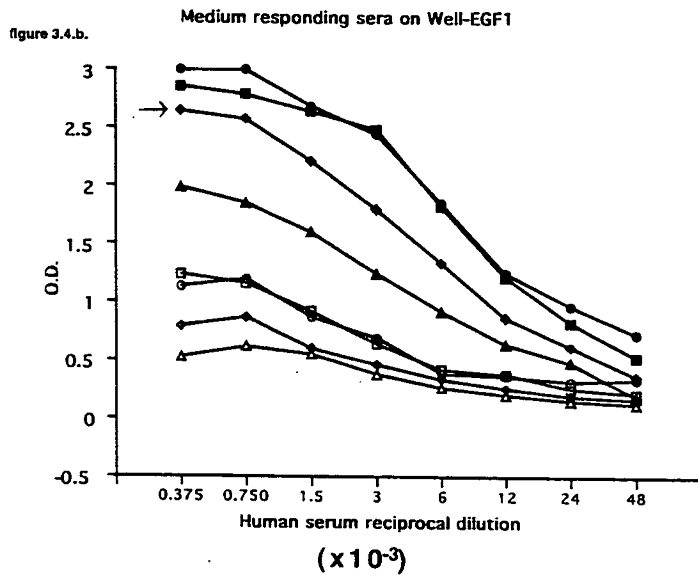


figure 3.4.c.

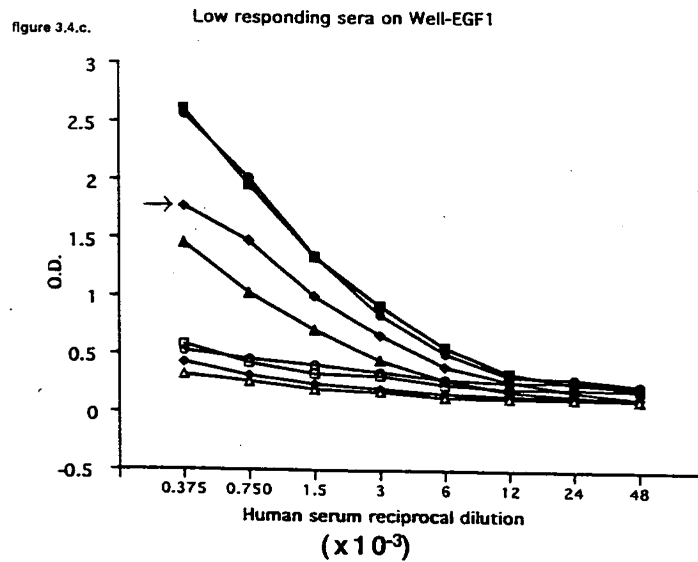


Figure 3.4. Titration of primary and secondary antibody to find optimal concentrations. Antibody is titrated on Well-EGF1 antigen saturated plates (solid symbols) or GST coated plates (hollow symbols). Titrations are carried out with a) high, b) medium and c) low titre sera, with different dilutions of the secondary antibody; ●1:500, ■1:1,000, ◆1: 5,000, ▲1:10,000. Hollow symbols are the same titrations on GST.

Arrow indicates optimum concentration chosen for subsequent assays.

dilutions resulted in very similar titration curves, which are steep and exceed the OD of 2 at lower concentrations of primary antibody.

(3) Optimisation of the primary antibody

The last layer of the ELISA to be optimised was the sample antibody. A dilution of primary antibody was chosen which gave an OD on the linear part of the titration curve, with the 1:5000 secondary antibody concentration.

For high, medium and low titre antibodies a 1:3000 dilution of primary antibody most consistently fell on the linear part of the titration curve, obtained with the 1:5000 dilution of secondary antibody (see figures 3.4.). OD levels reached the value of 2 with a 1:3000 dilution of some high titre sera, but most sera gave OD values within the accurate limitations of the assay, so comparisons of OD values would be possible.

(4) Time and temperature of incubation periods

Specific binding of different affinity antibodies is time-dependent (Davey and Steward 1988). The temperature during incubation also affects the rate of the reaction. Higher temperatures increase the rate of the reaction but also enhance non-specific binding and antigen desorption (Kemeny 1992). It has also been reported that the stoichiometric binding of the conjugated antibody is more likely to vary with long incubation periods (Venkatesan and Wakelin 1993). So I carried out a number of assays varying incubation times and temperatures. However, I found that the antibody incubation periods of 1 hour at 37°C, 3 hours at room temperature, or overnight at 4°C gave almost identical results and caused very little difference in background binding to non-specific antigen (see figure 3.5. for an example).

It has been suggested that the sensitivity of the assay can be increased by decreasing the temperature during the final step of the assay to prolong the substrate reaction (Portsmann and Kiessig 1992). As enzyme reactions are so dependent on temperature, I decided that this stage of the assay needed to be carried out at a controlled temperature to reduce day to day variation due differences in room temperature. Using a low temperature (4°C), I found that I could prolong the substrate reaction to 10 minutes, meaning that more ELISA plates could be developed in one batch.

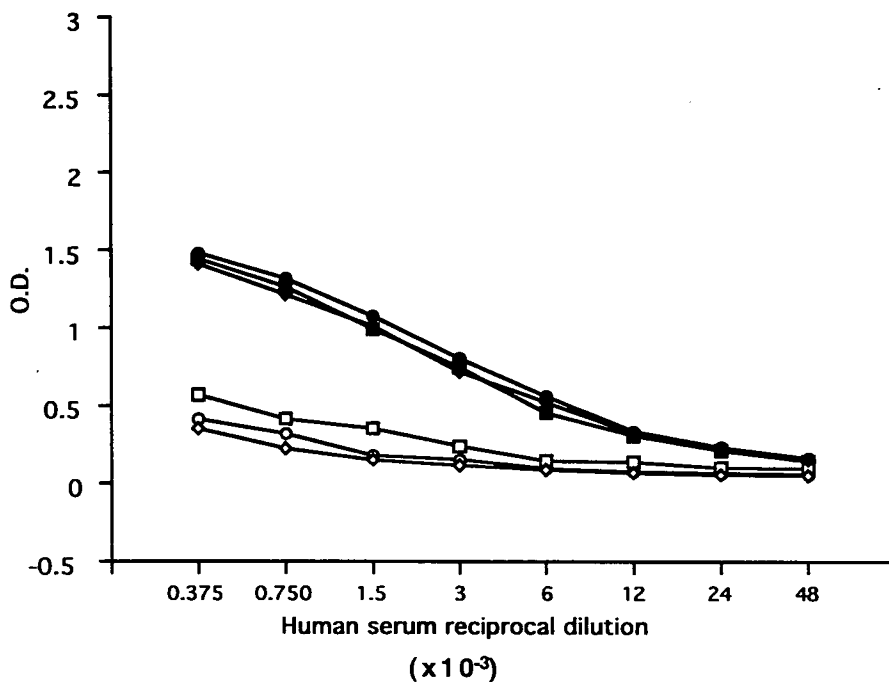


Figure 3.5. Titration of a serum sample under different incubation times and temperatures.

A serum sample was titrated on Well-19/GST antigen saturated plates, and underwent different incubatior conditions; ● overnight at 4°C, ■ 1 hour at 37°C, ◆ 3 hours at room temperature. Hollow symbols are the same titrations on GST.

(5) Measurement of the coefficient of variation

It is important to know the variation in OD values obtained within and between batches of ELISAs. This was worked out by carrying out an initial series of experiments. Samples are tested on the different antigens within a batch of ELISAs on separate plates and on assays carried out on separate days. The coefficient of variation is then worked out (using the formula in the introduction) to measure how much a single dilution of sample varies within a batch of ELISAs and between batches of ELISAs. Variation up to 20% is considered acceptable.

Variation within my initial test samples was between 3.8% and 10.2% (see tables 3.1 and 3.2.).

	Well-EGF1	MAD-EGF1	MAD-EGF2	MAD-19	MAD/Well-19	Well-19	Well-19/GST
plate 1	1.323	1.352	1.244	1.524	1.582	1.537	1.750
plate 2	1.363	1.389	1.289	1.686	1.650	1.595	1.651
plate 3	1.237	1.265	1.118	1.587	1.678	1.470	1.510
plate 4	1.285	1.267	1.103	1.410	1.560	1.484	1.571
plate 5	1.198	1.210	1.112	1.516	1.424	1.451	1.510
mean	1.283	1.267	1.173	1.545	1.579	1.507	1.598
SD	0.066	0.072	0.088	0.101	0.099	0.058	0.103
CV%	5.1	5.7	7.5	6.5	6.3	3.8	6.4

Table 3.1. The coefficient of variation for samples carried out within one assay on separate plates; intra batch variation.

	Well-EGF1	MAD-EGF1	MAD-EGF2	MAD-19	MAD/Well-19	Well-19	Well-19/GST
day 1	1.623	1.617	1.545	1.641	1.627	1.611	1.871
day 2	1.854	1.822	1.712	1.855	1.911	1.898	1.998
day 3	1.722	1.698	1.576	1.701	1.836	1.756	1.945
day 4	1.599	1.578	1.489	1.589	1.587	1.574	1.742
day 5	1.847	1.845	1.708	1.899	1.880	1.992	1.914
mean	1.729	1.712	1.606	1.737	1.768	1.766	1.894
SD	0.120	0.120	0.100	0.135	0.150	0.180	0.097
CV%	6.9	7.0	6.2	7.8	8.5	10.2	7.1

Table 3.2. The coefficient of variation for samples carried out in assays on separate days; inter batch variation.

Discussion

It is essential to optimise the ELISA protocol to the particular needs of each individual test. ELISAs produce vast quantities of data, but if careful thought is not put into the design of the ELISA, and measures are not taken to control its parameters, the data generated will be invalid, unreproduceable and incomparable between assays.

(1) Specificity of ELISA assays

Non-specific binding is a problem in IgG ELISAs as IgG has a high affinity for immobilised antigen (Kemeny *et al* 1985). Measures taken to decrease the background (such as reducing the incubation period or temperature) may also decrease the specific response (Avrameas 1992). Measures taken to increase specific binding only work if non-specific binding does not increase as well, i.e. the sound to noise ratio should be high.

One method to distinguish between specific and non-specific responses is to measure the binding of positive and negative sera to the antigen (Venkatesan and Wakelin 1993); conditions which result in a maximal difference between the OD of positive and negative sera should be chosen. A problem that many investigators have encountered in measuring antibody responses by ELISA, when applied to malarial infections, is that malaria causes polyclonal B cell activation which results in the increased production of non-specific antibody; hypergammaglobulinaemia. I was not convinced, therefore, that using European sera, as a means to measure the difference between specific and non-specific binding, would be appropriate for malaria-exposed sera. I thought that it may be more accurate, to maximise the difference between specific and non-specific binding through measuring the binding of malaria-exposed sera on specific and non-specific antigen. So I have chosen the concentration of primary and secondary antibody which maximises the difference between binding of the sera to the test antigen and the control antigen (GST).

(2) Quantitative aspects of the ELISA assay

Malaria immune responses can either be measured in ELISA as single 'spot check' dilutions to screen large numbers of sera for the presence or absence of specific antibodies, or by titration of each serum sample. Single OD values can

give an indication of differences in the antibody titre, i.e. a high OD indicates a greater antibody titre than a lower OD. Davey and Steward (1988) suggest that end point titrations are least affected by affinity of the antigen. At low dilutions of sera (antibody in excess), high affinity antibody will bind to the antigen preferentially. However, at high dilutions of sera (antigen in excess), low affinity antibody will also bind. End point titres can be obtained from serum titrations. This measures the least amount of antibody needed to complex a given amount of antigen. When specific binding reaches the background level, the end point is reached.

The choice of whether to measure antibody responses by single point dilution or by titration depends on the questions being asked of the assay. If hundreds of sera are being tested for the presence or absence of antibodies to an antigen, a spot check dilution is adequate, as long as the appropriate serum dilution is used. The appropriate dilution has to be determined by titration of a random sample of sera. An idea of relative antibody levels can be deduced from comparison of OD values. Then positive sera can be analysed in more detail by titration. This method is economical in time, antibody sample and costs (for reagents etc.).

Single dilutions are economical but ODs are subject to plate to plate, and day to day variability, which can be monitored by the coefficient of variation. My results indicate that results do not greatly differ between experiments, but as there is more variation in OD values obtained in ELISAs carried out on separate days, any samples which are to be compared with each other should be tested in the same assay. Caution should be taken in making comparisons of OD values obtained from samples tested in separate experiments.

Due to this day to day variation, some investigators compare the OD value obtained in that batch with a standard reference curve of IgG, where they convert the OD figure into antibody units, so antibody units can be compared between assays (for example; Blackman and Holder 1993). However, OD values can only be converted in this way if the standard reference curve has the same shape as the test sample i.e. the two curves are parallel (Kemeny 1992). Non-parallelism with the standard curve at low test sample concentration is due to differences in ionic strength (molarity) between the test sera and the reference antibody. The test serum titration curve flattens out (at the top) with high serum concentrations. If the undiluted sample falls outside the useful limits of the standard curve, converted values would not be valid. There is also a

basic assumption, in the use of a standard curve, that the standard antibody and the test serum have the same ability to bind to the solid phase receptor (Hamilton and Adkinson 1988). Antisera are a heterogeneous population of antibodies from different B cells with different affinities. Serum samples will also vary from one another due to differences in serum proteins, lipids and ions, which give rise to non-specific effects which alter the kinetics of the antigen/antibody binding and result in different plateaux for different antibodies. Thus, a single reference curve is not going to fit every test samples' protein matrix. From titrating many samples I have found that no two samples have exactly parallel titration curves. I therefore decided that this method was not appropriate for comparing my samples.

A major pitfall of ELISA is over interpretation of the results. ELISA is a good tool to provide qualitative information on the presence or absence of antibodies. However, the most desired end result is a measurement of how much analyte is present. Obtaining quantitative information in an indirect ELISA is not straight forward as antibody binding is being measured, not antibody levels. Antibody binding is a result of both antibody affinity and concentration, so an OD result does not give absolute quantitative information. Even end point titres are only measuring antibody binding. So only comparisons of antibody binding can be made. It is impossible to quantify antibody levels in ELISA, either by single point dilution or end point titration without making assumptions about the affinity of the antisera (Davey and Steward 1988).

(3) Statistical analysis of ELISA data

A cut off OD is needed to determine whether an individual is antibody positive or negative to any particular antigen. This cut off point between a specific and non-specific response is established by measuring antibody responses made by malaria-unexposed European sera. In this study, the mean plus two SD of European sera have been used to define the lower limit of a specific OD response. However this method has its limitations. As mentioned above, sera from malaria-exposed individuals will contain more non-specific antibodies than European sera; the cut off level between specific and non-specific responses defined by the European sera will be lower than that of malaria-exposed sera, so may result in false positive results. However, background/non-specific responses are accounted for by the subtraction of OD values obtained for the same sera on non-specific antigen (i.e. GST for GST fusion proteins). If a serum

sample has a high OD response for the non-specific antigen, the subtracted OD (ΔOD) will be counted as negative even though this sample may have contained specific antibody too, so resulting in a false negative result. In other words there is no perfect way to do this.

In some circumstances there is a clear difference between a group of responders and non-responders (see figure 3.6.). In these cases the two groups (responders and non-responders) can be distinguished visually; for example age groups <5, 6-8 and 9-10. In other cases, a purely statistical approach has to be taken. Although this may lead to two individuals with very similar OD values being classified as a responder or non-responder respectively, any other method would be arbitrary and subjective and thus susceptible to unintentional bias.

So the ELISA system has many advantages over other immunoassays in terms of speed, sensitivity, convenience and automation. However, it is important to realise that if the ELISA has not been properly optimised, this vast quantity of data produced will be unreliable. Another problem with ELISA is that it measures antibody binding, not antibody affinity, so the results must not be over-interpreted by making estimations of antibody concentrations. In this chapter I have optimised the ELISA to find the best concentration of components, and the best conditions, in which to perform reliable and reproducible ELISAs used in the following chapters.

figure 3.6.

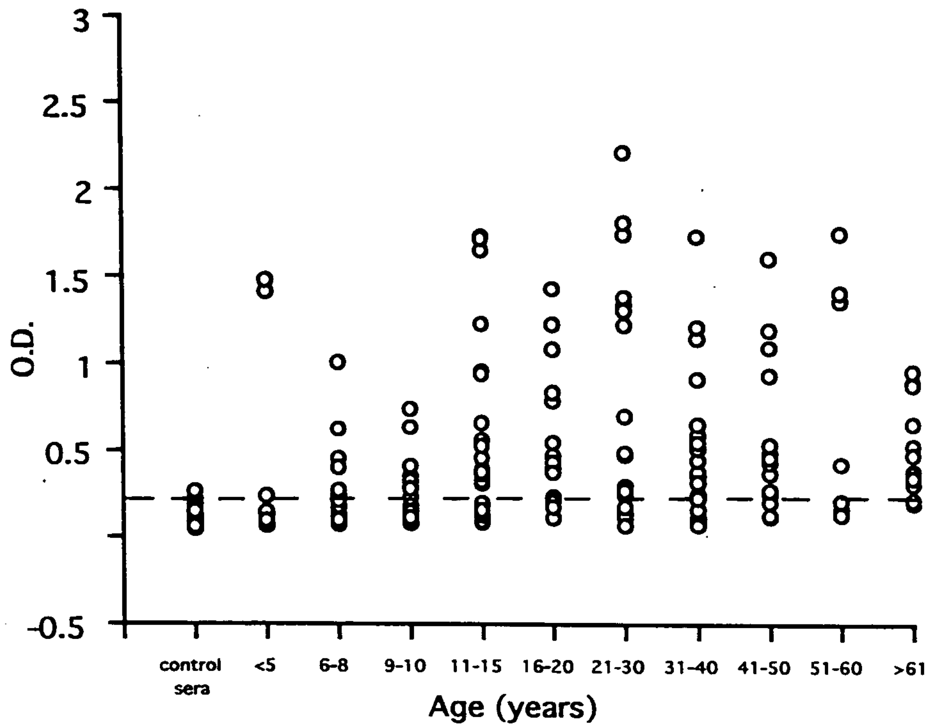


Figure 3.6. Dot plot demonstrating the difference between specific and non-specific antibody binding to a $PfMSP1_{19}$ antigen.

A cut off value between specific and non-specific antibody responses to, in this example, the MAD-19 antigen is determined by the mean plus 2 SD of the OD values obtained for European malaria-unexposed control sera. In some cases there is a clear difference between a group of responders and non-responders. For example for the age group 31 to 40, the upper limit defined by the European sera would be too low and result in false positive responders. In these cases, where there is a clear difference between the responders and non-responders, this cut off can be determined by eye. However, when this difference is not clear, the cut off is determined by the upper limit obtained by the European sera.

CHAPTER FOUR:
Characterising epitopes within the C-terminus of PfMSP1

Introduction

PfMSP1 is a large molecule (185-210kDa) so it would be impractical to produce the whole protein in native, recombinant or synthetic form. Therefore, antibody responses to constituent components of PfMSP1 need to be assessed in sera from malaria-exposed individuals in order to (1), discover whether they induce antibodies in a natural infection and (2), to assess whether this antibody response is associated with immunity to clinical malaria.

Apart from human vaccine trials that can directly measure the immunogenicity of candidate vaccine antigens, the only way to assess the immunogenicity of potential vaccine antigens in humans is to measure antibodies induced by natural infection.

PfMSP1 is considered to be an excellent candidate for inclusion in a malaria vaccine (reviewed by Diggs *et al* 1993). However, there have been relatively few studies of naturally acquired immune responses to it and there seems to be confusion over whether human antibodies predominantly recognise conserved or dimorphic regions within PfMSP1 (Muller *et al* 1989, Fruh *et al* 1991, Riley *et al* 1992a, 1993, Tolle *et al* 1993).

It has long been known that people living in malaria endemic areas possess antibodies to PfMSP1, indeed it was this high degree of antibody prevalence that added to its attractiveness as a vaccine candidate (Perrin and Dayal 1982, Hall *et al* 1984b, Holder and Freeman 1984). Using a panel of *E. coli*-derived recombinant proteins representing conserved and dimorphic regions within the Wellcome or MAD20 sequences of PfMSP1, Muller *et al* (1989) investigated human immune responses by Western blot in sera from 63 adults (aged 18-67 years), 35 children (aged 1-5 years) and 14 infants (aged 3-6 months) living in Burkino Faso, West Africa. Antibody responses to specific regions of the protein differed widely between individual sera, and there was no clear relationship between antibody responses and age. However, they did find that PfMSP1-specific antibodies were generally more prevalent in parasitised than in uninfected donors. In a continuation of this study, Fruh *et al* (1991), measured antibody responses, by Western blot, to the same panel of recombinant proteins in 37 adults (aged 18-73) and 57 children (aged 2-9) from Mali, West Africa. This study suggested that naturally acquired antibodies were predominantly directed against dimorphic sequences of PfMSP1. These immunodominant regions occurred in the C-terminus

of the $PfMSP1_{83}$ processing fragment (block 6), the N-terminus of $PfMSP1_{28-30}$ (blocks 6-8) and the dimorphic region of $PfMSP1_{42}$ (block 16), see figure 1.2.

Other investigators have found that the N-terminal region, containing conserved and dimorphic sequences from the $PfMSP1_{83}$ processing fragment, is the most antigenic part of the protein (Riley *et al* 1992a, 1993, Kramer and Oberst 1993). For example, immunoblotting experiments showed that all 726 individuals living in The Philippines had detectable antibodies to a yeast derived recombinant protein representing blocks 1-5 of $PfMSP1_{83}$ (Kramer and Oberst 1993). However, only individuals with high titre antibodies against PfMSP1 had detectable antibodies against the C-terminal protein, suggesting that the N-terminus of PfMSP1 is more antigenic than the C-terminus. However, the authors do point out that the yeast derived $PfMSP1_{42}$ construct may not have correctly formed the internal disulphide bonds which are vital to the antigenicity (McBride and Heidrich 1987) and immunogenicity (Chang *et al* 1992) of this region.

Previous investigations carried out in our laboratory have shown that serum antibodies to proteins representing highly conserved regions of the molecule were more prevalent than antibodies recognising dimorphic or polymorphic regions of PfMSP1 (Riley *et al* 1992a). However, the strain of parasite from which the recombinant constructs were derived (Wellcome) represents the dimorphic form of PfMSP1 which is not commonly found in The Gambia (Conway and McBride 1991), from where the serum samples were collected. Thus, it is likely that this study underestimated the prevalence of antibodies to dimorphic epitopes.

However, other investigators have found that, following experimental immunisation of animals with native PfMSP1, $PfMSP1_{19}$ is the most antigenic region of the molecule. For example, Cooper *et al* (1992) found that 12 out of 19 mAbs, raised in mice immunised with PfMSP1, bound to epitopes within $PfMSP1_{19}$. A number of studies by Hui and Chang have found that the antibody response of rabbits immunised with native PfMSP1 or baculovirus-derived $PfMSP1_{42}$ is focused on cross-reactive conformational determinates of $PfMSP1_{42}$ and suggest that the reduction-sensitive, conserved region (i.e. $PfMSP1_{19}$) is immunodominant (Chang *et al* 1992, Hui *et al* 1992, 1993). Kaslow *et al* (1994) demonstrated that the majority of rabbit serum antibodies raised to native PfMSP1 are specific for conserved epitopes within $PfMSP1_{19}$.

However, in malaria-exposed humans, the conserved region of the C-terminus of PfMSP1 appears to be poorly recognised (Muller *et al* 1989, Fruh *et al* 1991, Tolle *et al* 1993). The C-terminal *E. coli*-derived recombinant protein F10 used in these studies represents the Wellcome sequence of the dimorphic $PfMSP1_{33}$ and the conserved $PfMSP1_{19}$. It was assumed that F10 adopted a similar conformation to native $PfMSP1_{19}$, on the basis of reactivity with mAbs 2.2,

7.5 and 12.8, which only recognise the non-reduced $PfMSP1_{19}$ (McBride and Heidrich 1987). In Burkina Faso, the level of F10 recognition was slightly higher than in Mali but only 30% of adults were seropositive despite life long exposure (Muller *et al* 1989). The even lower prevalence of human antibodies to this Wellcome recombinant protein in Mali (25%) may result from lack of exposure of this population to Wellcome PfMSP1-expressing parasites (Fruh *et al* 1991). The recombinant protein M11 represents the MAD20 form of $PfMSP1_{33}$ and is immunodominant. This suggests that the low antibody prevalence to the F10 construct was due either to lack of exposure of the population to Wellcome parasites and/or, poor recognition of the conserved $PfMSP1_{19}$. Possible explanations for the low prevalence of antibodies to $PfMSP1_{19}$ include its close proximity to the merozoite surface, its potential concealment by the rest of the molecule and its small size (96 amino acids).

If antibodies are an important component of the protective immune response to malaria, it may be important that antibody levels remain high at all times, so that merozoites are targeted as soon as they leave the liver. It would therefore be important that the antigen induces a good memory response. This can be investigated by looking at antibody stability over time. Tolle *et al* (1993) found that children's antibody levels were not stable through a malaria transmission season. During the dry season their antibody levels are low, unlike adults, who have high antibody levels whether they are parasitised or not. During the wet season, children's antibody levels are boosted to levels similar to those of adults, but this increase in antibody response apparently has little effect on controlling parasitaemia. However, the antibody may come too late, i.e. pre-existing antibody at the time of sporozoite invasion is what is important. Baird and co-workers (reviewed in Baird 1995) suggest that antibody produced by children is unable to protect them against new malaria infections, due to an intrinsic difference in the immune response between adults and children, which results in the inability of children to control their malarial infection. This is presumably due to fundamental constitutional differences in the immune system of children and adults affecting antibody isotype, memory and affinity as well as the cellular component.

This chapter details the results of studies designed to address the questions of immunogenicity of $PfMSP1_{19}$, the stability of the antibody response and the effect of age on antibody responsiveness. In order to determine whether the conserved domain of the C-terminus of PfMSP1 is well recognised by antibodies induced by natural infection, or whether the dimorphic region is immunodominant, I have used ELISA assays to measure the prevalence of antibodies to recombinant proteins representing the Wellcome form of $PfMSP1_{42}$, the MAD20 form of $PfMSP1_{33}$ and the two allelic forms of $PfMSP1_{19}$ and its constituent EGF-like motifs, in a malaria-

exposed population. Sera were collected from individuals aged 1-70 years living in The Gambia, where malaria transmission is seasonally endemic.

In order to examine the memory response to malaria, I have measured antibody responses of 20 children and 22 adults in samples taken over three years, at the end of the rainy season (November) and at the end of the dry season (May).

I have examined the effect of age (and therefore malaria exposure) on antibody prevalence and I have used correlation analysis and competition ELISAs to determine whether the antibodies are strain-specific or whether they recognise epitopes which are present in both alleles, i.e. cross-reactive. Finally I have attempted to determine whether antibody responsiveness is influenced by genetic factors.

Methods and materials

(1) Antigens

Recombinant proteins used in this study have previously been described in chapter 2.

E. coli-derived GST fusion proteins representing the first EGF-like motifs of the Wellcome and MAD20 sequences of PfMSP1$_{19}$ (Well-EGF1, MAD-EGF1), the second EGF-like motif of MAD20 PfMSP1$_{19}$ (MAD-EGF2), the double EGF-like motif of Wellcome PfMSP1$_{19}$ (Well-19/GST) and MAD20 PfMSP1$_{33}$ (MAD-33) were used in this study.

Yeast recombinant proteins representing full length PfMSP1$_{19}$ were also tested; the MAD20 version of PfMSP1$_{19}$ (MAD-19), the Wellcome version (Well-19), and a hybrid of the first EGF-like motif of MAD20 and the second EGF-like motif of Wellcome (MAD/Well-19). The baculovirus recombinant protein representing the Wellcome version of PfMSP1$_{42}$ (Well-42) was also used in this study, see figure 2.1.

(2) Human sera from an age cross-sectional survey

Blood collected from people living in rural areas close to the town of Farafenni in The Gambia was the source of serum samples, and was provided by my supervisor, Dr. E. Riley. Malaria transmission in this area is seasonally endemic with new infections occurring during and immediately following the rainy season (July - December) (Greenwood *et al* 1987a). Serum samples were obtained at the end of the rainy season (October) from 195 children and adults (aged 1-70 years) and were stored at -20°C until used. Prior exposure to malaria was confirmed by serology; all sera had previously been shown to be positive (by immunofluorescence) for antibodies to acetone-fixed malaria schizonts with titres ranging from 1:160 to >1:20,000 (data not shown). Control serum samples were obtained from 28 European adults and 14 European children with no previous exposure to malaria.

(3) Human sera for longitudinal study of seasonal changes in antibody levels

Blood collected from 20 children (aged 3-7) from Farafenni, and 22 adults (aged 16-65) from inhabitants of a small Gambian village (Brefet), was provided by Dr. E. Riley. Serum samples were obtained at the end of the rainy season (November) and at the end of the dry season (May) over 3 years.

(4) Human sera from adult twins

Serum samples collected from 36 pairs of same-sex adult Gambian twins were kindly provided by Dr. A. Jepson (MRC Laboratories, The Gambia). By DNA probing, with five separate minisatelite probes, 15 pairs were shown to be monozygous (mz) and 21 pairs were dizygous (dz) (Jepson *et al* 1994). The twins were raised together and were still living in the same, or nearby, villages, so it was assumed that their past histories of malarial infections would be very similar.

(5) MAbs

The following $PfMSP1_{19}$-specific murine mAbs were used; 12.8 (McBride and Heidrich 1987), 111.4 (Holder *et al* 1985), and 14-1C (Dr. R. Reese, Agouron Institute, La Jolla, USA), see chapter 2.

(6) ELISA

Antibodies reacting with recombinant PfMSP1 proteins were detected by ELISA. Microtitre plates (Immulon 4; Dynatech) were coated overnight at 4°C with proteins diluted in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$ pH9.5). The saturating concentration of protein, determined by titration, was from 0.1µg/ml to 5µg/ml depending on the antigen, see chapter 3. Plates were washed three times in PBS/T (washing buffer), blocked with a 1% (w/v) solution of non-fat powered milk in PBS/T (blocking buffer) and washed again. 100µl of serum or mAb, diluted in blocking buffer, was added to duplicate wells and incubated overnight at 4°C. Optimal dilutions of serum or mAbs were determined by titration (see chapter 3). For all human sera, the steepest slope of the titration curve was observed at concentrations between 1:1000 and 1:10,000 (data shown in chapter 3). The optimum concentration of human sera for the assay was thus selected as 1:3000 (Kemeny 1992).

After washing, HRP-conjugated rabbit anti-human IgG (100µl diluted 1:5000 in PBS/T) or HRP-conjugated rabbit anti-mouse IgG (100µl at 1:1000 in PBS/T) (both Dako) were added to the plates, incubated for 3 hours at room temperature and developed with H_2O_2 and OPD (Sigma) for 10 minutes at 4°C. The reaction was stopped by the addition of 20µl of 2M H_2SO_4. Plates were read at 492nm. For GST fusion proteins the OD value of the GST control was subtracted from the OD of GST-recombinant fusion proteins, to give a specific OD (ΔOD) for the response to the PfMSP1 antigens.

(7) Competition ELISAs

To determine whether antibodies recognising individual recombinant proteins also recognised (cross-reacted with) other recombinant proteins, competition ELISAs were performed. The test serum was diluted in blocking buffer to which was added recombinant protein, at a range of concentrations. After incubation for 5 hours at room temperature, the blocked serum was added to immunoplates coated with a second recombinant protein. The remainder of the assay was as before.

(8) HLA class II determination of individuals from the age cross-sectional study

179 of the individuals from the age cross-sectional study had previously been tested for their HLA class II haplotype (Olerup *et al* 1991). HLA typing for DRB, DQA and DQB was performed by Southern blot analysis of *Taq 1* -cleaved DNA from peripheral blood leucocytes (Carlsson *et al* 1987).

(9) Statistical Methods

Specific OD values obtained for sera from malaria-unexposed European donors were used to establish a normal range for each antigen. The OD values for these control sera tended to be normally distributed; thus, Gambian sera giving an OD value greater than the mean plus 2SD of the European sera were considered to contain antibody specific for the relevant recombinant protein.

To determine whether prevalence of antibody to PfMSP1 proteins are associated with age, the number of seropositives for each antigen were compared between the age groups, using χ^2 for linear trend.

To determine whether levels of antibody of seropositive individuals to PfMSP1 proteins are associated with age, the distributions of OD values for each antigen were compared between the age groups using Kruskal-Wallis one-way analysis of variance. It was necessary to use a non-parametric test since the distributions of the OD values were highly skewed within each age group.

Antibody responses to the chimaeric MAD/Well-19 protein versus the MAD20 or Wellcome $PfMSP1_{19}$ proteins were compared by using the non-parametric Spearman's rank correlation coefficient, resulting in a r value.

To determine the association between HLA class II genotype and antibody response, individuals were classified as either responders or non-responders to each antigen and the probability of an immune response in individuals of any particular haplotype was estimated by multiple logistic regression allowing for potential confounding effects of age and sex. To allow for associations between

HLA genotype and malaria exposure within members of a single family group, the data was stratified by household and analysed by conditional logistic regression.

Fisher's exact test was used to compare the proportion of discordant twin pairs (i.e. where one twin was seronegative and the other seropositive for a particular antigen) between mz versus dz twins. Absolute differences in OD values for each twin pair (for each antigen) were calculated and the significance of the median difference between mz and dz pairs was assessed using a non-parametric Mann-Whitney test.

Results

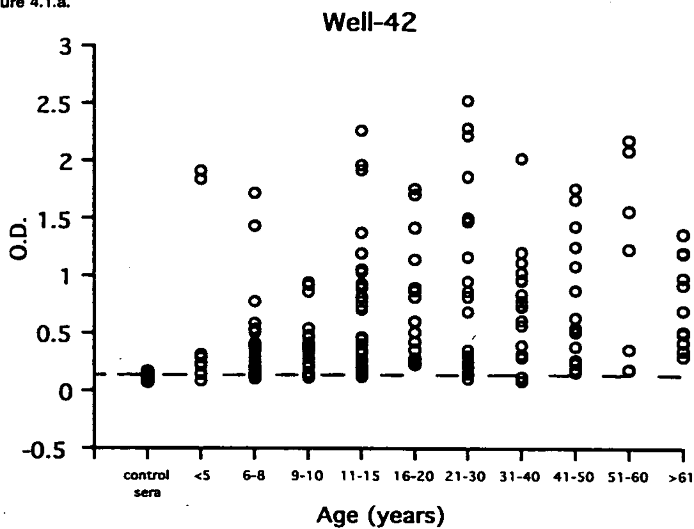
(1) Recognition of recombinant PfMSP1 proteins by human antibodies from an age cross-sectional survey

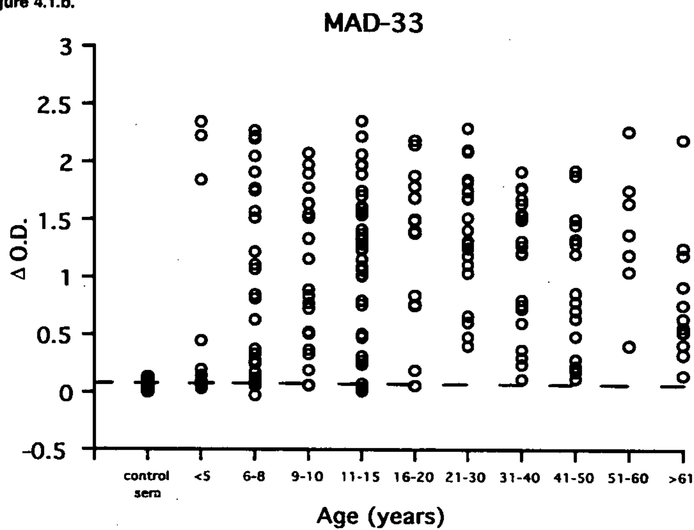
The mean plus 2SD of the European sera was used as a guide to distinguish between responders and non-responders. However, when there was an obvious gap between a group of responders and non-responders, the cut off OD value was determined visually. For example, in figure 4.1.a.; for the age group 41-50 there is no clear difference between a low antibody responder and a non-responder, so the mean plus 2SD OD value was used to distinguish this difference, while for the age group 21-30, there is a clear difference between these two populations of different responsiveness. The upper limit determined by European sera was occasionally too low, obviously including non-specific responses. Malarial infections induce high levels of non-specific antibody, which causes a problem in determining a 'true' antigen-specific response (see chapter 3). In these cases (see figure 4.1.f. for an example) I decided it would be more accurate to differentiate responsiveness visually.

All the PfMSP1 constructs were recognised by antibodies induced by natural malaria infection. Some sera showed high levels of antibodies to the C-terminal PfMSP1 constructs, with end point titrations of up to 1/48,000 (see figures 3.4.a.b.c. presented in chapter 3). However, the prevalence of antibodies to the different constructs varied considerably, see figures 4.1.a-i. and table 4.1. More than 60% of the sera contained antibodies that recognise Well-42. MAD-33 is recognised by 90% of individuals, indicating that this dimorphic region of PfMSP1 is immunodominant. The level of recognition of Well-42, which contains the Wellcome sequence of $PfMSP1_{33}$, is lower than the recognition of MAD-33. This lower prevalence of antibody responsiveness to Well-42 is probably due to lack of exposure to this sequence, as expression of the Wellcome PfMSP1 allele is rare in The Gambia (Conway and McBride 1991).The difference in the prevalence of antibodies to these two proteins indicates that the dimorphic regions of PfMSP1 are immunogenic, and even, immunodominant.

The prevalence of antibodies to the double EGF-like motif of $PfMSP1_{19}$ is approximately 40% (see table 4.2.). There is a close correlation between antibody responses to Well-42 and the two allelic forms of $PfMSP1_{19}$, indicating that much of the antibody response to Well-42 is directed to conserved epitopes within $PfMSP1_{19}$ (see figures 4.2.a.b.) and, once again, indicating that there is not much of an antibody response to the Wellcome dimorphic ($PfMSP1_{33}$) region of Well-42.

There are a few sera which recognise Well-42 but not $PfMSP1_{19}$, see figures 4.2.a.b. These sera probably recognise additional epitopes present in the





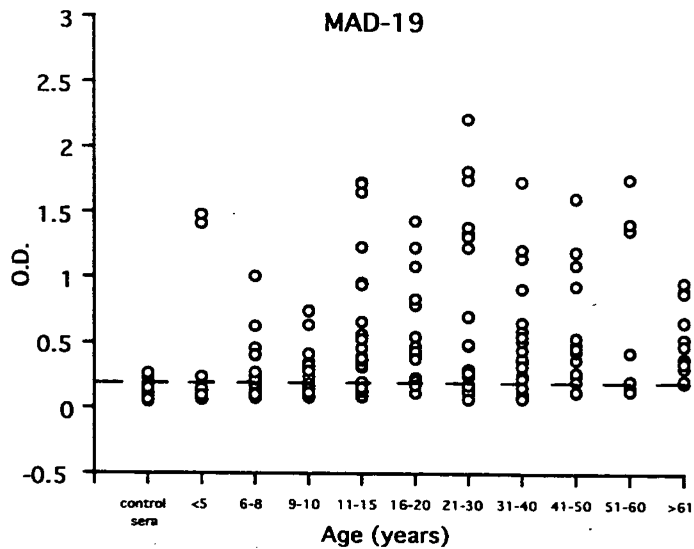


Figure 4.1. Dot plots showing human antibody binding (OD values) for 195 Gambian sera in an ELISA for recombinant PfMSP1 proteins:
a) Well-42, b) MAD-33, c) MAD-33, d) MAD/Well-19, e) Well-19, f) Well-19/GST, g) Well-EGF1, h) MAD-EGF1, i) MAD-EGF2.
OD values of 42 European control sera are also shown. All sera were tested at a dilution of 1:3000. (See text for details.)

figure 4.1.d.

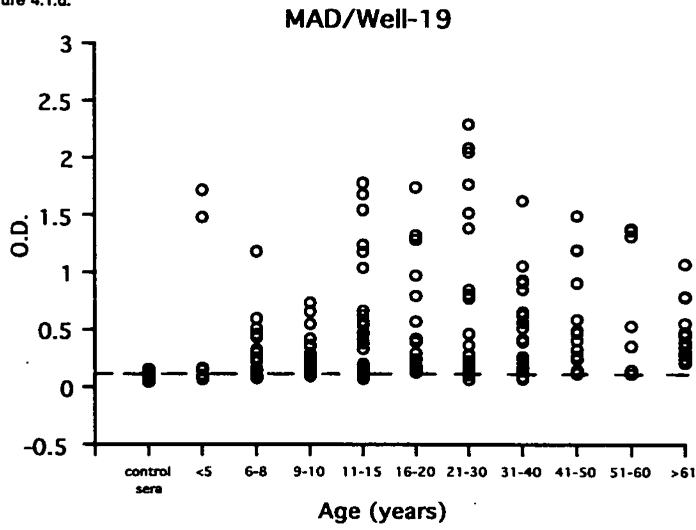


figure 4.1.e.

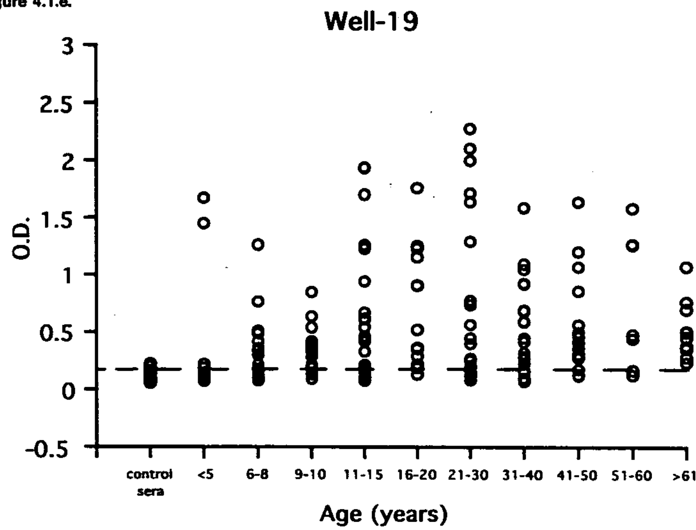


figure 4.1.f.

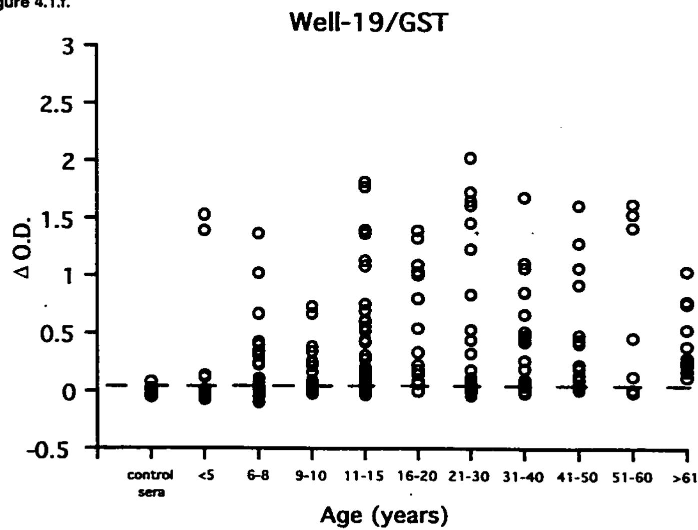


figure 4.1.g.

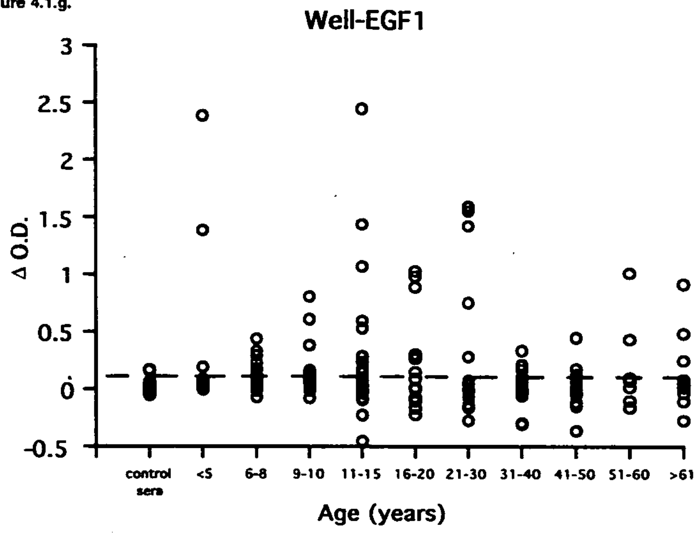


figure 4.1.h.

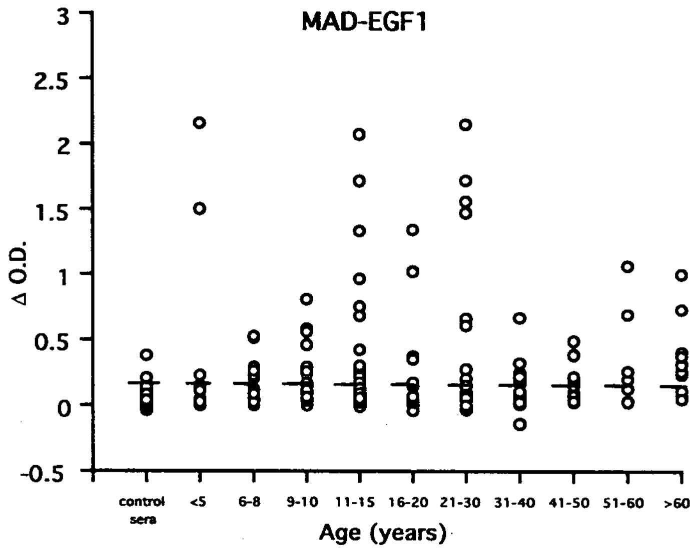
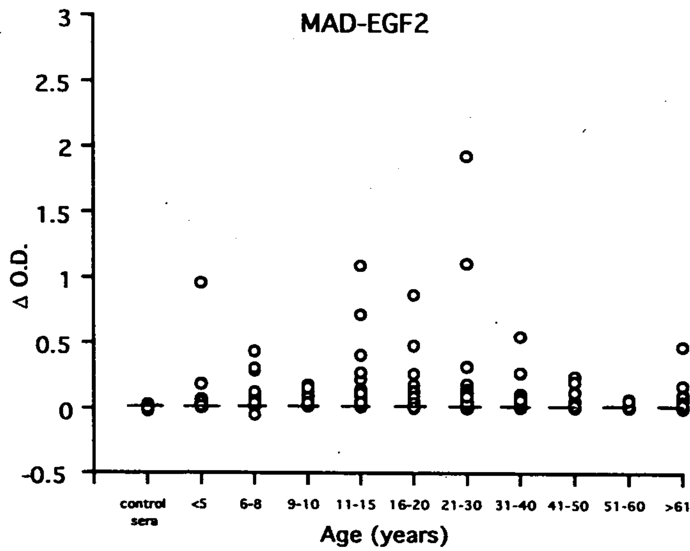


figure 4.1.i.



recombinant protein (MAD20)	n (%) seropositive n=195
EGF1 only	10 (5.1)
EGF2 only	8 (4.1)
both EGF1 and EGF2	1 (0.5)
19 only	26 (13.3)
both EGF1 and19	15 (15.8)
both EGF2 and 19	4 (2.1)
EGF1, EGF2 and 19	9 (4.6)
no response to any EGF epitope	122 (62.6)

Table 4.1. Antibody responses to MAD20 $PfMSP1_{19}$ and its constituent EGF-like motifs.

Number (percentage) of individuals with antibody responses to epitopes within the MAD20 form of $PfMSP1_{19}$.

Age (years)	3-8	9-15	>16	total	χ^2 trend	p
n	43	60	92	195		
Well-EGF1	5 (11.6)	8 (13.3)	19 (20.7)	32 (16.4)	2.10	0.15
MAD-EGF1	3 (7.0)	11 (18.3)	19 (20.7)	33 (16.9)	3.43	0.06
MAD-EGF2	6 (14.0)	5 (8.3)	13 (14.1)	24 (12.3)	0.07	0.79
MAD-19	4 (9.3)	12 (20.0)	38 (43.1)	54 (27.6)	16.90	0.00
MAD/Well-19	7 (16.3)	22 (36.7)	53 (57.6)	82 (42.1)	22.65	0.00
Well-19	7 (16.3)	20 (33.3)	50 (54.3)	77 (39.5)	18.98	0.00
Well-19/GST	11 (25.6)	25 (41.7)	43 (46.7)	79 (40.5)	4.97	0.03
MAD-33	30 (69.8)	55 (91.7)	90 (97.8)	175 (89.7)	3.21	0.07
Well-42	15 (34.9)	35 (58.3)	68 (73.9)	118 (60.2)	18.50	0.00

Table 4.2. The relationship between age and antibody responses to the C-terminal of PfMSP1.

Figures show the number (percentage) of antibody positive children ('responders') in each group, for each antigen. Responders are those where the specific OD was greater than the mean plus 2 SD of the control (malaria non-exposed) sera or determined visually. p value at 2 degrees of freedom.

figure 4.2.a.

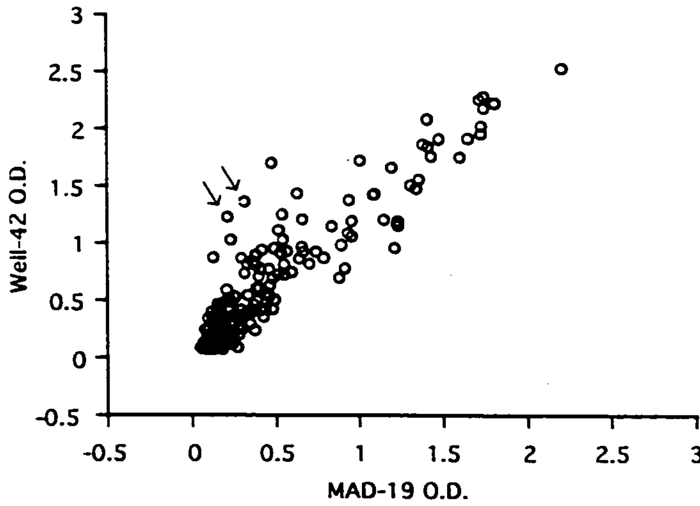


figure 4.2.b.

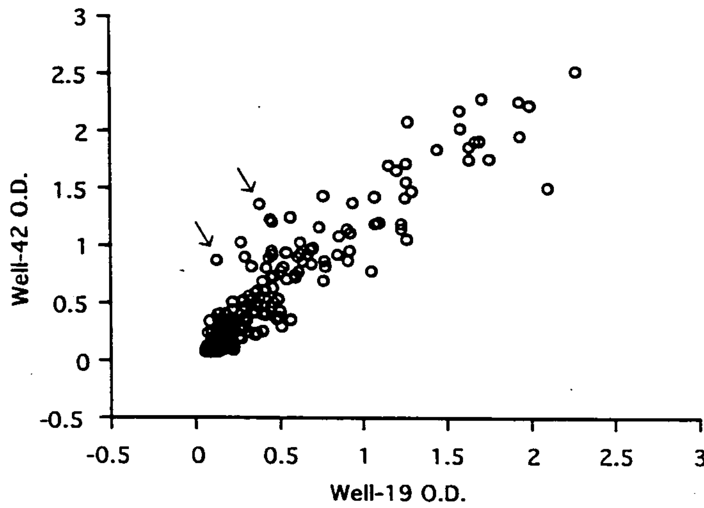


figure 4.2.c.

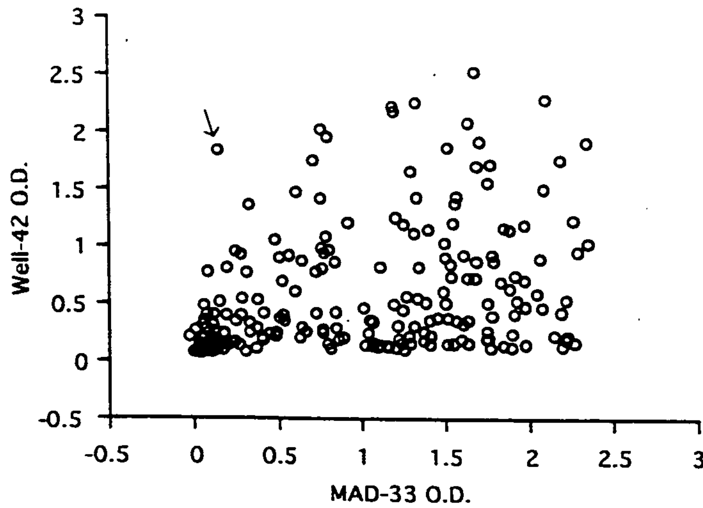


Figure 4.2. Comparison of antibody recognition of different C-terminal PfMSP1 sequences:
a) Well-42 vs MAD-19, b) Well-42 vs Well-19, c) Well-42 vs MAD-33.
Each dot represents a single serum sample, tested against each protein. Arrows indicate sera which are seropositive to Well-42 but seronegative to the double-motif protein, or MAD-33.

dimorphic $PfMSP1_{33}$ region of Well-42. If these individuals have not been exposed to Wellcome PfMSP1 expressing parasites, these epitopes may be cross-reactive with MAD20 $PfMSP1_{33}$ sequences. Figure 4.2.c. once again demonstrates the higher prevalence of antibody responsiveness to MAD-33 than Well-42 in this population, with few sera recognising epitopes exclusively in the Wellcome protein.

Less than 20% of sera show reactivity with the single EGF-like motif proteins, and the second EGF-like motif is particularly poorly recognised (table 4.2, figures 4.1.g.h.i.). Of the sera which recognise $PfMSP1_{19}$, approximately 50% recognise only the double-motif construct and did not recognise any of the single EGF-like motif proteins (see table 4.1.). This indicates that there are epitopes recognised by human sera in $PfMSP1_{19}$ that are not present when either EGF-like motif is expressed alone.

The prevalence of antibody responses to Well-42 and the double-motif proteins increases significantly with age (table 4.2.). A similar trend is seen for the EGF-1 proteins, and $PfMSP1_{33}$ although not statistically significant. There is no trend of increasing prevalence with age for EGF-2. In the majority of cases, once an individual has 'sero-converted' from a non-responder to a responder, the OD response of seropositive individuals does not significantly increase with age, but this result may the small number of seropositive individuals in each age group (see table 4.3.).

(2) Competition ELISAs to demonstrate the presence of new epitopes created by the double-motif construct

48% of the 54 sera which recognise the MAD20 double-motif do not recognise either of the MAD20 single motifs (table 4.1.). The remaining sera recognised both double- and single-motif proteins. To see if these sera recognise only sequences in the single-motif, or whether they recognise additional epitopes formed by the double-motif, I performed competition ELISAs between sera which recognise both the single- and double-motif proteins. Competition ELISAs carried out on eight sera showed that five recognise additional epitopes that are only present in the double-motif protein. Figure 4.3.a. shows an example of a serum where preincubation of this serum with MAD-EGF1 reduces the level of binding to MAD-19, but does not abolish it. So in addition to epitopes within the first EGF-like motif, this serum recognises an epitope(s) made up of sequences from both motifs. Another example (figure 4.3.b.) shows a serum for which preincubation with the single EGF-like motif has no effect on the recognition of the double-motif, so this serum recognises an additional epitope(s) that is distinct from the first-motif epitope(s).

Age (years)	median OD by age group for seropositives				Kruskal-Wallis test	
	3-8 n=43	9-15 n=60	>16 n=92	total n=195	H	p
Well-EGF1	0.385 (4)	0.802 (9)	0.750 (19)	0.678 (32)	1.82	0.39
MAD-EGF1	1.344 (2)	0.783 (12)	0.733 (19)	0.978 (33)	0.04	0.83
MAD-EGF2	0.285 (5)	0.561 (6)	0.322 (13)	0.319 (24)	2.97	0.23
MAD-19	1.008 (3)	0.942 (13)	1.022 (38)	0.958 (54)	0.04	0.98
MAD/Well-19	0.553 (6)	0.572 (23)	0.791 (53)	0.660 (82)	1.08	0.58
Well-19	0.636 (6)	0.621 (21)	0.816 (50)	0.763 (77)	0.76	0.69
Well-19/GST	0.532 (8)	0.614 (27)	0.889 (44)	0.730 (79)	5.85	0.05
MAD-33	0.959 (30)	1.325 (55)	1.252 (90)	1.246 (175)	1.55	0.46
Well-42	0.520 (14)	0.754 (36)	0.885 (68)	0.809 (118)	6.41	0.04

Table 4.3. The effect of age on median levels of anti-PfMSP1 antibodies produced by seropositive individuals.

Median OD levels of seropositive individuals to each antigen for each age group are shown.
Brackets=number of seropositive in each group for each antigen.
p value at 2 degrees of freedom.

Figure 4.3. Competition ELISAs demonstrate that additional epitopes are present within the double EGF-like motif protein (MAD-19) that are not present when either motif is expressed alone. Eight sera which react with the single EGF-like motif (MAD-EGF1) and with the double EGF-like motif (MAD-19) were tested; the results for two sera are shown here.

a) Serum 1493: preincubation of the serum with MAD-EGF1 reduces the level of binding to MAD-19 but does not abolish it. This serum may recognise an additional epitope in MAD-19 which overlaps with an epitope in MAD-EGF1.

b) Serum 1515: preincubation of the serum with MAD-EGF1 has no effect on the recognition of MAD-19.

This serum appears to recognise an epitope(s) in MAD-19 which is distinct from the epitope(s) in MAD-EGF1.

, preincubation with MAD-19, tested on MAD-19.

O, preincubation with MAD-19, tested on MAD-EGF1.

□, preincubation with MAD-EGF1, tested on MAD-EGF1.

; preincubation with MAD-EGF1, tested on MAD-19.

Antigen concentration represents pre-incubation conditions.

figure 4.3.a.

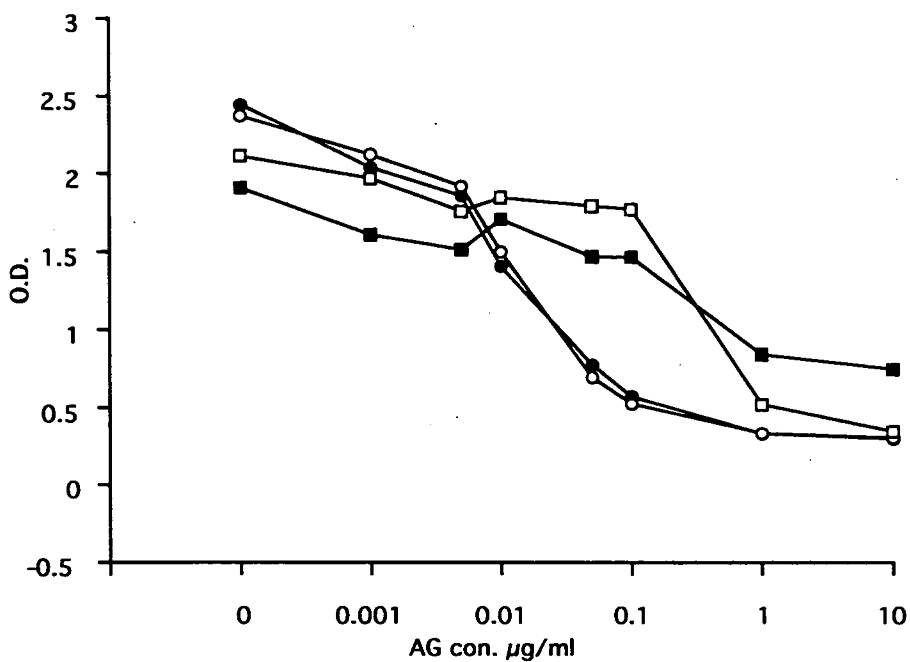
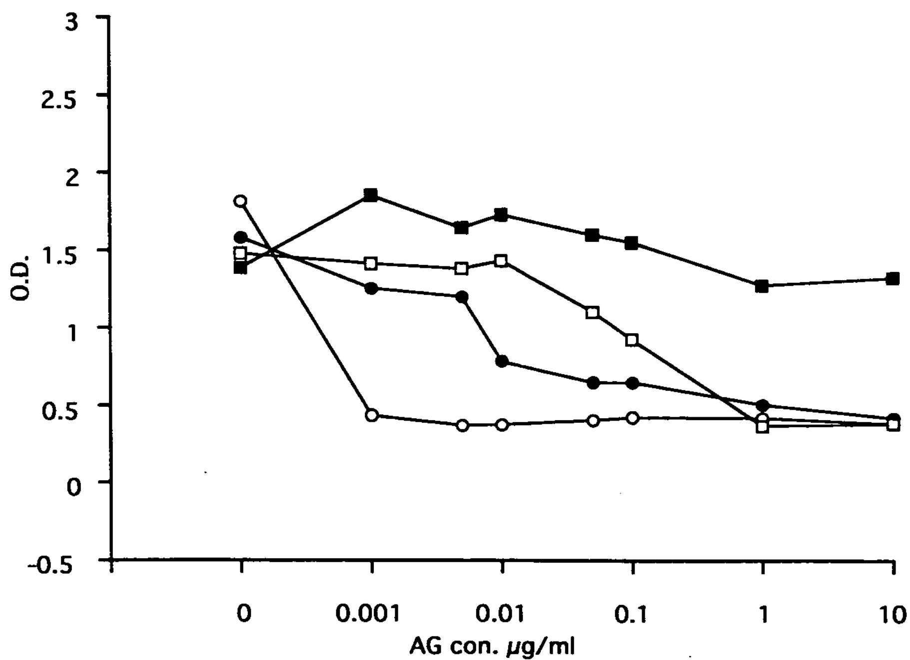


figure 4.3.b.



(3) Serological cross-reactivity between Wellcome and MAD20 PfMSP1 antigens in the age cross-sectional survey

Some murine mAbs can differentiate between the two allelic forms of $PfMSP1_{19}$ (figure 4.4.). Thus mAb 111.4 (derived from a mouse immunised with merozoites of the Wellcome isolate) recognises only the Wellcome type of the first EGF-like motif, whereas mAb 14-1C recognises both types equally. MAb 12.8 (from a mouse immunised with a MAD20-type isolate) recognises both forms but has a higher affinity for the MAD20 type than for the Wellcome type. Due to this ability of mAbs to differentiate between the two allelic sequences of $PfMSP1_{19}$, I decided to compare human polyclonal antibody responses to proteins representing the two different forms of $PfMSP1_{19}$ to see if variant epitopes are a major component in the polyclonal antibody response.

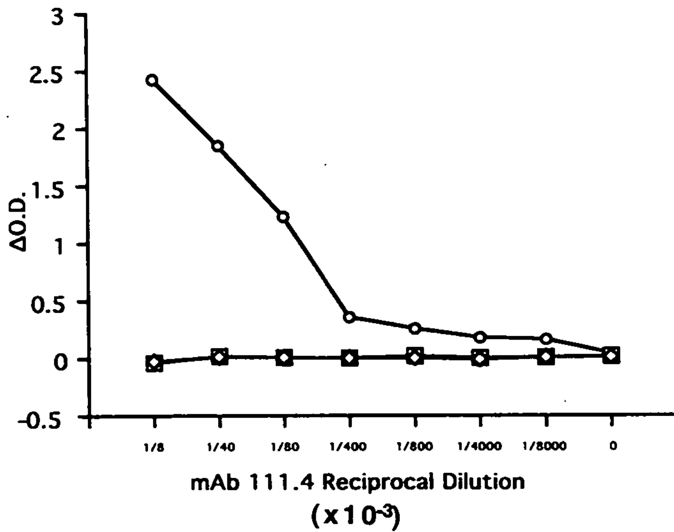
(3a) Comparison of antibody recognition of proteins representing different $PfMSP1_{19}$ sequences

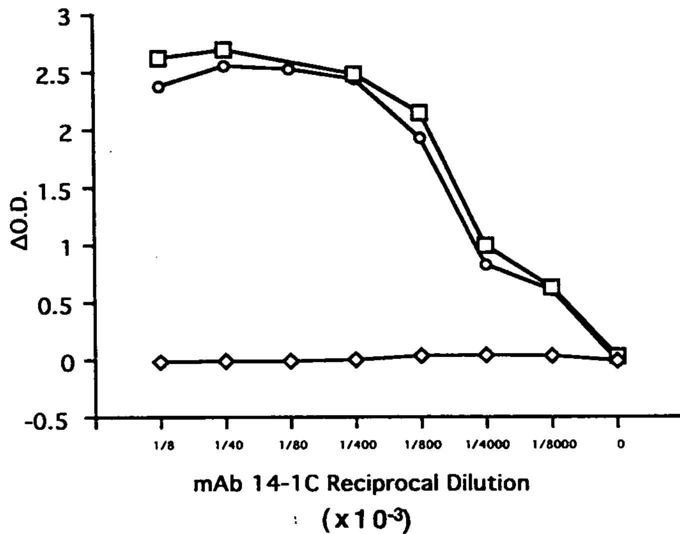
Sera which give a high OD for the Wellcome proteins also tended to give a high OD to the MAD20 proteins. When recognition of two double-motif proteins is directly compared, there is a high degree of correlation between them (see figure 4.5.a.). However, occasional sera do react more strongly with one sequence than the other (arrow). This seems to be due to differential recognition of the three dimorphic amino acid residues in the second EGF-like motif. Figures 4.5.b. and c. show OD values obtained for the Wellcome and MAD20 forms of $PfMSP1_{19}$ compared with OD values obtained for the chimaeric protein MAD/Well-19. The correlation is much closer where proteins share the same second EGF-like motif (figure 4.5.b.) than where they share the first-motif but differ at the second-motif (figure 4.5.c.)

When sera are compared for recognition of single or double EGF-like proteins, they fall into three groups (figure 4.5.d.). Some sera react with both the double and the single (◉) (see figure 4.5.d.), whereas other sera recognise only the double-motif (●). The remaining sera recognise neither (o). Similarly, occasional sera (◉) recognise both the double-motif and the second single-motif (figure 4.5.e.), and a very few sera (◉) recognise both the first- and second-motifs (figure 4.5.f.).

(3b) Titration of individual human sera against the $PfMSP1_{19}$ proteins

The above data seem to suggest that most human antibodies do not differentiate between the variant sequences of $PfMSP1_{19}$. The two allelic forms of the first EGF-like motif differ in sequence by only one amino acid (glutamine in the Wellcome form and glutamate in the MAD20 form at position 14, figure 1.3.); it is thus likely that the two sequences are immunologically cross-reactive.





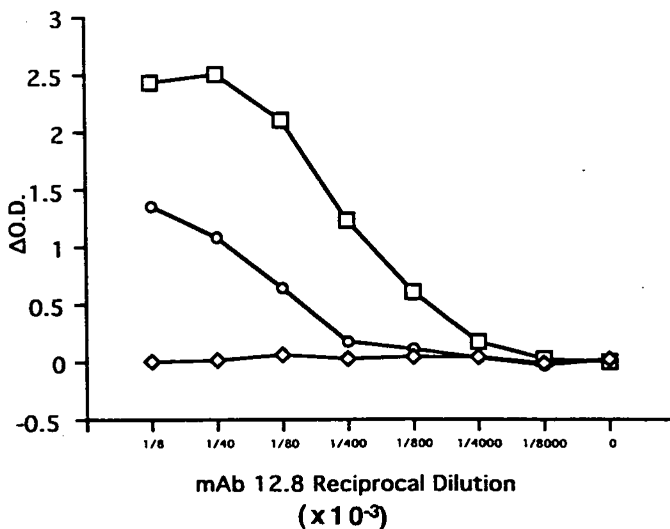


Figure 4.4. MAbs recognise both variant-specific and cross-reactive epitopes between MAD20 and Wellcome sequences of PfMSP1$_{19}$:

a) mAb 111.4 recognises only the Wellcome first-motif.

b) mAb 14-1C recognises both sequences of the first EGF-like motif.

c) mAb 12.8 recognises both sequences, but the MAD20 to a higher degree.

O, Well-EGF1, □, MAD-EGF1, ◇, MAD-EGF2.

Figure 4.5. Comparison of antibody recognition of different $PfMSP1_{19}$ sequences:

a) Well-19 vs MAD-19; Wellcome sequence versus MAD20 sequence. Overall correlation is high ($r=0.901$, $n=195$, $p<0.001$) but some sera (arrowed) clearly differentiate between the two sequences.

b) MAD/Well-19 vs Well-19; proteins share the Welcome sequence at the second EGF-like motif, but differ at the first motif. Correlation is high ($r=0.983$, $n=195$, $p<0.001$) showing that the single amino acid change (E to Q) is not recognised by polyclonal sera.

c) MAD/Well-19 vs MAD-19; proteins share the MAD20 sequence at the first EGF-like motif but differ in the second motif. The correlation ($r=0.965$, $n=195$, $p<0.001$) is similar to that in (a) but lower than in (b). This indicates that some sera (arrowed) clearly differentiate between the KNG and TSR sequences.

figure 4.5.a.

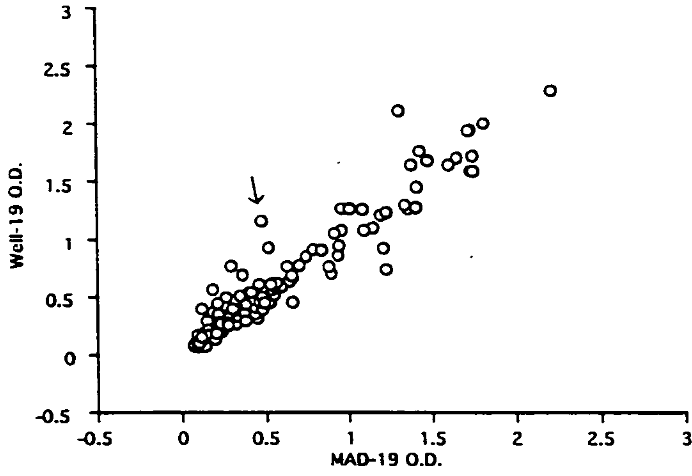


figure 4.5.b.

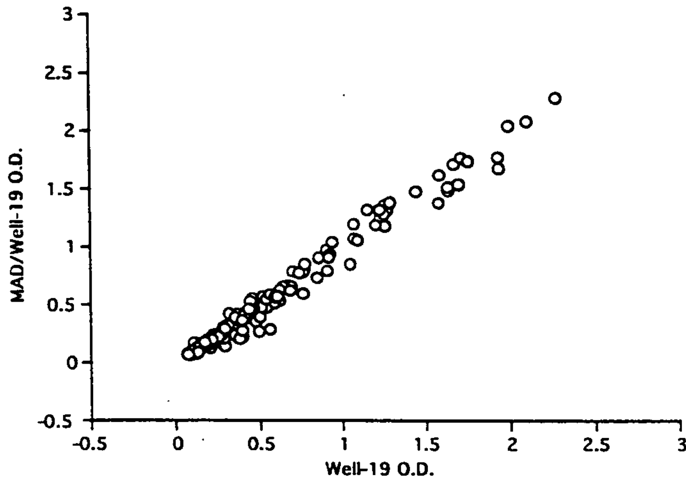


figure 4.5.c.

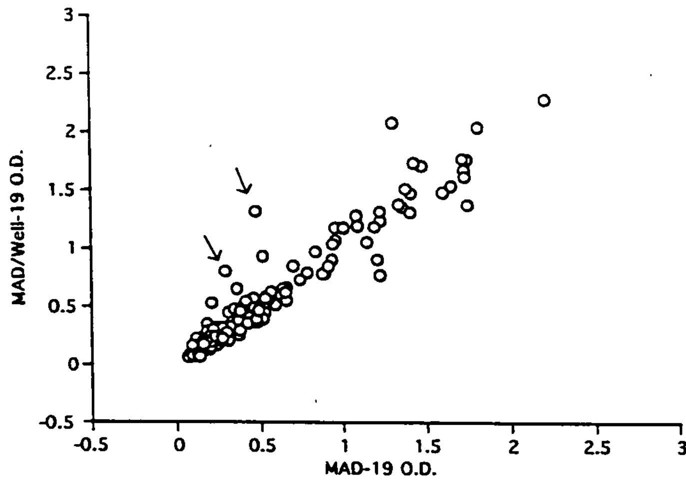


Figure 4.5. Comparison of antibody recognition of different PfMSP1$_{19}$ sequences:

d) MAD-19 vs MAD-EGF1; proteins share the MAD20 first EGF-like motif sequence. The correlation is low ($r=0.403$, $n=195$, $p<0.001$) as approximately 50% of the seropositive sera recognise epitopes in the double-motif protein not present in the first-motif protein.

= sera that react with the double-motif protein only,

◉ = sera that react with both the double- and first-motif proteins,

O = sera seronegative for MAD20 PfMSP1$_{19}$.

e) MAD-19 vs MAD-EGF2; proteins share the MAD20 second EGF-like motif sequence. The correlation is low ($r=0.322$, $n=195$, $p<0.001$) as the majority of seropositive sera recognise epitopes in the double-motif protein.

= sera that react with the double-motif protein only/

◉ = sera that react with both the double- and second-motif proteins.

O = sera seronegative for MAD20 PfMSP1$_{19}$.

f) MAD-EGF1 vs MAD-EGF2; proteins represent either the first or second EGF-like motif. The correlation is low ($r=0.335$, $n=195$, $p<0.001$). There are very few sera which recognise both the first- and second-motif, and even less that recognise the second-motif exclusively.

= sera that react with the first-motif protein only.

◉ = sera that react with both the first- and second-motif proteins.

⊙ = sera that react with the second-motif protein only.

O = sera seronegative for MAD20 PfMSP1$_{19}$.

figure 4.5.d.

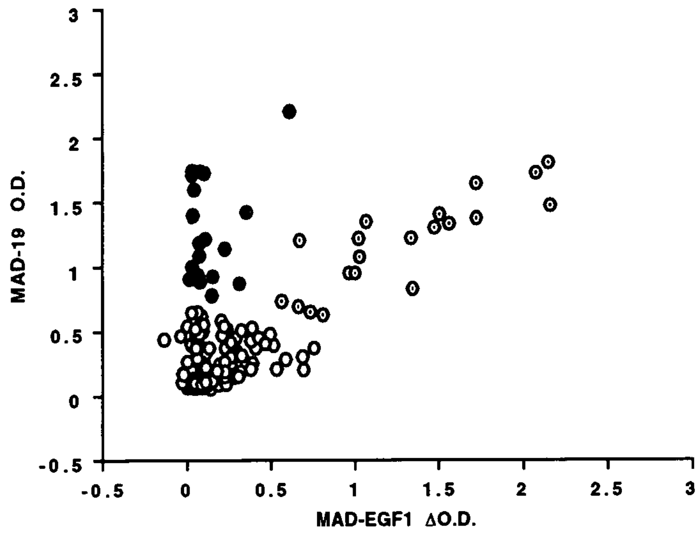


figure 4.5.e.

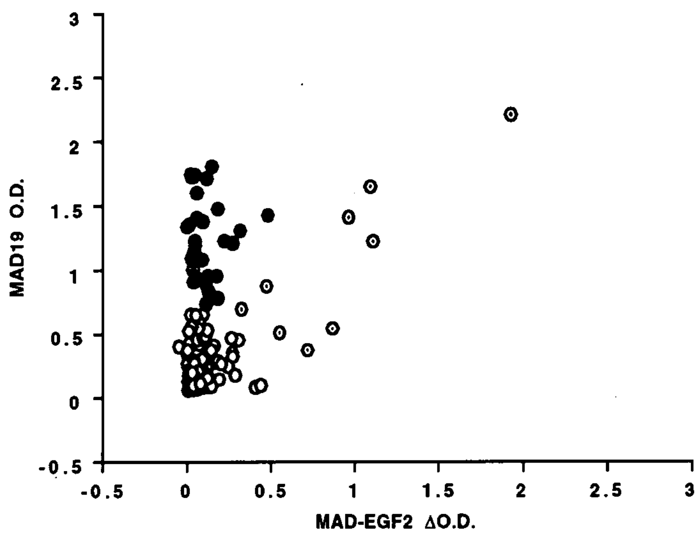
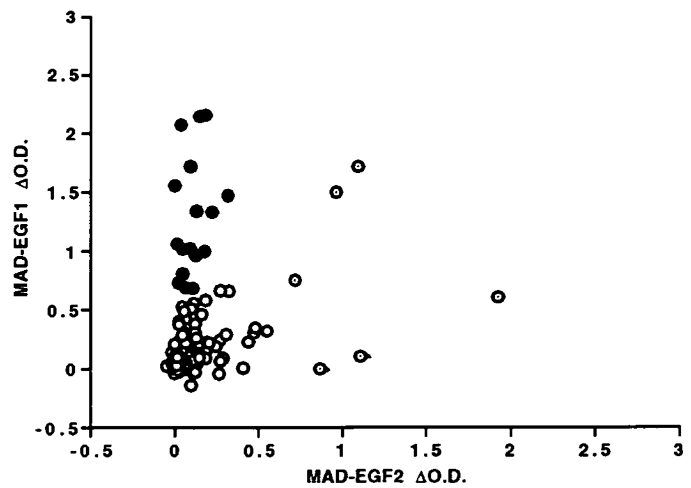


figure 4.5.f.



To confirm that these antibodies were recognising both sequences with the same avidity and affinity, six sera, previously shown to contain antibodies to the first EGF-like motif, were titrated against the three single EGF-like motif proteins. All six sera gave identical titration curves for the two allelic forms of the first EGF-like motif (an example is shown in figure 4.6.a.). They also gave identical titration curves for the two allelic forms of the double-motif protein (an example is shown in figure 4.6.b.). This confirms that these human polyclonal sera do not differentiate between the two allelic forms of $PfMSP1_{19}$.

(3c) Polyclonal sera contain cross-reactive population of antibodies

Finally, to confirm that polyclonal human sera contain a single population of antibodies that recognise both sequences, competition ELISAs were performed.

Individual sera which recognised MAD20 and Wellcome derived proteins with equal avidity were selected. As shown in figure 4.7.a. (serum 1592), preincubation of the serum with 1 to 10 μg of either the Wellcome or MAD20 first EGF-like motif protein per ml inhibits subsequent binding of that serum to plates coated with either of the two of these proteins. For another serum (1614, figure 4.7.b.) inhibition was obtained by preincubation with less than 0.1μg of protein per ml.

(4) Seasonal changes in antibody responses

To determine whether antibody levels are affected by boosting from infection during the wet season, antibody responses were measured in 20 children and 22 adults over three malaria transmission seasons.

Antibody responses to MAD-EGF1, Well-19 and MAD-33 were measured in serum samples taken every six months i.e. wet and dry seasons. Responsiveness and OD levels to PfMSP1 proteins were more stable in adults than in children over the three wet and dry seasons, see table 4.4. Despite life long exposure to these PfMSP1 proteins, some adults were persistently antibody negative to these proteins over the three year period. Individual children showed clear evidence of seasonal variation of the antibody response (for example; E24009, Q03005).

(5) HLA analysis of serological responsiveness to PfMSP1 proteins in the age cross-sectional study

Data from the three year longitudinal study shows that some adults are persistently antibody negative to C-terminal PfMSP1 recombinant proteins, and data from the age cross-sectional study show that 40% of adults do not possess antibodies to the conserved $PfMSP1_{19}$ despite life-long exposure. To determine whether this non-responsiveness is due to their HLA class II genetic make up, the relationship

Figure 4.6. Human sera recognise epitopes which are cross-reactive between MAD20 and Wellcome sequences of $PfMSP1_{19}$:

a) human serum 1275 recognises both sequences of the first EGF-like motif but not the second-motif.

b) human serum 1579 recognises all three yeast double-motif $PfMSP1_{19}$ constructs.

O, Well-EGF1,□, MAD-EGF1,◇, MAD-EGF2,●, MAD-19, Δ, Well-19,■, MAD/Well-19.

figure 4.6.a.

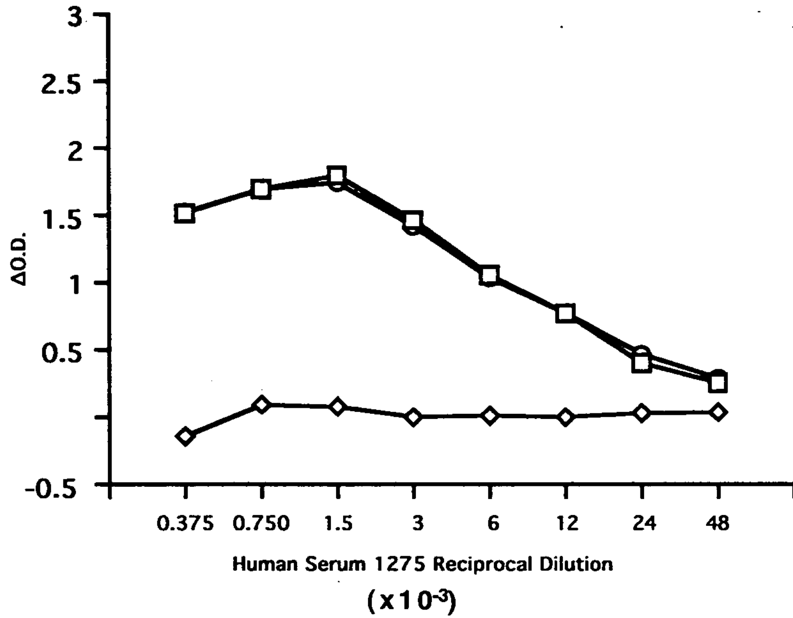


figure 4.6.b.

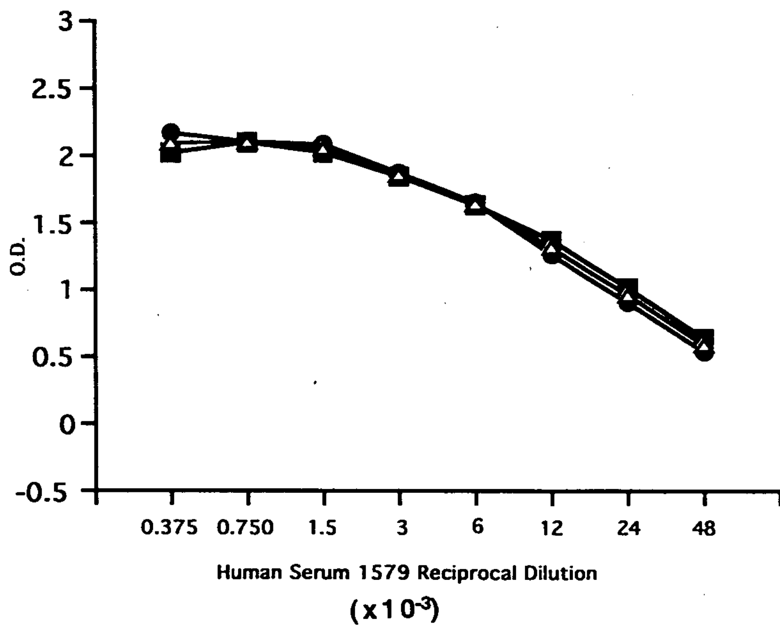


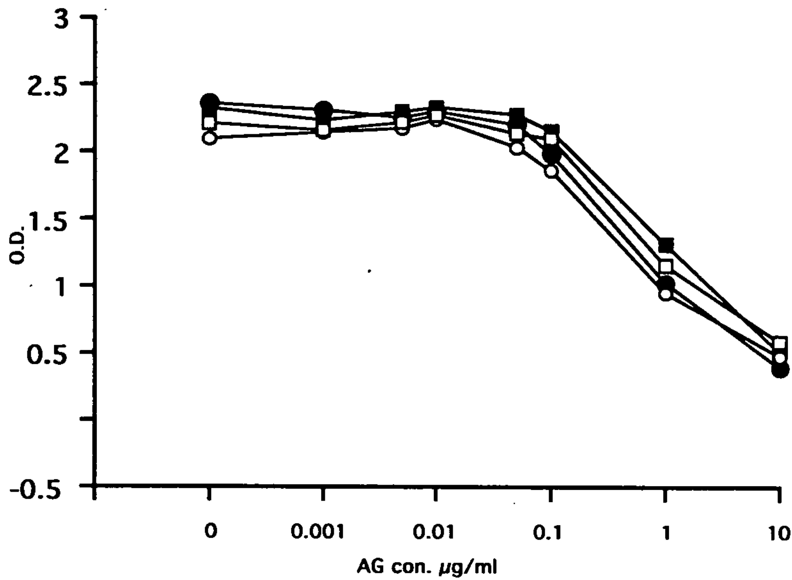
Figure 4.7. Competition ELISAs to confirm the presence of cross-reactive antibodies. Preincubation of human serum a)1592 or b)1614 with 1μg/ml of either protein Well-EGF1 or protein MAD-EGF1 inhibits subsequent binding of that serum to plates coated with either MAD-EGF1 or Well-EGF1 respectively .

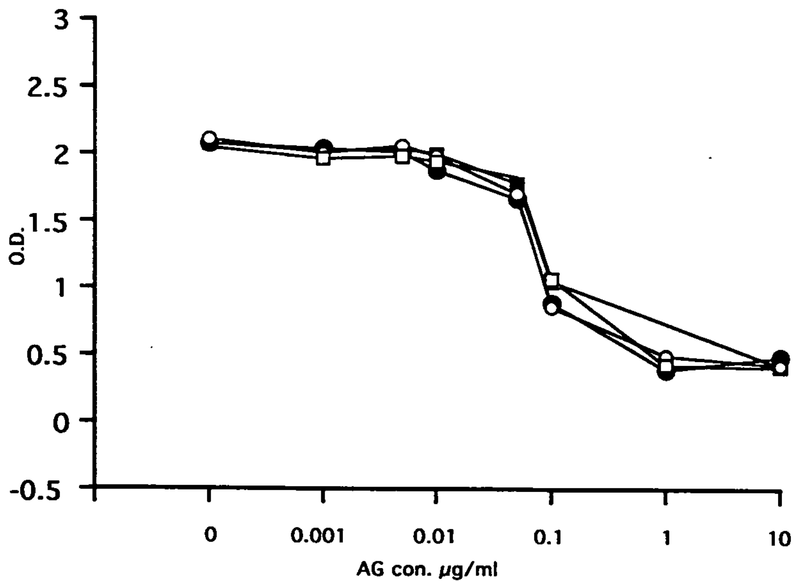
, serum preincubated with Well-EGF1 on Well-EGF1-coated plates.

, serum preincubated with Well-EGF1 on MAD-EGF1-coated plates.

O, serum preincubated with MAD-EGF1 on Well-EGF1-coated plates.

☐, serum preincubated with MAD-EGF1 on MAD-EGF1-coated plates.





donor	age	MAD-EGF1	MAD-19	MAD-33
E07008	5	- - + - - -	- - + - - -	- + + - - -
E08010	7	+ - + + - +	- + + - + -	+ + - - - -
E09009	3	- - - - -	- - - - -	- - - - -
E17004	3	- - - - - -	- - - - - -	- - - - - -
E18003	6	- + - - - +	- + - - - +	+ + + + - +
E20015	5	- - - - -	- - + - -	- + + + +
E24008	6	- - + - - -	- - + - - -	+ - - - -
E24009	4	- - - - - -	- - - - - +	- + - + - +
E25007	3	- - - - - -	- - - - - -	- - - - - -
E25009	6	- - - - - -	- + - - - -	+ - - + + -
N05034	3	- - - -	- - - -	- - - -
P06008	7	- - - - -	- - - - -	- - - - -
P06009	4	- - - - - -	+ - - - - -	- + - - - -
P06010	3	- - - - -	- - - + +	- + - - + +
P10016	6	- - - - - -	- + - - - -	+ + + + + +
P10017	3	- - - - - +	- - - - +	+ + + - +
P11005	6	- - - -	- - - -	- - - -
P12009	5	- + - - -	- + - - - -	+ + - + +
Q01008	6	- - - +	- - - +	+ + + +
Q03005	4	- - - + - +	- - - + - +	- - + - +
12	60	- - - - -	- - - - -	+ + + + +
13	55	+ + + + +		
45	45	- - - - -	- - + - -	+ + + + +
50	38	- - - - -	- - - - -	+ + + + +
79	50	+ + + + +	+ + + + +	+ + + + +
110	17	+ + + + +	+ + + + +	+ + + + +
167	16	- - - - -	- - - - -	+ + + + +
109	16	- - - -	- + - +	- - - -
31	44	- - - + +	+ - + + +	+ + + + +
104	16	- - - + +	- - - + +	+ + + + +
53	60	- - - - - -	- - - - - -	+ + + + + +
15	45	- +	+ + + +	+ + + +
120	65	- - - - - -	- - - - - -	- - - - - -
95	41	- - - - -	+ + + + +	+ + + + +
4	34	+ + + + + +	+ + + + + +	+ + + + + +
117	17	- - + - - -	- - + - - -	+ + + + + +
28	55	- - - - - -	- - - - - -	+ + + + + +
69	35	- - - - -	- - - - -	- - - + -
116	28	- - - - -	- - - - -	+ + + + +
125	28	+ - + - +	+ - - - +	+ + + + +
112	18	- - - - -	- - - - -	+ + + + +
105	24	+ - - - -	+ + - - -	+ + + + +

Table 4.4. Longitudinal survey of antibody responses to PfMSP1 proteins in children (upper half) and adults (lower half).

Each symbol represents a separate serum sample. Samples were collected at six month intervals.
-- = antibody negative,
+ = antibody positive (higher than mean plus 2 SD of 24 European sera),
+ = strongly antibody positive (>mean plus 6 SD).
Blank space indicates either no sample or no data.
First, third and fifth samples = wet season.
Second, fourth and sixth samples = dry season.

between HLA class II haplotype and antibody responsiveness to MAD-EGF1, MAD-EGF2, MAD-19 and MAD-33 was analysed for the cross-sectional cohort of Gambian donors (table 4.5.). Only those haplotypes which were present in at least 10 of the 195 individuals tested were included in the analysis. No significant associations were seen between any of the C-terminal PfMSP1 recombinant proteins and any HLA class II antigen. Importantly, individuals with identical HLA class II haplotypes could have different antibody responses.

(6) Serological responsiveness to PfMSP1 proteins in adult twins

Another method of looking for genetically-determined differences in antibody responsiveness is to compare antibody responses in genetically identical individuals (i.e. mz twins) and non-identical (i.e. dz) twins. Antibody responses were measured in 15 pairs of adult mz twins and 21 pairs of adult dz twins to Well-EGF1, MAD-EGF2, MAD-19, Well-19 and MAD/Well-19.

Overall, the level of concordance of antibody responsiveness is high (36 pairs tested against 5 antigens = 180 comparisons, of which 139 were concordant, = 77%), this presumably reflects the similarity of past malaria exposure within pairs. 23% of comparisons were discordant, with one twin being seropositive to a particular antigen while the other twin was seronegative. If these differences were the result of genetic differences between twins, it would be expected that more dz than mz pairs would be discordant, but this is not the case (see table 4.6.). There was no significant difference between the number of discordant pairs between the mz and dz twins, (Fisher's exact test, $p>0.05$ for all antigens) or the median difference in OD values (Mann-Whitney; $p>0.07$ for all antigen). So mz twins are no more alike in their antibody responsiveness than dz twins, and genetically identical mz twins can have very different antibody responses.

Haplotype no.	Serological specificity		n	MAD-EGF1		MAD-EGF2		MAD-19		MAD-33	
	DR	DQ	n	n	%	n	%	n	%	n	%
1	1	w5	7	1	0.14	6	0.86	5	0.71	5	0.71
2	w15	w6	4	2	0.50	3	0.75	4	1.00	4	1.00
7	w17	w2	7	4	0.57	5	0.71	7	1.00	7	1.00
8	w18	w4	1	1	1.00	1	1.00	1	1.00	1	1.00
9	3	w4	6	1	0.17	4	0.67	2	0.33	6	1.00
11	4	w8	4	2	0.50	3	0.75	4	1.00	2	0.50
12	4	w2	1	1	1.00	1	1.00	1	1.00	1	1.00
14	7	w2	10	3	0.30	10	1.00	8	0.80	10	1.00
15	7	w2	2	1	0.50	2	1.00	2	1.00	2	1.00
17	w8	w7	4	2	0.50	3	0.75	4	1.00	4	1.00
18	w8	w7	3	2	0.67	3	1.00	3	1.00	3	1.00
21	9	w2	10	3	0.30	9	0.90	9	0.90	9	0.90
22	w10	w5	16	5	0.31	14	0.88	13	0.81	15	0.94
23	w11	w7	3	1	0.33	3	1.00	3	1.00	3	1.00
26	w11	w7	4	2	0.50	2	0.50	3	0.75	3	0.75
29	w11	w7	54	22	0.41	46	0.85	42	0.78	53	0.98
34	w13	w6	4	0	0.00	3	0.75	2	0.50	4	1.00
37	w13	w6	3	0	0.00	3	1.00	3	1.00	3	1.00
38	w13	w6	27	10	0.37	23	0.85	24	0.89	27	1.00
39	w13	w7	9	3	0.33	7	0.78	6	0.67	9	1.00
χ^2				2.82		6.13		2.79		4.44	
p				0.73		0.29		0.73		0.49	

Table 4.5. The association between HLA class II haplotype and the serological responsiveness to PfMSP1 proteins.

Number /percentage of individuals expressing each haplotype who were seropositive for each antigen. χ^2 is conditional logistic regression statistic for the overall association between HLA type and immune response.
p value at 5 degrees of freedom.

	Well-EGF1		MAD-EGF2		MAD-19		Well-19		MAD/Well-19	
mz1	0.170	0.065	0.069	0.135	0.123	0.138	0.127	0.155	0.126	0.146
mz2	0.065	0.127	0.045	0.120	0.769	0.822	0.766	0.905	0.828	0.930
mz3	0.035	0.049	0.034	0.147	0.114	0.163	0.130	0.177	0.115	0.198
mz4	0.011	0.011	0.048	0.037	0.128	0.106	0.134	0.354	0.114	0.086
mz5	0.009	0.006	0.030	0.057	0.289	0.138	0.227	0.149	0.173	0.140
mz6	1.059	0.210	0.702	0.025	1.220	0.482	1.241	0.539	1.288	0.501
mz7	0.513	0.021	0.503	0.062	0.047	0.681	0.048	1.403	0.066	1.373
mz8	0.013	1.978	0.073	0.499	1.358	1.738	1.648	1.811	1.565	1.870
mz9	0.018	0.012	0.024	0.019	0.079	0.341	0.076	0.380	0.091	0.407
mz10	0.036	0.030	0.049	0.057	0.075	0.085	0.069	0.089	0.065	0.089
mz11	0.038	0.010	0.028	0.025	0.315	0.129	0.313	0.148	0.356	0.141
mz12	0.126	0.276	0.017	0.029	0.457	0.843	0.446	0.777	0.380	0.717
mz13	0.021	0.034	0.108	0.017	0.153	0.205	0.133	0.138	0.132	0.119
mz14	0.044	0.359	0.003	0.053	0.230	0.571	0.171	0.454	0.210	0.541
mz15	0.140	0.072	0.033	0.016	0.225	0.103	0.215	0.104	0.212	0.099
dz1	0.090	0.061	0.055	0.099	0.171	0.139	0.199	0.183	0.211	0.177
dz2	0.033	0.022	0.077	0.036	0.339	0.147	0.354	0.150	0.323	0.141
dz3	0.035	0.039	0.071	0.149	0.146	0.157	0.159	0.168	0.150	0.157
dz4	0.011	0.048	0.043	0.026	0.099	0.106	0.111	0.126	0.106	0.156
dz5	0.002	0.029	0.021	0.075	0.105	0.256	0.114	0.304	0.100	0.167
dz6	0.004	0.008	0.037	0.037	0.140	0.123	0.163	0.156	0.162	0.160
dz7	0.022	0.082	0.040	0.018	0.369	0.806	0.509	0.951	0.448	0.888
dz8	0.022	0.024	0.033	0.033	0.096	0.082	0.104	0.085	0.095	0.080
dz9	0.166	0.144	0.009	0.061	0.222	0.390	0.246	0.404	0.275	0.489
dz10	0.043	0.049	0.033	0.042	0.097	0.076	0.088	0.094	0.081	0.100
dz11	0.163	0.034	0.091	0.014	1.414	0.197	1.432	0.237	1.317	0.211
dz12	0.093	0.063	0.052	0.061	0.348	0.145	1.119	0.138	0.959	0.122
dz13	0.060	0.081	0.044	0.062	0.579	0.607	0.525	0.621	0.573	0.626
dz14	0.035	0.109	0.037	0.445	0.181	0.332	0.153	0.302	0.165	0.383
dz15	0.015	0.001	0.010	0.006	0.073	0.168	0.073	0.223	0.063	0.178
dz16	0.043	0.028	0.004	0.019	0.123	0.115	0.102	0.119	0.111	0.116
dz17	0.014	0.023	0.011	0.000	0.067	0.077	0.071	0.081	0.069	0.078
dz18	0.003	0.010	0.017	0.034	0.086	0.080	0.083	0.106	0.084	0.093
dz19	0.001	0.026	0.027	0.023	0.332	0.167	0.242	0.164	0.145	0.157
dz20	0.024	0.013	0.018	0.052	0.086	0.163	0.091	0.159	0.096	0.151
dz21	0.011	0.016	0.012	0.009	0.061	0.061	0.060	0.064	0.057	0.058

	Well-EGF1	MAD-EGF2	MAD-19	Well-19	MAD/Well-19
*>	0.058	0.028	0.132	0.110	0.098
Comparison of proportion discordant:					
%'s[2]	27% vs. 5%	40% vs. 38%	40% vs. 19%	20% vs. 14%	20% vs. 14%
p-value[1]	0.138	0.817	0.260	0.677	0.677
Comparison of average within-twin difference:					
median's[2]	0.062 vs. 0.021	0.050 vs. 0.023	0.151 vs.0.032	0.163 vs. 0.068	0.113 vs.0.050
W statistic[3]	320.5	313.0	333.5	331.5	334.0
p-value[2]	0.17	0.26	0.08	0.09	0.07

[1] Fisher's exact test (two-sided p-value)
[2] Monozygous first
[3] Mann-Whitney W test

Table 4. 6. Comparison of antibody responses to PfMSP1$_{19}$ antigens in adult monozygous and dizygous Gambian twins.

The first figure in each column represents the OD value for one twin, the second figure is the OD value for the other twin in the pair. Discordant results (one above and one below the cut off) are underlined. * Cut off values for positive responses (mean plus 2SD of control sera OD).

Discussion

I have screened sera from a population of malaria-exposed individuals from The Gambia for antibodies recognising recombinant proteins representing the Wellcome form of $PfMSP1_{42}$, the MAD20 form of $PfMSP1_{33}$ and the two allelic forms of the single and double EGF-like motifs of $PfMSP1_{19}$.

This study has shown that; 1) $PfMSP1_{33}$, $PfMSP1_{19}$ and the constituent EGF-like motifs are naturally immunogenic in humans, 2) the prevalence of antibodies increases with age, 3) the antibodies tend to recognise epitopes that are conserved or cross-reactive between the two allelic sequences, 4) antibody responses are stable in adults and 5) antibody responses do not appear to be genetically restricted. The prevalence of individuals who are seropositive to PfMSP1 proteins increases with age (though not always statistically significant), which may suggest that multiple infections are required to initiate an antibody response.

Approximately 50% of the individuals seropositive for $PfMSP1_{19}$ had antibodies to the single first EGF-like motif; recognition of the second EGF-like motif is much lower. As the number of individuals seropositive to the single first- and second-motif proteins do not equal the number seropositive to the double-motif protein, this indicates that a major epitope(s) of $PfMSP1_{19}$ requires the presence of both EGF-like motifs, and is missing when either motif is expressed alone. Approximately 90% of individuals have antibodies to $PfMSP1_{33}$, 40-50% have antibodies to $PfMSP1_{19}$ and 12-17% have antibodies to the individual EGF-like motifs.

Udhayakumar *et al* (1995) have measured antibody responses of malaria-exposed adults to recombinant proteins representing $PfMSP1_{19}$. They found 70-90% of individuals were seropositive to $PfMSP1_{19}$, which is much higher than reported here. It is possible that this is due to the higher malaria transmission intensity in Kenya, but I suspect that differences in their ELISA protocol, compared to the one used here, may contribute to the high seroprevalence claimed. Sera were tested at a very high concentration (1:100 compared to 1:3000 tested here). The concentration of sera used here has been optimised to detect fine differences in antibody binding and give low level non-specific binding (see chapter 3). Measuring antibody responses at such a high concentration (as in the Udhayakumar study) suggests that antibody responses were measured at an insensitive region of the antibody titration curve (see figures 3.4.). In fact sera may have been barely positive and their measurements probably resulted from non-specific binding. Many of these sera would probably have been deemed negative in the ELISA protocol used here. Non-specific binding does appear to be a problem in the Udhayakumar study, as cut off values of up to 0.7 OD have been

used, which is very high. Also, direct comparisons of OD responses from different batches of ELISAs have been made despite the evidence that OD values varied widely from batch to batch; cut off OD values for the same antigen, tested in different batches, varied from 0.1 to 0.7. Due to reasons discussed above (insensitivity of the ELISA protocol used) their results may be inaccurate and are a classic example of the pitfalls of the ELISA technique (Venkatesan and Wakelin 1993).

Although more than 95% of parasites transmitted in The Gambia are of the MAD20 MSP1 genotype (Conway and McBride 1991), constructs representing the alternative Wellcome sequence of PfMSP1$_{19}$ were well recognised by Gambian sera. To determine whether the human antibody response to PfMSP1$_{19}$ is allele-specific, responses to constructs representing the two alternative sequences were directly compared. I found that PfMSP1$_{19}$-specific antibodies in human sera tend to recognise epitopes which are conserved or cross-reactive between variant sequences. Although occasional sera were shown to contain two separate, non-cross-reacting populations of antibodies, the majority of positive sera clearly recognise an epitope which is conserved or cross-reactive between the two sequences. A few sera contain antibodies which appear to bind with higher avidity to one sequence of the protein than to the other sequence. This was particularly apparent when antigens which differed in the sequence of the second EGF-like motif (where there are three amino acid differences) were compared, suggesting that there is a minor epitope in the second EGF-like motif which involves the dimorphic residues. In contrast, substitution of glutamate for glutamine in the first EGF-like motif has very little affect on recognition by human antibodies. In this respect, human antibodies differ substantially from murine mAbs (such as 111.4) which recognise epitopes involving dimorphic amino acid residues. This cross-reactivity bodes well for the development of a malaria vaccine which could provide protection against all variants of the parasite.

However, Udhayakumar *et al* (1995) claim that variant epitopes in PfMSP1$_{19}$ result in differential recognition by some malaria-exposed sera; actually, only 7 of the 65 individuals tested in ELISA did not cross-react between the two prototype sequences (E-TSR; MAD20 and Q-KNG; Wellcome) of PfMSP1$_{19}$. Data presented here demonstrate that individuals are almost always antibody positive for the two allelic forms, but may have a higher OD to one allelic form.

Antibody responses to PfMSP1$_{19}$ and Well-42 are highly correlated. This suggests that the majority of Well-42's recognition is due to conserved epitopes within PfMSP1$_{19}$. Serum from 74% of adult donors aged 16 years and above contained antibodies which recognised the Well-42 construct and 60% of adult sera recognised the double EGF-like motif. Therefore only 14% of adults exclusively recognised dimorphic epitopes in Well-42. However as the Well-42 construct

represents the allele which is not commonly expressed in The Gambia this is, perhaps, not surprising.

Individuals that recognise the Wellcome $PfMSP1_{42}$ but not $PfMSP1_{19}$, may have been exposed to parasites expressing the Wellcome PfMSP1 allele. On the other hand they may recognise conserved or cross-reactive epitopes within the $PfMSP1_{33}$ part of Well-42. However, there are clearly epitopes within $PfMSP1_{33}$ that are not cross-reactive between the two dimorphic sequences, as almost 90% of individuals were seropositive for epitopes within the MAD20 $PfMSP1_{33}$. This data confirms that the dimorphic region of $PfMSP1_{33}$ is immunodominant and that the Well-42 construct under-estimates antibody prevalence to $PfMSP1_{42}$ in The Gambia. This is in agreement with observations by Hui and Chang (Hui *et al* 1992, 1993) who suggest that antibodies to the dimorphic $PfMSP1_{33}$ are not cross-reactive while antibodies to the conserved $PfMSP1_{19}$ are.

It has been suggested by Fruh *et al* (1991) that antibody responses to PfMSP1 are strain-specific, however their data do not really support this conclusion. They showed that there is an unequal distribution of the two allelic forms of PfMSP1 in Mali, as none of the eight parasite isolates tested expressed the Wellcome allele. Accordingly, they found that Wellcome recombinant proteins were not well recognised by this population. A recombinant protein representing the Wellcome C-terminal region, including $PfMSP1_{19}$, was recognised by this population, but only at a low prevalence; this was assumed to be due to infrequent exposure to Wellcome-type parasites. However, I would speculate, from my own observations, that if they had measured antibody responses to a MAD20 version of $PfMSP1_{19}$, they would have found a similar (low) level of responsiveness as for their Wellcome $PfMSP1_{19}$ construct.

Another area of confusion is whether or not antibody responses to PfMSP1 are transient or long-lived. Data presented here show that antibody responses to the C-terminus of PfMSP1 are not stable in children but are very stable in adults. My data show that antibody prevalence varies with season in children but stabilises in adults, with little seasonal variation in immune individuals over the age of 18 years. In Mali, it has been reported that the prevalence of anti-PfMSP1 responses increases in the wet (transmission) season and declines in the dry season (Fruh *et al* 1991). The authors conclude, therefore, that antibody responses are short-lived. However, closer analysis of their data indicates that (1), changes in antibody prevalence occur only for children and only for antibody to dimorphic epitopes and (2), that approximately half of the children remain seropositive from one wet season to the next. The data is not divided into small age groups, so it is not possible to tell whether the stable responses are seen in older children, but from my data, I would predict that this might be the case.

Riley *et al* (1993) carried out a similar study of antibody prevalence through a malaria transmission season in adults. It was found that antibody responses to native PfMSP1$_{83}$ or Well-42 did not fluctuate during the year. There was a slightly higher OD response in the wet season than in the dry season, but these differences were not significant. When antibody responses to native PfMSP1$_{83}$ and Well-42 (Riley *et al* 1993) were compared at the end of the rainy season in 32 rural and 29 urban dwellers, it was found that mean levels of antibody were slightly higher in the rural group, but again these differences were not significant, despite the difference in the parasite transmission rates between the two environments. This implies that in adults, intermittent exposure to infection may be sufficient to maintain serum antibody titres.

In contrast, Brown *et al* (1991) demonstrated that the half life of antibodies to the N-terminus of PfMSP1 in non-immune soldiers, recovering from their first malarial infection, is less than one month. The pattern of antibody responsiveness in these non-immune adults is similar to that in Gambian children. Thus, it seems that effective long-term memory responses to PfMSP1$_{19}$ develop only after repeated exposure to malaria.

This study also indicated that some adults remain persistently non-responsive to essentially conserved C-terminal PfMSP1 antigens over the three years, despite life-long exposure. Also, in some children antibody responsiveness to MAD-19 was not boosted during the rainy season even though boosting occurred for MAD-33. To determine whether persistent non-responder status is genetically determined, the effects of HLA class II genes and non-HLA genes on antibody responsiveness were examined. However, no significant associations were found. This confirms previous studies indicating that immune responses to PfMSP1 are not HLA restricted (Guttinger *et al* 1991, Riley *et al* 1992b).

The reason why 40% of adult donors from the cross-sectional study did not possess antibodies to PfMSP1$_{19}$ is not known. All donors are known to have been exposed to malaria over many years and had high titres of antibody to malaria schizonts. Analysis of the data with respect to the known HLA class II genotype or non-HLA genes of the donors does not indicate that the response to PfMSP1$_{19}$ is genetically restricted. It is possible that the seronegative donors may have antibody to other regions of PfMSP1 which inhibit the formation of antibodies to PfMSP1$_{19}$. Steric interference has been demonstrated between mAbs binding to apparently distant PfMSP1 epitopes (Wilson *et al* 1987) suggesting that the N-terminal region of PfMSP1 may physically obscure PfMSP1$_{19}$ in the native protein. An alternative, but unlikely, explanation is that seronegative individuals may not have been recently infected with malaria and, in the absence of boosting, antibody may have fallen below detectable levels.

Another possibility for the selective antibody responsiveness to $PfMSP1_{19}$ is clonal imprinting, or original antigenic sin. The term 'original antigenic sin' describes the phenomenon where higher affinity antibodies are produced to strains (of an infecting agent) seen prior to the vaccinating strain. In a primary infection, naive T and B cells proliferate and transform into memory cells. Memory B cells have a higher avidity for antigen than do naive B cells as they express more surface Ig (sIg) and somatic mutation of Ig genes results in expression of sIg of higher affinity (Vitetta *et al* 1991). On secondary infection, these memory B cells compete successfully with naive B cells for antigens which cross-react with the original infecting strain, so antibodies of the original infecting specificity are produced. Epitopes which were not present in the original infection, or which induced memory cells of low affinity, are effectively ignored.

There are many features of malaria infection which make clonal imprinting a possible explanation for the selective antibody responses seen in malaria-immune individuals, 1) malarial antigens are made up of conserved, semi-conserved and polymorphic epitopes (McCutchan *et al* 1988), so cross-reactive immune responses would be expected, 2) people are infected many times with malaria, and polymorphism within malaria parasites is extensive, so it is highly likely that successive infections would be of differing genotypes, and 3), in clinically immune individuals, blood stage infections are of very low density so that competition for antigen may occur between B cells of differing affinity. If for some reason, $PfMSP1_{19}$ is not very antigenic and/or induces low affinity antibody, it may be ignored in favour of immunodominant regions. High affinity responses would be boosted and low affinity responses would eventually be lost.

Other possible explanations for the low prevalence of anti-$PfMSP1_{19}$ antibodies include conformational changes in the protein after secondary processing, such that antibodies raised against unprocessed $PfMSP1_{42}$ can no longer bind the cleaved $PfMSP1_{33}$ and $PfMSP1_{19}$. This secondary processing may have destroyed epitopes and created new epitopes through confirmational changes. So there may be a different repertoire of antibodies recognising $PfMSP1_{42}$ from those that recognise $PfMSP1_{33}$ and $PfMSP1_{19}$.

Similarly, infrequent recognition of the EGF-like motifs by immune sera may be a functional consequence of the processing of PfMSP1. Although the initial processing of PfMSP1 occurs during the later stages of schizogony, such that mature merozoites carry only the processed version of PfMSP1, the secondary processing which cleaves $PfMSP1_{42}$, producing the membrane bound $PfMSP1_{19}$ and $PfMSP1_{33}$ which is shed, seems to occur immediately prior to merozoite invasion (Blackman *et al* 1991b). Thus $PfMSP1_{19}$ may only be exposed to the immune system for a very short period of time. The protease responsible for this

secondary processing is parasite-derived and the cleavage site is within a highly conserved region of the molecule, suggesting this secondary processing is under functional constraint (Blackman *et al* 1992, 1993). A possible function of $PfMSP1_{33}$ could be to provide protection of $PfMSP1_{19}$ from the immune system, by for example physically shielding it, or by providing epitopes with high affinity for B cells, thereby diverting antibody responses away from $PfMSP1_{19}$. However, I found that antibody responses to MAD-19 do not negatively correlate with antibody responses to MAD-33 (data not shown).

The merozoite invasion rate of RBCs is very low (Dr. N. Brown, NIMR, personal communication). If those merozoites which failed to invade RBCs have processed $PfMSP1_{19}$ on their surface, then the immune system will be extensively exposed to this protein. If, on the other hand, the reason for these merozoites' failure to invade RBCs is because they have not undergone secondary processing, these merozoites will not have processed $PfMSP1_{19}$ exposed on their surface.

In conclusion, for whatever reason, life long exposure to the conserved protein, $PfMSP1_{19}$, can be insufficient to induce an antibody response. The low positivity rate is unlikely to be due to polymorphism of protective epitopes since most sera tend to recognise epitopes which are cross-reactive between the two prototypes. $PfMSP1_{19}$ is recognised less frequently and less rapidly than other merozoite proteins (for example PfMSP2, R. Taylor *et al*, in press), suggesting that $PfMSP1_{19}$ is not particularly immunogenic. If this is due to a lack of exposure to epitopes within the processed $PfMSP1_{19}$, then immunisation with a recombinant protein may induce a significantly better antibody response than that induced by native protein during natural infection. There is evidence from mouse models that experimental vaccination with recombinant proteins does indeed induce a qualitatively and quantitatively better protective response than that induced with native MSP1 (Ling *et al* 1994). The fact that the naturally immunodominant epitopes within $PfMSP1_{19}$ are cross-reactive between the two prototypes of the protein suggests that if a protective vaccine based on $PfMSP1_{19}$ could be produced, it would protect against all strains of *P. falciparum*.

CHAPTER FIVE:
Characterisation of the biological significance of C-terminal PfMSP1-specific antibodies

Introduction

The preceding chapter describes the prevalence of antibodies to epitopes within the C-terminus of PfMSP1. However, the presence of antibodies to malarial antigens does not necessarily correlate with protective immunity (examples; Druilhe and Khusmith 1987, Riley *et al* 1992a). Individuals who develop clinical symptoms of malaria may have high levels of antimalarial antibodies. A more accurate way to identify the antibody responses involved in protective immunity is to assess the quality rather than the quantity of the antibody produced to malarial antigens. Thus, the next logical step in evaluating antibody responses to $PfMSP1_{19}$ was to assess the biological function of the antibody produced.

The EGF-like motifs are found in MSP1 in all species of malaria parasites sequenced to date (David *et al* 1984, Lewis 1989, Deleersnijder *et al* 1990, del Portillo *et al* 1991), suggesting that the structure is under some functional constraint. Many proteins containing these EGF-like motifs are involved in cell surface interactions such as receptor binding, protein adhesion, or signalling (Appella *et al* 1988, Engel 1989). If $PfMSP1_{19}$ fulfils one of these functions, this would explain why the structure is conserved.

There is evidence to suggest that the EGF-like motifs are an important target of protective immunity. In studies using the rodent malaria models *P. yoelii* and *P. chabaudi*, three MSP1-specific mAbs (Py302, PccNIMP23 and Pca5C10/66) passively protected mice against challenge infection with homologous blood-stage parasites (Boyle *et al* 1982, Majarian *et al* 1984, Lew *et al* 1989a). Two of these mAbs (Py302 and PccNIMP23) bind to epitopes within the EGF-like motifs (Burns *et al* 1988, Burns *et al* 1989a, McKean *et al* 1993a). Mice immunised with this cysteine-rich region of *P. yoelii* MSP1, expressed as a fusion protein in *E. coli*, were partially or completely protected against challenge infection with *P. yoelii* parasites (Daly and Long 1993, 1995, Ling *et al* 1994). Additionally, all PfMSP1-specific mAbs which inhibit the growth of the parasites *in vitro* (Pirson and Perkins 1985, Blackman *et al* 1990, Cooper *et al* 1992) have been shown to bind to conserved, conformation-dependent epitopes within $PfMSP1_{19}$ (McBride and Heidrich 1987, Cooper *et al* 1992, Chappel and Holder 1993,

Burghaus and Holder 1994). Finally, serum from rabbits immunised with a correctly folded, insect cell-expressed, $PfMSP1_{42}$ protein (but not with an identical but incorrectly folded yeast cell product) completely inhibited *P. falciparum* growth *in vitro* (Chang *et al* 1992). The biological activities of these $PfMSP1_{42}$-specific antibodies were cross-reactive as sera inhibited the *in vitro* growth of homologous and heterologous parasites with equal efficiency (Hui *et al* 1993).

Native, recombinant and synthetic polypeptides representing PfMSP1, or fragments of it, have been shown to induce partial or complete protection, from subsequent homologous and heterologous challenge infection in monkeys (Hall *et al* 1984a, Perrin *et al* 1984, Cheung *et al* 1986, Patarroyo *et al* 1987a, 1987b, Siddiqui *et al* 1987, Holder *et al* 1988, Herrera *et al* 1990, Rodriguez *et al* 1990, Etlinger *et al* 1991, Herrera *et al* 1992, Kumar *et al* 1995). In one study, carried out by Hui and Siddiqui (1987), where *Aotus* monkeys were vaccinated with mAb affinity purified native PfMSP1, complete protection from an otherwise lethal challenge was obtained. The mAb used to purify native PfMSP1 is specific for a conformational epitope within $PfMSP1_{42}$, so the antigen preparation will have been highly enriched for the C-terminal processing fragment. Kaslow and co-workers immunised *Aotus* monkeys with a yeast recombinant protein representing $PfMSP1_{19}$ which completely protected two of the four monkeys from a lethal challenge infection. A third monkey had a prolonged prepatent period but eventually required treatment, the fourth monkey was unprotected (Kumar *et al* 1995). In the Siddiqui study, a correlation was seen between protection in PfMSP1-immunised monkeys and the ability of their serum antibodies to inhibit parasite growth *in vitro* ; such a correlation was not seen in the Kumar study.

In collaborative work, Dr. R. Ballou (Walter Reed Army Institute of Research, Washington DC, USA) and Dr. A. Holder have immunised *Aotus* monkeys with an *E. coli*-derived, *P. falciparum* double EGF-like motif GST fusion protein (Well-19/GST). No protection was observed in any of the animals, relative to the GST controls, for either homologous or heterologous challenge infections. 3 of the 8 monkey sera were able to inhibit parasite invasion of RBCs in *vitro* (Burghaus *et al*, manuscript in preparation).

Although the antigenic specificity of the antibody produced will be important for protection, i.e. directed to protective epitopes, the functional capacity of this antibody can be compromised if it is not of a suitable subclass for the function required from it. Studies have shown that, when exposed to malaria infection, non-immune donors produced predominantly

non-cytophilic IgG2 antibodies but that immune adults have high levels of cytophilic antimalarial IgG1 and IgG3 antibodies (Wahlgren *et al* 1983, Bouharoun-Tayoun and Druilhe 1992). Bouharoun-Tayoun and Druilhe have shown that antibodies of different IgG subclass can have the same epitope specificity and suggest that competition for epitope-binding can cause a blocking effect, where non-cytophilic classes (IgG2 and IgM) preferentially bind to the epitope preventing the binding of cytophilic (and presumably functional) antibodies (IgG1 and IgG3). They suggest that the long time taken to develop effective antimalarial immunity may correspond to a progressive modification of the immune response from non-protective antibody to protective antibody. They have previously shown that antibodies, by themselves, are unable to directly limit parasite growth, but act indirectly by stimulating blood monocytes via their Fc receptor after binding to their parasite target (Bouharoun-Tayoun *et al* 1990). They found that IgG from immune people co-operated efficiently with monocytes while IgG from non immunes did not.

In this chapter I report experiments to determine the biological function of human and monkey polyclonal antibodies to $PfMSP1_{19}$;

1) I have performed competition ELISAs to investigate whether human sera and vaccinated monkey sera recognise the same epitopes as growth inhibitory mAbs;

2) I have characterised human anti-$PfMSP1_{19}$-specific and anti-$PfMSP1_{33}$ antibodies in terms of their IgG subclass;

3) I have affinity selected antibodies from pooled adult malaria-immune IgG to the double-motif and to the second EGF-like motif of $PfMSP1_{19}$ to determine whether antibodies to this region are able to inhibit the *in vitro* parasite invasion of RBCs either directly or in a cell-mediated, antibody-dependent manner.

Methods and materials

(1) Antigens

Recombinant proteins used in this study have previously been described in chapter 2. *E. coli*-derived GST fusion proteins representing the first EGF-like motif of Wellcome (Well-EGF1) and MAD20 (MAD-EGF1) sequences of $PfMSP1_{19}$, the second EGF-like motif of the MAD20 $PfMSP1_{19}$ (MAD-EGF2), the double EGF-like motif of the Wellcome $PfMSP1_{19}$ (Well-19/GST) with GST cleaved and uncleaved and, the MAD20 $PfMSP1_{33}$ (MAD-33), were used.

(2) Sera

Human sera from the age cross-sectional survey, previously described in chapter 4, were used.

Monkey sera was obtained from 14 *Aotus* monkeys. Three monkeys had undergone a malaria infection and had been drug cured. Three monkeys had been immunised with GST-cleaved $PfMSP1_{19}$ (Well-19/GST) and underwent a homologous challenge infection with the FVO strain of *P. falciparum* (Wellcome-like). Three monkeys were immunised with Well-19/GST and underwent a heterologous challenge infection with the CAMP strain of parasites (MAD20-like). Five monkeys were immunised with GST and challenged with either FVO or CAMP parasites. Sera was collected prior to immunisation (Burghaus *et al* manuscript in preparation).

(3) MAbs

The following $PfMSP1_{19}$-specific murine mAbs were used: 2.2, 7.5, 12.8, and 12.10 (McBride and Heidrich 1987); 111.2 and 111.4 (Holder *et al* 1985); 5B1 (Pirson and Perkins 1985); and 14-1C (R. Reese, Agouron Institute, La Jolla, California), see table 2.1.

(4) ELISA

Previously described in chapter 4.

(5) Titration ELISAs of affinity purified IgG and vaccinated monkey sera

Monkeys were immunised at day 1, 30 and 60 and challenged at day 67 with 10^5 parasitised RBCs (Burghaus *et al* manuscript in preparation). Each dose contained 74mg of Well-19/GST or 40mg of GST in liposome adjuvant absorbed to alum prior to injection. Sera were collected prior to challenge

infection (on day 67). Monkeys were challenged with either homologous (Wellcome-like [FVO]) or heterologous (MAD20-like[CAMP]) parasites.

To assess the purity, specificity and titre of affinity selected IgG to the first, second- and double-motif proteins, all the antibodies were titrated from high to low concentrations on Well-EGF1, MAD-EGF2, cleaved Well-19/GST, and GST. The rest of the ELISA was performed as previously described in chapter 4.

(6) MAb competition ELISAs

Competition ELISAs were performed to determine whether epitopes recognised by human antibodies or monkey sera were similar to those recognised by murine mAbs. To see if the human and monkey antibodies could inhibit binding of the mAbs, plates coated with cleaved Well-19/GST, were first incubated with varying concentrations of serum. After extensive washing, mAb (at a dilution which results in an OD just at the top of the sigmoidal titration curve) was added to the plate, incubated for 3 hours and developed with HRP-anti-mouse IgG and OPD. The reciprocal experiment was also performed to see if mAbs could inhibit binding of the sera. In this case, antigen coated plates were first incubated with varying concentrations of mAb, then with serum and, finally, with HRP-anti-human IgG. Anti-human IgG cross-reacts with *Aotus* IgG: OD values for HRP-anti-human IgG and anti-*Aotus* IgG were identical for monkey sera (data not shown); I was therefore able to carry out all ELISAs with anti-human IgG.

(7) Purification of human IgG

Human IgG, derived from clinically immune adults from The Ivory Coast where malaria is endemic, was kindly provided by Dr. P. Druilhe (Institut Pasteur, Paris, France). 400-500ml of blood was collected from 333 adults aged 19-23. IgG was separated by the Cohn ethanol method and further treated by mild pepsin digestion at pH 4.0 (Sabchareon *et al* 1991). European IgG, from heat inactivated sera of healthy blood group A+ donors, provided by Dr. A. Holder, was used as a negative control (Eur-IgG).

(8) Affinity purification of PfMSP1$_{19}$ double-motif and second EGF-like motif-specific IgG

IgG was affinity purified on PfMSP1$_{19}$ fusion proteins immobilised on an insoluble matrix (Harlow and Lane 1988). Purified fusion protein (30mgs of MAD-EGF2 or 10mgs of Well-19/GST) was bound to cyanogen bromide activated Sepharose (Pharmacia) at 2mg of protein/ml swollen gel,

according to manufacturer's instructions. Purified total-IgG was passed over a MAD-EGF2 and then a Well-19/GST affinity column at a rate of 30ml/hour for three hours (this total-IgG had previously been passed over a Well-EGF1 column by Dr. J. Chappel). IgG which passed through both columns, and therefore was regarded as being non-PfMSP1$_{19}$-specific, was collected (void-IgG). The column was washed over night in 100mM, then 10mM, Tris-HCl pH 8.0. Bound IgG was eluted from each column separately with two gel volumes of 100mM glycine pH 2.5 into 1/10 final volume 1M Tris-HCl pH 8.0, to restore neutral pH (OD's of eluate samples were measured by spectrophotometer; elution ceased when OD reached a base level).The columns were then washed with 100mM Tris-HCl pH 8.0 with 0.02% (w/v) azide to regenerate the column and stored at 4°C.

Eluted IgG was dialysed into 100mM Tris-HCl pH 8.0 and then concentrated to 1-2mg/ml in an ultrafiltration unit (Amicon, PM10 filter). IgG purity was assessed by analysis of Coomassie blue stained SDS PAGE gels, and IgG yield was quantified assuming that 1mg/ml IgG equals an OD of 1.4 at A_{280} (Harlow and Lane 1988).

The following prefixes will be used; 'total-' refers to the total IgG present before the affinity purification; 'AP-' refers to the affinity purified IgG to a specific antigen; 'void-' refers to the remaining IgG after depletion of IgG specific to the PfMSP1$_{19}$ antigens; 'Eur-' refers to European control IgG.

(9) *In vitro* parasite culture

P. falciparum clones 3D7 and K1 were maintained in O+ RBCs at 6% haematocrit in complete RPMI (RPMI 1640 medium [Gibco] supplemented with 25mM HEPES, 24mM $NaHCO_3$, 25mg/ml gentamicin, 50g/l hypoxanthine, 10% [v/v] human O+ serum). Culture medium was changed daily. Cultures were grown in a CO_2 incubator at 37°C. Thin blood film smears (methanol fixed and Giemsa stained) were made daily. Parasite cultures were synchronised by a combination of sorbitol which effectively kills all stages apart from the 'rings' (Lambros and Vanderberg 1979) and percoll (Sigma) (Holder and Freeman 1982) when appropriate.

(10) *In vitro* parasite invasion inhibition assay and antibody-dependent cellular-inhibition assay

Test IgG's were dialysed into incomplete RPMI, under sterile conditions, to give a concentration of 2mg/ml in 0.5ml final volume. The antibodies were filter sterilised (0.2µm pore size, micro-centrifuge tube filter, Whatman) before being aliquoted into 96-well round bottomed microtitre plates. 3D7

and K1 late trophozoites/early schizonts were purified, from high parasitaemia cultures, and diluted with RBCs to give a 1% parasitaemia and 4% haematocrit. Parasites were purified on a 60% percoll gradient where 'segmenters' are retained in the top band.

Test IgG's were used at three concentrations; 1mg/ml, 100µg/ml and 10µg/ml (final concentration in the well). Control wells contained either 5mM EGTA, pH8.0, final concentration, or complete RPMI only. EGTA at this concentration effectively inhibits merozoite invasion of RBCs (McCallum-Deighton and Holder 1992). Double the final concentration of the test IgG or EGTA in (50µl complete RPMI) was added to each well. To this was added 50µl of the parasite/RBC mix to equal a final concentration of 0.5% parasitaemia and 2% haematocrit. Late trophozoites/early schizonts were used to be certain that no schizonts had ruptured and invaded new RBCs before being added to the test wells.

Antibody-dependent cellular inhibition assays (see figure 5.1. for schematic diagram of this assay) were run in parallel to the invasion inhibition assays, where adherent mononuclear cells from healthy European blood donors were added to wells containing test IgG plus schizonts and RBCs: 10µl of $1x10^7$/ml monocytes were added to each well, i.e. 10^5 cells per well. (10µl less of complete RPMI was added to these wells to equal a final volume of 100µl/well as before.) Control wells contained monocytes, parasites and RBCs without antibody.

Each test was performed in four identical wells. After 36 hours growth, at 37°C in a CO_2 incubator, duplicate thin blood smears were made from each well of the invasion inhibition assay. Smears were made after 4 days for the antibody-dependent cellular-inhibition assay. The final parasitaemia was determined by microscopic examination of the number of ring stage parasites within 35 fields or approximately, on average, 2000 RBCs per methanol-fixed, Giemsa-stained smear. Multiple ring parasites within one RBC were recorded as a single infected cell.

(11) ELISA to determine IgG subclass

ELISA assays were performed as usual except that monoclonal, subclass-specific, mouse anti-human IgG was used as the second step reagent. Subclass-specific assays were optimised by titration (by Dr. R. Taylor, University of Edinburgh, UK), such that the titration curves for each reagent were co-incident: i.e. that for a given absolute concentration of IgG1, IgG2, IgG3 or IgG4, the same OD value was obtained. The reagents used were mouse monoclonal anti-human IgG1(1:2000; code 1170317; Boehringer,

Mannheim, Germany), IgG2 (1:500; code 1170309; Boehringer), IgG3 (1:1000; code MCA516; Serotec, Oxford, UK) and IgG4 (1:500; code 1170287; Boehringer).

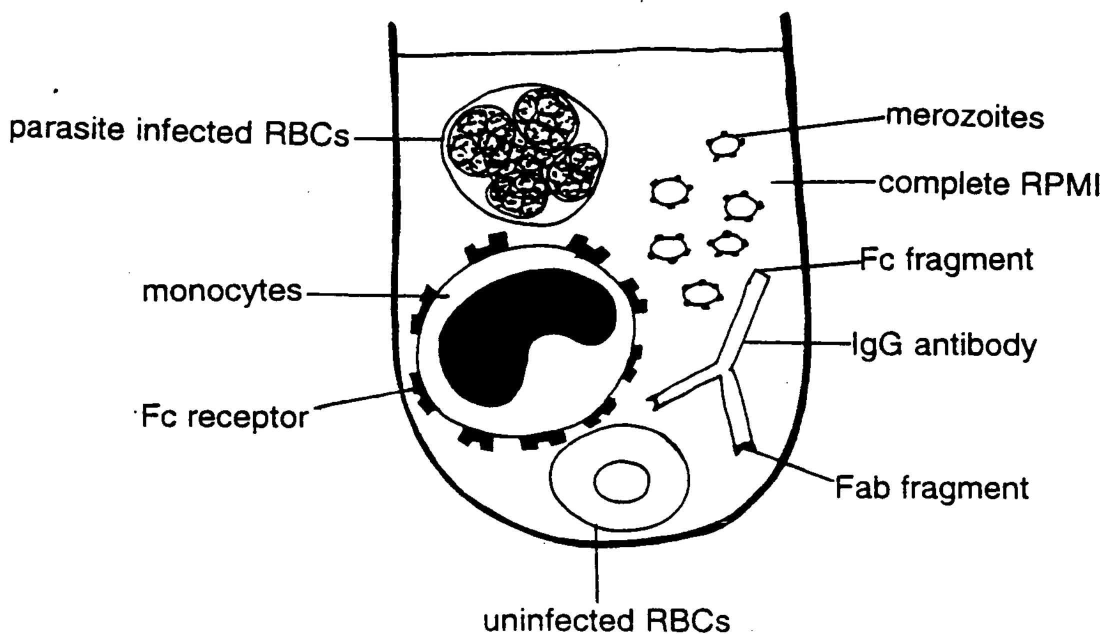


Figure 5.1. Schematic diagram of antibody-dependent cellular-inhibition assay. A 96-well microtitre plate contains human peripheral blood monocytes, uninfected RBCs, parasitised RBCs (and subsequently merozoites), and IgG antibody. Merozoites express $PfMSP1_{19}$ on their surface. The Fab fragment of the IgG may bind to this antigen, and so expose the Fc fragment to monocytes which have Fc receptors on their surface. Monocyte Fc receptors bind the antibody bound to the merozoite and either phagocytose the complex, or release toxic radicals which kill the parasite.

Results

(1) Characterisation of antibodies which compete with mAb binding

(1a) Human polyclonal antibodies inhibit the binding of invasion inhibitory mAbs

In order to determine whether antibodies induced by malaria infection in humans recognise the same epitopes as mAbs (one of which, 12.8, inhibits merozoite invasion of RBCs), competition ELISAs were performed. Three mAbs were tested: 111.4 recognises Well-EGF1; 12.8 and 14-1C both recognise conserved epitopes in the first EGF-like motif (Well-EGF1 and MAD-EGF1) (Chappel and Holder 1993, Burghaus and Holder 1994). Results are summarised in table 5.1.

Four human sera with high titres of antibodies to $PfMSP1_{19}$ were tested and all were able to block the binding of all three mAbs in a dose-dependent manner (figures 5.2.a. and c. show two examples) indicating that the epitopes recognised by these mAbs are also recognised by human sera. However, in the reciprocal experiments, mAbs were not able to block binding of human antibodies (figures 5.2.b. and d.) suggesting that human sera recognise many epitopes in addition to those recognised by mAbs.

(1b) Characterisation of serum from $PfMSP1_{19}$-vaccinated monkeys by ELISA

Sera from immunised monkeys were assessed in ELISA to determine the specificity of the antibodies to epitopes within $PfMSP1_{19}$. The titres of $PfMSP1_{19}$-specific antibodies in the monkey serum samples were determined on the *E. coli*-derived, cleaved Well-19/GST, (figure 5.3.). All Well-19/GST-vaccinated monkey sera (except number 11) gave very similar titration curves and had end point titres of >1/48,000 to $PfMSP1_{19}$. There were no differences in antibody titre in monkeys who were given a homologous challenge compared to those given heterologous challenge. One vaccinated monkey (number 11) had a significantly lower antibody titre with an end point titre of 1/6000. Also included were three unvaccinated monkeys which had undergone malarial infection and been drug cured, and so would be expected to be immune on rechallenge (Diggs *et al* 1995). These monkeys had the lowest antibody titres (<1/3000) to $PfMSP1_{19}$, with one monkey (number 12) almost seronegative for $PfMSP1_{19}$. Five monkeys were vaccinated with the GST fusion partner of the protein alone (monkeys 1,2,3,7,8), and had no $PfMSP1_{19}$-specific antibodies.

human serum	50% reduction in OD for mAb 14-1C		50% reduction in OD for mAb 111.4		50% reduction in OD for mAb 12.8	
	on Well-EGF1	on MAD-EGF1	on Well-EGF1	on MAD-EGF1	on Well-EGF1	on MAD-EGF1
1001	1/1500-1/3000	1/750+	1/1500-1/3000	-	1/6000	1/750-1/1500
1120	1/1500-1/3000	1/750+	1/1500	-	1/6000	1/500-1/750
1019	1/400-1/800	1/200	1/100+	-	1/200-1/400	-
1189	1/750+	1/750+	1/750+	-	1/750-1/1500	1/750-1/1500

Table 5.1. Human antibodies block the binding of $PfMSP1_{19}$-specific mAbs to recombinant $PfMSP1_{19}$.

4 human sera were tested at varying concentrations. The concentration of serum which resulted in a 50% decrease in OD of the mAb is shown.

Figure 5.2. Competition ELISAs.

Human serum 1120 was able to inhibit the binding of mAbs in a dose-dependent manner, but mAbs were not able to inhibit binding of human antibody.

a) Serum 1120 inhibits binding of mAb 111.4 (mAb 111.4 recognises only Well-EGF1).

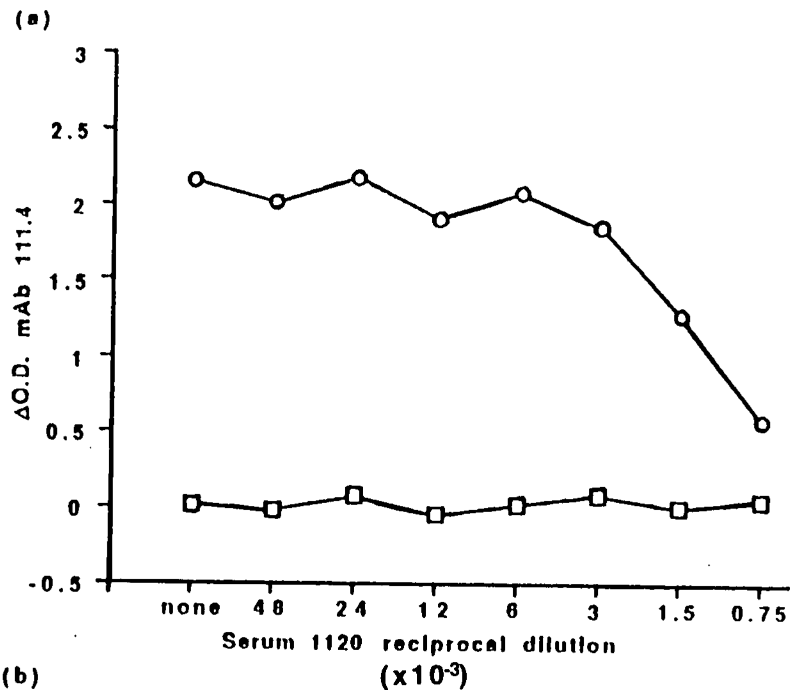
b) MAb 111.4 does not inhibit binding of serum 1120.

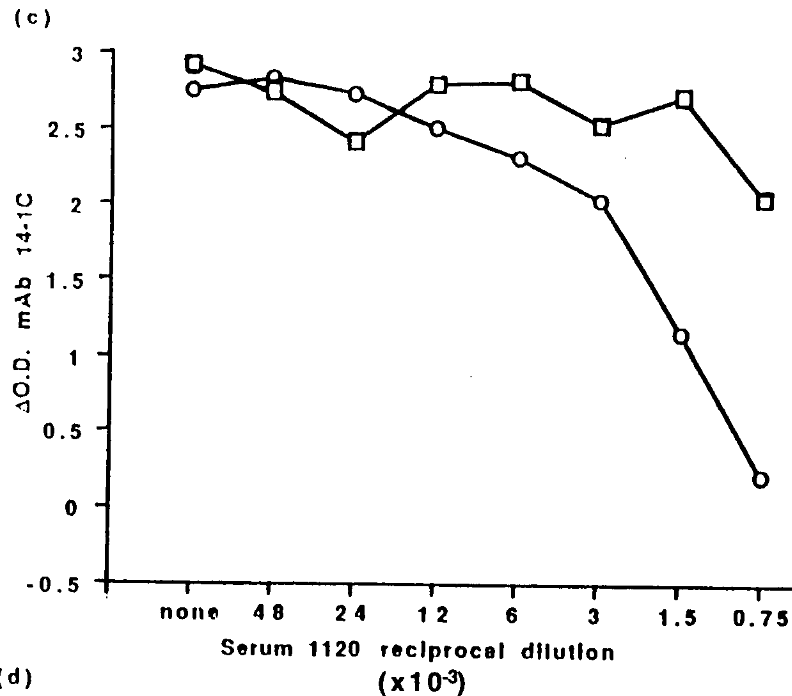
c) Serum 1120 inhibits binding of mAb 14-1C.

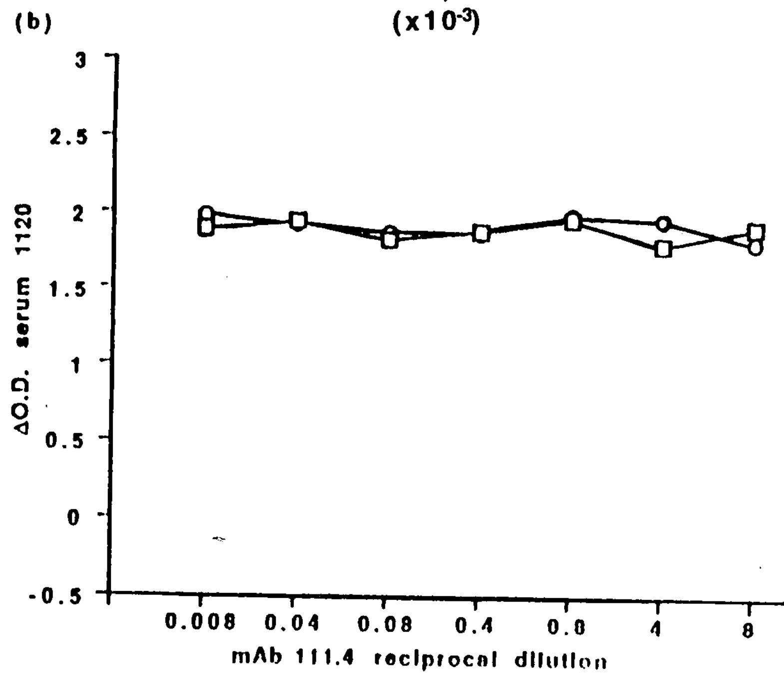
d) MAb 14-1C does not inhibit binding of serum 1120.

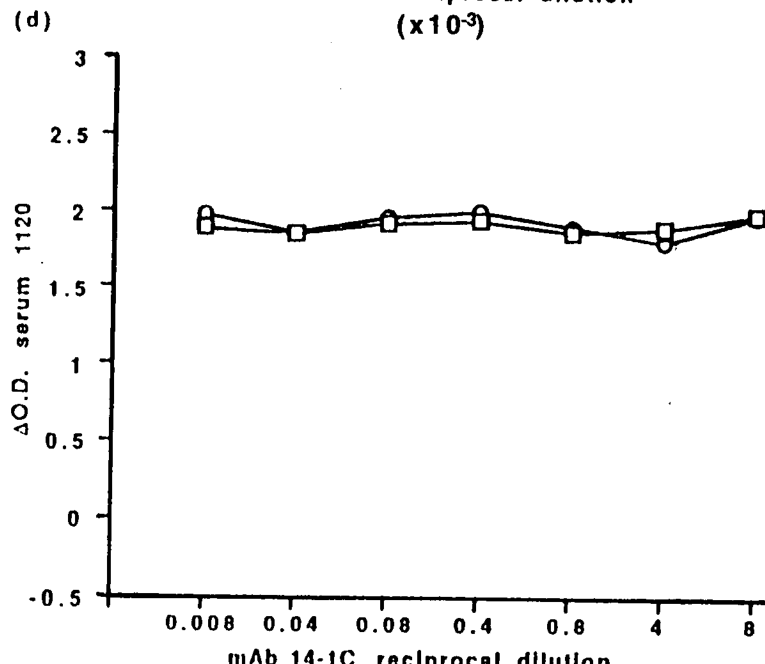
O, Well-EGF1,□, MAD-EGF2.

figure 5.2.









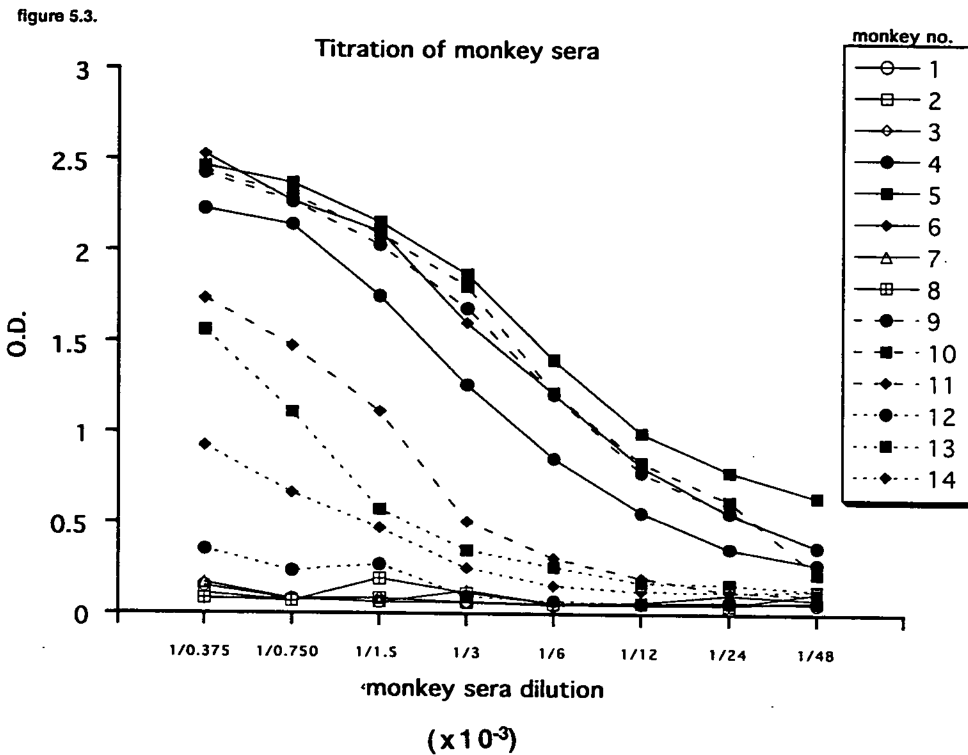


Figure 5.3. Titration of monkey sera on cleaved Well-19/GST.

Titrations were carried out to determine the serum dilution needed to obtain an OD at the top of the sigmodial titration curve for subsequent competition ELISAs.

monkeys 4, 5, 6 = vaccinated heterologous challenge infection

monkeys 9, 10, 11 = vaccinated homologous challenge infection

monkeys 12, 13, 14 = infected and cured

monkeys 1,2,3,7,8 = GST control infection

(1c) Sera from PfMSP1$_{19}$-vaccinated monkeys compete with protective mAbs for antigen binding

All 14 monkey sera were assayed for their ability to block the binding, to cleaved Well-19/GST, of mAbs 12.8 and 12.10 which are invasion inhibitory; 111.2 which does not inhibit invasion; and 2.2 and 7.5 which block the invasion inhibitory effect of 12.8 and 12.10. Results are summarised in table 5.2.

MAb 12.10 recognises the double EGF-like motif. The sera from vaccinated monkeys are able to compete out the binding of this mAb, but only at very high serum concentrations (1/25-1/50), (figure 5.4.a.).

MAb 12.8 recognises the first EGF-like motif. The sera from vaccinated monkeys are able to compete out the binding of this mAb, (figure 5.4.b.); inhibition begins at a dilution of around 1/250 (compared to 1/50 for mAb 12.10). Serum number 11, which had a low titre to Well-19/GST, began to compete with mAb 12.8 at a higher concentration than the others, but resulted in a similar level of inhibition at a dilution of 1/25; i.e. reduction in OD from 2.5 to background level of 0.5.

Serum from the vaccinated and challenged monkeys competed out the binding of the mAbs more effectively than the infected and cured monkey sera. Infected and cured monkey sera had little effect on either 12.8 or 12.10 mAb binding, except for monkey number 13 which resulted in approximately a 40% reduction in OD at 1/25 sera dilution for both mAbs.

The monkey sera were also tested for their ability to inhibit the binding of a non-invasion inhibitory mAb (111.2), and two mAbs which have been shown to block the inhibitory affect of 12.8 and 12.10 (2.2 and 7.5). For mAb 111.2, once again the sera from the vaccinated monkeys caused the greatest reduction of mAb binding. Sera from monkeys given a homologous challenge caused inhibition at a lower concentration than did sera from monkeys given heterologous challenge (figure 5.4.c.) suggesting that this mAb epitope includes a dimorphic amino acid residue. Sera from infected and cured monkeys inhibited binding of mAb 111.2 but at much higher concentrations than for vaccinated animals and serum 12 caused no reduction in mAb binding.

In the case of mAb 2.2, homologous challenged monkey sera competed at lower concentrations than the heterologous challenged monkeys, but both vaccinated groups totally inhibited mAb binding at higher concentrations (figure 5.4.d.). Infected and cured monkey sera were unable to inhibit this mAb from binding. Inhibition of mAb 7.5 binding by the monkey

treatment	monkey sera number	mAb 12.10		mAb 12.8		mAb 111.2		mAb 2.2		mAb 7.5	
		beginning of inhibition	% decrease in OD at 1/25 dilution	beginning of inhibition	% decrease in OD at 1/25 dilution	beginning of inhibition	% decrease in OD at 1/25 dilution	beginning of inhibition	% decrease in OD at 1/25 dilution	beginning of inhibition	% decrease in OD at 1/25 dilution
vaccinated heterologous challenge	4	1/50-1/250	67	1/250-1/500	100	1/500-1/2500	60	1/250-1/500	100	1/25-1/50	11
	5	1/50-1/250	50	1/500-1/2500	96	1/500-1/2500	56	1/500-1/2500	93	1/500-1/2500	48
	6	1/50-1/250	78	1/250-1/500	87	1/500-1/2500	100	1/500-1/2500	91	1/500-1/2500	75
vaccinated homologous challenge	9	1/50-1/250	33	1/250-1/500	87	1/250-1/500	64	1/2500-1/5000	84	1/500-1/2500	44
	10	1/50-1/250	73	1/500-1/2500	97	1/500-1/2500	78	1/2500-1/5000	94	1/500-1/2500	54
	11	1/50-1/250	68	1/50-1/250	81	1/250-1/500	78	1/500-1/2500	94	1/50-1/250	33
infected and cured	12	-	0	-	0	-	0	-	0	1/25-1/50	18
	13	1/25-1/50	35	1/25-1/50	43	1/50-1/250	69	1/25-1/50	11	1/50-1/250	90
	14	1/25-1/50	5	1/50-1/250	10	1/50-1/250	34	-	0	1/25-1/50	49

Table 5.2. Vaccinated monkey sera block the binding of $PfMSP1_{19}$-specific mAbs to $PfMSP1_{19}$ recombinant proteins.

The lowest concentration of serum which caused some inhibition of mAb binding is shown.

The % decrease in OD is calculated as

$$\frac{\text{OD with 1/25 serum - background OD}}{\text{maximum OD (no serum) - background OD}}$$

GST vaccinated monkeys resulted in no inhibition and are not shown in the table.

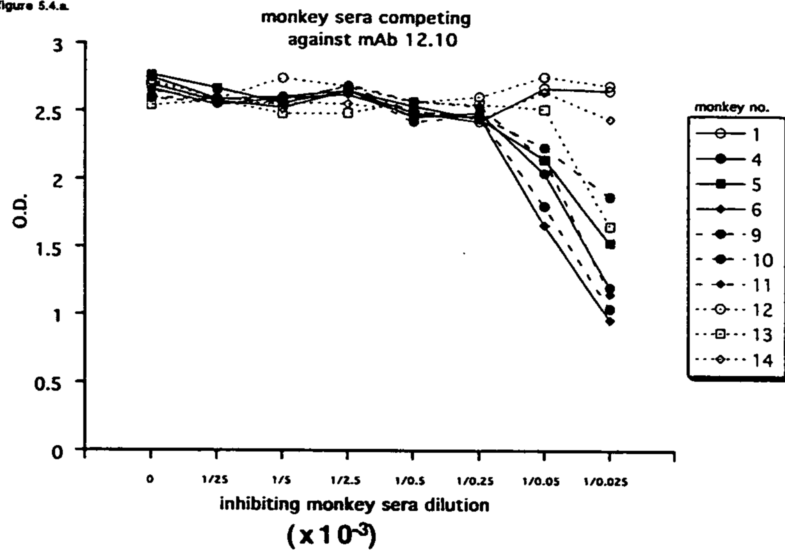


figure 5.4.a.
monkey sera competing
against mAb 12.10
3
2.5
2
1.5
1
0.5
0
O.D.
0
1/25
1/5
1/2.5
1/0.5
1/0.25
1/0.05
1/0.025
inhibiting monkey sera dilution
(x10-3)
monkey no.
1
4
5
6
9
10
11
12
13
14

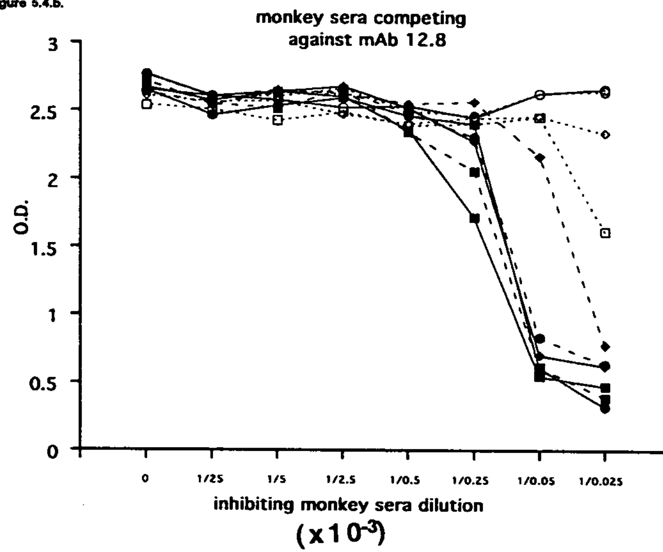


figure 5.4.b.
monkey sera competing
against mAb 12.8
3
2.5
2
1.5
1
0.5
0
O.D.
0
1/25
1/5
1/2.5
1/0.5
1/0.25
1/0.05
1/0.025
inhibiting monkey sera dilution
(x10-3)

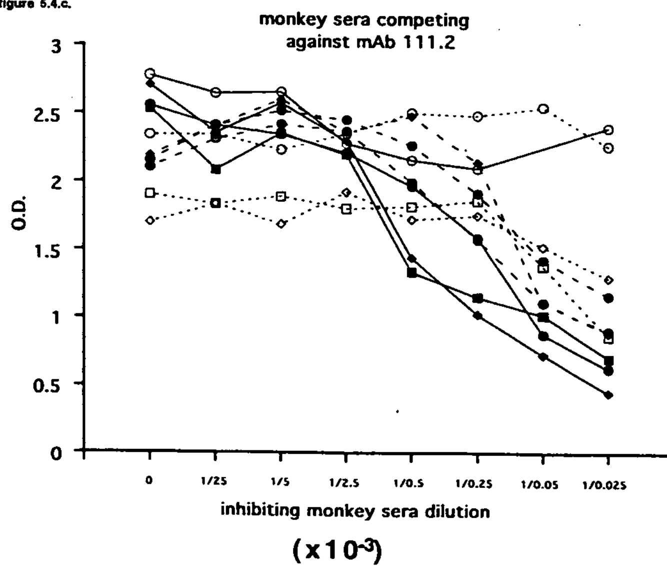
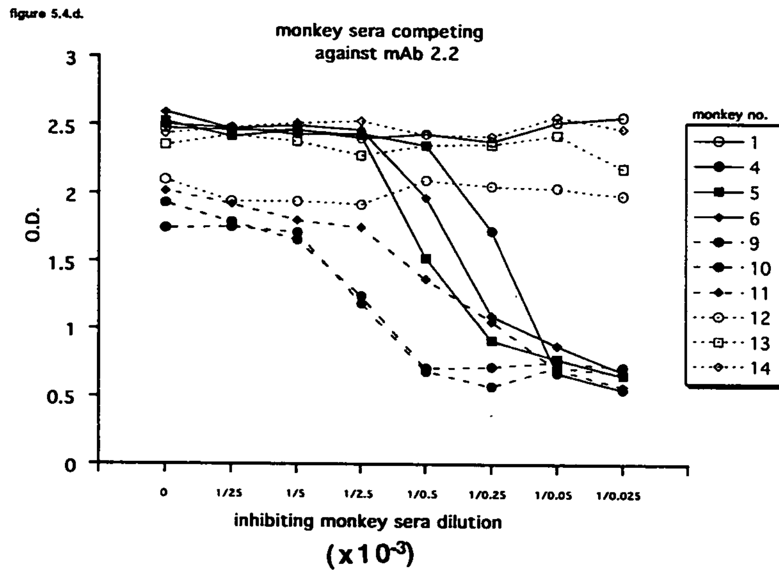


figure 5.4.c.
monkey sera competing
against mAb 111.2
3
2.5
2
1.5
1
0.5
0
O.D.
0
1/25
1/5
1/2.5
1/0.5
1/0.25
1/0.05
1/0.025
inhibiting monkey sera dilution
(x10-3)



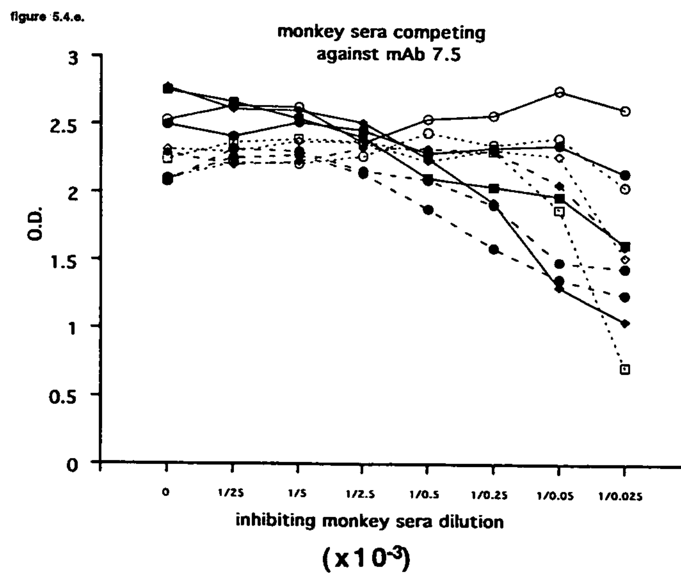


Figure 5.4. Competition ELISAs of monkey sera with mAbs.

Cleaved Well-19/GST coated plates were preincubated with monkey sera. Subsequent inhibition of binding by mAbs is measured.

a) mAb 12.10, b) mAb 12.8, c) mAb 111.2, d) mAb 2.2, and e) mAb 7.5.

monkeys 9, 10, 11 = vaccinated homologous challenge infection

monkeys 12, 13, 14 = infected and cured

monkeys 1 = GST control infection

sera was mixed, with each test group resulting in a high, medium or low inhibitory effect (figure 5.4.e.).

In summary, these experiments show that antibodies competing with inhibitory, non-inhibitory and blocking mAbs are stimulated during both vaccination and 'natural' infection, although lower antibody concentrations and antibody with lower affinity, are produced in 'natural' infection than with vaccination (see figure 5.3).

In the reciprocal experiments, the mAbs were unable to compete with the binding of the monkey sera, indicating that the monkey sera contain antibodies to a number of epitopes, of which those recognised by these mAbs may be only minor components (figures 5.5.a.b.).

(2) Characterising the human polyclonal IgG subclass response to the C-terminus of PfMSP1

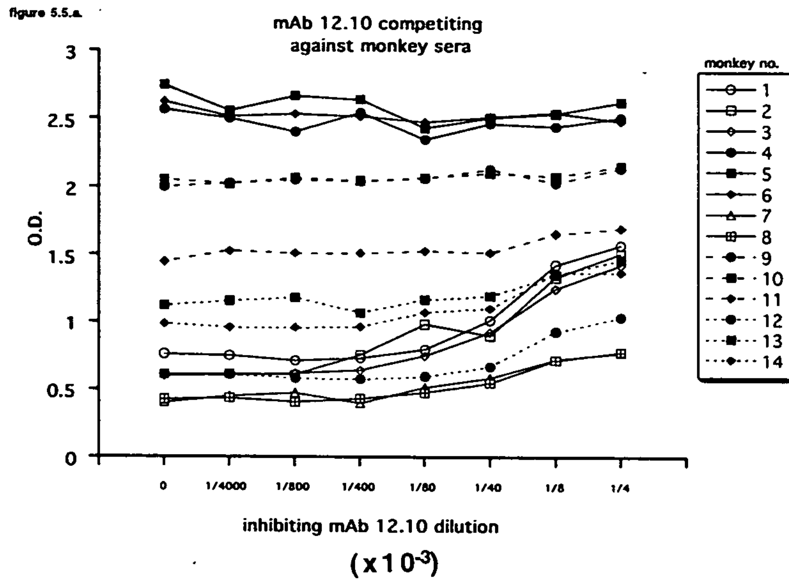
IgG subclass was determined by subclass-specific ELISA for all individuals, in the study population who were seropositive to proteins representing $PfMSP1_{19}$ (see previous chapter). I also measured the IgG subclass of antibodies against $PfMSP1_{33}$.

The IgG response to the $PfMSP1_{19}$ proteins was predominantly of the IgG1 subclass (figures 5.6.a-d. and table 5.3.). Occasionally individuals produced IgG3 antibodies instead of, or as well as, IgG1. The predominant IgG subclass did not vary among persons of different ages. However, both IgG1 and IgG3 antibodies were produced against $PfMSP1_{33}$, with an occasional IgG4 response, see figure 5.6.a.

(3) Invasion inhibition/parasite growth assays

(3a) Affinity purification of $PfMSP1_{19}$-specific IgG

Having shown that some of the human antibody response is similar to the invasion inhibitory mAbs 12.8 and 12.10 (section 1a), I determined whether human antibodies can inhibit RBC invasion *in vitro*, using antibodies affinity selected to $PfMSP1_{19}$. IgG specific to epitopes within Well-EGF1, MAD-EGF2 and Well-19 were absorbed on to an affinity column from a sample of 900mg of IgG which had been purified from 178 adult inhabitants of The Ivory Coast (Sabchareon *et al* 1991). Antibody was then eluted off the column and used for growth inhibition assays and competition ELISAs. Dr. J. Chappel affinity purified antibodies to the first EGF-like motif (APEGF1-IgG). I used the APEGF1-IgG-depleted IgG to affinity purify antibodies to the second EGF-like motif (APEGF2-IgG) and then to the double-motif protein (AP19-IgG).



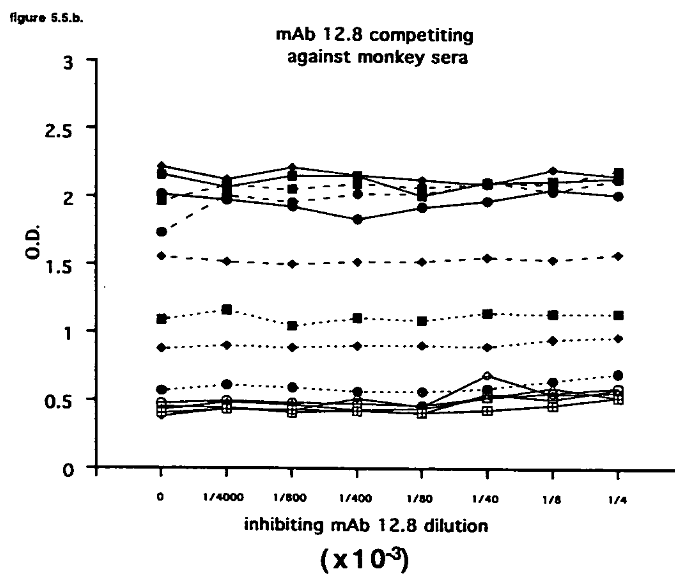


Figure 5.5. Competition ELISAs of mAbs with monkey sera.

Cleaved Well-19/GST coated plates were preincubated with mAbs. MAbs were unable to inhibit the binding of monkey sera.

a) mAb 12.10 and b) mAb 12.8.

monkeys 9, 10, 11 = vaccinated homologous challenge infection

monkeys 12, 13, 14 = infected and cured

monkeys 1 = GST control infection

figure 5.6.a.

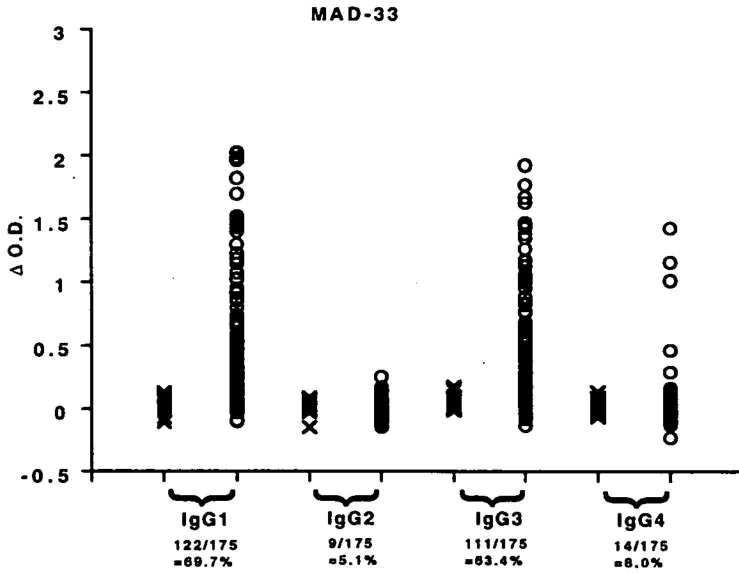


figure 5.6.b.

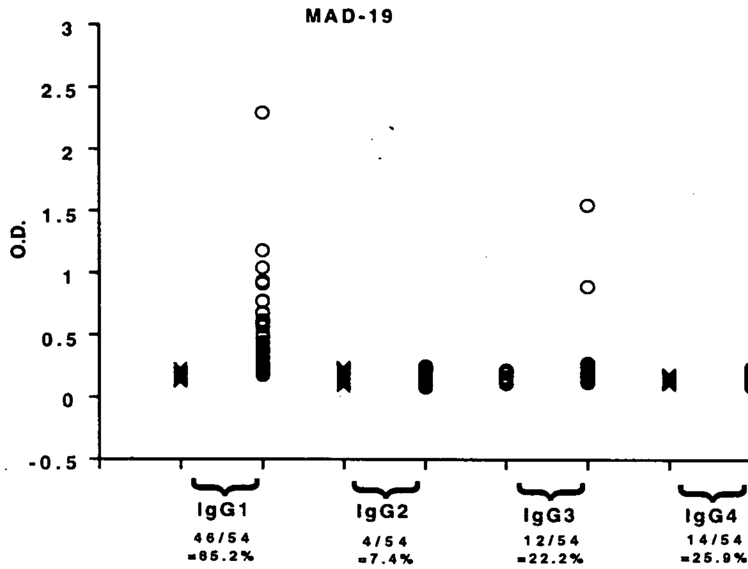
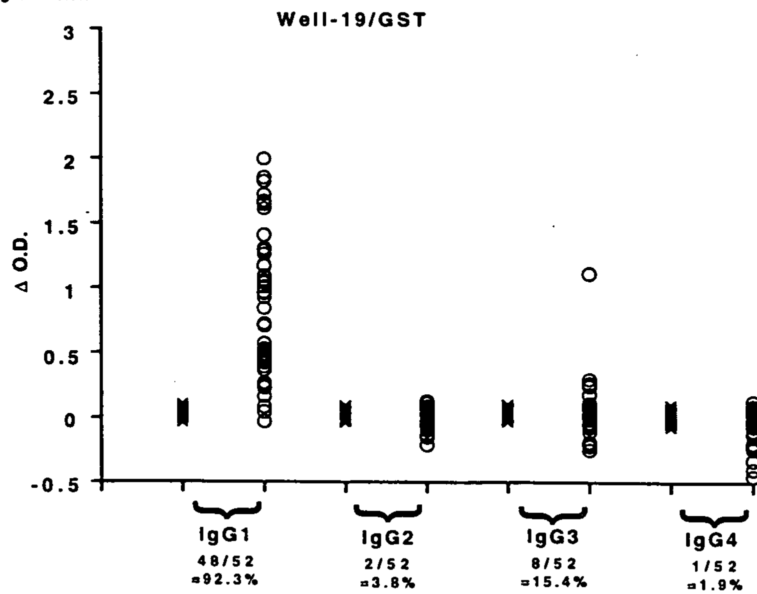
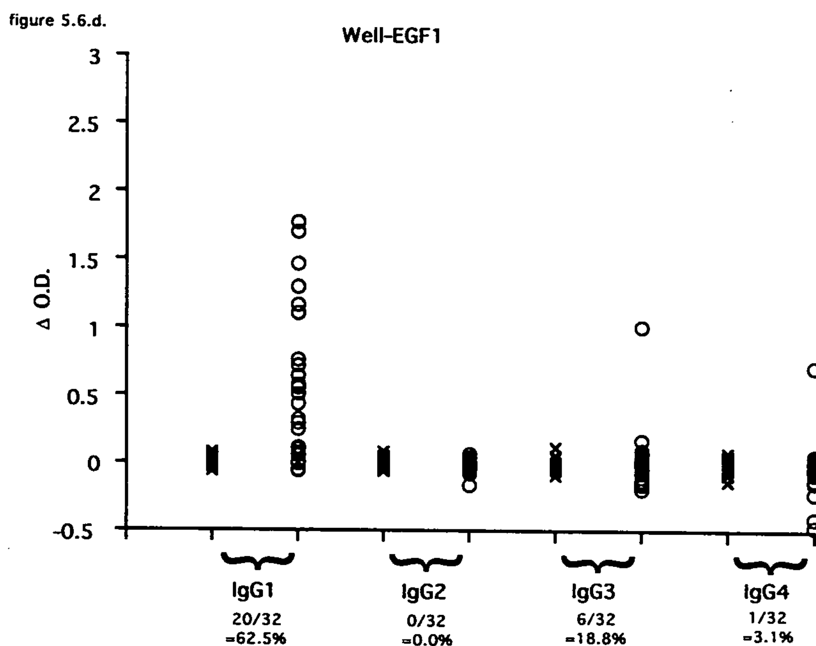


figure 5.6.c.





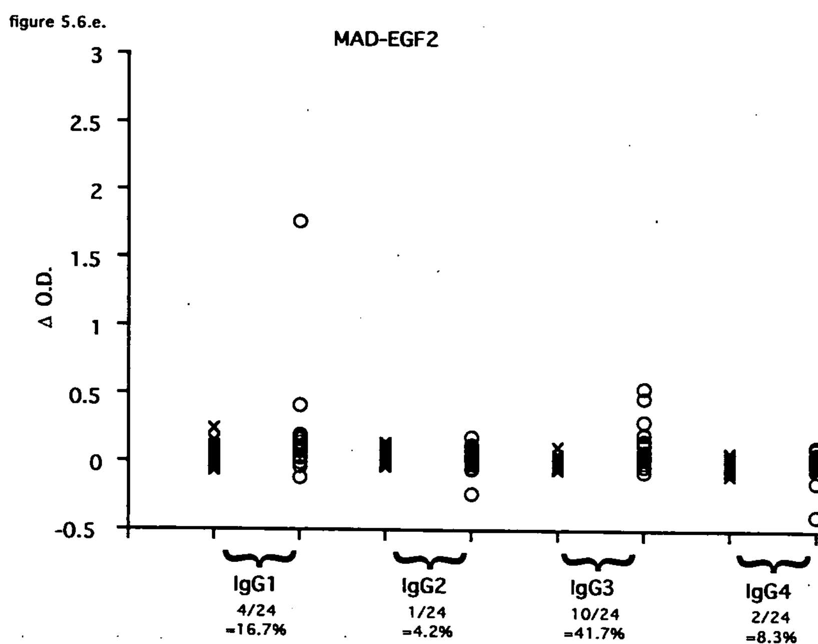


Figure 5.6. Dot plots showing IgG subclass-specific responses.

Only IgG positive Gambian sera were tested; 24 European sera are shown as controls. All sera were tested at a dilution of 1:1000.

a) MAD-33; 175 Gambian sera tested.

b) MAD-19; 54 Gambian sera tested.

c) Well-19/GST; 52 Gambian sera tested.

d) Well-EGF1; 32 Gambian sera tested.

e) MAD-EGF2; 24 Gambian sera tested.

Number and percentage of the sera with IgG subclass antigen specific antibody above the mean plus 2 SD of control (malaria non-exposed) sera are shown.

X, European sera; O, Gambian sera.

	n	IgG1 only	IgG3 only	IgG4 only	IgG1+2	IgG1+3	IgG1+4	IgG2+3	IgG1,2 +3	IgG1,2+ 4	IgG1,3 +4	IgG1,2, 3+4
Well-EGF1	21	14 (66.7)	1 (4.8)	0 (0.0)	0 (0.0)	5 (23.8)	1 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MAD-EGF2	12	1 (8.3)	7 (58.3)	1 (8.3)	2 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)
Well-19/GST	48	39 (79.6)	1 (2.0)	0.0 (0)	1 (2.0)	6 (12.2)	1 (2.0)	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
MAD-19	49	27 (56.3)	1 (2.1)	1 (2.1)	0 (0.0)	6 (12.5)	6 (12.5)	0 (0.0)	0 (0.0)	2 (4.2)	3 (6.3)	2 (4.2)
MAD-33	156	38 (24.4)	32 (20.5)	0 (0.0)	1 (0.6)	65 (41.7)	5 (3.2)	1 (0.6)	5 (3.2)	1 (0.6)	7 (4.5)	1 (0.6)

Table 5.3. IgG subclass response to the C-terminus of PfMSP1.

IgG subclass antibodies were measured in individuals who were seroposive to each antigen. Figures show the number (percentage) of IgG subclass 'responders' to each antigen, indicating how many individuals produced different combinations of IgG subclass antibodies. None of the sera contained only IgG2 antibodies. Responders are those where the specific OD of the IgG subclass antibody response to each antigen was greater than the mean plus 2 SD of the control (malaria non-exposed) sera.

(3b) Characterisation of affinity purified human antibodies by ELISA

Affinity selected antibodies were tested in ELISA to demonstrate the specificity, and to quantify the amount, of PfMSP1$_{19}$-specific antibodies in the AP-IgG in comparison with other IgG preparations (figure 5.7.). Differences in IgG concentrations were estimated by comparison of the parallel titration curves (Kemeny 1992).

APEGF1-IgG was estimated to contain 100-fold more Well-19/GST-specific IgG than the void-IgG. APEGF2 and AP19-IgG contained 10-fold more Well-19/GST-specific IgG than the void-IgG.

I also determined the IgG subclass of the PfMSP1$_{19}$ affinity preparations, and found they contained only IgG1 antibodies (ODs above background level only for IgG1; APEGF1=1.810, APEGF2=0.938, AP19=0.345) (data not shown).

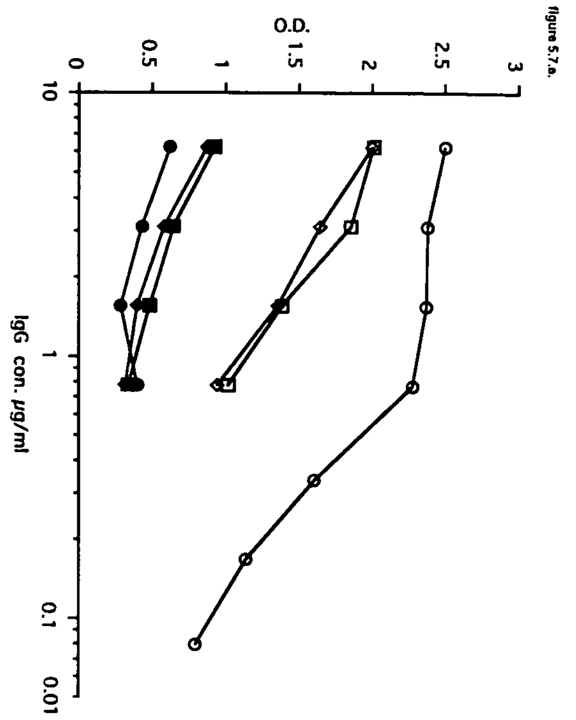
(3c) Affinity purified antibodies compete with protective mAbs for antigen binding

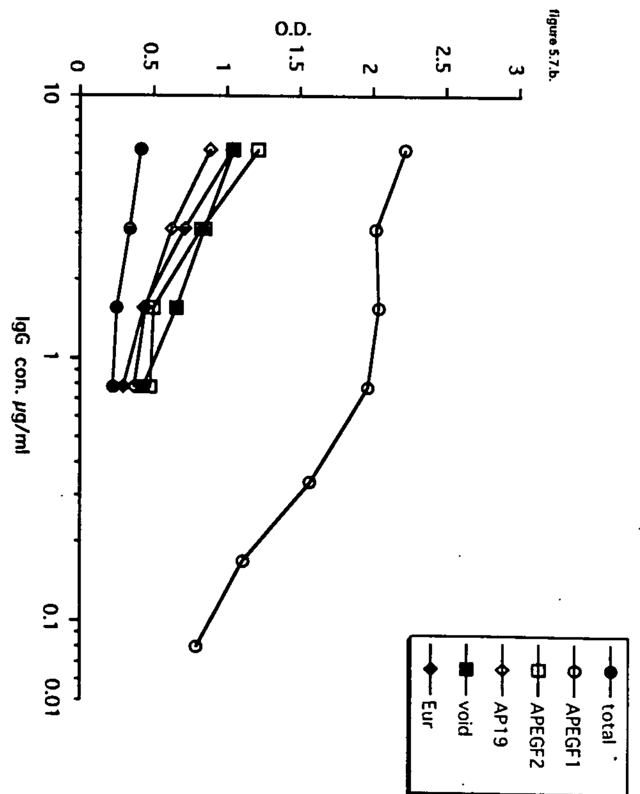
The different IgG preparations were assayed for their ability to block the binding of mAbs; 12.8, 12.10 and 5B1 which are invasion inhibitory; 111.2 which does not inhibit invasion; and 2.2 and 7.5 which block the invasion inhibitory effect of 12.8, 12.10 and 5B1 (figure 5.8.).

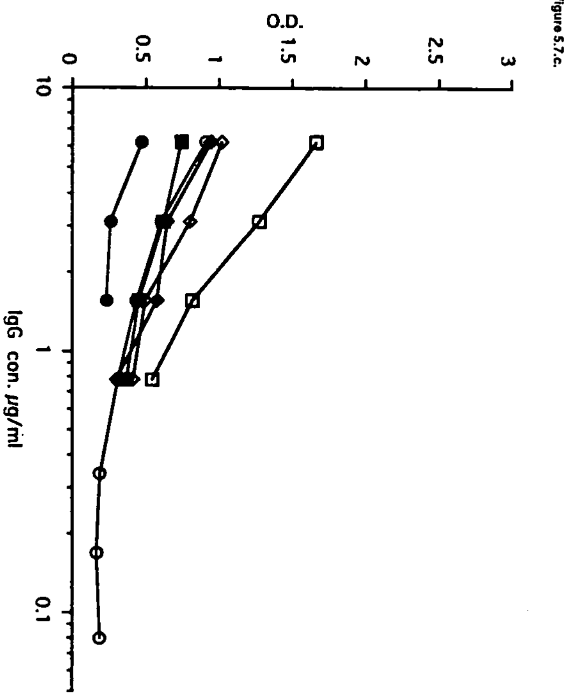
The APEGF1-IgG fraction was able to compete with all of the mAbs which bind to the first EGF-like motif (i.e. 12.8, 5B1, 2.2 and 7.5) but was especially effective at inhibiting 12.8 and 2.2. Inhibition of binding of all 4 of these mAbs began at between 2-4µg/ml of APEGF1-IgG. 10µg/ml of IgG resulted in 90-100% reduction in OD of mAbs 2.2 and 12.8, but caused only a 30% and 20% reduction of mAbs 7.5 and 5B1 respectively. This suggests that APEGF1-IgG has a higher affinity for the 12.8 and 2.2 -like epitopes. As expected, none of the other IgG preparations were able to inhibit binding of these four mAbs.

AP19-IgG was able to compete with mAbs 111.2 and 12.10 which bind to epitopes formed by sequences from both EGF-like motifs (figures 5.9.a.b.). Inhibition of binding began between 2-4µg/ml of IgG resulting in 80-100% reduction in OD at 4µg/ml. The other IgG preparations were not able to inhibit.

No mAbs directed to epitopes of the second EGF-like motif were available at the time to carry out competition ELISAs.







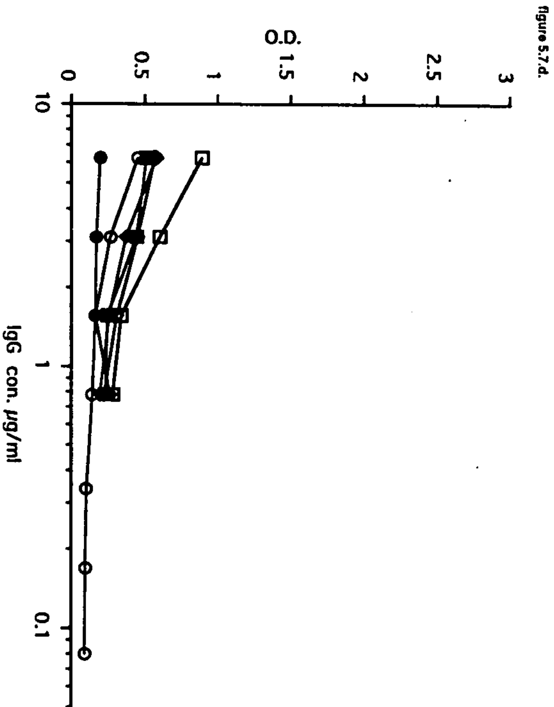
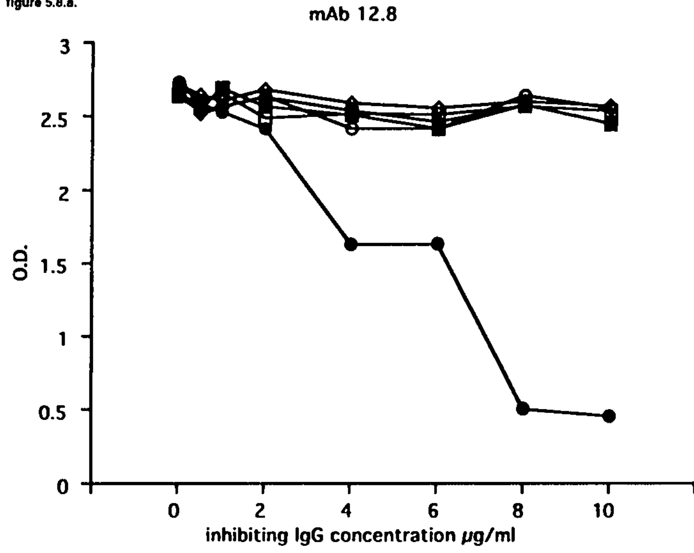
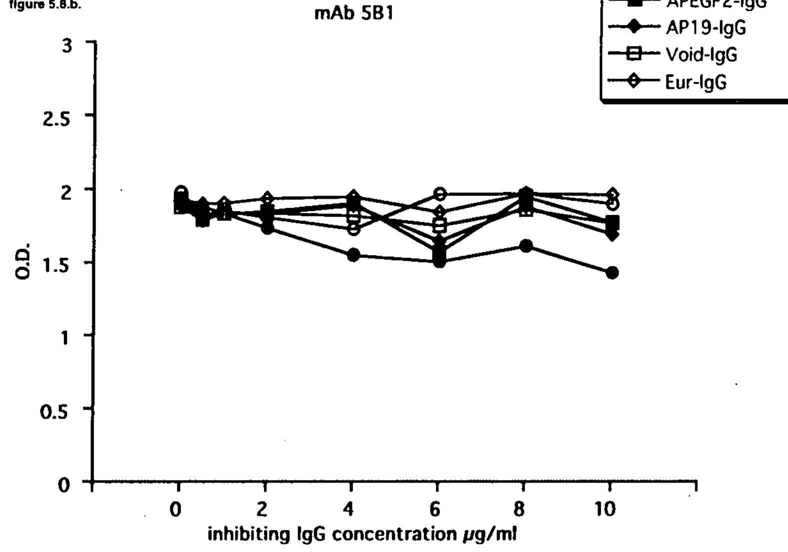


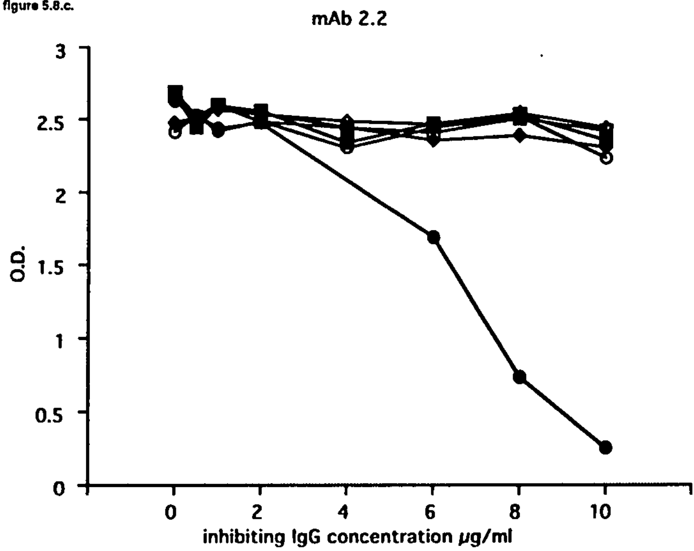
Figure 5.7. Titration of human IgG preparations.

Titrations were carried out to assess AP-IgG specificity and to determine the IgG concentration of each preparation needed to obtain an OD at the top of the sigmodial titration curve for subsequent competition ELISAs.

IgG titrated on; a) cleaved Well-19/GST; b) Well-EGF1; c) MAD-EGF2; d) GST.







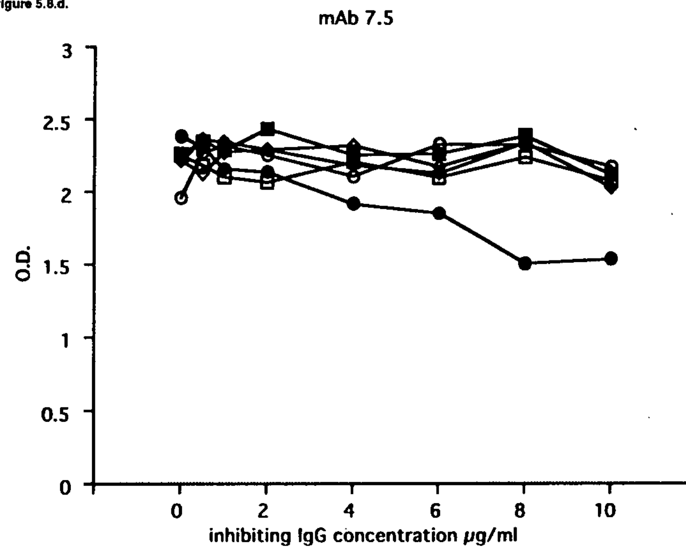
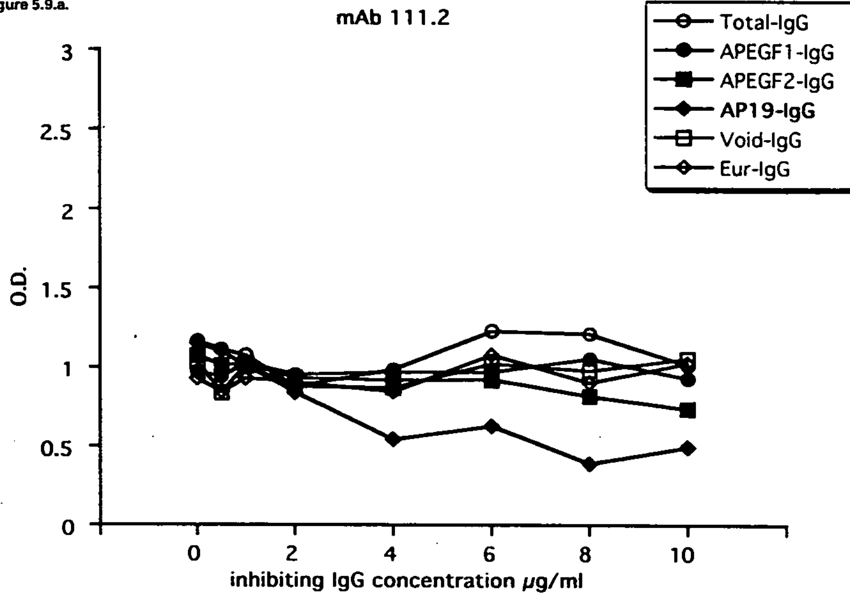


Figure 5.8 . Competition ELISAs to determine whether affinity purified human IgG preparations inhibits binding of mAbs.

Cleaved Well-19/GST coated plates were preincubated with human IgG. Subsequent inhibition of binding by mAbs (at optimal concentration) is measured.



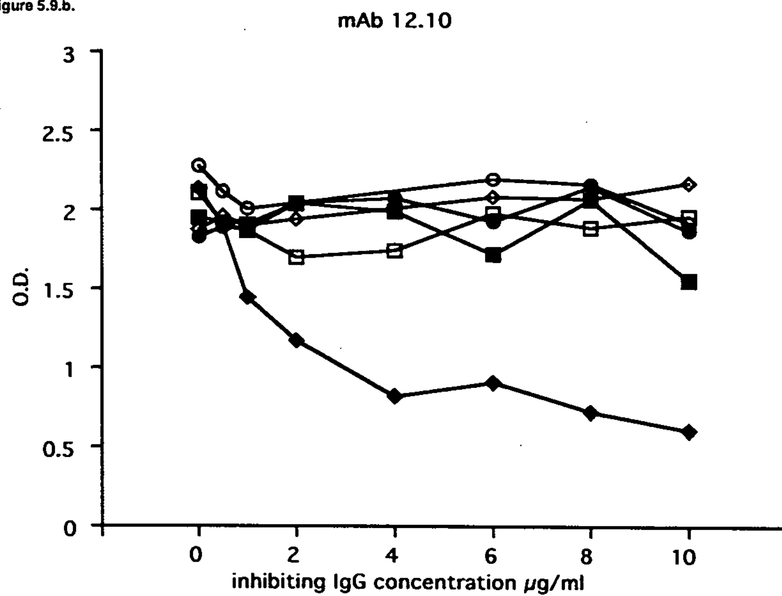


Figure 5.9. Competition ELISAs to determine whether affinity purified human IgG preparations inhibits binding of mAbs.

Cleaved Well-19/GST coated plates were preincubated with human IgG. Subsequent inhibition of binding by mAbs (at optimal concentration) is measured.

(3d) Parasite *in vitro* invasion assay

Highly synchronous late trophozoites/early schizonts were incubated with the various IgG preparations at a parasitaemia of 0.5%. Parasitaemias were determined after 36 hours, thus giving parasites ample time to rupture from the old RBCs and to allow reinvasion of new RBCs. The parasitaemia might be expected to increase approximately six fold in one reinvasion cycle, so should equal 3% if no inhibition of invasion had occurred. In the case of the K1 strain, parasites grown in complete RPMI, European-IgG, total-IgG and void-IgG resulted in a parasitaemia of approximately 3% (table 5.4.). The 3D7 strain, however, grew much more slowly in culture and only reached a parasitaemia of approximately 1.2% in complete RPMI and in European-IgG. EGTA was very effective in blocking invasion, resulting in 70-80% reduction in parasitaemia.

Parasites grown in human malaria-immune IgG affinity selected to $PfMSP1_{19}$ and the second EGF-like motif (but not the first EGF-like motif, see Chappel *et al* 1993) resulted in significantly lower parasitaemias compared to those obtained for parasites grown in complete RPMI (see figures 5.10.a.b.). Percentage inhibition of parasite invasion is highly statistically significant: $PfMSP1_{19}$-specific antibodies were able to inhibit parasite growth by up to 70%. Test IgG's were used at three concentrations; 1mg/ml, 100µg/ml and 10µg/ml. The most highly significant inhibition obtained with 1mg/ml of antibody. However, significant inhibition was obtained with as little as 10µg/ml of antibody (table 5.4.).

Antibodies selected to the Wellcome $PfMSP1_{19}$ and the MAD20 second EGF-like motif were equally effective against parasites from either PfMSP1 family. The morphology of the remaining parasites was normal on Giemsa-stained smears for all cultures, and no agglutinated clusters of merozoites were observed, indicating that the antibody was probably acting at the level of merozoite invasion.

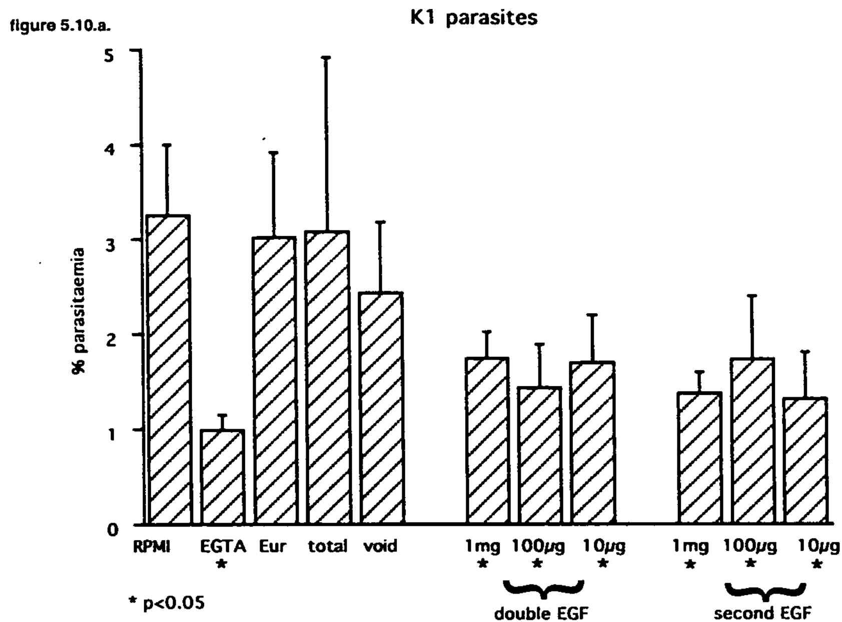
(3e) Antibody-dependent cellular-inhibition (ADCI) assay

Results from the ADCI assay are less easy to interpret. There was considerable variation between the replicates of each treatment leading to wide standard deviations (table 5.5.). In the 3D7 cultures (but not the K1 cultures) there was some evidence that the control IgG (void-IgG and Eur-IgG) preparations actually enhanced parasite growth when compared with cultures without IgG, but this was not statistically significant.

		1mg/ml		100µg/ml		10µg/ml	
		% parasitaemia	% inhibition	% parasitaemia	% inhibition	% parasitaemia	% inhibition
K1	European-IgG	3.02 + 0.89	7.23	2.17 + 0.84	33.38	2.23 + 0.51	31.34
	total-IgG	3.08 + 1.83	5.14	2.79 + 0.68	14.22	2.22 + 0.55	31.75
	void-IgG	2.43 + 0.75	25.14	2.30 + 0.32	29.14	2.17 + 0.52	33.23
	AP-Well-19/GST	1.74 + 0.28**	46.62*	1.43 + 0.46**	56.00**	1.69 + 0.51**	47.91*
	AP-MAD-EGF2	1.37 + 0.22***	57.94***	1.73 + 0.66*	46.77*	1.31 + 0.49**	59.60**
	EGTA	0.99 + 0.16***	69.3***				
	RPMI	3.25 + 0.75					
3D7	European-IgG	1.07 + 0.39	11.00	0.94 + 0.71	21.42	1.23 + 0.62	+2.33
	total-IgG	0.61 + 0.63	48.92	0.70 + 0.57	41.83	0.80 + 0.52	33.33
	void-IgG	0.66 + 0.24	45.00	0.74 + 0.27	38.03	0.63 + 0.55	47.50
	AP-Well-19/GST	0.35 + 0.24**	71.25**	0.47 + 0.25**	60.58**	0.60 + 0.11*	50.00*
	AP-MAD-EGF2	0.49 + 0.19**	59.33**	0.63 + 0.23	47.92	0.59 + 0.21*	50.83*
	EGTA	0.26 + 0.22**	78.3**				
	RPMI	1.20 + 0.39					

Table 5.4. In vitro parasite invasion inhibition assay.

Percentage parasitaemia (plus SD) and percentage inhibition of parasite growth relative to parasites grown in RMPI only.
*** = p<0.005
** = p<0.02
* = p<0.05



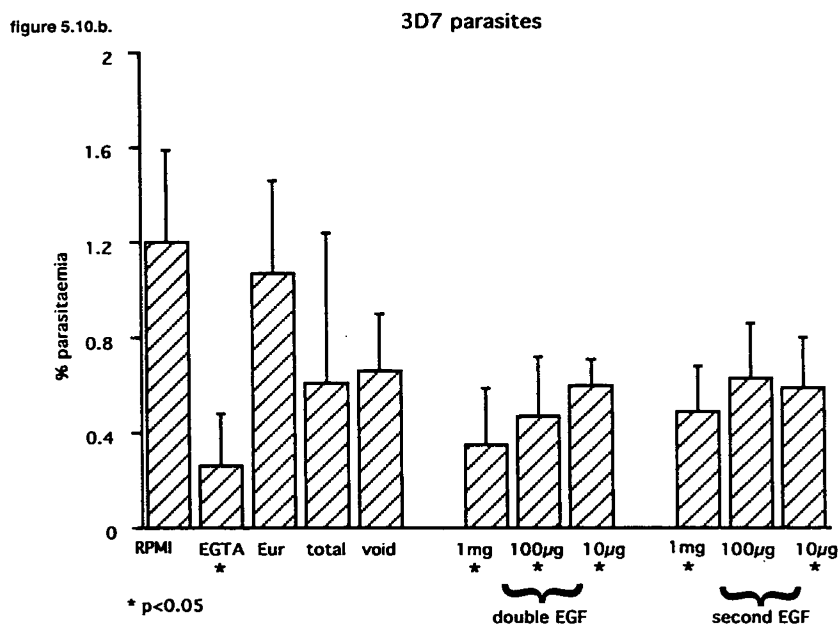


Figure 5.10. Bar chart of invasion inhibition assay.

Bars on chart represent mean final percentage parasitaemias, error bars indicate the standard deviation between parasitaemias for each test sample.

a) K1 parasites, b) 3D7 parasites.

		1mg/ml		100µg/ml		10µg/ml	
		% parasitaemia	% inhibition	% parasitaemia	% inhibition	% parasitaemia	% inhibition
K1	European-IgG	1.07 + 0.59		1.58 + 0.70		1.62 + 0.71	
	total-IgG	1.82 + 0.96	+70.84	1.69 + 1.34	+6.65	1.69 + 0.72	+4.64
	void-IgG	1.36 + 0.78	+27.10	2.76 + 0.14	+74.36	1.93 + 1.40	+19.16
	AP-Well-19/GST	1.22 + 0.37	+13.55	1.17 + 0.56	25.76	1.54 + 0.63	4.51
	AP-Well-EGF1	nd	nd	0.67 + 0.22	57.91	1.12 + 0.35	30.78
	AP-MAD-EGF2	1.71 + 1.22	+60.09	1.05 + 0.51	33.67	1.61 + 1.37	0.62
	RPMI	nd					
	RPMI+monocytes	1.96 + 1.12					
3D7	European-IgG	1.51 + 0.39		2.12 + 0.44		1.62 + 0.75	
	total-IgG	0.39 + 0.25***	74.50***	1.60 + 1.14	24.35	nd	nd
	void-IgG	1.30 + 0.45	13.90	1.19 + 0.39	43.97	1.34 + 0.35	17.56
	AP-Well-19/GST	0.62 + 0.27***	58.94***	1.49 + 1.11	29.55	0.79 + 0.09	51.51
	AP-Well-EGF1	nd	nd	0.61 + 0.36***	71.16***	0.60 + 0.20*	63.15*
	AP-MAD-EGF2	1.47 + 0.55	2.78	1.61 + 0.83	23.8	nd	nd
	RPMI	0.66 + 0.23					
	RPMI+monocytes	0.90 + 0.51					

Table 5.5. Antibody-dependent cellular -inhibition assay.

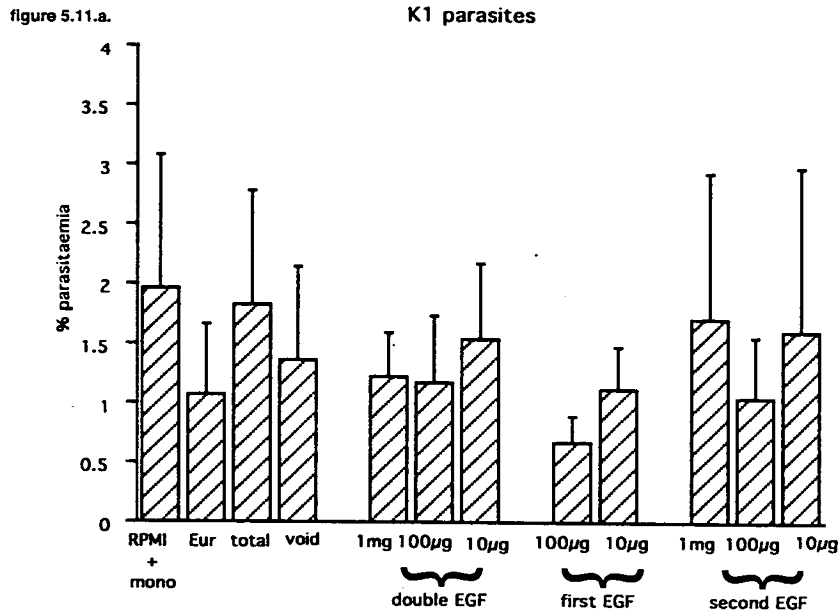
Percentage parasitaemia (plus SD) and percentage inhibition of parasite growth obtained in the presence of different IgG preparations relative to parasites grown in European IgG.

*** = $p<0.005$

* = $p<0.05$

In K1 cultures, 100μg/ml APEGF1-IgG reduced parasite growth by approximately 60% compared to Eur-IgG but, due to interassay variation, this was not statistically significant (figure 5.11.a.). However, APEGF1-IgG inhibited growth of 3D7 by 71%, this was dose-dependent and highly significant ($p<0.005$) (figure 5.11.b.). Total-IgG and AP19-IgG were able to significantly inhibit growth of 3D7 (but not K1) at a concentration of 1mg/ml but had no effect at lower concentrations. APEGF2-IgG had no significant effect on either K1 or 3D7.

Therefore, 3D7 seemed to be more susceptible to ADCI reactions than K1. Total-IgG and AP19-IgG were able to mediate ADCI only at relatively high concentration whilst APEGF1-IgG was able to mediate ADCI at antibody concentrations of 10-100μg/ml. The inhibition by AP19-IgG may be a residual effect of its invasion inhibitory activity. In contrast, APEGF1-IgG had no effect in the absence of monocytes and the ADCI effect is thus real.



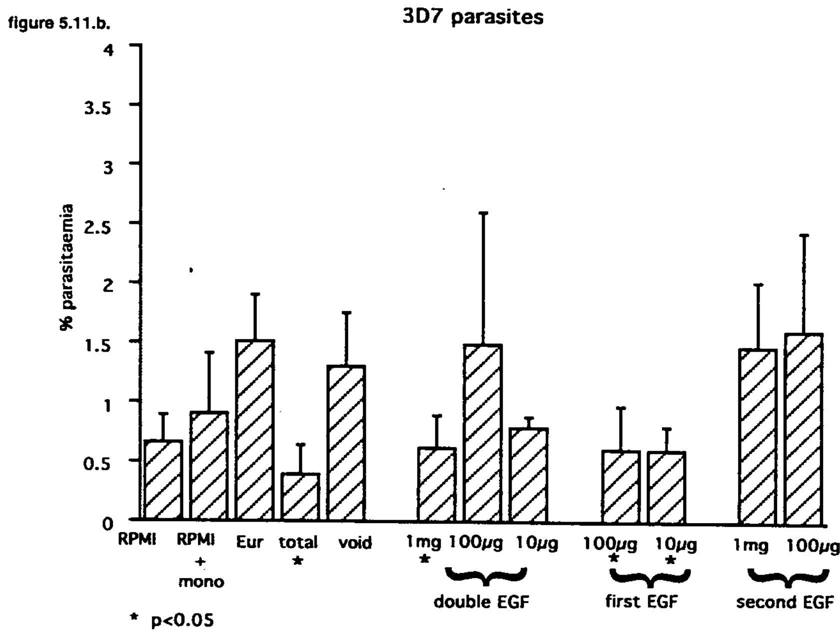


Figure 5.11. Bar chart of antibody-dependent cellular-inhibition assay.

Bars on chart represent mean final percentage parasitaemias, error bars indicate the standard deviation between parasitaemias for each test sample.

a) K1 parasites, b) 3D7 parasites.

Discussion

The mechanism of protection against clinical malaria remains unclear, but it has been demonstrated that immunity can be passively transferred by the administration of IgG from immune adults to young children who have not yet developed an effective immune response (Cohen *et al* 1961, Edozien *et al* 1962, McGregor *et al* 1963). However, the mechanism by which antibody protects an immune person from clinical symptoms of malaria is unknown. Blackman *et al* (1990) suggest that antibody may neutralise the ability of merozoites to penetrate erythrocytes by blocking the secondary processing of PfMSP1$_{42}$, which is thought to be a prerequisite for RBC invasion. Or perhaps antibody may cause the agglutination of merozoites, at the time of schizont rupture, preventing merozoites from invading new RBCs (Epstein *et al* 1981, Lyon *et al* 1989): anti-PfMSP1 antibodies are found in polyspecific sera which mediate the formation of clusters of agglutinated merozoites. Perkins and co-workers have demonstrated that unprocessed PfMSP1 can bind to RBCs, and that a mAb to PfMSP1$_{19}$ can inhibit this binding, suggesting that antibody may inhibit merozoite attachment to RBCs (Perkins and Rocco 1988). Druilhe and Bouharoun-Tayoun (Bouharoun-Tayoun *et al* 1990) have shown that antibodies which have proved to be protective *in vivo* do not directly limit parasite growth but act indirectly by stimulating blood monocytes via their Fc receptors after binding to the parasite target. This could be tested by comparing IgG Fab fragments with intact Ig. The lack of the Fc region would prevent co-operation with monocytes.

Competition ELISAs demonstrate that polyclonal, malaria-immune human sera can inhibit the binding of mAbs whose epitopes map to either single or double EGF-like motif proteins. However, none of the mAbs could inhibit binding of human antibodies suggesting that human sera may recognise a number of epitopes within PfMSP1$_{19}$, only some of which are recognised by mAbs. Some of the epitopes which are recognised by human polyclonal sera include those recognised by invasion inhibitory mAbs. Human polyclonal sera contain antibodies which recognise the same epitopes as invasion inhibitory mAbs (12.8 and 12.10) but many sera also contain antibodies which recognise the same epitopes as blocking mAbs. The same pattern was seen for the vaccinated monkey sera and the affinity purified EGF-1 IgG. Presence of both inhibitory and blocking antibody may be the reason why sera had no protective effect *in vitro*, or *in vivo* (in monkeys).

It is possible that human polyclonal antibodies with similar biological properties to these inhibitory mAbs may be responsible, at least in part, for protection observed *in vitro*. The total-IgG preparation used here had no direct inhibitory effect on either parasite growth or invasion *in vitro* at concentrations of 1-5mg/ml (Bouharoun-Tayoun *et al* 1990), but was able to inhibit parasite growth *in vitro* in co-operation with monocytes and was very effective *in vivo*, drastically reducing asexual parasitaemia when administered to human malaria patients (Sabchareon *et al* 1991).

Antibodies to the first EGF-like motif were affinity purified by Dr. J. Chappel. From 900mgs of total-IgG, 2.9mgs of EGF-1-specific IgG were selected by affinity chromatography. Assuming that the APEGF1-IgG was wholly specific for $PfMSP1_{19}$, approximately 0.3% of the total-IgG population was specific for epitopes within the first EGF-like motif. Similarly, 3.2mgs or 0.35% of the total-IgG population (once antibodies to the first EGF-like motif had been depleted out) was specific for epitopes within the second EGF-like motif and 1.7mgs or 0.2% of the total-IgG (once antibodies to the two single EGF-like motifs had been depleted out) was specific for additional epitopes within $PfMSP1_{19}$. Taking into consideration the fact that higher-affinity antibodies will remain bound to the column during the elution process (Chappel *et al* 1993), the results suggest that a substantial proportion of the IgG is directed against $PfMSP1_{19}$. However some of the IgG selected here could be specific for GST which makes up part of the fusion proteins.

Murine mAbs mapping to epitopes within $PfMSP1_{19}$ have been shown to directly inhibit *in vitro* parasite invasion of RBCs in the absence of monocytes. Invasion inhibition is believed to be due to mAb inhibition of the secondary processing of $PfMSP1_{42}$ to $PfMSP1_{19}$ (Blackman *et al* 1990). The invasion inhibition assay used here was similar to the one used by Blackman *et al* (1990) in which merozoite invasion was significantly inhibited by mAb at a concentration of 100µg/ml. In this study, affinity purified antibodies to $PfMSP1_{19}$ were able to block the binding of the invasion inhibitory mAbs, and in *in vitro* invasion inhibition assays, purified antibodies to $PfMSP1_{19}$ and the second EGF-like motif (but not the first, see Chappel *et al* 1993) were able to inhibit parasite growth by up to 70%. Significant inhibition was obtained with as little as 10µg/ml of antibody. Antibodies selected to either of the two allelic forms of $PfMSP1_{19}$ (i.e. Well-19/GST and MAD-EGF2) were equally effective against parasites from either PfMSP1 allelic family suggesting this invasion inhibitory antibody is cross-reactive for the two allelic forms of PfMSP1.

Pretreating merozoites with some $PfMSP1_{19}$-specific mAbs blocks the effect of invasion inhibitory mAbs by blocking the binding of mAbs which inhibit secondary processing (Blackman *et al* 1994). Through competition ELISAs, I have been able to show that the AP-IgG preparations also contain blocking type antibodies, i.e. AP-IgG competes with mAbs 2.2 and 7.5.

These results suggest that several different anti-$PfMSP1_{19}$ antibodies may be produced during natural infection or immunisation with $PfMSP1_{19}$, only a subset of which exert protective biological effect(s). An effective $PfMSP1_{19}$ vaccine will have to induce primarily invasion inhibitory, rather than blocking, antibody. One way of assessing the efficacy of $PfMSP1_{19}$ vaccination is by use of primate models. *Aotus* monkeys are the best monkey models for human malaria because as well as having evolutional kinship to humans, they support the development of high density of *P. falciparum* parasites and are available in relatively large numbers (Anonymous 1988).

Aotus monkeys immunised with the *E. coli*-derived Well-19/GST of *P. falciparum* were not protected from either heterologous or homologous challenge (R. Ballou, A. Holder *et al*). I have conducted ELISAs to determine whether antibodies induced in this vaccination experiment were able to compete with invasion inhibitory mAbs and/or blocking (non-invasion inhibitory) mAbs. I found that sera from vaccinated monkeys competed with all these mAbs, indicating that the monkey sera contain all of these types of antibodies. It took very high concentrations of monkey sera to inhibit mAb binding. This suggests that the target epitopes of these invasion inhibitory mAbs are not particularly immunodominant in monkeys. The relative concentrations and affinities of the inhibitory versus the blocking antibodies may determine the protective effect of serum antibodies. The presence of relatively high titre blocking antibody in the vaccinated monkey serum may explain why the monkeys were not protected. There does not seem to be any difference, by titration or competition ELISA, in the antibody titres of vaccinated monkeys which underwent a heterologous or homologous challenge infection.

I decided that it would be useful to determine the IgG subclass of human polyclonal antibodies produced to $PfMSP1_{19}$ in natural malarial infections, as work by Bouharoun-Tayoun and Druilhe has emphasised the need to determine the biological function of antibody responses produced in malaria. I found that predominantly IgG1 antibodies are produced to $PfMSP1_{19}$, suggesting that C-terminal PfMSP1-specific antibodies may play a role in opsonisation or complement-mediated lysis of free merozoites.

However, these antibodies were able to inhibit merozoite invasion in the absence of either complement or monocytes, indicating that (despite their subclass) they were in fact acting primarily by physically blocking active sites on the $PfMSP1_{19}$ molecule.

So in conclusion, the data presented in this chapter indicate that natural malarial infections in humans, and artificial vaccination of monkeys with $PfMSP1_{19}$, induces invasion inhibitory type antibodies, as shown by their ability to compete with invasion inhibitory mAbs. Affinity purified human antibodies to $PfMSP1_{19}$, and to its constituent second EGF-like motif are able to inhibit the *in vitro* parasite invasion of RBCs. However, human antibody affinity purified to the first EGF-like motif, and antibody induced by vaccination of monkeys with a $PfMSP1_{19}$ recombinant protein, has no protective effect *in vitro* or *in vivo.* This may be due to the presence of other $PfMSP1_{19}$-specific antibodies which block the effect of invasion inhibitory antibodies. This may explain the paradoxical findings that, despite the presence of high levels of serum antibodies to PfMSP1, many individuals in malaria-endemic areas have parasites in their peripheral blood (Riley *et al* 1992a). The relative concentrations and affinities of inhibitory and blocking antibodies may determine whether secondary processing of the MSP1 complex occurs which, in turn, will affect the ability of the merozoites to invade RBCs. Obtaining an appropriate balance between inhibitory and blocking antibodies will determine whether $PfMSP1_{19}$ vaccination is successful or not.

CHAPTER SIX:
Identification of C-terminal PfMSP1-specific human serum antibodies associated with resistance to clinical malaria

Introduction

The previous chapter suggests that antibodies to the C-terminus of PfMSP1 may mediate a protective function *in vitro*. The next step was to look at naturally occurring antibodies in a population living in a malaria endemic area, to determine whether there is an association between the prevalence of antibodies to the C-terminus of PfMSP1 and protection from clinical symptoms of malaria.

There is no direct relationship between levels of antimalarial antibodies and resistance to clinical malaria. It seems that much of the antibody response is probably not protective, and in some cases may even be associated with an increased risk of developing clinical symptoms (Tolle *et al* 1993, Riley *et al* 1990). (It has been noted that the *in vitro* growth of parasites may even be enhanced in immune serum suggesting that some antibody specificities may facilitate parasite growth or invasion into RBCs [Bouharoun-Tayoun *et al* 1990].) Individuals with high levels of antimalarial antibodies often have high parasitaemias. The reason for these paradoxical findings is not understood, but part of the antibody response may be a form of immune evasion by the parasite. It has been suggested that some regions of malarial antigens contain immunodominant B-cell epitopes for this precise reason (Day and Marsh 1991, Schofield 1991).

Many immunodominant epitopes are within areas of antigenic diversity, i.e. polymorphic or dimorphic amino acid sequences. An individual may be confronted with so many variants of these epitopes that the immune response becomes overloaded and responds poorly to critical protective epitopes, effectively causing a 'smoke screen' effect. Many malarial antigens are highly repetitive in their amino acid sequence (Weber 1988), and it has been suggested that these repeats have evolved to frustrate the selection of high affinity antibody responses by somatic mutation of immunologlobulin genes (Anders *et al* 1988). Schofield (1991) has suggested that repeat regions contribute to the lack of effective immunity to malaria, as they are effectively

'seen' by the immune system as thymus-independent antigens (analogous to polysaccharides) and so result in thymus-independent antibody formation, which would lead to failures in T cell help, antibody affinity maturation and memory formation.

These lines of thought would suggest that, 1) variable/polymorphic regions of antigens would be immunodominant, 2) conserved regions may be under some functional and/or structural constraint but have evolved to be poorly immunogenic, and 3) antibody responses to the former are not protective, and may even be associated with an increase risk of clinical infection, whereas antibody responses to the latter, though rare, are associated with protection. In the case of PfMSP1, both conserved (recombinant protein p190L representing conserved block 3) (Riley *et al* 1992a) and dimorphic (recombinant proteins M6, M7 and M11 representing blocks 6, 6-8 and 16 respectively) (Tolle *et al* 1993) regions have been shown to be immunodominant, and are also associated with protection from clinical malaria.

The two longitudinal protection studies of antibodies to PfMSP1 (Riley *et al* 1992a, Tolle *et al* 1993), were carried out in West Africa, where parasites predominantly express the MAD20 allelic prototype (Conway and McBride 1991, Fruh *et al* 1991). In this region, the so called 'variable', i.e. dimorphic, regions of the protein will essentially be 'seen' as conserved regions by the host population. MAD20 dimorphic recombinant proteins were predominantly recognised in the Tolle study. Wellcome sequence proteins were used to detect antibody responsiveness in the study by Riley *et al,* so responses to dimorphic proteins were underestimated. The only truly polymorphic region of PfMSP1 is the repeat sequence of block 2, corresponding to the N-terminal $PfMSP1_{83}$ processing fragment. Tolle *et al* (1993) have found that antibody responses to this region are associated with an increased risk of infection and inability to control parasitaemia. However, this work, and their previous findings (Muller *et al* 1989, Fruh *et al* 1991), suggests that the polymorphic repeat region is not immunodominant. (Also noted in our laboratory; Dr. D. Cavanagh, personal communication.) Tolle *et al* (1993) suggest that this association of antibodies with polymorphic regions and increased risk of clinical malaria may reflect the number of infections a person has had, in that the sequences are so variable that the chance that a person would have antibodies to any particular variant would be increased in someone who has experienced many infections, and so may be indicative of a person who is particularly susceptible to infection.

There seems to be some disagreement about whether antibody responses to the PfMSP1_{83} processing fragment are protective. It has been noted that unparasitised individuals have higher levels of antibodies to PfMSP1_{83} than do parasitised individuals (Chizzolini *et al* 1988, Kramer and Oberst 1993). Antibody responses to native PfMSP1_{83} tended to be associated with resistance to clinical malaria in partially immune children in one study, though this was not statistically significant (Riley *et al* 1992a), but in a later study (Riley *et al* 1993) it was found that parasitised susceptible adults had higher levels of anti-PfMSP1_{83} antibodies, this may reflect recent exposure (again this was not significant, possibly due to the small sample size). However, PfMSP1_{83} has been shown to contain both protective regions, e.g. block 3 (Riley *et al* 1992a) and blocks 6-8 (Tolle *et al* 1993) and non-protective regions e.g. block 4 (Tolle *et al* 1993).

Antibody responses to the C-terminal PfMSP1_{42} have also been demonstrated to be associated with resistance to clinical malaria in partially immune children (Riley *et al* 1992a). However prevalence of antibodies to this protein is low in children, and not particularly high in adults (see chapter 4). This association of antibody responses to PfMSP1_{42} with protection was also observed in a later study where it was found that susceptible urban dwelling adults had lower levels of PfMSP1_{42} antibodies than healthy adults living in rural areas (Riley *et al* 1993). Again, the recombinant protein used in this study represents the (Wellcome) allelic form of PfMSP1_{42} rarely expressed by parasites in this study area, so the antibody assay would have measured responses to epitopes which are conserved between the two allelic forms of PfMSP1, in either the conserved PfMSP1_{19} region and/or in the dimorphic PfMSP1_{33} region, which make up PfMSP1_{42}. I have carried out further studies to investigate the association between antibody responses to PfMSP1_{42} and resistance to clinical malaria. I have evaluated the antibody response (in a population mainly exposed to parasites expressing the MAD20 allele of PfMSP1), to the complete version of the MAD20 allelic form of the dimorphic PfMSP1_{33} and to both sequences of PfMSP1_{19} and to its constituent EGF-like motifs, to determine whether naturally acquired immunity to malaria involves antibody responses to this region of PfMSP1. The study was conducted using samples from two different regions of West Africa; The Gambia, where malaria is seasonally endemic, and Sierra Leone, where perennial malaria transmission occurs.

Methods and materials

(1) Study population and study design

The study concentrated on children who were actively acquiring immunity to malaria (aged 3-8 years in The Gambia, n=327; 0-8 years in Sierra Leone, n=645). In The Gambia, malaria transmission is seasonally endemic (Greenwood *et al* 1987a), with most new infections occurring during the three month long rainy season (July to October). Children receive between 1 and 5 infective mosquito bites per year (Lindsay *et al* 1989), and become clinically immune to malaria by the age of 8-10 years (Greenwood *et al* 1987a).

In the southern region of Sierra Leone, annual rainfall is higher than in The Gambia, extending over a period of 8-9 months each year, with inhabitants receiving approximately 30 infective mosquito bites per year (Barnish *et al* 1993a, 1993b, Bockarie *et al* 1994). As a result, malaria transmission is perennial. Due to the high intensity of malaria in this region, antimalarial immunity develops rapidly; most children are clinically immune to malaria by the age of 5 years (Barnish *et al* in press). Immediately prior to the annual rainy season (May 1988 in The Gambia and March 1990 in Sierra Leone), blood samples were taken. Blood smears were made, stained with Giemsa and examined for malaria parasites and serum was retained for immunological assays. A morbidity survey was carried out every week for the duration of the malaria transmission season in The Gambia, and for one whole year in Sierra Leone. During these weekly surveys, blood smears were taken from all febrile children (axillary temperature 37.5°C or greater). During the weekly survey in the Sierra Leonean study, 30 randomly chosen, afebrile children were monitored for parasites to give a more accurate measure of the prevalence of asymptomatic malaria in this study group.

Children were re-examined at the end of the study. Blood smears were taken to detect malaria parasitaemia, and children were classified into one of four morbidity groups depending on their experience of malaria during this malaria transmission season. Children who had a febrile episode (≥37.5°C) coincident with parasitaemia of 5000 or more *P. falciparum* parasites per µl of blood, were classed as having had an attack of clinical malaria. Children who had any level of *P. falciparum* parasites in their blood smear but experienced no fever in the study period were classed as having had an asymptomatic infection. Children who experienced fever associated with low parasitaemia

(indeterminate group) or had no evidence of infection (no infection group) were not included in the statistical analysis of morbidity as it is not certain that the former children were ill from malaria or whether the latter children were exposed to malaria.

Control European serum samples were obtained from 28 adults and 14 children with no previous exposure to malaria. All serum samples were stored at -20°C until used.

(2) Antigens

The recombinant proteins used in this study have previously been described in chapter 2. *E. coli* derived GST fusion proteins representing; the first EGF-like motif of Wellcome (Well-EGF1) and MAD20 (MAD-EGF1) sequences of $PfMSP1_{19}$, the second EGF-like motif of the MAD20 $PfMSP1_{19}$ (MAD-EGF2) and, the MAD20 $PfMSP1_{33}$ (MAD-33), were used in this study to detect PfMSP1 antibody responses.

Yeast recombinant proteins representing full length $PfMSP1_{19}$ were also tested; 3D7 represents the MAD20 version of $PfMSP1_{19}$ (MAD-19), and FVO/E is a hybrid of the first EGF-like motif of MAD20 and the second EGF-like motif of Wellcome (MAD/Well-19).

(3) ELISA

Previously described in chapter 4.

(4) ELISA to determine IgG subclass

Previously described in chapter 5.

(5) Statistical methods

Specific OD values obtained for sera from malaria-unexposed European donors were used to establish a normal range for each antigen. The OD values for these control sera tended to be normally distributed; thus, African sera giving an OD value greater than the mean plus 2SD of the European sera were considered to contain antibody specific for the relevant recombinant protein.

χ^2 test for linear trend was used to assess age-related trends in prevalence of clinical malaria and in the prevalence of antibody response. χ^2 was used to detect differences in antibody responsiveness between different groups.

To determine the relationship between anti-PfMSP1 antibodies and malaria morbidity, only children experiencing either clinical or asymptomatic malaria were compared, since the clinical status of children in the other two morbidity categories could not be ascertained with any degree of certainty. The association between clinical status and possession of antibodies to a certain protein was calculated as an odds ratio (OR), i.e. the likelihood that an antibody positive child would experience a clinical infection compared to the likelihood of experiencing an asymptomatic infection. An OR of less than 1.0 is indicative of a decreased risk of infection.

Confounding factors which might have an effect on PfMSP1 antibody prevalence included; sex of a child, carriage of the sickle cell trait, age, and in the Gambian study; ethnic group and the use of bed nets. The appropriate significant confounders were allowed for by logistic regression analysis before calculating the OR.

Statistical analysis was done by D. McGuinness (University of Edinburgh, Scotland, UK) and J. Morris (London School of Tropical Medicine, London, UK).

Results

(1) Relationship between age and malaria morbidity

The prevalence of asymptomatic infections, measured here as a positive *P. falciparum* parasitaemia in the absence of fever, increased with age in both study groups, see table 6.1. This increase was more apparent in the Sierra Leonean children than in Gambian children (χ^2 for trend; The Gambia χ^2=3.5, p=0.06; Sierra Leone χ^2=78.2, p=0.00). The prevalence of clinical disease declined with age in a similar fashion. These results confirm that the age groups being studied were those in which clinical immunity was developing. In the Sierra Leonean study, more than 20% of the children below the age of one showed no evidence of infection, compared with 5-10% of children aged 2-5 years. This apparent resistance of infants to malaria infection probably reflects a combination of factors; 1) infants will probably have a lower exposure rate to infective mosquitoes due to wrapping of the infants and constant physical proximity to the mother, 2) the protective effects of maternal Ig transferred via the placenta (McGregor 1986), 3) a milk diet; 'breast milk' is deficient in paramino benzoic acid (PABA), a vital growth factor for malaria parasites; experiments with rodent malarias show that parasitaemia develops more slowly in mice fed a PABA-deficient diet (Wernsdorfer and McGregor 1988) and 4) foetal haemoglobin (Pasvol *et al* 1976). However, infants who did become infected were more likely to be symptomatic than were older children; 103/131 (79%) of infected 0-1 year olds were symptomatic compared with 90/147 (61%) of 2-3 year olds and 51/149 (34%) of 4-5 year olds, indicating that infants have little 'anti-disease' immunity (Playfair *et al* 1990).

The geometric mean level of parasitaemia was significantly lower in children with asymptomatic infections than in children with clinical infections (table 6.4.; Student's t test, for The Gambia, t=12.99, df=198, p<0.0001; for Sierra Leone, t=24.2, df=506, p<0.0001), confirming that children who experience asymptomatic infections are able to control parasite growth.

(2) Relationship between anti-PfMSP1 antibodies and age

Up to 13% of the Gambian children and 20% of Sierra Leonean children had antibodies to recombinant proteins representing the double-motif $PfMSP1_{19}$ protein at the beginning of the study, see table 6.2. 5-6% of the Gambian children and up to 13% of Sierra Leonean children had antibodies to the first

	The Gambia						Sierra Leone						
age (years)	3-4	5-6	7-8	total	χ^2 trend	p	0-1	2-3	4-5	6-8	total	χ^2 trend	p
n	120	110	97	327			167	162	157	159	645		
clinical	42 (35.0)	42 (38.2)	25 (25.8)	109 (33.3)	1.8	0.18	103 (61.7)	90 (55.6)	51 (32.5)	25 (15.7)	269 (41.7)	86.0	0.00
asymptomatic	29 (24.2)	27 (24.5)	35 (36.0)	91 (27.8)	3.5	0.06	19 (11.4)	51 (31.5)	83 (52.9)	86 (54.1)	239 (37.1)	78.2	0.00
indeterminate	8 (6.6)	13 (11.8)	15 (15.5)	36 (11)	4.3	0.04	9 (5.4)	6 (3.7)	15 (9.6)	12 (7.5)	42 (6.5)	2.00	0.16
no infection	41 (34.2)	28 (25.5)	22 (22.7)	91 (27.0)	3.7	0.06	36 (21.6)	15 (9.3)	8 (5.1)	36 (22.6)	95 (14.7)	0.02	0.89

Table 6.1. Malaria morbidity in the Gambian and Sierra Leonean cohorts.

The children were categorised by age (years) and malaria morbidity. Figures show the number (percentage) of children in each group.
No effect of sex or sickle cell gene carriage was observed in relation to either malaria morbidity or antibody responses (data not shown).

	The Gambia						Sierra Leone						
Age (years)	3-4	5-6	7-8	total	χ^2 trend	p	0-1	2-3	4-5	6-8	total	χ^2trend	p
n	120[a]	110[b]	97[c]	327[d]			167[e]	162[f]	157[g]	159[h]	645[i]		
MAD-19	6 (5)	8 (7.3)	14 (14.4)	28 (8.6)	5.9	0.01	11 (6.6)	31 (19.1)	25 (15.9)	43 (27.0)	110 (17.1)	19.6	<0.001
MAD/Well-19	9 (7.5)	15 (13.6)	19 (19.6)	43 (13.1)	6.9	0.01	17 (10.2)	36 (22.2)	30 (19.1)	49 (30.8)	132 (20.5)	17.4	<0.001
Well-EGF1	5 (4.2)	6 (5.4)	7 (7.2)	18 (5.5)	0.9	0.33	15 (9.0)	20 (12.3)	11 (7.0)	21 (13.2)	67 (10.4)	0.5	0.49
MAD-EGF1	6 (5.0)	7 (6.4)	7 (7.2)	20 (6.1)	0.5	0.49	15 (9.0)	28 (17.3)	15 (9.5)	25 (15.7)	83 (12.9)	1.2	0.28
MAD-EGF2	1 (0.8)	3 (2.7)	7 (7.2)	11 (3.4)	6.5	0.01	1 (0.6)	3 (1.8)	7 (4.5)	11 (6.9)	22 (3.4)	11.4	0.001
MAD-33	26 (22.2)	40 (37.0)	38 (40.0)	104 (32.5)	7.9	0.005	45 (30.4)	72 (56.7)	90 (70.9)	90 (78.9)	297 (57.6)	70.7	0.000

Table 6.2. The relationship between age and antibody responses to the C-terminal of PfMSP1.

Figures show the number (percentage) of antibody positive children ('responders') in each group, for each antigen.
Responders are those where the specific OD was greater than the mean plus 2 SD of the control (malaria non-exposed) sera.
n values for MAD-33 are smaller: a=117, b=108, c=95,d=320, e=148, f=127, g=127, h=114, i=516.

EGF-like motif proteins. Only 3.4% of children in both study groups have antibodies to the second EGF-like motif. 30% of Gambian children and 60% of Sierra Leonean children have antibodies to $PfMSP1_{33}$. (Dot plots showing antibody responses to MAD-19 and MAD-33 are shown in figure 6.1.) These findings are broadly similar to those seen in chapter 4.

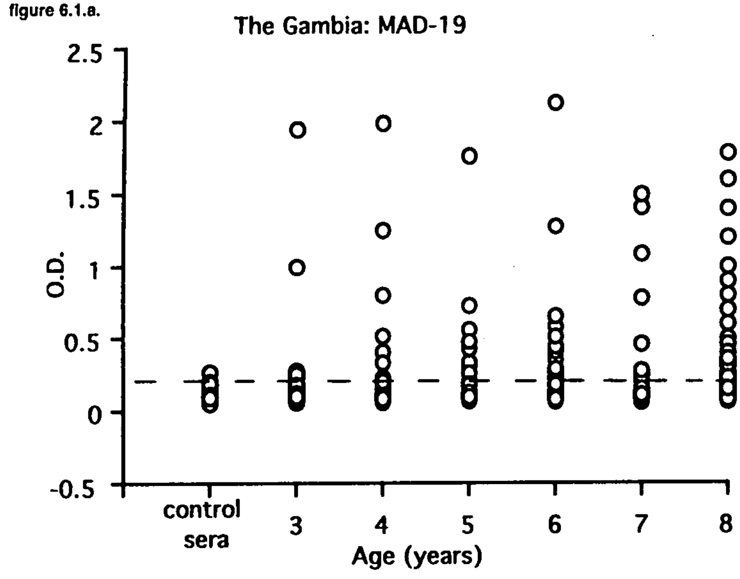
The prevalence of antibody responses to MAD-33, $PfMSP1_{19}$ and the MAD-EGF2 increases with age in both study groups (table 6.2.). Antibody responses to EGF1 do not increase with age. These age differences in antibody responsiveness to the PfMSP1 proteins agree with my previous findings in chapter 4 (except for MAD-EGF2; but this may be due to the larger sample size in each age group in this study). Again, antibody responses to the two allelic forms of the $PfMSP1_{19}$ and the first EGF-like motif proteins are highly correlated (data not shown).

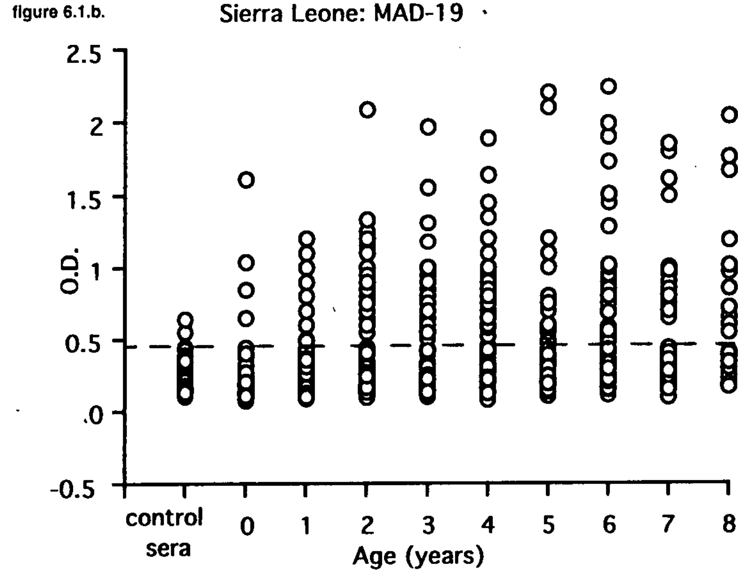
(3) Relationship between anti-PfMSP1 antibodies and ethnic group

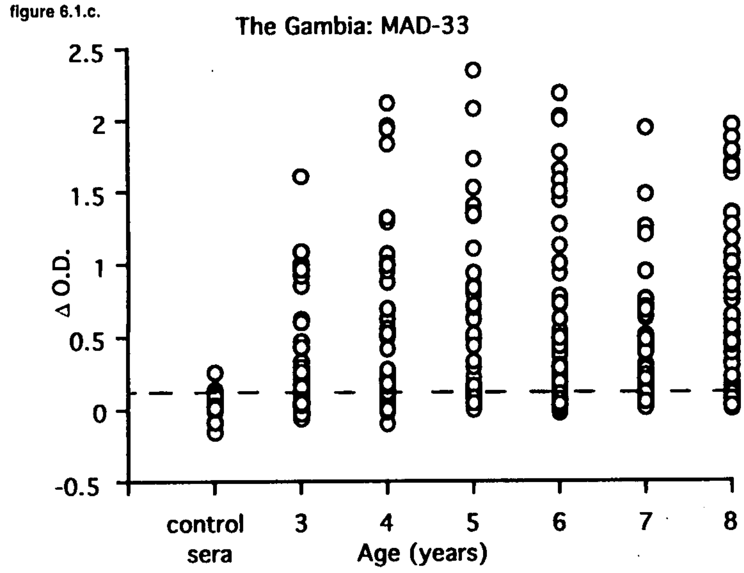
The vast majority of Sierra Leonean children come from the same ethnic group (Mende, 94%) (Barnish *et al* 1993a), whilst the Gambian children came from three separate, and genetically distinct ethnic groups (Olerup *et al* 1991). In The Gambia, malaria morbidity was found to differ between children of different ethnic group, in that Fula children are less likely to be infected (table 6.3.a.). The prevalence of antibodies to the double-motif $PfMSP1_{19}$ proteins differed significantly between the three groups, being higher in children of the Fula ethnic group than in Mandinka or Wollof children (table 6.3.b.)

(4) Relationship between anti-PfMSP1 antibodies and malaria morbidity

Antibodies measured in the children's sera at the beginning of the malaria transmission season were compared with their subsequent malaria morbidity, after allowing for confounding effects of age and, in The Gambia, ethnic group. In both the Gambian and the Sierra Leonean studies, the prevalence of antibodies to epitopes within the C-terminus of PfMSP1 was higher in children who experienced only asymptomatic infections than in children who experienced clinical infections (except for antibody responses by Gambian children to the first EGF-like motif proteins). However, the odds ratio for this difference is not always significant, see table 6.4.







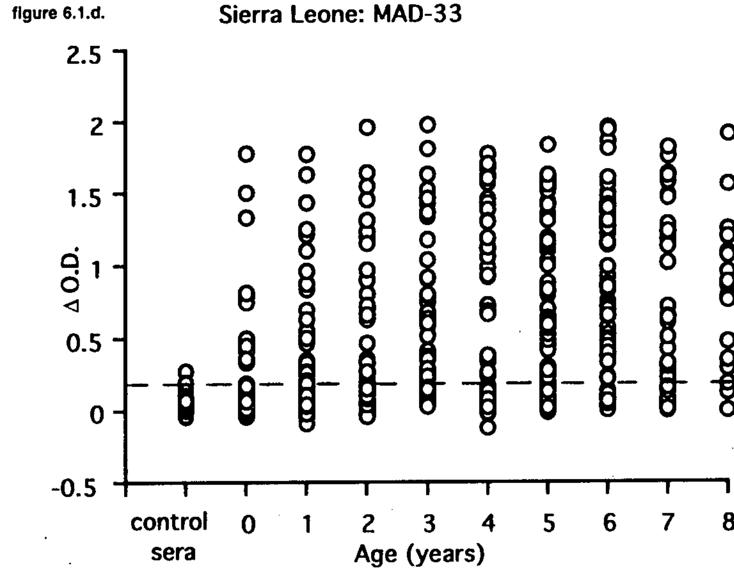


Figure 6.1. Dot plots showing human antibody binding (OD values) for sera in an ELISA for recombinant PfMSP1 proteins: MAD-19;

a) 327 Gambian sera; b) 645 Sierra Leonean sera, and MAD-33: c) 320 Gambian sera; b) 516 Sierra Leonean sera.

OD values of 42 European control sera are also shown. All sera were tested at a dilution of 1:3000. (See text for details.)

n	Fula	Mandinka	Wollof	χ^2	p
	157	83	78		
clinical	45 (28.7)	34 (41.0)	52 (33.3)	31.1	0.000
asymptomatic	40 (25.5)	21 (25.3)	25 (32.1)	1.31	0.518
indeterminate	14 (8.9)	9 (10.8)	13 (16.7)	3.14	0.208
no infection	58 (36.9)	19 (22.9)	14 (17.9)	11.01	0.004

Table 6.3.a. Malaria morbidity in the Gambian in relation to ethnic group.

The children were categorised by ethnic group and malaria morbidity.
Figures show the number (percentage) of children in each group.
p value at 2 degrees of freedom.

ethnic group	Fula	Mandinka	Wollof	χ^2	p
	n=165[a]	n=83	n=79[b]		
MAD-19	20 (12.1)	3 (3.6)	5 (6.3)	5.8	0.06
MAD/Well-19	31 (18.8)	6 (7.2)	6 (7.6)	9.3	0.01
Well-EGF1	11 (7.2)	2 (2.4)	3 (3.8)	2.0	0.37
MAD-EGF1	12 (7.3)	2 (2.4)	4 (5.1)	1.1	0.57
MAD-EGF2	5 (3.0)	0 (0.0)	0 (0.0)	4.5	0.11
MAD-33	44 (28.0)	30 (36.1)	28 (35.9)	2.3	0.31

Table 6.3.b. The association between ethnicity and antibody responses to the C-terminus of PfMSP1 in The Gambian cohort.

Figures represent the number (percentage) of children with detectable antibody to each antigen. For MAD-33 n is smaller; a=157, b=78. p value at 2 degrees of freedom.

	The Gambia				Sierra Leone			
% (n)	asymptomatic (n=91)	clinical (n=109)	OR for clinical malaria* (95% CI)	p	asymptomatic (n=239)	clinical (n=269)	OR for clinical malaria** (95% CI)	p
MAD-19	11 (12.1)	8 (7.3)	0.7 (0.3-2.0)	0.52	53 (22.2)	34 (12.6)	0.7 (0.4-1.2)	0.16
MAD/Well-19	16 (17.6)	12 (11.0)	0.7 (0.3-1.6)	0.35	65 (27.2)	40 (14.9)	0.6 (0.4-1.0)	0.06
Well-EGF1	5 (5.5)	8 (7.3)	1.7 (0.5-5.7)	0.38	26 (10.9)	24 (8.9)	0.9 (0.5-1.8)	0.79
MAD-EGF1	5 (5.5)	9 (8.3)	1.8 (0.6-5.9)	0.31	32 (13.4)	32 (11.9)	1.0 (0.5-1.7)	0.90
MAD-EGF2	10 (11.0)	0 (0.0)	0.0 (0.0-0.5)	<0.001	12 (5.0)	4 (1.5)	0.4 (0.1-1.4)	0.14
MAD-33	40 (46.5)	27 (25.7)	0.4 (0.2-0.8)	<0.05	121 (70.8)	114 (48.7)	0.8 (0.5-1.3)	0.37
geometric mean parasitaemia	411.8/µl (206.2-821.2)	32,112/µl (2,6871-38,376)			831.1/µl (666.6-1042.8)	40,644.3/µl (37,008-44,606)		

Table 6.4. The relationship between antibody responses to the C-terminal of PfMSP1 and malaria morbidity.

Figures show the number (percentage) of antibody positive children ('responders') in the clinical and asymptomatic morbidity groups for each antigen.
* Odds ratio adjusted for age and ethnic group
** Odds ratio adjusted for age
p value represents significance of likelihood ratio test, obtained using logistic regression.
n values for MAD-33 are smaller: The Gambia; asymptomatic=86, clinical=105, Sierra Leone; asymptomatic=171, clinical=234.

In Gambian children, the prevalence of antibodies to MAD-EGF2 was significantly higher in children with asymptomatic infections than in those experiencing clinical malaria. The significance of this association remained high ($p<0.001$) even after adjusting for the confounding factors of age and ethnic group. In the Sierra Leonean study there was a suggestion of a reduced risk of clinical malaria associated with antibody responses to MAD-EGF2, but this was not significant since four antibody positive children experienced clinical infections.

In both populations, there seems to be an association of antibody prevalence to the double-motif $PfMSP1_{19}$ proteins and protection. In the Sierra Leonean children, responses to the MAD/Well-19 proved to be associated with protection, reducing the risk of clinical malaria by 40%.

Antibody responses to MAD-33 are also associated with protection from clinical malaria in Gambian children, resulting in a 60% reduced risk of developing clinical malaria.

(5) Comparison of antibody responses in Gambian and Sierra Leonean children

The prevalence of antibody responses to all of the C-terminal PfMSP1 proteins (except MAD-EGF2) was higher in Sierra Leonean children than in Gambian children, see table 6.2. This may reflect the higher intensity of malaria transmission in Sierra Leone. The longer rainfall season in Sierra Leone results in a longer malaria transmission season, but also the level of rainfall is very high, producing a year-round habitat for mosquito breeding, resulting in a higher incidence of infective mosquito bites.

In Sierra Leone, at the time of blood sample collection, children were monitored for the presence of parasites. Significant associations were observed between the presence of asymptomatic parasitaemia at the time of serum sample collection and the presence of antibody to the double EGF-like $PfMSP1_{19}$ proteins and $PfMSP1_{33}$, but not to the single EGF-like proteins (table 6.5.). This suggests that recent boosting may be required to maintain significant antibody titres to $PfMSP1_{19}$, and may partially explain the low prevalence of anti-$PfMSP1_{19}$ antibodies in children, especially in The Gambia, as these serum samples were taken at the end of the dry season.

	parasitaemia n=365[a]	no parasitaemia n=279[b]	χ^2	p
MAD-19	72 (19.1)	38 (13.6)	4.16	0.041
MAD/Well-19	87 (23.8)	45 (16.1)	5.75	0.016
Well-EGF1	39 (10.7)	28 (9.4)	0.16	0.686
MAD-EGF1	45 (12.3)	38 (12.8)	0.00	0.951
MAD-EGF2	15 (4.1)	7 (2.4)	1.07	0.301
MAD-33	196 (83.4)	39 (32.8)	44.12	0.000

Table 6.5. The association between concurrent parasitaemia and antibody responses to the C-terminal of PfMSP1 in the Sierra Leonean cohort.

Figures represent the number (percentage) of children with or with out parastaemia at time of blood sample collection.
For MAD-33 n is smaller; a=286, b=119. p value at 2 degrees of freedom.

(6) IgG subclass response of C-terminal PfMSP1 seropositive children

The IgG subclass of MAD-19-specific antibodies in seropositive individuals in Sierra Leone was determined by subclass specific ELISA. Mainly IgG1 antibodies reacted with MAD-19. IgG subclass response was not associated with an increased or reduced risk of clinical malaria (see table 6.6.).

n (%)	asymptomatic (n=239)	clinical (n=269)	OR for clinical malaria*	
			(95% CI)	p
IgG1	37/53 (69.8)	23/35 (65.7)	0.9 (0.4-2.3)	0.83
IgG2	0/53 (0.0)	0/35 (0.0)		
IgG3	18/53 (34.0)	6/35 (17.1)	0.5 (0.2-1.4)	0.18
IgG4	10/53 (18.9)	11/35 (31.4)	2.2 (0.8-6.2)	0.13

Table 6.6. The relationship between IgG subclass antibody responses to $PfMSP1_{19}$ (MAD-19) and malaria morbidity in Sierra Leone.

Figures show the number (percentage) of IgG subclass antibody positive children in the clinical and asymptomatic morbidity groups for the total IgG antibody positive children to MAD-19.
* Odds ratio adjusted for age.
p value represents significance of likelihood ratio test, obtained using logistic regression.

Discussion

Epidemiological studies cannot demonstrate absolute correlations between a particular immune response to an antigen and protection from clinical malaria, but these associations can give an indication of antigens which may be important in the induction of clinical immunity. The interpretation of epidemiological studies is complicated by difficulties of defining morbidity caused by malaria. Here, clinical malaria is defined as a child who has at least one episode of fever associated with a parasitaemia of 5000 or more parasites per µl of blood. Asymptomatic malaria is defined here as a child who has parasites in their blood film at least once during the study period, with any level of parasitaemia, but no clinical symptoms.

The relationship between fever, parasitaemia and clinical malaria has recently been re-examined for areas of high malaria transmission intensity where the majority of the population harbour parasites at any one time (Armstrong-Schellenberg *et al* 1994, Smith *et al* 1995). These authors are in agreement that the method used above is adequate for children over the age of one year. However, in children ill with malaria under this age, temperature did not correlate with parasite levels. Also fevers last for a shorter duration and so may go unmonitored. So this method of diagnosis will probably underestimate the number of cases of clinical malaria in infants. In addition, as many children have a parasitaemia, to say that any fever a child experiences is due to malaria will over estimate the cases of clinical malaria in older children. However, there is evidence that parasitaemia decreases in the prevalence of fever (Kwiatkowski 1995), which would result in an increase in the indeterminate group and an under estimation of clinical malaria (Armstrong-Schellenberg *et al* 1994). Many children will have been classed as having no infection, as they had no detectable parasites in their peripheral blood smear, however, blood films will underestimate the prevalence of sub clinical infections (Hang *et al* 1995). So if anything, these definitions will underestimate asymptomatic malaria and overestimate clinical malarial cases. Nevertheless, data from this chapter confirms previous findings of Riley *et al* (1992a) that there is a significant association between antibody responses to epitopes within the C-terminus of PfMSP1 and resistance to clinical malaria.

Data presented here suggest that antibody responses to epitopes within both the conserved $PfMSP1_{19}$ protein, and the dimorphic $PfMSP1_{33}$, are

associated with the ability to control clinical symptoms of malaria. The significance of this association is enhanced by the fact that the same observations, though not always significant, have been made in two distinct populations with different genetic backgrounds, from areas of differing malarial intensity, on samples collected two years apart. The association of the protective effect of these antibodies is in marked contrast with the lack of such an association with antibodies to other malarial antigens with protection tested in a similar fashion (Riley *et al* 1990).

As in chapter 4, I have noted that the prevalence of antibodies to $PfMSP1_{19}$ and MAD-33 increase with age. The age correlation of acquisition of antibodies suggests repeated exposure to infection may be required to develop sufficient memory. With the case of $PfMSP1_{19}$, this may possibly reflect increased recognition of a conserved region which is exposed to the immune system for a short time during RBC invasion, and at other times may be hidden by the rest of the molecule.

As in chapter 5, mainly IgG1 antibodies were detected to $PfMSP1_{19}$ epitopes. I was unable to demonstrate an association between subclass and protection from clinical malaria in this study since nearly all positive sera contained predominantly IgG1 antibodies. A similar study was conducted in Madagascar, measuring IgG subclass of antibodies to Pf155/RESA in adults newly exposed to malaria. They also found that predominantly IgG1 and IgG3 antibodies were produced but the level of Pf155/RESA-specific IgG tended to be higher in non-protected, rather than in protected, individuals (Dubois *et al* 1993).

The highest degree of protection was observed with antibody responses to MAD-EGF2. Even though the number of seropositive children is low, all ten Gambian children with antibodies to this protein prior to the malarial transmission season, experienced only asymptomatic malarial infections during the following months. This indicates that these children were able to control their parasitaemia to below levels at which clinical symptoms are manifest. A similar trend was seen for the Sierra Leonean children, however, of the 16 children which had antibodies to MAD-EGF2, 4 (25%) did succumb to clinical infections. The second EGF-like motif is more polymorphic than the first, with three dimorphic amino acid differences compared to one in the first EGF-like motif. There is the possibility that the non-protected Sierra Leonean children were infected with parasites expressing the alternative sequence. Some

antibody responses to PfMSP1_{19} may be sequence-specific, as Tolle *et al* (1993) have shown that dimorphic areas may be critical in the induction of protective immunity. However, Riley *et al* (1992a), have shown, using recombinant proteins representing Wellcome sequences, that there is a certain degree of strain-transcending immunity, as antibodies to two conserved regions, block 3 of PfMSP1_{83} and block 17 of PfMSP1_{42}, were associated with protection from clinical malaria in Gambian children, an area where expression of the Wellcome allele is rare. This suggests that there is strain-transcending immunity, caused by epitopes within conserved sequences. Alternatively, in susceptible children, the second EGF-like motif-specific antibodies may have been of a non-protective IgG subclass or may have been of a non-invasion inhibitory type (see chapter 5).

A short and relatively conserved (dimorphic) antigen, which is the target of protective anti-malarial immune responses, would be an ideal component for a subunit malaria vaccine which could be manufactured using recombinant DNA technology. One potential problem for the use of PfMSP1_{19} in a vaccine, is its apparently poor immunogenicity, especially of the second EGF-like motif. Despite multiple infections, only 27-30% of Sierra Leonean children aged 6-8 years possessed antibodies to PfMSP1_{19}, and only 7% possessed antibodies to MAD-EGF2. Only 60% and 14% of Gambian adults (chapter 4) are seropositive to PfMSP1_{19} and MAD-EGF2 respectively, indicating that even life long exposure to this essentially conserved protein may be insufficient to induce an antibody response.

Reasons for this non-responsiveness are unknown. Data from chapter 4 indicates that antibody responses to PfMSP1_{19} are not limited by HLA class II genotype and genetic background is not a major factor in determining responsiveness. However, there is a suggestion, from a larger study of Gambian twins (Jepson *et al*) that antibody responses to PfMSP1_{42} (which contains PfMSP1_{33} and PfMSP1_{19}) may be regulated by non-MHC genes. If this antigen is to be used as a vaccine it will have to be confirmed that individuals of different genotypes can successfully be immunised.

From work presented here, ethnic background appears to affect antibody responsiveness to PfMSP1_{19} (in The Gambia), however when ethnic group was allowed for in multiple logistic regression, the associations of antibody responsiveness to PfMSP1_{33} and the second EGF-like motif of PfMSP1_{19} with resistance to clinical malaria were still highly significant. Whether this

difference in antibody responsiveness between the ethnic groups is genetic or environmental is unknown. However, it is known that malaria transmission is higher in Fula villages, primarily due to village location and housing conditions (Greenwood *et al* 1987b).

Recent boosting appeared to account for elevated levels of antibody responsiveness to $PfMSP1_{19}$ and $PfMSP1_{33}$ in Sierra Leone, suggesting that antibodies levels to these proteins do not remain stable throughout the year in children (in confirmation of data presented in chapter 4), and may explain the low prevalence of antibodies in Gambian children as their antibody responses were measured at the end of the dry season. Successful immunisation with these proteins may require T cell help to maintain the production of protective antibody (as has been demonstrated in mouse models; Daly and Long 1995).

CHAPTER SEVEN:
T cell responses to PfMSP1$_{19}$

Introduction

Despite evidence of the importance of B cell/antibody responses in the development of immunity against malaria (see chapter 4), there is evidence that cell-mediated immunity may be just as important.

Freeman and Holder (1983) immunised BALB/c mice with affinity purified PyMSP1 and found that vaccinated mice were significantly protected against homologous challenge infection. However, despite obtaining high titre antibodies specific to PyMSP1, the passive transfer of serum from these resistant animals to naive recipients had no protective effect against subsequent challenge infection. This suggested to the authors that the protective response observed was, to a major extent, cell-mediated. This inability of sera from immune hosts to transfer protection has been noted by other investigators (reviewed in Weidanz and Long 1988). Other evidence for the importance of cell-mediated immunity in rodent malaria includes; 1) passive transfer of immune sera to splenectomised or T cell-deprived recipients proves not to be protective; 2) B cell deficient mice can spontaneously resolve malarial infections or resist reinfection; 3) immunity to *P. chabaudi adami* can be transferred to nude mice using immune T cells but not with B cells; 4) immunity can be transferred using antigen-specific T cell lines and clones against *P. chabaudi adami*; 5) mice which lack B cells are more sensitive to primary malarial infections, however the function of B cells is not only to produce Ig but also to act as antigen presenting cells (APCs), so this increased sensitivity to infection may be a result of a lack of T cell activation; 6) μ-suppressed mice can be made immune to malaria by infection followed by drug cure; 7) thymectomised mice are unable to make a protective response to *P. yoelii* or *P. berghei*, these mice were unable to control parasitaemia after drug cure, unlike mice which received a thymus tissue graft or immune serum; 8) Playfair *et al* (1985) found that stimulation of T cells resulting in delayed hypersensitivity (rather than stimulation of T helper cells resulting in antibody production) correlated with protection of mice from *P. yoelii* following immunisation with purified PyMSP1; the classes of Ig responsible for the passive transfer of

immunity in mice are of the IgG1 and IgG2 subclasses, production of which is T cell dependent. Nude mice fail to produce antibodies and die, suggesting that T cell help is important for the production of protective antibodies. However, optimal protection against *P. yoelii* appears to depend on the cooperation of immune T and B cells, whereas immunity to *P. chabaudi adami* appears to be less dependent on antibody (see Del Giudice *et al* 1988, Troye-Blomberg and Perlmann 1988, Weidanz and Long 1988 for reviews).

The extent to which these rodent model systems mimic human responses in malaria is unclear. To measure T cell responses of malaria-immune individuals, antigens are added to peripheral blood mononuclear cells *in vitro.* T cells will proliferate if the antigen contains appropriate T cell epitopes and is processed by APCs, which present peptide fragments on their surface in conjunction with a major histocompatibility complex (MHC) molecule. Only if the T cell receptor (TCR)-antigen complex recognises both the antigen fragment and the MHC molecule will the T cell proliferate.

T cell proliferation assays only measure whether T cells have been activated to undergo clonal expansion. They give no indication of which subset of T cells has been stimulated. This, however, can be determined by analysis of cytokines produced by the T cell population. There are two main subsets of T cells; CD4+ cells and CD8+ cells. CD8+ T cells are cytotoxic, recognise MHC class I molecules, are involved in the intracellular killing of infected cells and have been shown to be important in immunity against the intracellular/liver stage of the parasites life cycle (Schofield *et al* 1987). CD4+ T cells are MHC class II restricted and perform regulatory, helper functions in immunity. CD4+ T helper cells (Th cells) can be further categorised as either Th1 cells which are involved in the regulation of cell-mediated immunity through the release of such cytokines as interferon-gamma (IFNγ) and interleukin-2 (IL-2), or Th2 cells which are involved in the regulation of B cells and control the IgG subclass response through the release of such cytokines as IL-4 (Cox and Liew 1992).n However the production of these cytokines is not absolute proof of aTh1 or Th2 response as other cell can also produce these cytokines, for example natural killer cells also produce IFNγ.

In humans, the association of T cell responses (proliferation or cytokine production) with immunity to blood stage parasites has been demonstrated (Riley *et al* 1992a). It is thought that CD4+ T cells contribute to immunity against blood stage parasites both by helping antibody responses (Troye-

Blomberg *et al* 1990) and by activating macrophages. It would be necessary to include T cell epitopes in a vaccine against blood stage parasites to provide T cell help to induce optimal antibody synthesis and long term immunological memory as well as antibody-independent effector mechanisms. It would be advantageous to select conserved T cell epitopes for inclusion within a vaccine.

T cell epitopes of PfMSP1 were first identified by screening for human T cell proliferation to a recombinant protein (190-L) representing the highly conserved block 3 region (Sinigaglia *et al* 1988). In total, four separate T cell epitopes have been identified in this conserved region (Crisanti *et al* 1988, Rzepczyk *et al* 1989). T cell clones, were reactive with purified PfMSP1 from three *P. falciparum* isolates (Rzepczyk *et al* 1989), and uncloned T cells from the majority of individuals tested proliferated to peptides representing these conserved epitopes (Rzepczyk *et al* 1989, Simitsek *et al* 1990). This is encouraging for vaccine development, as despite the marked polymorphism of PfMSP1, immunisation with a conserved T cell epitope may be sufficient to induce T cell help, and would be boosted after exposure to parasites expressing the alternative PfMSP1 allele from that used for immunisation.

Research for vaccines against blood stage parasites has centred on humoral immunity, with only limited success (Hall *et al* 1984a, Perrin *et al* 1984, Siddiqui *et al* 1987, Patarroyo *et al* 1987a, 1987b, 1988). However, once it was realised that T cell epitopes should also be included the immunogenicity and protective effect of these antibody inducing vaccine antigens was improved. Herrera *et al* (1990) immunised monkeys with the 190-L recombinant protein construct and only obtained partial protection despite the presence of four known T cell epitopes. They repeated the study, but immunised monkeys with the recombinant protein 190-N (which contains the 190-L protein) fused with a universal T cell epitope (CS.T3) from the circumsporozoite protein of *P. falciparum* (Herrera *et al* 1992). This epitope is recognised by T cells in association with many different MHC class II molecules (Sinigaglia *et al* 1988). Improved protection correlated with the levels of IFNγ produced, suggesting that cell-mediated immunity rather than antibody was responsible for protection.

Antibody responses to $PfMSP1_{19}$ appear to be associated with protection from clinical malaria (see chapters 5 and 6) but the prevalence of such antibodies is low. It is possible that this is due to a lack of T cell helper epitopes in the native $PfMSP1_{19}$ protein. Immunisation of mice with $PyMSP1_{15}$ protected

8 out of 11 from a lethal challenge infection (Daly and Long 1993). However, this protection was only obtained with the $PyMSP1_{15}$ GST fusion protein, and not with the GST-cleaved protein even though both constructs induced antibodies which recognise native PyMSP1. This suggests that GST may contain T cell epitopes which provide T cell help for the production of protective antibodies. However, GST would not be an appropriate carrier for a malaria vaccine as natural infection with malaria parasites would not boost GST T cell responses. In a recent study (Ling *et al* 1994)15 out of 16 mice immunised with GST-cleaved $PyMSP1_{15}$ were protected from a lethal infection, and 15 out of 15 mice immunised with the uncleaved protein were also protected; in this case, T cell help may have been provided by Freund's complete adjuvant which is unacceptable for use in humans. However, mice immunised with a smaller fragment of the C-terminus of PyMSP1 ($PyMSP1_{11}$) were completely protected against challenge infection and passive transfer of their serum significantly delayed the onset of parasitaemia in naive mice (Daly and Long 1995). The authors show that CD4+ T cells become important in immunity 12 days after immunisation, suggesting that CD4+ T cells are important in maintaining high levels of antibody. CD4+ T cells may also play an auxiliary role in protection through the production of cytokines resulting in the activation of other effector mechanisms of immunity. The authors proposed that in the shortened $PyMSP1_{11}$ construct, cryptic T cell epitopes were unveiled which were able to provide the necessary T cell help for protective antibody responses to develop.

Immunisation with $PfMSP1_{19}$-GST fusion proteins did not protect monkeys against challenge infection (Burghaus *et al* manuscript in preparation), while immunisation of monkeys with yeast recombinant proteins representing the same protein protected 2 out of the 4 vaccinated monkeys from a lethal challenge infection (Kumar *et al* 1995). Both vaccination schemes induced invasion inhibitory type antibodies (see chapter 5); 3 of 5 sera in the Burghaus study were able to inhibit parasite invasion of RBCs *in vitro* , non of the sera from the Kumar study were able to inhibit. Possibly the lack of protection with the bacterial proteins was due to insufficient T cell help for the induction of a protective antibody response by antibody affinity maturation or isotype switching. This may result from post-translational differences between proteins formed in the two different expression systems, or may once again be due to the choice of adjuvant used (Kumar *et al* used Freund's adjuvant, while Burghaus *et al* used liposomes and alum as an alternative to Freund's).

I have found that in individuals naturally exposed to malaria, $PfMSP1_{19}$ is poorly antigenic compared to other regions of the molecule (see chapter 4). This low prevalence may be due to few people ever making antibodies to $PfMSP1_{19}$, or it may be that lots of people make antibodies, but memory is poor so antibody levels fall quickly and lots of people are seronegative at any one time. So, in this chapter, I have investigated whether this poor antibody response to $PfMSP1_{19}$ is due to lack of T cell help. I have considered that this may be due to a lack of T cell epitopes *per se* and that the presence of disulphide bonds within $PfMSP1_{19}$ may hinder linearisation of the peptides for presentation within the peptide binding groove of MHC molecules, thus preventing T cell recognition. In this study I have measured peripheral lymphocyte proliferative responses in malaria immune adults to recombinant proteins representing $PfMSP1_{19}$ in a native and a reduced form.

Methods and materials

(1) Antigens

Recombinant proteins used in this study have previously been described in chapter 2. *E. coli*-derived GST fusion proteins representing the first EGF-like motif of the Wellcome and MAD20 sequences of $PfMSP1_{19}$ (Well-EGF1, MAD-EGF1), the second EGF-like motif of MAD20 $PfMSP1_{19}$ (MAD-EGF2), and the double EGF-like motif of Wellcome $PfMSP1_{19}$ (Well-19/GST) were used. Yeast recombinant proteins representing the full length MAD20 sequence of $PfMSP1_{19}$ were also tested (MAD-19), see figure 2.1. Proteins were used in a reduced and non reduced form at 1µg/ml (see chapter 2).

T cell proliferative responses were also measured to the mitogen Phytohemagglutinin (PHA; Difco Laboratories, Detroit, Mich.; 2µg/ml final concentration), Tuberculin purified protein derivative (PPD; Evans Medical Ltd, Leatherhead, England; 1µg/ml final concentration) and to the disulphide-constrained tetanus toxoid (Wellcome; .4µg/ml final concentration). These proteins acted as positive controls in the assays.

(2) Study population

Blood was collected from 19 adults living in a rural area of The Gambia. Malaria transmission in this area is seasonally endemic during and immediately following the rainy season (July - December) (Greenwood *et al* 1987a). Samples were obtained prior to (March) the rainy season.

(3) T cell proliferation assay

Cells from non-exposed European donors were used to test the toxicity of the proteins and to determine the extent of non-specific T cell activation.

20mls of blood were collected from each donor. Plasma was separated from heparinised blood by centrifugation, aliquoted and stored at -20°C. Mononuclear cells (MNC) were isolated by density dependent gradient centrifugation (Lymphoprep, Nycomed, Norway). Cells were washed and suspended in RPMI supplemented with 2mM L-glutamine, 100u/ml penicillin and streptomycin, 30mM HEPES, 0.22% (v/v) sodium bicarbonate and 10% (v/v) non-immune human A+ serum (heat inactivated). MNC (10^5 per well) were added to 96-well round bottomed microtitre plates in a total volume of 200µl.

Triplicate wells contained optimal concentrations of antigen. Cells were grown for seven days in a CO_2 (5%) incubator at 37°C.

The cells were left 7 days to proliferate as this has previously been determined to be the optimal culture period time from kinetic experiments carried out by Riley *et al* (1993). Eighteen hours prior to harvesting; 100µl of cell free supernatant was removed from each well and 100µl of complete RPMI containing 1µCi ^{3}H-thymidine was added to each well. Supernatants were collected for the measurement of IFNγ responses after 6 days as this is when their concentration is highest (Riley *et al* 1993). Cells were harvested and incorporation of ^{3}H-thymidine was measured by liquid scintillation counting. The geometric mean counts per minute (cpm) of triplicate wells was determined for each antigen and the mean stimulation index (SI) was calculated as shown;

$$SI = \frac{\text{geometric mean triplicate antigen cpm}}{\text{geometric mean triplicate control cpm}}$$

where the control is either the unstimulated (background) count for non-fused proteins, i.e. MAD-19, or the value obtained with the GST fusion partner alone for the GST fusion proteins (Bennett and Riley 1992).

(4) Interferon γ ELISA

IFNγ in culture supernatants was measured by a two site ELISA assay (Andersson *et al* 1989) using two mouse mAbs to human recombinant IFNγ (Chromogenix, Molndal, Sweden). IFNγ concentration was determined by reference to the international human IFNγ standard GG23-901-530 (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). The IFNγ ELISAs were performed by Dr. E. Riley, (University of Edinburgh, Scotland, UK).

(5) Statistical methods

To determine whether T cell proliferative responses to the PfMSP1 proteins increased with reduction and alkylation of the proteins, a non parametric Wilcoxon signed rank test was carried out measuring differences in the cpm obtained for the two versions of the protein.

Results

(1) Toxicity testing of PfMSP1$_{19}$ proteins

European cells were tested to see if antigens were toxic and to see whether they activate non-immune cells. T cell proliferation was measured in samples containing 1µg and 10µg/ml of non-reduced antigen. As cpm were similar or greater than RPMI alone it was evident that the proteins were not toxic (table 7.1.). As high cpm was obtained to the positive control PPD it was evident that the cells were alive and activated. At 10µg/ml the antigens caused some non-specific activation of non-immune cells; thus, for subsequent studies, PfMSP1$_{19}$ proteins were used at 1µg/ml only.

(2) Lymphoproliferative responses to native PfMSP1$_{19}$

Background lymphoproliferative responses in control wells, containing either GST or RPMI alone, are at an acceptable level (table 7.2.). Lymphoproliferative responses to positive control proteins are high (100% respond to PHA) indicating that the assay was working and that the cells were alive and activated (see table 7.3. and figures 7.1.a. and 7.2.).

Responses to PfMSP1$_{19}$ are extremely low, with no positive SI response to any of the PfMSP1$_{19}$ proteins apart from the Well-EGF1 protein (a positive response is considered to be a SI greater than 3). SI produced to the EGF-1 and double-motif proteins appear to be cross-reactive for the two allelic forms of the protein.

For individual results of the proliferation assays see appendix 1.

(3) Lymphoproliferative responses to reduced PfMSP1$_{19}$

Proteins were reduced and alkylated to test whether the disulphide structure of PfMSP1$_{19}$ may be responsible for lack of responsiveness to this protein (see figure 7.3. for confirmation of protein reduction). For the majority of individuals, reduction and alkylation of the disulphide-constrained PfMSP1$_{19}$ proteins did result in an increase in proliferation, significantly increasing T cell proliferative responses to the reduced proteins (see table 7.2. and figures 7.1 and 7.2.). T cell responses seemed to increase more for the MAD20 proteins; The Gambia is an area where the MAD20 form of PfMSP1 predominates (Conway and McBride 1991), so people may recognise this form of the protein more.

Donor	Antigen	1μg/ml		10μg/ml	
		mean cpm	SI	mean cpm	SI
SD	Well-EGF1	1518.7	0.8	3857.7	3.5
	MAD-EGF1	1616.7	0.9	2082.7	1.9
	MAD-EGF2	779.0	0.4	4944.0	4.5
	Well-19/GST	628.7	0.3	1278.4	1.2
	MAD-19	693.7	0.6	631.4	0.5
	GST	1829.4	1.6	1096.7	0.9
	PPD	44705.0	38.5		
	RPMI	1160.7			
KS	Well-EGF1	1819.0	2.3	2132.0	1.4
	MAD-EGF1	1283.0	1.6	1464.7	1.0
	MAD-EGF2	977.4	1.3	5583.7	3.8
	Well-19/GST	715.7	0.9	1427.7	1.0
	MAD-19	782.7	0.9	679.0	0.8
	GST	781.0	0.9	1480.0	1.6
	PPD	31522.7	34.8		
	RPMI	905.3			

Table 7.1. Mean cpm and SI obtained from two representative European donors.

	mean, (range), [SE] cpm		mean, (range), [SE] SI		n (%) with SI greater than 3		increase or decrease (*) in response to reduced protein	
	non reduced	reduced	non reduced	reduced	non reduced	reduced	Wilcoxon Statistic	p valve
Well-EGF1	1618.1 (148-3805) [264.3]	1535.9 (81-12445) [634.0]	1.5 (0.8-3.5) [0.2]	1.9 (0.7-5.9) [0.3]	2 (10.5)	4 (21.1)	144.0	0.05
MAD-EGF1	1100.8 (159-2606) [164.6]	2988.3 (87-10236) [692.9]	1.1 (0.4-2.2) [0.1]	2.4 (0.6-7.2) [0.5]	0 (0.0)	6 (31.6)	189.0	0.00
MAD-EGF2	988.3 (77-3365) [173.4]	2416.2 (93-8047) [502.8]	1.0 (0.3-2.1) [0.1]	2.1 (0.6-7.1) [0.4]	0 (0.0)	5 (26.3)	190.0	0.00
Well-19/GST	1176.7 (147-2768) [167.5]	2597.6 (109-14419) [739]	1.1 (0.7-2.3) [0.1]	1.9 (0.5-5.1) [0.3]	0 (0.0)	4 (21.1)	176.0	0.00
MAD-19	221.3 (74-1069) [50.9]	1315.6 (127-7492) [483.3]	0.8 (0.4-1.6) [0.1]	5.2 (0.7-39.9) [2.1]	0.0 (0)	6 (31.6)	190.0	0.00
PPD	25123.0 (103-78889) [5952.2]	13089.1 (189-42889) [3102.0]	102.4 (0.5-475.8) [29.1]	54.8 (1.3-234.8) [15.4]	19 (100)	18 (94.7)	13.0*	0.00
TT[ø]	12719.0 (98-35891) [4672.9]	50.8 (0.77-159.4) [19.7]	3239.8 (342-13832) [1491.1]	13.3 (1.8-73.6) [6.9]	7 (70.0)	6 (60.0)	6.0*	0.03
GST	1111.1 (127-2798) [167.5]	1624.9 (1161-7799) [4455.7]	5.0 (1.0-14.9) [1.0]	7.12 (0.9-41.5) [2.3]	9 (47.4)	11 (57.9)	119.0	0.34
PHA	128690.2 (31503-1161100) [57678]	-	547.6 (129.2-5151.5) [257.2]	-	19 (100)	-	-	-
RPMI	279.5 (124-744) [32.8]	-	-	-	-	-	-	-

Table 7.2. T cell proliferative responses for $PfMSP1_{19}$ recombinant proteins.

n=19, [ø] n=10

figure 7.1.a.

unreduced antigen

SI: 1000, 100, 10, 1, 0.1

Well-EGF1 MAD-EGF1 MAD-EGF2 Well-19/GST MAD-19 PPD TT GST

antigen

figure 7.1.b.

reduced antigen

SI: 1000, 100, 10, 1, 0.1

Well-EGF1 MAD-EGF1 MAD-EGF2 Well-19/GST MAD-19 PPD TT GST

antigen

Figure 7.1. Lymphoproliferative responses (SI) to; a) non reduced and b) reduced PfMSP1$_{19}$ recombinant proteins and control proteins.

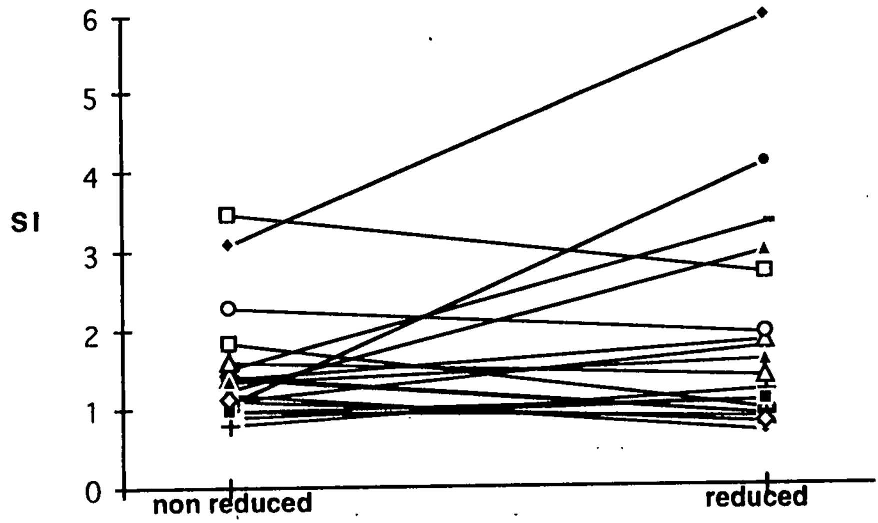


figure 7.2.a.

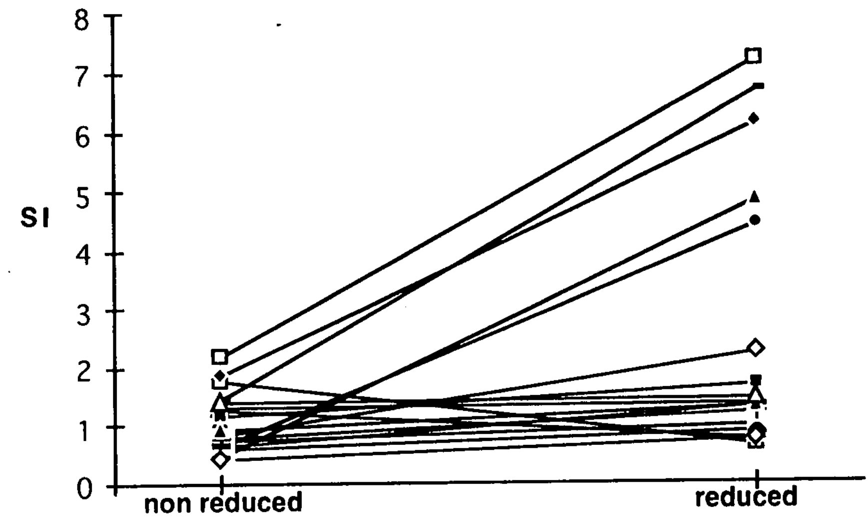


figure 7.2.b.

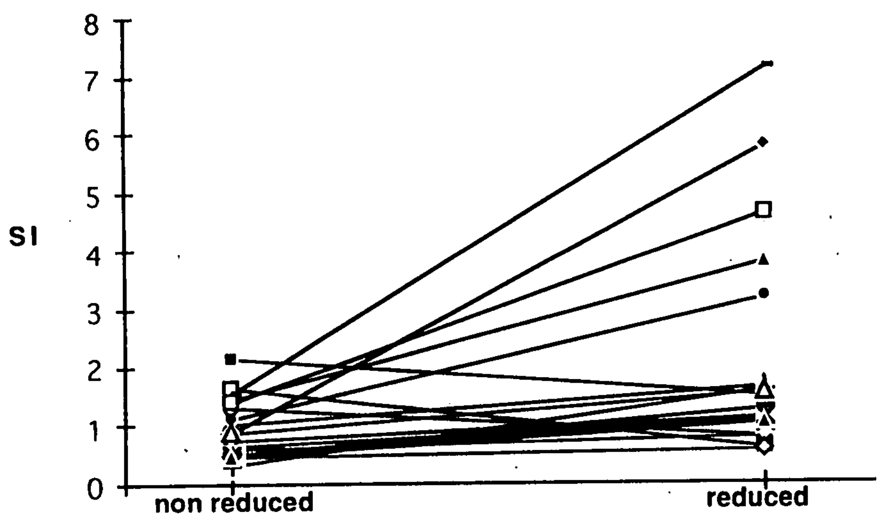


figure 7.2.c.

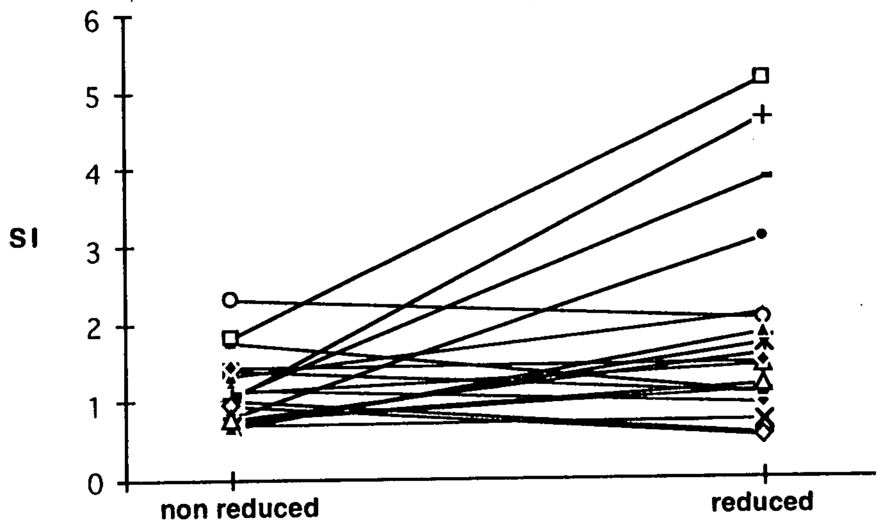
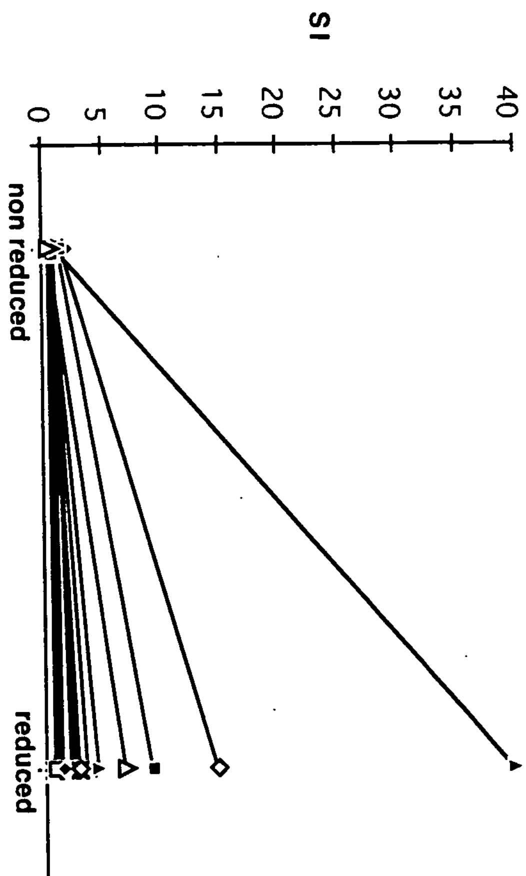
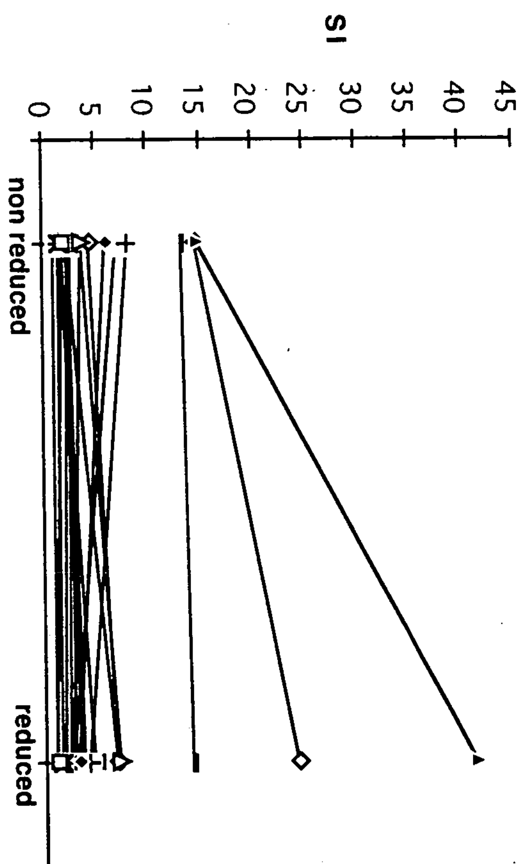
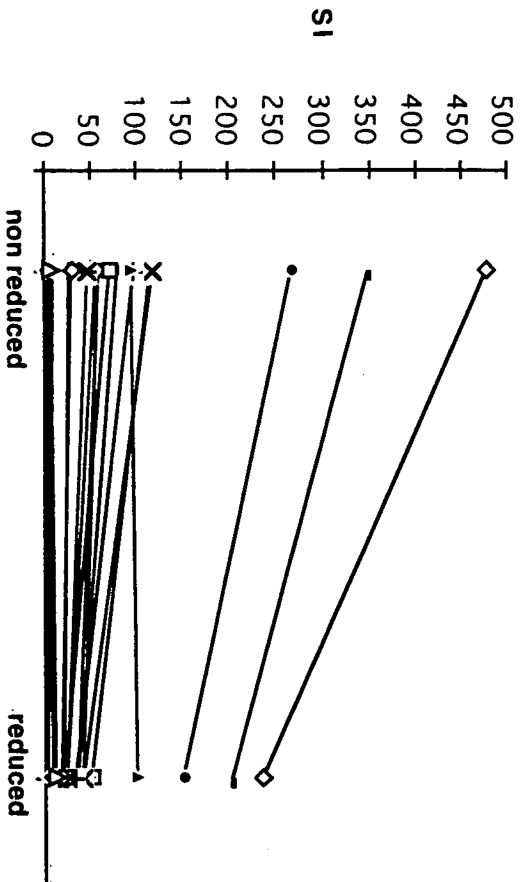


figure 7.2.d.







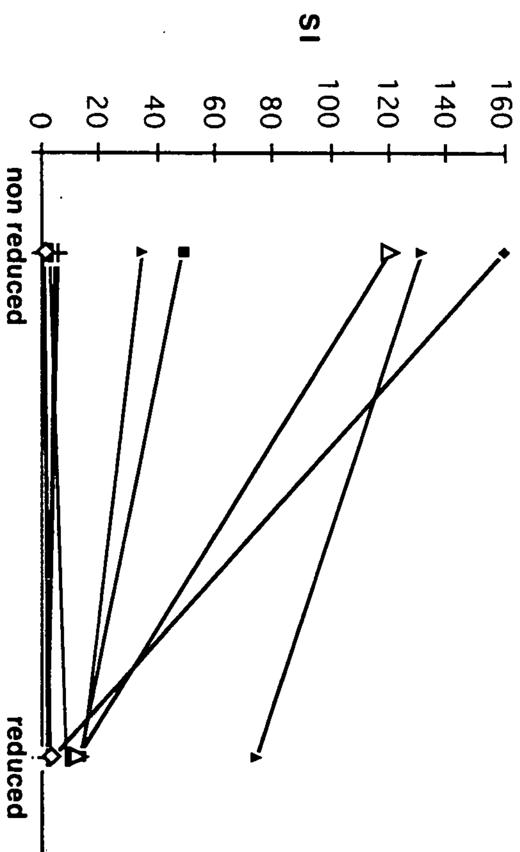


Figure 7.2. lymphoproliferative responses to the PfMSP1_{19} and control proteins demonstrating either an increase or decrease in the SI obtained by an individual to the reduced versus the non reduced form of the protein.

a) Well-EGF1, b) MAD-EGF1, c) MAD-EGF2, d) Well-19/GST,

e) MAD-19, f) GST, g) PPD and h) TT.

Figure 7.3. SDS-PAGE gel of reduced and non reduced PfMSP1$_{19}$ and control proteins.

a) lane1=MW markers in kDa
lane 2=Well-EGF1 reduced
lane 3=Well-EGF1 non reduced
lane 4=MAD-EGF1 reduced
lane 5=MAD-EGF1 non reduced
lane 6=MAD-EGF2 reduced
lane 7=MAD-EGF2 non reduced
lane 8=GST reduced
lane 9=GST non reduced
lane 10=MW markers

b) lane1=MW markers in kDa
lane 2=MAD-19 reduced
lane 3=MAD-19 non reduced
lane 4=
lane 5=
lane 6=
lane 7=
lane 8=PHA reduced
lane 9=PHA non reduced
lane 10=MW markers

c) lane1=MW markers in kDa
lane 2=MAD-19 reduced
lane 3=MAD-19 non reduced
lane 4=MAD/Well-19 reduced
lane 5=MAD/Well-19 non reduced
lane 6=Well-19 reduced
lane 7=Well-19 non reduced
lane 8=Well19/GST reduced
lane 9=Well-19/GSTnon reduced
lane 10=

figure 7.3.a.

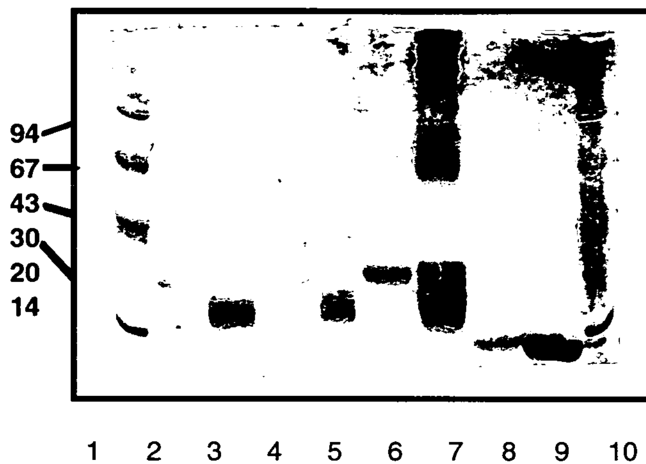


figure 7.3.b.

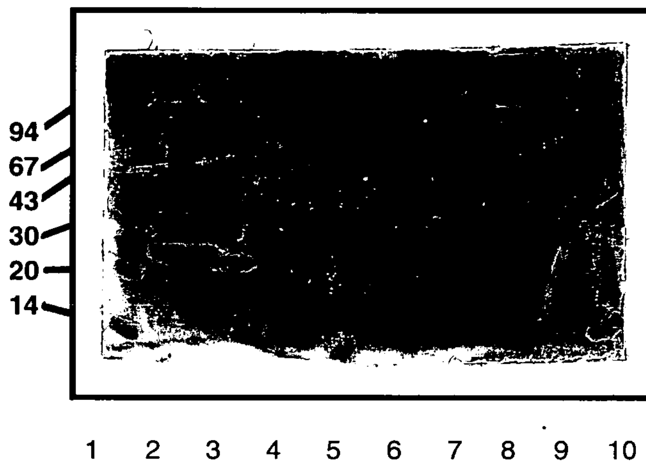
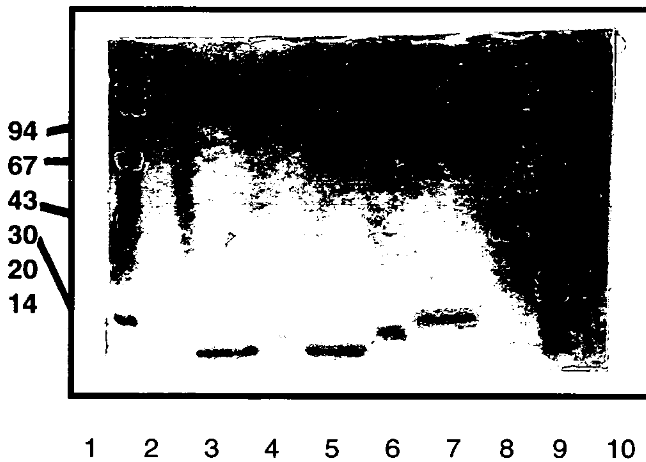


figure 7.3.c.



Reduction and alkylation of the non disulphide-constrained proteins (GST and PPD) did not result in an increase in proliferation in the majority of cases, and actually caused a significant reduction in responses to PPD and TT (see figures 7.1 and 7.2. and table 7.2.). TT is a large disulphide-constrained molecule. Reduction of this protein mainly resulted in a decreased proliferative response. This may be due to its large size; most of its epitopes need the disulphide structure to stimulate proliferation.

However, lymphoproliferative responses to the reduced disulphide-bonded PfMSP1$_{19}$ recombinant proteins are still low compared to the positive control proteins PPD and TT. Very few individuals' MNCs proliferated to the reduced form of the protein; prevalence of responsiveness ranging from 20 to 30% of adults (table 7.2.).

(4) IFNγ responses to PfMSP1$_{19}$

There is a linear relationship between the OD obtained in the IFNγ ELISAs and log IFNγ units (U), so OD values can be converted into U/ml of IFNγ by comparison with a standard curve (see figure 7.4.). IFNγ responses to the positive controls PHA and PPD are high (100% for PHA), demonstrating that the assay worked and that the cells produced IFNγ if appropriately stimulated.

IFNγ responses to PfMSP1$_{19}$ recombinant proteins are extremely low, in both prevalence and levels; EGF-2 seems to be particularly poor at inducing IFNγ responses (see table 7.3.). However, individuals that did respond to EGF-1 and/or the double-motif proteins, produced IFNγ to both the Wellcome and MAD20 versions indicating that IFNγ stimulating T cell epitopes are cross-reactive for the two forms of PfMSP1$_{19}$. Some donors who produced IFNγ in response to EGF-1 did not respond to the double-motif protein, indicating that processing of the molecule may be important for T cell recognition.

In the case of IFNγ, there is no evidence that reduction of disulphide bonds affected the response, either for the PfMSP1 proteins or the control proteins.

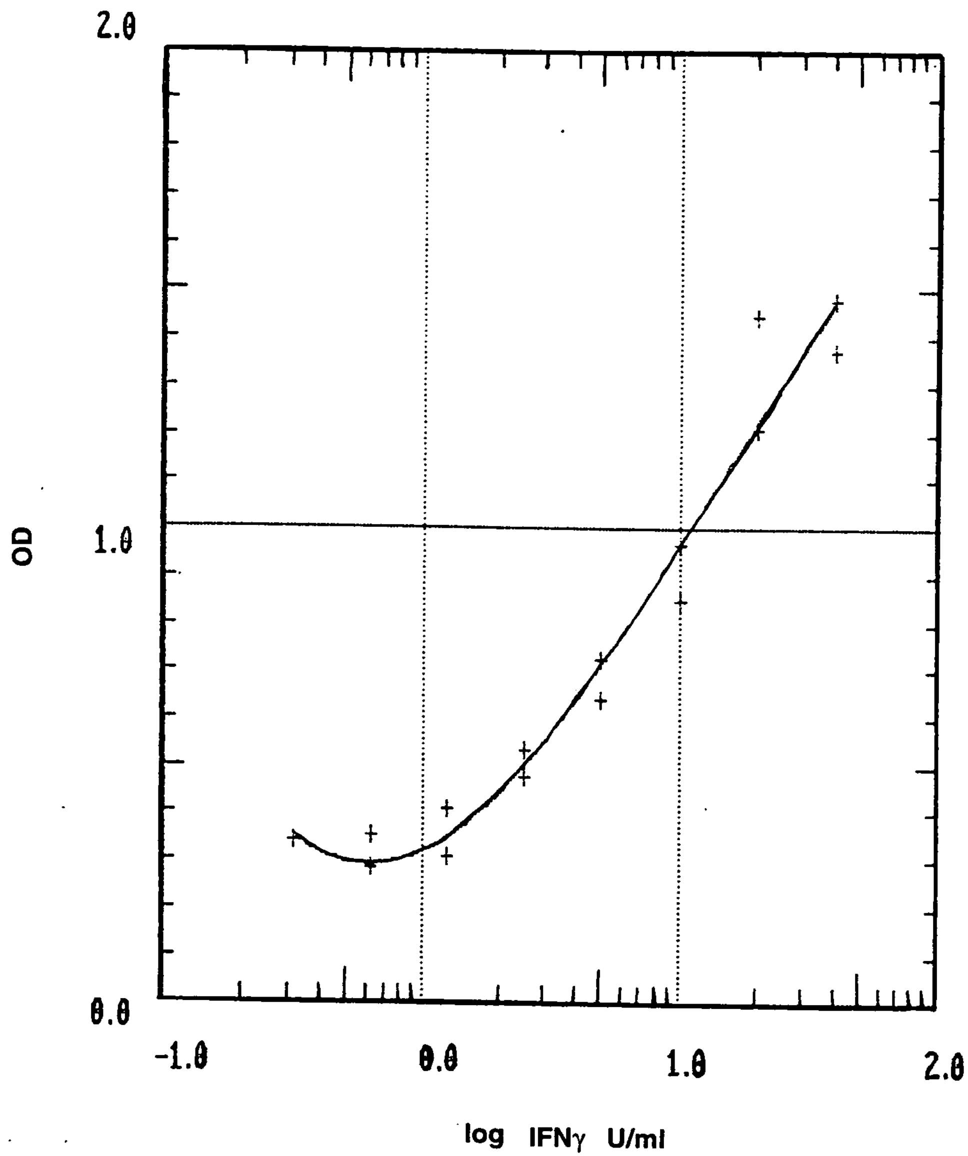


Figure 7.4. Standard curve of OD and IFNγ U/ml.

Antigen	Donor																			n (%)
	4	12	13	15	35	45	50	53	69	79	95	104	110	112	116	117	120	125	167	responders
RPMI																				0 (0.0)
Well-EGF1				3							4					6		2		4 (21.1)
Well-EGF1 R				3														23		2 (10.5)
MAD-EGF1											3					3				2 (10.5)
MAD-EGF1 R				3							3							7		3 (15.8)
MAD-EGF2																				0 (0.0)
MAD-EGF2 R																		3		1 (5.3)
Well-19/GST																				0 (0.0)
Well-19/GST R											2							8		2 (10.5)
MAD-19																				0 (0.0)
MAD-19 R			3															3		2 (10.5)
GST	2																	4		2 (10.5)
GST R											6							3		2 (10.5)
PPD	13	28		40+		9	40+	8	31	9	10	16			7	3		5		13 (68.4)
PPD R	5	10		40+		3	40+		25	17	4	8			3			11		11 (57.9)
TT																3				1 (5.3)
TT R																		6		1 (5.3)
PHA	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	40+	19 (100)

Table 7.3. IFNγ responses (U/ml).

R=reduced form of the protein.
Measured in international units/ml.
Blank space equals an IFNγ response <2U/ml.

Discussion

Previous studies carried out in our laboratory show that lymphoproliferative responses to recombinant proteins representing $PfMSP1_{42}$ (Well-42), but not other regions of the PfMSP1 molecule, are associated with resistance to clinical malaria in children (Riley *et al* 1992a). To further analyse this response, I have used recombinant proteins representing $PfMSP1_{19}$ and its constituent EGF-like motifs to try and map T cell epitopes within this region using peripheral blood T cells from malaria immune adult Gambian donors.

There are several limitations to the measurement of T cell responses to malarial antigens using this method (i.e. monitoring peripheral T cell proliferation) and these limitations should be taken in to account when assessing non-responsiveness. Only small volumes of peripheral blood are collected to perform the T cell assay, and only small numbers of T cells are tested (10^5 cell/well). Also many crucial cellular interactions occur deep within the body (i.e. within the spleen), so cells isolated from the peripheral blood at any one time may only represent a subset of the total malaria-reactive pool (Riley *et al* 1993). It has also been demonstrated that the number of responding individuals will be seriously underestimated unless multiple parameters of T cell activation are measured, such as measuring cytokine production (Troy-Blomberg *et al* 1990). Furthermore, responsiveness of individuals varies greatly from wet to dry seasons, and year to year, so total responsiveness cannot be predicted from only one survey (Riley *et al* 1993). A possible drawback of stimulating T cell proliferation *in vitro* is that the low responsiveness seen may actually be an artefact of inappropriate processing of the protein by the APCs present in the *in vitro* experiment.

After *in vitro* culture with recombinant proteins, and appropriate control proteins, T cells from very few individuals either proliferated or produced IFNγ in response to $PfMSP1_{19}$. However, I did not measure IL-4 responses, so I may have underestimated T cell responses (Troy-Blomberg *et al* 1990). Assays were performed on 19 adult donors in the low malaria transmission season. This is an appropriate time of year to measure T cell proliferative responses to malarial antigens as it has been demonstrated that T cells are depleted from the peripheral circulation during episodes of acute malaria, so there is a decrease in proliferative responses during and at the end of the malaria transmission season (Hviid and Theander 1993). T cell proliferative responses were

subsequently measured in 16 adults during the rainy season and, as expected, responsiveness was found to be much lower than during the dry season (Dr. M. Waterfall, University of Edinburgh, personal communication).

A recent study has also noted low T cell responsiveness to $PfMSP1_{19}$ (Udhayakumar *et al* 1995). In this study, T cell responsiveness of malaria-exposed Kenyan adults was measured to 8 synthetic peptides representing linear epitopes of $PfMSP1_{19}$. Only 3 of the 8 peptides induced T cell proliferative responses in more than 30% of the study population (n=40-104). This essentially confirms previous data by Riley *et al* (1992a, 1993) that, although there are well recognised T cell epitopes within the N-terminus region of $PfMSP1_{42}$, there are few such epitopes within $PfMSP1_{19}$.

Poor T cell responsiveness to $PfMSP1_{19}$ in African adults may be due to down regulation of T cell responses in immune adults (reviewed in Riley *et al* 1994). Both cell-mediated and antibody-mediated mechanisms of immunity are thought to contribute to immunity against malaria, but also contribute to the pathology caused by malaria. T cells produce IFNγ which activate macrophages to produce tumour necrosis factor-alpha (TNFα) which can result in damage to the host as well as the parasite (reviewed in Taverne 1993). Also unregulated activation of B cells can result in hypergammaglobulinaemia and hyper-reactive malaria splenomegaly (Crane 1986). So immune responses to malaria need to be carefully regulated to maintain the delicate balance between protection from infection and immune-mediated pathology. CD8+ T cells have been reported to suppress CD4+ T cell responses to PfMSP1, probably to avoid pathological consequences of persistent immune activation (Hviid and Theander 1993). Measuring T cell responses of partially-immune children or malaria-exposed travellers, where down regulation of immune responses would not have developed, may circumvent this problem.

However, a factor attributing to the poor recognition of $PfMSP1_{19}$ may be its possession of disulphide bonds which may hinder the linearisation of peptides by APCs for their presentation within the MHC peptide binding groove. It has been demonstrated with other disulphide-bonded proteins that reduction of the bonds and unfolding of the protein is a prerequisite for antigen processing. This allows proteolytic processing enzymes access to the protein so forming linear peptides with exposed cysteine residues for T cell recognition (Collins *et al* 1991). Reduction and alkylation of $PfMSP1_{19}$ significantly

increased the proliferative response indicating that processing of the protein may be a problem.

However, correct tertiary structure of a protein has also been demonstrated to be important for recognition of the protein. Vidard *et al* (1992) demonstrated that reduction and alkylation of cysteine residues within ovalbumin (OVA) drastically reduced its capacity to stimulate some OVA-specific T cell clones. They found that presentation of some epitopes was not influenced by denaturation, while others were either increased or decreased. Similarly, Atassi *et al* (1989) demonstrated that T cell lines responded well to native lysozyme but poorly to an unfolded version of the protein. As APCs internalise and digest the protein to peptide fragments, it would be expected that T cell recognition would be independent of tertiary structure of the protein. However in these cases, presentation of the tertiary structure to the APC is important for correct antigen presentation. In summary, alterations in the native form of an antigen can affect its processing pattern. Whether this is important for the presentation of T cell epitopes to PfMSP1_{19} *in vitro* is unknown.

Even with reduction of the protein, T cell lymphoproliferative responses to PfMSP1_{19} are still low (20 to 30%). An effect of the disulphide structure on processing was also suggested by Udhayakumar *et al* (1995), however, they measured T cell responses to linear peptides, so this does not explain the low responsiveness found in their study. So, even though antigen processing of the disulphide bonded antigen by APCs appears to be a limiting factor, the main reason for lack of T cell responsiveness to PfMSP1_{19} appears to be that this region lacks T cell epitopes. Using a computer algorithm for predicting T cell activating epitopes, the above authors were unable to find any predicted T cell epitope within PfMSP1_{19}, emphasising this possibility. This does not bode well for a PfMSP1_{19} subunit vaccine. However, the inclusion of known helper T cell epitopes from elsewhere may allow an effective antibody response to be induced.

Hui *et al* (1994a) found that immunisation of rabbits with recombinant proteins representing PfMSP1_{42} induces antibodies with strong inhibitory activity against parasite growth *in vitro*. These antibodies were specific for PfMSP1_{19} as depletion of PfMSP1_{19}-specific antibodies on an affinity column abolished the growth inhibitory activity of the serum. However, immunisation of rabbits with PfMSP1_{19} induced antibodies which did not inhibit parasite growth *in vitro*. This suggests that PfMSP1_{19} contains epitopes for the production of

specific antibodies, but that T cell epitopes within $PfMSP1_{19}$ do not induce effective inhibitory antibodies, while presumably T cell epitopes within the N-terminus of $PfMSP1_{42}$ construct, i.e. $PfMSP1_{33}$, do. The authors conclude that the nature/specificity of T helper epitopes is crucial to the induction of biologically relevant/protective antibodies. On the other hand, secondary processing of $PfMSP1_{42}$ may result in a conformational change in $PfMSP1_{42}$ compared to $PfMSP1_{19}$, which could affect the specify of $PfMSP1_{19}$-specific antibodies.

However, it seems possible that the induction of protective antibodies to $PfMSP1_{19}$ is dependent on T cell epitopes located within $PfMSP1_{33}$. Secondary processing of $PfMSP1_{42}$ to $PfMSP1_{33}$ and $PfMSP1_{19}$, may mean there is no cognate interaction between T and B cells to form an adequate antibody response to $PfMSP1_{19}$. In the Udhayakumar study (1995), it was found that T cell proliferative responses to synthetic peptides representing $PfMSP1_{33}$ were more prevalent than peptides representing $PfMSP1_{19}$.

In a continuation of their work, Hui *et al* (1994b) tried to further characterise T helper cell epitopes within $PfMSP1_{42}$. Mice were primed with a sub-optimal dose of native PfMSP1 or baculovirus $PfMSP1_{42}$, and boosted with homologous or heterologous native PfMSP1 or $PfMSP1_{42}$. Cross priming with heterologous PfMSP1 was as effective as homologous PfMSP1 at inducing antibody of similar titre to PfMSP1 suggesting conserved or cross-reactive epitopes provide T cell help. Data presented here indicates that T cell epitopes in $PfMSP1_{19}$ are cross-reactive. However, these authors found $PfMSP1_{42}$ was not as effective at inducing $PfMSP1_{42}$-specific antibodies as native PfMSP1. This lead the authors to suggest that either recombinant $PfMSP1_{42}$ does not properly represent native $PfMSP1_{42}$ or dominant T helper cell epitopes for $PfMSP1_{42}$ antibody production do not come from $PfMSP1_{42}$.

The ability of vaccination of mice with GST-cleaved recombinant $PyMSP1_{15}$ protein to induce a protective immune response against challenge infection (Ling *et al* 1994) contrasts with the inability of $PfMSP1_{19}$ immunisation of rabbits to induce biologically relevant antibodies (Hui *et al* 1994a). However, there are crucial differences between $PfMSP1_{19}$ and its analogue $PyMSP1_{15}$. Although most of the cysteine residues are conserved between $PfMSP1_{19}$ and $PyMSP1_{15}$, the intervening amino acid sequence is not. Also, the first cysteine pair of the first EGF-like motif of $PyMSP1_{15}$ is missing, suggesting that there is a structural/conformational difference between $PfMSP1_{19}$ and

PyMSP1$_{15}$. This may result in differences in antigen processing by APCs, leading to different immune responses. Also, the dissimilarities in amino acid sequences may result in differences in the repertoire of T and B cell epitopes.

An alternative explanation is that relevant T helper epitopes may have been present in the PfMSP1$_{19}$ construct, but may have been rendered non-immunogenic due to immunisation conditions. Adjuvant has been demonstrated to be important to both immunogenicity and protection (reviewed in Hui *et al* 1994a). Protection of animals immunised with PyMSP1$_{15}$ is dependent upon the adjuvant used (C. A. Long, Hahnemann University, Philadelphia, PA, personal communication in Hui *et al* 1994a). Different adjuvant formulations may alter the selection/inhibition of different proteases or processing pathways, or activate/inhibit different types of APC which influence the generation of T cell epitopes. Interactions between adjuvant and genetic background have also been demonstrated. Various combinations of mouse strains and adjuvants affected the production of growth inhibitory antibodies, possibly as a result of differences in processing of the antigen, resulting in the generation of different peptide fragments, able to interact with different sets of MHC class II antigens (Hui and Chang 1992, Chang *et al* 1994, Golding *et al* 1994).

Differences in MHC haplotype could be a reason for T cell non-responsiveness to PfMSP1$_{19}$. The influence of MHC haplotype on immune responsiveness in mice has been reported for several malarial antigens, e.g. the circumsporozoite antigens of *P. falciparum* and *P. vivax* (Del Giudice *et al* 1988), RESA (Lew *et al* 1989b), and gamete surface antigens (Good *et al* 1988b). Genetic responsiveness to PfMSP1$_{19}$ has been noted in mice, as BALB/c mice make antibodies to this protein while C57B1/10 mice do not (Hui *et al* 1994a).

Even though there appears to be genetic restriction of T cell responsiveness in different inbred mouse strains, MHC restriction of T cells has been difficult to demonstrate in out bred human populations. Guttinger *et al* (1991) found that the T cell epitopes of the conserved block 3 of PfMSP1 can be recognised in association with many different MHC class II molecules. Heterogeneity of the human class II gene complex and the fact that T cell epitopes can associate with several different class II molecules makes it unlikely that class II determined non-responsiveness will prove to be a problem in the development of a subunit vaccine for use in out bred human populations (Riley

et al 1991, 1992b). Data presented in chapter 4 and in Taylor *et al* (in press) suggests that antibody responses to PfMSP1$_{19}$, by malaria-exposed individuals, is not genetically determined. However, I have only tested the Mandinka ethnic group. So in future work, may be the Fula and Wollof ethnic groups should also be tested. However, data presented here is similar to Udhayakumar *et al* (1995) where they looked at genetically different populations in Kenya, which may mean that T cell responses are not genetically restricted.

Previous data from our laboratory demonstrate that human T cells preferentially recognise protein constructs representing the N- rather than the C-terminus of PfMSP1 (Riley *et al* 1993). My own findings show that there is poor T cell proliferative responses to this region. Strategies are needed to increase T cell help to result in the production of effective antibody responses against PfMSP1$_{19}$. The fact that the highly conserved T cell epitopes of p190L (Sinigaglia *et al* 1988), recognised in association with many different HLA class II molecules (Guttinger *et al* 1991), are recognised by a high proportion of Gambian adults (Riley *et al* 1992a, 1993) is very promising for vaccine development. Inclusion of this region of PfMSP1 in a vaccine as well as PfMSP1$_{19}$ may induce T cell help for the production of antibody in most vaccinated individuals, but not necessarily antibodies of biological significance, i.e. protective/growth inhibitory antibodies. More attention will have to be paid to delivery systems, i.e. adjuvants.

CHAPTER EIGHT:
Discussion

1. PfMSP1$_{19}$ as a vaccine candidate

Antigens on the merozoite surface are obvious candidates for inclusion within a blood stage vaccine. There is a growing body of evidence that immune responses, mainly antibody-mediated, to the major surface antigen on the merozoite, MSP1, confer protection from clinical symptoms of malaria. This evidence comes from immunisation of animals with native or recombinant MSP1, passive transfer of immune serum from animals immunised with MSP1 and the association of antibody to PfMSP1 with clinical immunity to malaria in semi-immune children. The highly conserved C-terminus of the molecule, PfMSP1$_{19}$, is the target of the majority of PfMSP1-specific mAbs which inhibit merozoite invasion *in vitro*. Also, serum from rabbits immunised with PfMSP1$_{42}$ was able to inhibit the *in vitro* growth of parasites from either PfMSP1 family suggesting, due to the dimorphic nature of PfMSP1$_{33}$, that the target(s) of the inhibitory antibody was located within the highly conserved PfMSP1$_{19}$ region.

A major problem in the development of a vaccine against malaria is the extensive polymorphism of malaria antigens. PfMSP1$_{19}$, however, is a highly conserved protein; only four out of its 96 amino acid residues are not conserved and these are dimorphic. PfMSP1$_{19}$-specific growth inhibitory antibodies, monoclonal or polyclonal, are effective against parasites expressing either PfMSP1 allele, which is encouraging evidence that a recombinant vaccine consisting of the highly conserved EGF-like motif region of PfMSP1$_{19}$ may be effective in vaccination against all parasite strains.

Data presented here lend substantial additional support that PfMSP1$_{19}$ is a good candidate antigen for a blood stage vaccine:

(1) I have found that PfMSP1$_{19}$ is naturally antigenic in a malaria-exposed human population and that antibody responses to the two allelic forms of PfMSP1$_{19}$ are cross-reactive. Despite the low prevalence of the Wellcome allele in The Gambia (<5%, Conway and McBride 1991), Gambian antibody responses to the two allelic forms of PfMSP1$_{19}$ are highly correlated, suggesting that immunisation with one allelic form of PfMSP1$_{19}$ can induce an antibody response against the other allelic form. This is encouraging for vaccine

development as antibody responses would be boosted by infection even if the infecting strain was not the same as the vaccinating strain.

(2) I have found that the prevalence of antibodies to the second and the double EGF-like motif proteins is higher in children who experience asymptomatic infections than in children who develop clinical malaria. I found that antibody responses to $PfMSP1_{33}$ are also associated with resistance to clinical malaria in Gambian children.

(3) Finally, I have found that human serum antibodies are able to inhibit the binding of invasion inhibitory mAbs to recombinant $PfMSP1_{19}$ in competition ELISAs and that human polyclonal IgG, affinity purified to $PfMSP1_{19}$, is able to inhibit the *in vitro* growth of parasites. This inhibition was effective at physiological Ig levels and was functionally cross-reactive for the two allelic forms of PfMSP1.

Taken together, my data strongly support a continued interest in $PfMSP1_{19}$ as a component of a malaria vaccine. However, there are a number of questions which remain to be answered, and a number of potential complications.

2. Immune selection pressure within $PfMSP1_{19}$

Although the C-terminus of PfMSP1 is highly conserved, it has been suggested that selection through immune pressure may result in mutations leading to amino acid diversity in protective epitopes (Miller *et al* 1993). This may have important consequences. For example, the change in a single amino acid residue in the $PfMSP1_{19}$ analogue in *P. yoelii* ($PyMSP1_{15}$) destroys the protective effect of the mAb 302 (Burns *et al* 1989b), and the antigen could no longer be immunoprecipitated by this mAb. The protective effect of mAb302 varies for different *P. yoelii* cloned lines of the same sequence (Daly *et al* 1992).

MAb NIMP M23, which binds to an epitope within the C-terminus of PcMSP1 (McKean *et al* 1993a), significantly delays the onset of parasitaemia of *P. chabaudi chabaudi* (Boyle *et al* 1982). The protection elicited by this mAb is strain-specific (Brown *et al* 1985); a single amino acid determines this specificity (McKean *et al* 1993b). Parasites can develop resistance to the inhibitory effect of NIMP M23 when grown *in vivo* in mAb-secreting hybridoma mice (Wood *et al* 1989), although the C-terminal amino acid sequences of the susceptible and resistant parasites were not different (unpublished data cited in

McKean *et al* 1993b). However, antibody resistance is not always associated with changes in amino acid sequence (Parry *et al* 1990, Sadziene *et al* 1992) and may be due to other factors such as post-translational modifications.

This may not bode well for a vaccine based on $PfMSP1_{19}$. When the majority of the population has become immune to the parasite, immune pressure may select for parasites carrying point mutations in $PfMSP1_{19}$ that are no longer recognised by the host's protective antibodies. On the other hand, it is not clear that the findings in these two rodent malarias have direct implications for *P. falciparum*. MAb NIMP M23 is not a particularly protective mAb, delaying the onset of parasitaemia rather than preventing parasite growth completely. Also the sequences of the C-terminus of *P. chabaudi chabaudi* AS and CB are so dissimilar (only 78% homology) that hyper immune serum raised against one strain fails to cross-react with the heterologous strain (McKean *et al* 1993b), so it is not surprising that strain-transcending immunity was not induced by the passive transfer of the mAb NIMP M23. There is also little sequence homology between the C-terminus of *P. yoelii* strains (77%) (Daly *et al* 1992).

Until recently, the C-terminal sequence had only been determined for four *P. falciparum* isolates, and these were laboratory cultured lines, grown in a uniform environment in the absence of immune pressure (Blackman *et al* 1991a). The situation in the field may be different, with the presence of more diversity in this region of the molecule due to selection of new variants through immune pressure. However, a study carried out by Jongwutiwes *et al* (1993) demonstrated limited diversity in the field. Of 19 Thai wild isolates, the authors identified 18 nucleotide substitutions in the C-terminus of PfMSP1, 9 of which were in $PfMSP1_{19}$, all resulting in dimorphic amino acid substitutions. They found that residues at the $PfMSP1_{42}$ and $PfMSP1_{19}$ cleavage sites were conserved, as were all 12 cysteine residues.

However, the question is: would this limited amount of variation within $PfMSP1_{19}$ be enough to impede the production of protective immunity? The four amino acid differences between the two allelic forms of $PfMSP1_{19}$ do not appear to alter the ability of $PfMSP1_{42}$-specific antibody to inhibit parasite invasion *in vitro*, as antibody raised to parasites of one allelic form inhibits parasite growth of either allelic family (Chang *et al* 1992 and data presented in chapter 5). Also, all of the four $PfMSP1_{19}$-specific invasion inhibitory mAbs bind to both allelic forms of $PfMSP1_{19}$. These invasion inhibitory mAbs bind to different epitopes within $PfMSP1_{19}$; 12.8 and 5B1 bind to the first EGF-like motif

(Chappel and Holder 1993), 4H9/19 binds to the second EGF-like motif (Cooper *et al* 1992) and 12.10 binds to the double EGF-like motif protein (Chappel and Holder 1993). It seems therefore that as there are multiple protective epitopes within $PfMSP1_{19}$; variation in sequence, if it develops, is unlikely to develop in every protective epitope. The use of a protein with multiple protective epitopes lessens the likelihood of the vaccine becoming ineffective through mutations in protective epitopes. Miller *et al* (1993) point out that variation with polio strains is not a problem for the polio vaccine as there are also invariant epitopes which are fixed by structural requirements, and this could be the case with $PfMSP1_{19}$. Immunity based on a epitope which is conserved for structural and/or functional reasons means that problems due to antigenic diversity through point mutations are less likely to occur. $PfMSP1_{19}$ is likely to be under structural constraint (due to disulphide bonding and formation of the two putative EGF-like motifs), suggesting that the sequence of $PfMSP1_{19}$ may be able to vary only within very limited constraints.

3. Strain-specificity within $PfMSP1_{19}$

Despite the fact that invasion inhibitory mAbs recognise the two allelic forms of $PfMSP1_{19}$, and that polyclonal sera cross-react in their inhibitory action on merozoite invasion, it has not been unequivocally demonstrated that immunisation of animals with one allelic form of PfMSP1 protects animals from parasites expressing the heterologous allele. Etlinger *et al* (1991) demonstrated that total protection from heterologous challenge infection could be induced in vaccination with PfMSP1. However, the vaccinating strain (K1) and the challenge strain (FUP; MAD20 type) share 25% homology in the N-terminus of PfMSP1 so the protection observed could not be definitely attributed to epitopes within conserved regions of the C-terminus of the molecule. Apart from the Patarroyo SPf66 vaccine (contains block 1 of PfMSP1), humans have not been vaccinated with PfMSP1, so it is not known if a vaccine based on $PfMSP1_{19}$ would induce strain-specific immunity. It is not surprising that animals vaccinated with a recombinant protein representing $PyMSP1_{15}$ were not protected against a heterologous challenge infection as this is a region of heterogeneity in *P. yoelii.* The question of whether vaccination of monkeys with $PfMSP1_{19}$ would provide protection against a heterologous challenge infection has only once been addressed (Burghaus *et al* manuscript in preparation). In this study neither homologous nor heterologous challenge was controlled. In

the Kumar study (1995), protection was obtained with $PfMSP1_{19}$ vaccination, however, a heterologous challenge was not tested.

It has been suggested that anti-malarial immunity is strain-specific in humans, as seen with the experimental infection with malaria for the treatment of neurosyphilis. The effect of a primary infection on the subsequent infection was monitored. It was found that prior infection did not totally protect individuals from a subsequent infection, but that homologous reinfections induced less clinical symptoms of malaria than a heterologous challenge (Jeffery 1966). However, it has also been demonstrated that a similar level of immunity to that obtained against homologous strains can be obtained against the heterologous strain (Boyd and Kitchen 1945). Complete susceptibility to heterologous strains has been reported (James *et al* 1932, Boyd *et al* 1936), but this has also occurred with secondary homologous infections (James and Shute 1926). In 1936, Boyd and Kitchen reported that *P. falciparum* induced immunity to homologous, but not to heterologous strains; but in 1945 they changed their opinion (reviewed in Baird 1995). So, there appears to be a degree of uncertainty as to whether immunity to malaria in humans is strain-specific or not.

The fact that pooled IgG from immune individuals, passively transferred into non-immune recipients, enables these individuals to control their parasitaemias to levels below which clinical symptoms occur (Cohen *et al* 1961, McGregor *et al* 1963, Sabchareon *et al* 1991) suggests that there is a broad strain-transcending antibody response made in immune individuals. On the other hand, *in vitro* studies have shown that human immune sera inhibit parasite growth in a strain-specific manner (Vernes *et al* 1984, Haynes *et al* 1987, Sy *et al* 1990). It is difficult to correlate these results with PfMSP1-specific immunity due to the potential contribution of other malarial antigens, which may provide strain-specific immunity. However, PfMSP1-specific antibody, polyclonal and monoclonal, inhibits merozoite invasion of parasites expressing either allele (see chapter 5).

4. Antigenic integrity of the $PfMSP1_{19}$ vaccine antigen

Siddiqui *et al* (1987), found that monkeys immunised with native PfMSP1 were completely protected from challenge infection. The native PfMSP1 used for vaccination was affinity purified on a chromatography column using a mAb specific for the C-terminus of PfMSP1, so the vaccinating preparation would have been enhanced with C-terminal proteins (Chang *et al* 1992). It is

encouraging for vaccine development that immunisation of animals with recombinant proteins representing $PfMSP1_{19}$ or its homologue in *P. yoelii*, protects animals from a lethal challenge infection. However, not all vaccinated animals are protected.

A major factor in antigen quality may be the expression system used for the production of the recombinant protein. Correct disulphide linkages, within $PyMSP1_{15}$, have been demonstrated to be vital for inducing, and being recognised by, protective antibody (Ling *et al* 1994). Immunisation of rabbits with $PfMSP1_{42}$ produced in a baculovirus expression system induces antibodies which inhibit merozoite invasion *in vitro*, but $PfMSP1_{42}$ produced in a yeast expression system does not (Chang *et al* 1992). Production of the double EGF-like motif in a bacterial expression system results in an antigen which can protect mice from *P. yoelii* infection (Daly and Long 1993,1995, Ling *et al* 1994) but a similar product does not protect monkeys from *P. falciparum* infection (Burghaus *et al* manuscript in preparation). $PfMSP1_{42}$ produced in a bacteria expression system did not protect monkeys from infection, but $PfMSP1_{19}$ produced in yeast protected two of four vaccinated monkeys from a lethal challenge infection (Kumar *et al* 1995). Proteins produced in different expression systems may have different post-translational modifications which could affect immunogenicity and production of protective immune responses.

However, the difference in protection obtained in these immunisations may be due to the different adjuvants used rather than differences in the protein produced by the different expression systems. The choice of adjuvant has a profound effect on the protection obtained. Immunisation with MSP1 proteins in Freund's adjuvant seems to result in protection against challenge infection, whereas immunisation with the same protein in a different adjuvant does not (Daly and Long 1993, Ling *et al* 1994, C. Long personal communication cited in Hui *et al* 1994a, Kumar *et al* 1995, Burghaus *et al* manuscript in preparation). However, Freund's adjuvant is not safe to use in humans.

5. Imprinting $PfMSP1_{19}$ immunogenicity

Another potential problem of the use of $PfMSP1_{19}$ in a vaccine is its apparently poor immunogenicity: that despite being a conserved protein, only 60% of an adult, malaria-exposed population possess antibodies to this protein. The immunogenicity of the protein will have to be increased for an effective immune response to be induced by vaccination. There are many theories as to why

$PfMSP1_{19}$ is poorly antigenic (see chapter 4), but hopefully the provision of Th epitopes and the high concentration of $PfMSP1_{19}$ in the immunogen will circumvent these problems. Vaccination of mice with the recombinant protein representing $PyMSP1_{15}$ provided better protection than with the whole PyMSP1 molecule, suggesting that the immune response was concentrated on the critical epitopes for the induction of immunity (Ling *et al* 1994). Also, monkeys immunised with $PfMSP1_{19}$ plus T cell epitopes from tetanus toxoid were protected from challenge infection (Kumar *et al* 1995), while monkeys immunised with $PfMSP1_{19}$ alone were not (Burghaus *et al* manuscript in preparation). It may be possible to enhance the immunogenicity of $PfMSP1_{19}$ by the inclusion of cytokines such as IL-2 (Kawamura *et al* 1985, Good *et al* 1988a), by the use of immunomodulating adjuvants (Hui *et al* 1991, Hui and Chang 1992), and by conjugating the immunogen to a larger protein (Ballou *et al* 1985, Herrington *et al* 1987, Daly and Long 1993), see chapter 7.

6. Should other epitopes of PfMSP1 be included in a vaccine?
MSP1 has been shown to contain other protective regions. Passive transfer of the mAb Pca5C10/66, which maps to epitopes within blocks 4 and 8, protects mice against challenge infection with *P. chabaudi* (Lew *et al* 1989, 1990). However, its epitope is in a region of microheterogeneity, and so is likely to be strain-specific (Lew and Beck 1990).

A small sequence from block 1 is included in the SPf66 synthetic peptide vaccine, and has been suggested to confer some degree of protection (Molano *et al* 1992). Antibody responses to the N-terminus of PfMSP1 have been demonstrated to be associated with resistance to clinical malaria in malaria-exposed individuals (Riley *et al* 1992a, Tolle *et al* 1993) and vaccination of monkeys with synthetic peptides representing $PfMSP1_{83}$ induced partial protection in monkeys (Cheung *et al* 1986, Patarroyo *et al* 1987a, 1987b).

However, bacterial recombinant proteins representing $PfMSP1_{83}$ did not protect monkeys from infection (Knapp *et al* 1988). Rabbit antibodies raised to native $PfMSP1_{83}$ were unable to inhibit parasite growth *in vitro*. As this region of the molecule is shed from the merozoite before RBC invasion, it has been suggested that it may not be involved in the invasion process and may even protect fragments of the molecule which are important to the invasion process (Strych *et al* 1987). It has been demonstrated that certain antibodies specific to $PfMSP1_{83}$ can block the binding of antibodies that bind to $PfMSP1_{19}$,

suggesting that these N-terminal antibodies may contribute to immune evasion by blocking the binding of protective/functional antibodies to the C-terminus (Wilson *et al* 1987).

So other regions of the PfMSP1 molecule appear to confer some degree of protection, and appear to be important in inducing T cell help to produce protective/functional $PfMSP1_{19}$-specific antibody (Hui *et al* 1994a). However, careful choice will have to be made for the inclusion of other epitopes from the PfMSP1 molecule as it has been demonstrated that non-protective PfMSP1-specific antibodies can inhibit the binding of protective antibodies (Wilson *et al* 1987), and that immunodominant epitopes may divert antibody production away from protective epitopes (Schofield 1991).

7. Mechanisms of immunity induced by $PfMSP1_{19}$

The mechanism by which $PfMSP1_{19}$-specific antibodies inhibit merozoite invasion is unknown. MSP1-specific antibodies have been shown to agglutinate merozoites (Epstein *et al* 1981) and MSP1 antibodies are found in immune clusters of merozoites formed in the presence of polyspecific antibody (Lyon *et al* 1989). My data (chapter 5) show that antibody to $PfMSP1_{19}$ prevents merozoite invasion. If secondary processing of $PfMSP1_{42}$ is a prerequisite for invasion (see below), $PfMSP1_{19}$-specific antibodies may sterically hinder the binding of the protease which causes the this proteolysis. Or, if the EGF-like motifs bind to an EGF receptor on the RBC, $PfMSP1_{19}$-specific antibodies may sterically hinder this binding. As the majority of $PfMSP1_{19}$-specific human sera antibodies are of the IgG1 subclass, it is possible that antibody may opsonise merozoites to be destroyed by phagocytes and compliment. I have not measured the effect of complement, but there is an indication of antibody-dependent cellular-inhibition (ADCI) as there is a reduction in parasitaemia of cultures grown in the presence of monocytes and $PfMSP1_{19}$-specific (first EGF-like motif) Ig compared with parasites grown in antibody without monocytes (see chapter 5).

From data presented here, antibody responses to $PfMSP1_{33}$ also appear to be associated with protection from clinical malaria, although only in the Gambian study (see chapter 6). The reason this was seen in The Gambia, and not in Sierra Leone, may be due to the high frequency of this allele (MAD20) in The Gambia (antibody responses were measured using recombinant protein representing the MAD20 version of $PfMSP1_{33}$). The frequency of expression of

this allele in Sierra Leone has not been measured. However, recognition of $PfMSP1_{33}$ by Sierra Leonean sera is high, so it is not known why it is associated with protection in one study and not in another. The function of $PfMSP1_{33}$-specific antibodies is unknown, but could include such mechanisms as the inhibition of secondary processing of $PfMSP1_{42}$. $PfMSP1_{33}$ may also provide T cell help for B cell production of protective/functional antibody. There is evidence that there are T cell epitopes within $PfMSP1_{33}$ that are important for protection elicited by $PfMSP1_{19}$. Hui *et al* (1994a) demonstrated that serum from rabbits immunised with $PfMSP1_{19}$ was unable to inhibit parasite growth *in vitro*, while serum from rabbits immunised with $PfMSP1_{42}$ could. The antibodies responsible for the growth inhibitory action could be absorbed out of the preparation on a $PfMSP1_{19}$ affinity column. This suggests that $PfMSP1_{19}$-specific antibodies are responsible for growth inhibition, but that epitopes within $PfMSP1_{33}$ may be important for their induction. It would be interesting to measure T cell responses to $PfMSP1_{33}$ and see, through which cytokines are produced, whether a Th response is evoked. It is possible that antibody responses to $PfMSP1_{33}$ may also be important in the growth inhibitory effect of antibodies raised to $PfMSP1_{42}$.

Contrary to a previous study (Freeman and Holder 1983), recent work by Daly and Long (1995) demonstrated that the passive transfer of serum from mice vaccinated with MSP1 (native MSP1-specific antibody was used in the Freeman and Holder 1983 study and $PyMSP1_{15}$-specific antibody in the Daly and Long 1995 study) conferred protection to naive recipients, suggesting that protection was antibody mediated.

It has been demonstrated by Blackman *et al* (1994) that inhibitory mAbs inhibit the secondary processing of $PfMSP1_{42}$ to $PfMSP1_{33}$ and $PfMSP1_{19}$ and that non-inhibitory mAbs can block the inhibition of secondary processing. This suggests that the specificity of the antibody induced determines its effectiveness. This may explain why vaccination of animals with $PfMSP1_{19}$ or $PyMSP1_{15}$ does not induce protection in all vaccinated animals; the relative concentrations and affinities of the blocking versus the inhibitory antibodies will determine whether the antibody response is protective or not.

In the morbidity survey reported in chapter 6, the prevalence of antibody responsiveness to the double and second EGF-like motif proteins was always higher in children who experienced asymptomatic infections than in children who experienced clinical infections. However, there was never a 100%

association of antibody responsiveness with protection (except for Gambian children to the second EGF-like motif). This may be due to the presence of non-protective, blocking $PfMSP1_{19}$ antibodies (the ELISA method used does not differentiate between inhibitory and blocking antibody). There is also the possibility that these unprotected children may have produced IgG antibodies of an inappropriate subclass, although, IgG antibodies of any subclass should be able to physically block the binding of other antibodies.

Thus, a major concern, for the use of $PfMSP1_{19}$ in a vaccine, is that the correct specificity of antibody is produced. It is possible, and needs to be investigated, that this specificity may be controlled by Th epitopes.

8. The function of MSP1

As yet, no functional role has been attributed to MSP1. It has been postulated that MSP1 may be involved in a receptor/ligand interaction, binding to RBCs in a sialic-acid dependent manner (Perkins and Rocco 1988). MAb 5B1, which binds to the first EGF-like motif (Chappel and Holder 1993), can inhibit the binding of MSP1 to the RBC, and can also inhibit RBC invasion *in vitro*, suggesting that the function of MSP1 may be to attach to the merozoite to the RBC so that invasion can occur. However, only intact MSP1 binds to the RBC, but the protein on the merozoite surface is fully processed, so there is some controversy whether MSP1 is important for binding to the RBC.

However, $PfMSP1_{19}$ is taken into the RBC bound to the surface of the merozoite, and it has been suggested that the secondary processing event of $PfMSP1_{42}$ is a pre-requisite for parasite invasion (Blackman *et al* 1991b). Comparison of the amino acid sequence of PfMSP1 identified two putative epidermal growth factor (EGF)-like motifs in $PfMSP1_{19}$ (Blackman *et al* 1991a). EGF-like motifs have been found in a variety of diverse proteins, their common feature is that they are extracellular and are thought to be involved in cell-cell interactions (reviewed in Appella *et al* 1988). EGFs interact with specific receptor proteins; it has been suggested that bacteria and viruses use growth receptors to enter cells (Isberg 1991, Eppstein *et al* 1985), and EGF receptors have been found on the RBC surface (Engelmann *et al* 1992). EGFs are usually activated by proteolytic processing (Davis 1990), suggesting that the secondary processing of $PfMSP1_{42}$ may activate some function mediated through the EGF-like motifs of $PfMSP1_{19}$. This idea is supported by the finding that inhibition of secondary processing inhibits RBC invasion (Blackman *et al*

1994). The majority of PfMSP1-specific mAbs which inhibit merozoite invasion *in vitro* bind to epitopes within the two EGF-like motifs of $PfMSP1_{19}$ (Copper *et al* 1992, Chappel and Holder 1993).

Alternatively, it has been suggested that $PfMSP1_{19}$ may have other biochemical functions such as signal transduction, once inside the RBC, allowing differentiation to occur, and that this signalling may be inhibited by antibody binding (Holder and Blackman 1994). It is not known why primary and secondary processing occurs, but it has been suggested that they might induce conformational changes in the protein that result in activation or acquisition of function (Holder and Blackman 1994). It has also been suggested that primary processing may be a form of immune evasion, directing antibodies to immunodominant regions; the immune complex is then shed at invasion (Strych *et al* 1987). Secondary processing is carried out by a parasite protease (Blackman and Holder 1992, Blackman *et al* 1993), and these authors speculate that the function of this processing may be to expose $PfMSP1_{19}$ so that it can interact with a receptor on the RBC surface. However, attempts to answer this question, by demonstrating the binding of $PfMSP1_{19}$ on liposomes to a receptor on the RBC surface, have been unsuccessful (Holder and Blackman 1994).

9. Closing comments

Data presented here is in agreement with the general consensus that $PfMSP1_{19}$ is a very promising candidate antigen for a blood stage vaccine against malaria (Diggs *et al* 1993). Obstacles to be overcome in the future include; 1) to make $PfMSP1_{19}$ more immunogenic, probably by the inclusion of relevant T cell epitopes and choice of adjuvant, 2) to develop strategies to control the fine specificity of the antibody induced as, $PfMSP1_{19}$ can also induce non-protective antibodies which can compete with protective antibodies, and 3) to determine whether immune pressure will select different $PfMSP1_{19}$ sequences which would reduce the efficacy of the vaccine in subsequent years.

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Appendix 1

Extra data for chapter 7

Donor	Antigen	cpm (SI) unreduced		cpm (SI) reduced		Δcpm (ΔSI) reduced-unreduced	
4	Well-EGF1	935.5	(1.1)	1181.0	(1.8)	245.5	(0.6)
	MAD-EGF1	675.6	(0.8)	794.2	(1.2)	118.6	(0.7)
	MAD-EGF2	554.5	(0.7)	896.9	(1.1)	342.4	(0.6)
	Well-19/GST	640.9	(0.8)	1049.9	(1.6)	409.0	(0.5)
	MAD-19	143.8	(0.6)	547.8	(2.3)	404.0	(0.3)
	PPD	19187.7	(78.7)	8191.8	(33.6)	-10995.9	(2.3)
	TT	1233.0	(5.1)	451.7	(1.9)	-748.3	(2.7)
	GST	848.1	(3.5)	666.8	(2.7)	-181.3	(1.3)
	RPMI	243.9					
	PHA	31503.2	(129.2)				
12	Well-EGF1	921.2	(1.8)	1481.5	(1.0)	560.3	(1.9)
	MAD-EGF1	895.5	(1.8)	967.4	(0.6)	71.9	(2.8)
	MAD-EGF2	826.9	(1.6)	940.9	(0.6)	114.0	(2.6)
	Well-19/GST	698.9	(1.4)	1635.4	(1.1)	936.5	(1.3)
	MAD-19	204.4	(0.7)	1080.3	(3.4)	875.9	(0.2)
	PPD	36127.5	(114.9)	15436.3	(49.1)	-20691.2	(2.3)
	TT						
	GST	507.2	(1.6)	1506.8	(4.8)	999.6	(0.3)
	RPMI	314.5					
	PHA	48222.5	(153.3)				
13	Well-EGF1	148.2	(1.2)	80.6	(0.7)	-67.6	(1.7)
	MAD-EGF1	158.6	(1.3)	87.2	(0.8)	-70.8	(1.7)
	MAD-EGF2	76.5	(0.6)	92.8	(0.8)	16.3	(0.8)
	Well-19/GST	147.1	(1.2)	108.5	(1.0)	-38.6	(1.3)
	MAD-19	74.3	(0.6)	126.7	(1.0)	52.4	(0.6)
	PPD	1025.3	(7.8)	188.8	(1.4)	-836.5	(5.4)
	TT						
	GST	127.3	(1.0)	116.1	(0.9)	-11.2	(1.1)
	RPMI	130.8					
	PHA	52611.7	(402.2)				
15	Well-EGF1	3432.2	(1.4)	3690.8	(0.9)	258.6	(1.6)
	MAD-EGF1	1992.0	(0.8)	9002.4	(2.2)	7010.4	(0.4)
	MAD-EGF2	1833.0	(0.8)	4444.5	(1.1)	2611.5	(0.7)
	Well-19/GST	1788.0	(0.8)	4428.2	(1.1)	2640.5	(0.7)
	MAD-19	265.8	(1.6)	2430.5	(14.7)	2164.7	(0.1)
	PPD	78888.6	(475.8)	38927.6	(234.8)	-39960.9	(2.0)
	TT						
	GST	2399	(14.5)	4035.2	(24.34)	1636.2	(0.6)
	RPMI	165.8					
	PHA	50104.4	(302.2)				
35	Well-EGF1	682.2	(1.2)	1834.1	(3.0)	1151.9	(0.4)
	MAD-EGF1	358.0	(0.5)	2978.2	(4.8)	2620.2	(0.1)
	MAD-EGF2	797.8	(1.5)	2328.2	(3.8)	1530.4	(0.4)
	Well-19/GST	721.7	(1.3)	1310.4	(2.1)	588.7	(0.6)
	MAD-19	158.7	(0.8)	933.3	(4.4)	774.6	(0.2)
	PPD	12534.2	(59.5)	7226.8	(34.3)	-5307.4	(1.7)
	TT	7213.5	(34.3)	3033.1	(14.4)	-4180.4	(2.4)
	GST	551.9	(2.6)	619.9	(2.9)	68.0	(0.9)
	RPMI	210.5					
	PHA	73522.2	(349.3)				
45	Well-EGF1	659.9	(1.4)	1385.2	(1.8)	725.3	(0.8)
	MAD-EGF1	608.0	(1.3)	997.4	(1.3)	389.4	(1.0)
	MAD-EGF2	484.0	(1.0)	1243.4	(1.7)	759.4	(0.6)
	Well-19/GST	528.8	(1.1)	1074.2	(1.4)	545.4	(0.8)
	MAD-19	116.1	(0.6)	241.8	(1.2)	125.7	(0.5)
	PPD	12090.5	(58.0)	8470.3	(40.7)	-3620.2	(1.4)
	TT						
	GST	475.4	(2.3)	754.9	(3.6)	279.5	(0.6)
	RPMI	208.3					
	PHA	59154.9	(284.0)				
50	Well-EGF1	2167.2	(1.1)	3167.4	(4.1)	1000.2	(0.3)
	MAD-EGF1	1321.1	(0.7)	3422.3	(4.4)	2101.2	(0.2)
	MAD-EGF2	2240.3	(1.1)	2438.3	(3.2)	198.0	(0.4)
	Well-19/GST	1609.2	(0.8)	2379.8	(3.1)	770.6	(0.3)
	MAD-19	229.2	(0.8)	600.3	(2.1)	371.1	(0.4)
	PPD	76678.3	(267.5)	42351.7	(147.8)	-34326.6	(1.8)
	TT						
	GST	2018.7	(7.0)	773.8	(2.7)	-1244.9	(2.6)
	RPMI	286.6					
	PHA	53447.3	(186.5)				

Donor	Antigen	cpm (SI) unreduced		cpm (SI) reduced		Δcpm (ΔSI) reduced-unreduced	
53	Well-EGF1	1960.8	(2.3)	2961.0	(2.0)	1000.2	(1.2)
	MAD-EGF1	785.3	(0.9)	1954.8	(1.3)	1169.5	(0.7)
	MAD-EGF2	1131.8	(1.3)	1194.8	(0.8)	63.0	(1.7)
	Well-19/GST	1967.1	(2.3)	3071.3	(2.3	1104.2	(1.1)
	MAD-19	404.7	(0.9)	569.6	(1.3)	164.9	(0.7)
	PPD	25046.6	(55.4)	10928.1	(24.2)	-14118.5	(2.3)
	TT						
	GST	857.1	(1.9)	1520.1	(3.4)	663.0	(0.6)
	RPMI	451.9					
	PHA	59251.8	(131.1)				
69	Well-EGF1	747.4	(1.0)	613.3	(0.9)	-134.1	(1.1)
	MAD-EGF1	462.5	(0.6)	629.5	(0.9)	167.0	(0.7)
	MAD-EGF2	403.5	(0.5)	732.5	(1.0)	329.0	(0.5)
	Well-19/GST	543.4	(0.7)	534.1	(0.7)	-9.3	(1.0)
	MAD-19	184.8	(0.6)	841.4	(2.7)	656.6	(0.2)
	PPD	37204.8	(117.8)	12821.2	(40.6)	-24383.6	(2.9)
	TT						
	GST	777.7	(2.5)	723.8	(2.3)	-53.9	(1.1)
	RPMI	315.8					
	PHA	101755.3	(322.2)				
79	Well-EGF1	659.2	(0.9)	685.5	(1.1)	26.3	(0.8)
	MAD-EGF1	562.4	(0.8)	639.9	(1.0)	77.5	(0.8)
	MAD-EGF2	391.4	(0.5)	733.5	(1.1)	342.1	(0.5)
	Well-19/GST	512.6	(0.7)	1118.9	(1.7)	606.3	(0.4)
	MAD-19	204.7	(0.4)	311.1	(0.7)	106.4	(0.7)
	PPD	20858.1	(45.2)	10843.4	(23.5)	-10014.7	(1.9)
	TT						
	GST	745.5	(1.6)	651.1	(1.4)	-94.4	(1.2)
	RPMI	461.2					
	PHA	106701.3	(231.4)				
95	Well-EGF1	1434.8	(0.8)	1151.5	(1.2)	-283.3	(0.7)
	MAD-EGF1	1997.1	(1.1)	5827.6	(6.1)	3830.5	(0.2)
	MAD-EGF2	617.6	(0.3)	1496.0	(1.6)	878.4	(0.2)
	Well-19/GST	1874.4	(1.0)	4424.6	(4.7)	2550.2	(0.2)
	MAD-19	176.7	(0.8)	281.7	(1.3)	105.0	(0.6)
	PPD	21405.9	(95.0)	7107.8	(31.5)	-14298.1	(3.0)
	TT	1261.4	(5.6)	458.3	(2.0)	-803.1	(2.8)
	GST	1823.1	(8.1)	951.1	(4.2)	-872.0	(1.9)
	RPMI	225.4					
	PHA	1161149.2	(5151.5)				
104	Well-EGF1	754.6	(1.5)	1243.4	(3.3)	488.8	(0.5)
	MAD-EGF1	722.4	(1.4)	2495.8	(6.7)	1773.4	(0.2)
	MAD-EGF2	763.4	(1.5)	2667.5	(7.1)	1904.1	(0.2)
	Well-19/GST	550.4	(1.1)	1436.1	(3.8)	885.7	(0.3)
	MAD-19	80.2	(0.4)	243.1	(1.1)	162.9	(0.3)
	PPD	74490.5	(348.1)	42889.5	(200.4)	-31601	(1.7)
	TT	142.4	(0.7)	384.6	(1.8)	242.2	(0.4)
	GST	503.1	(2.4)	374.0	(1.8)	-129.1	(1.3)
	RPMI	214.0					
	PHA	84290.5	(393.9)				
110	Well-EGF1	2294.7	(1.4)	1535.9	(0.9)	-758.8	(1.6)
	MAD-EGF1	1066.3	(0.6)	2229.3	(1.3)	1163.0	(0.5)
	MAD-EGF2	867.0	(0.5)	2143.0	(1.2)	1276.0	(0.4)
	Well-19/GST	1670.9	(1.0)	947.1	(0.6)	-723.8	(1.8)
	MAD-19	106.7	(0.9)	301.3	(2.4)	194.6	(0.4)
	PPD	3063.0	(24.7)	2629.5	(21.2)	-433.5	(1.2)
	TT	315.3	(2.5)	1003.3	(8.1)	688.0	(0.3)
	GST	1655.2	(13.4)	1731.3	(14.0)	76.1	(1.0)
	RPMI	123.9					
	PHA	48454.3	(391.1)				
112	Well-EGF1	1522.4	(1.0)	5449.0	(1.1)	3926.6	(0.9)
	MAD-EGF1	1842.1	(1.2)	8384.7	(1.7)	6542.6	(0.7)
	MAD-EGF2	3364.4	(2.1)	7474.8	(1.5)	4110.4	(1.5)
	Well-19/GST	2768.0	(1.8)	5417.6	(1.1)	2649.6	(1.6)
	MAD-19	1069.0	(1.4)	6672.3	(9.0)	5603.3	(0.2)
	PPD	12052.0	(16.2)	15621.2	(21.0)	3569.2	(0.8)
	TT	35891.5	(48.2)	9839.8	(13.2)	-26051.7	(3.7)
	GST	1569.6	(2.1)	5083.8	(6.8)	3514.2	(0.3)
	RPMI	744.3					
	PHA	133317.4	(179.1)				

Donor	Antigen	cpm (SI) unreduced		cpm (SI) reduced		Δcpm (ΔSI) reduced-unreduced	
116	Well-EGF1	2961.6	(3.5)	1540.1	(2.7)	-1421.5	(1.3)
	MAD-EGF1	1876.8	(2.2)	4085.9	(7.2)	2209.1	(0.3)
	MAD-EGF2	1188.8	(1.4)	2612.4	(4.6)	1423.6	(0.3)
	Well-19/GST	1553.6	(1.8)	2915.5	(5.1)	1361.9	(0.4)
	MAD-19	272.7	(0.5)	390.6	(0.8)	117.9	(0.7)
	PPD	35278.7	(69.2)	10704.6	(21.0)	-24574.1	(3.3)
	TT						
	GST	855.1	(1.7)	568.8	(1.1)	-286.3	(1.5)
	RPMI	510.0					
	PHA	104038.1	(204.0)				
117	Well-EGF1	3787.5	(3.1)	3904.2	(5.9)	116.7	(0.5)
	MAD-EGF1	2260.5	(1.8)	4035.5	(6.1)	1775.0	(0.3)
	MAD-EGF2	1072.1	(0.9)	3797.0	(5.8)	2724.9	(0.02)
	Well-19/GST	1765.0	(1.4)	966.0	(1.5)	-799.0	(1.0)
	MAD-19	102.9	(0.5)	237.9	(1.3)	135.0	(0.4)
	PPD	8402.1	(40.9)	4342.6	(21.1)	-4059.5	(1.9)
	TT	32778.2	(159.4)	699.5	(3.4)	-32078.7	(46.9)
	GST	1234.0	(6.0)	657.7	(3.2)	-576.3	(1.9)
	RPMI	205.7					
	PHA	42622.6	(207.2)				
120	Well-EGF1	696.5	(1.1)	760.1	(0.8)	63.6	(1.4)
	MAD-EGF1	264.0	(0.4)	690.1	(0.7)	426.1	(0.6)
	MAD-EGF2	298.2	(0.5)	540.6	(0.6)	242.4	(0.8)
	Well-19/GST	586.5	(0.9)	512.3	(0.5)	-74.2	(1.8)
	MAD-19	86.9	(0.6)	387.5	(2.7)	300.6	(0.2)
	PPD	4000.4	(28.1)	2476.1	(17.4)	-1524.3	(1.6)
	TT	98.2	(0.7)	342.1	(2.4)	243.9	(0.3)
	GST	628.3	(4.4)	962.4	(6.8)	334.1	(0.7)
	RPMI	142.2					
	PHA	74239.6	(552.1)				
125	Well-EGF1	3805.0	(1.4)	12445.4	(1.6)	8640.4	(0.9)
	MAD-EGF1	2606.2	(0.9)	10236.2	(1.3)	7630.0	(0.7)
	MAD-EGF2	1219.6	(0.4)	8047.1	(1.0)	6827.5	(0.4)
	Well-19/GST	1875.2	(0.7)	14419.3	(1.9)	12544.1	(0.4)
	MAD-19	234.3	(1.3)	7492.2	(39.9)	7257.9	(0.03)
	PPD	17392.4	(92.5)	18411.3	(97.9)	1018.9	(0.9)
	TT	24690.3	(131.3)	13832.5	(73.6)	-10857.8	(1.8)
	GST	2797.9	(14.9)	7799.2	(41.5)	5001.3	(0.4)
	RPMI	188.0					
	PHA	69528.9	(369.8)				
167	Well-EGF1	1172.9	(1.6)	1878.4	(1.4)	705.5	(1.2)
	MAD-EGF1	1024.0	(1.4)	1994.7	(1.5)	970.7	(1.0)
	MAD-EGF2	637.2	(0.9)	2110.0	(1.5)	1472.8	(0.6)
	Well-19/GST	564.4	(0.8)	1605.3	(1.2)	1040.9	(0.7)
	MAD-19	89.1	(0.5)	1306.5	(6.7)	1217.4	(0.1)
	PPD	895.9	(4.6)	2168.0	(11.0)	1272.1	(0.4)
	TT	23569.7	(119.9)	2353.3	(12.0)	-21216.4	(10.0)
	GST	736.3	(3.8)	1375.4	(7.0)	639.1	(0.5)
	RPMI	196.6					
	PHA	91180.6	(463.8)				

Appendix 2

Published papers

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Serum Antibodies from Malaria-Exposed People Recognize Conserved Epitopes Formed by the Two Epidermal Growth Factor Motifs of $MSP1_{19}$, the Carboxy-Terminal Fragment of the Major Merozoite Surface Protein of *Plasmodium falciparum*

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The major merozoite surface protein of *Plasmodium falciparum* (PfMSP1) is a candidate antigen for a malaria vaccine. A 19-kDa C-terminal processing product of PfMSP1 ($PfMSP1_{19}$) is composed of two domains sharing a cysteine-rich motif with epidermal growth factor (EGF) and is the target of monoclonal antibodies which block erythrocyte invasion in vitro. We have evaluated human antibody responses to $PfMSP1_{19}$ by using recombinant proteins representing the EGF motifs encoded by the two main alleles of the *MSP1* gene. We find that both EGF motifs are antigenic but that only 10 to 20% of malaria-exposed individuals have serum antibodies that recognized either of the motifs. When both EGF motifs were expressed together as a single protein, they were recognized by more than 40% of sera from malaria-exposed individuals. Major epitopes recognized by human antibodies are dependent upon the correct tertiary structure of the protein and are cross-reactive between the different allelic sequences of $PfMSP1_{19}$. This suggests that antibodies induced by vaccination with one or the other allelic forms of the protein could recognize all strains of *P. falciparum*. Immunoglobulin G (IgG) subclass-specific enzyme immunoassays indicate that $PfMSP1_{19}$ antibodies are predominantly of the IgG1 subclass.

Clinical symptoms of malaria are associated with the asexual ıltiplication of merozoites within erythrocytes. A prophylac- malaria vaccine would need to block this stage of the par- te's life cycle. The major merozoite surface protein-1 (16, , 21) of *Plasmodium falciparum* (PfMSP1) is considered an portant vaccine candidate because monoclonal antibodies IAbs) against it can block erythrocyte invasion in vitro (1, 11, , 30, 32) and primates immunized with either purified native MSP1 or recombinant proteins or synthetic peptides repre- nting parts of the protein are partially or completely pro- cted against experimental infections with *P. falciparum* (9, 13, , 17, 28, 29, 33).

PfMSP1 is the precursor to several major surface proteins of e merozoite (4, 14, 18, 26). After synthesis as a single pro- n, PfMSP1 is processed to produce four major fragments fMSP1$_{83}$, PfMSP1$_{28}$, PfMSP1$_{38}$, and PfMSP1$_{42}$). At the time merozoite release, the PfMSP1$_{42}$ fragment undergoes sec- dary processing to form a 33-kDa (PfMSP1$_{33}$) product, iich is shed, and a 19-kDa fragment (PfMSP1$_{19}$), which re- ains on the merozoite surface during erythrocyte invasion d is present on ring stage parasites (1). It has been suggested at this secondary processing of PfMSP1$_{42}$ to PfMSP1$_{19}$ is a prerequisite for erythrocyte invasion (1, 2, 4). The C-terminal fragments of PfMSP1 are of particular interest with respect to vaccine development, since naturally acquired antibodies to PfMSP1$_{42}$ are associated with resistance to clinical malaria in Gambian children (31) and MAbs mapping to the PfMSP1$_{19}$ fragment inhibit merozoite invasion in vitro (1, 11, 26, 30). In addition, an MAb recognizing an epitope within the C terminus of the PfMSP1 homolog of *Plasmodium yoelii* (PyMSP1) is able to passively protect mice against this malaria infection (6).

PfMSP1$_{19}$ consists of two domains, each with six highly conserved cysteine residues which are characteristic of epidermal growth factor (EGF) motifs (3, 10). The primary sequence of PfMSP1$_{19}$ is highly conserved except for four amino acid residues that are subject to dimorphic substitutions (22, 27). In the Wellcome sequence, these four residues are Q in the first motif and K-N-G in the second motif, while in the MAD20 sequence, they are E and T-S-R, respectively (Fig. 1).

In this study, we have evaluated the serological recognition of recombinant proteins representing the double and separate EGF motifs of PfMSP1$_{19}$ by antibodies from people living in a malaria-endemic area. We conclude that the major epitopes within PfMSP1$_{19}$ that are recognized by human antibodies are conformation dependent and require the presence of both EGF motifs. Furthermore, we report that human antibodies recognize epitopes which are conserved, or cross-reactive, between the two commonly occurring allelic forms of PfMSP1$_{19}$ and are mainly of the immunoglobulin G1 (IgG1) subclass.

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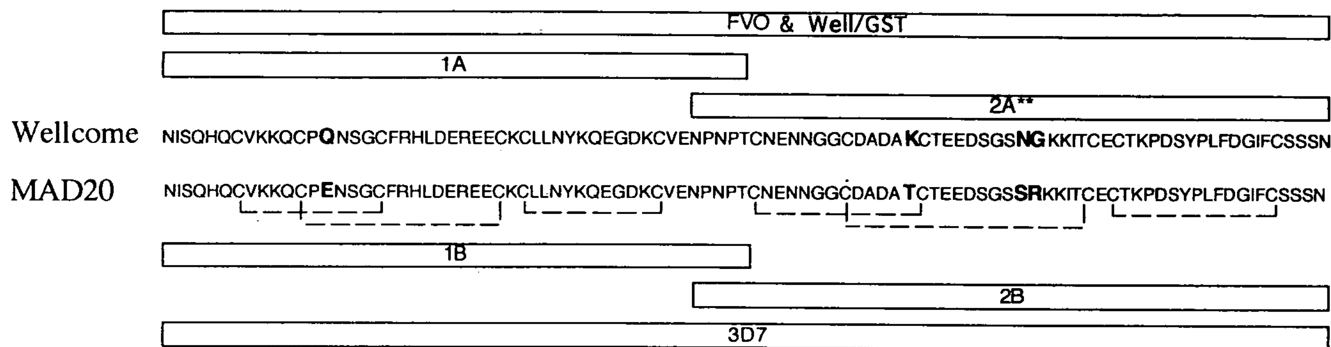


FIG. 1. Partial sequences of the Wellcome and MAD20 allelic forms of $PfMSP1_{19}$, showing the positions of sequence dimorphism, putative disulfide bonds (3, 22), and the recombinant proteins used. Dimorphic amino acids are shown in boldface type; putative positions of disulfide bonds are indicated by dashed lines. Proteins 1A, 2A, FVO, and Well/GST represent the Wellcome allelic sequence; proteins 1B, 2B, and 3D7 represent the MAD20 sequence. The first EGF motif is represented by 1A and 1B; the second motif is represented by 2A and 2B. FVO (Wellcome $MSP1_{19}$), 3D7, and Well/GST represent both EGF motifs of $PfMSP1_{19}$. FVO and Well/GST have the same $PfMSP1_{19}$ sequence but differ in the expression system used to produce them (*S. cerevisiae* and *E. coli*, respectively). The protein FVO/E represents a recombinant form of $PfMSP1_{19}$ with the first motif of MAD20 and the second motif of Wellcome (MAD/Well $MSP1_{19}$) (not shown). **Protein 2A was found to be insoluble and thus was not used in ELISAs.

MATERIALS AND METHODS

Recombinant PfMSP1 proteins. Primary sequences of the recombinant proteins are shown in Fig. 1. Single first and second EGF motifs of the two allelic prototypes of PfMSP1, represented by the MAD20 and Wellcome isolates of *P. falciparum* (27), and the double EGF motif of the Wellcome form were expressed as glutathione *S*-transferase (GST) fusion proteins (34) in *Escherichia coli* transformed with recombinant pGEX3 plasmids (8). As a control, GST was produced by using the pGEX3 plasmid without any insert. The double EGF motif and three of the four single EGF motif constructs were expressed as soluble fusion proteins. The second EGF motif of the Wellcome allele (2A in Fig. 1) was insoluble in aqueous solution and only slightly soluble in 8 M urea and thus was not used. All the GST fusion proteins were recognized by conformation-dependent MAbs, indicating that the proteins assumed an approximately native conformation, with correctly formed disulfide bonds (5, 26).

Proteins representing three different versions of $PfMSP1_{19}$ were produced in recombinant *Saccharomyces cerevisiae* (23), the MAD20 and Wellcome allelic prototypes (3D7 and FVO) and a recombinant form (MAD20 first EGF motif with Wellcome second EGF motif [FVO/E]; see Fig. 1). The addition of a histidine tag to the C terminus of these fusion proteins enabled them to be purified on nickel-nitrilo-triacetic acid-agarose (23).

The purity of the fusion proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein yield was estimated by comparison with bovine serum albumin standards.

Human sera. Blood was collected from people living in rural areas close to the town of Farafenni in the Gambia. Malaria transmission in this area is seasonally endemic, with new infections occurring during and immediately following the rainy season (July to December) (15). Serum samples were obtained at the end of the rainy season (October) from 195 children and adults (aged 1 to 70 years) and were stored at −20°C until used. Prior exposure to malaria was confirmed by serology: all sera were positive (by immunofluorescence) for antibodies to malaria schizonts, with titers ranging from 1:160 to >1:20,000. Control serum samples were obtained from 28 European adults and 14 European children with no previous exposure to malaria.

MAbs. The following $PfMSP1_{19}$-specific murine MAbs were used: 7.5, 12.8, and 12.10 (26); 111.2 and 111.4 (20); 6E2/53 and 4H4/34 (11) (a kind gift of A. Saul, Queensland Institute of Medical Research, Brisbane, Australia); and 14-1C (a kind gift of R. Reese, Agouron Institute, La Jolla, Calif.).

Immunoassays. (i) ELISA. Antibodies reacting with recombinant PfMSP1 proteins were detected by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Immulon 4; Dynatech, Billingshurst, United Kingdom) were coated overnight at 4°C with proteins diluted in carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$ [pH 9.5]). The saturating concentration of protein, determined by titration, was from 0.1 to 5 μg/ml, depending on the antigen. Plates were washed three times in phosphate-buffered saline (PBS, pH 7.2)-Tween 20, blocked with a 1% solution of nonfat powdered milk in PBS-Tween 20 (blocking buffer), and washed again. Then 100 μl of serum or MAb was diluted in blocking buffer, incubated at room temperature for 5 h, added to duplicate wells, and incubated overnight at 4°C. Optimal dilutions of serum or MAbs were determined by titration. For all human sera, the steepest slope of the titration curve was observed at concentrations between 1:1,000 and 1:10,000 (data not shown). The optimum concentration of human serum for the assay was thus selected as 1:3,000 (24).

After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (100 μl of 1:5,000) or HRP-conjugated rabbit anti-mouse IgG (100 μl of 1:1,000) (both from Dako Ltd., High Wycombe, United Kingdom) were added to the plates, incubated for 3 h at room temperature, and developed with H_2O_2 and *o*-phenylenediamine (Sigma, Poole, United Kingdom) for 10 min at 4°C. The reaction was stopped by the addition of 20 μl of 2 M H_2SO_4. Plates were read at 492 nm. For GST fusion proteins, the optical density (OD) value of the GST control was subtracted from the OD of GST recombinant fusion proteins to give a specific OD (ΔOD) for the response to the PfMSP1 antigens. For yeast-derived proteins, when no fusion protein was involved, the original OD values are shown.

(ii) Competition ELISA. To determine whether antibodies recognizing individual recombinant proteins also recognized (cross-reacted with) other recombinant proteins, competition ELISAs were performed. The test serum was diluted in blocking buffer to which was added recombinant protein at a range of concentrations. After incubation for 5 h at room temperature, the blocked serum was added to immunoplates coated with a second recombinant protein. The remainder of the assay was performed as above.

Competition ELISAs were also performed to determine whether epitopes recognized by human antibodies were similar to those recognized by murine MAbs. To see if human antibodies could inhibit binding of the MAbs, plates coated with an antigen were first incubated with various concentrations of human serum. After extensive washing, a dilution of an MAb was added to the plate, incubated for 3 h, and developed with HRP-anti-mouse IgG and *o*-phenylenediamine. The reciprocal experiment was also performed to see if MAbs could inhibit binding of the human sera. In this case, antigen-coated plates were first

TABLE 1. Prevalence of antibodies to $PfMSP1_{19}$ in the Gambia

Age group	No.	No. (%) of sera recognizing recombinant protein[a]:						
		Wellcome 1st motif	MAD20 1st motif	MAD20 2nd motif	MAD20 $MSP1_{19}$	MAD/Wellcome $MSP1_{19}$	Wellcome $MSP1_{19}$	Wellcome/GST $MSP1_{19}$
Children (<8 yr)	33	5 (15.2)	3 (9.1)	6 (18.2)	4 (12.1)	7 (21.2)	7 (21.2)	11 (33.3)
Adolescents (9–15 yr)	60	8 (13.3)	11 (18.3)	5 (8.3)	12 (20)	22 (36.7)	20 (33.3)	25 (41.7)
Adults (>16 yr)	92	19 (20.7)	19 (20.7)	13 (14.1)	38 (43.1)	53 (57.6)	50 (54.3)	43 (46.7)
Total	195	32 (16.4)	33 (16.9)	24 (12.3)	54 (27.6)	82 (42.1)	77 (39.5)	79 (40.5)

[a] A positive response is one which is greater than the mean plus 2 SDS of the OD values for 42 control (malaria nonexposed) sera.

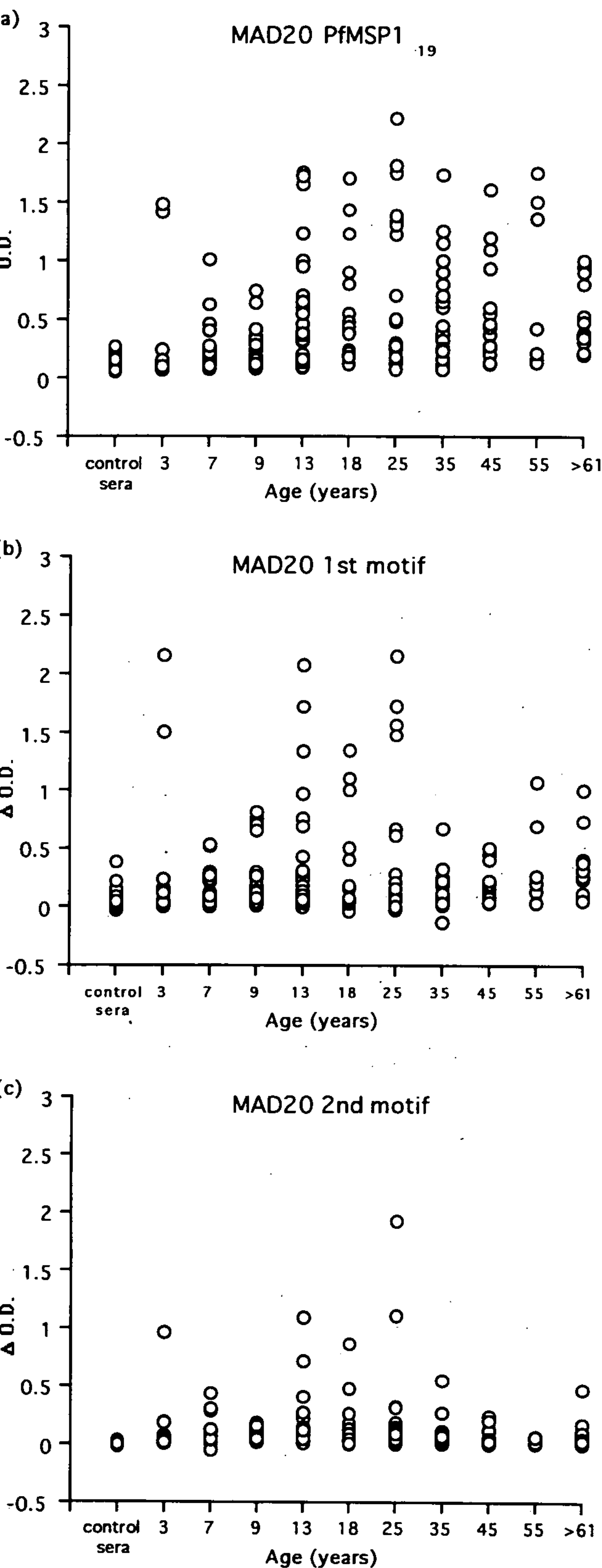


FIG. 2. Dot plots showing human antibody binding for 195 Gambian sera in ELISA for recombinant PfMSP1 proteins. (a) MAD20 $PfMSP1_{19}$ (3D7), OD values; (b) MAD20 first motif (1B), ΔOD values (see text for details); (c) MAD20 second motif (2B), ΔOD values. The OD values of 42 European control sera are also shown. All sera were tested at a dilution of 1:3,000.

TABLE 2. Effect of age on median levels of anti-PfMSP1 antibodies[a]

Antigen	Median OD by age group			Kruskal-Wallis test	
	<8 yr (n = 43)	9–15 yr (n = 59)	>16 yr (n = 92)	χ^2	P
Wellcome 1st domain	0.047	0.028	0.019	3.9	0.141
MAD20 1st domain	0.084	0.096	0.138	4.5	0.103
MAD20 2nd domain	0.042	0.057	0.058	1.7	0.428
MAD20 $MSP1_{19}$	0.124	0.182	0.413	40.3	<0.001
Wellcome $MSP1_{19}$	0.143	0.215	0.448	35.5	<0.001
MAD20/Wellcome $MSP1_{19}$	0.120	0.200	0.411	36.0	<0.001
Wellcome/GST $MSP1_{19}$	0.024	0.128	0.307	23.1	<0.001
GST	0.111	0.115	0.135	12.7	0.002

[a] Sera were diluted 1:3,000.

incubated with various concentrations of MAb, then with human serum, and finally with HRP-anti-human IgG.

(iii) ELISA with reduced and nonreduced antigen. To determine whether epitopes recognized by human antibodies were dependent on the presence of intact disulfide bonds, ELISAs were performed with reduced and alkylated or nonreduced recombinant antigens. To prepare reduced protein, 0.5 mg of an antigen was incubated at 37°C for 1 h in 0.5 M Tris-HCl (pH 8.1) containing 2 mM EDTA and 60 mM dithiothreitol (Sigma). Iodoacetic acid, dissolved in 1 M NaOH, was then added at a 2.5-fold molar excess over dithiothreitol. The samples were kept in the dark, the pH was monitored and maintained at 8.1 for 30 min, and then the proteins were dialyzed overnight at 4°C. Plates were coated for ELISA with the appropriate saturating concentration of the reduced protein and used as above.

(iv) ELISA to determine IgG subclass. ELISAs were performed as above (1) except that monoclonal, subclass-specific, mouse anti-human IgG was used as the second-step reagent. Subclass-specific assays were optimized by titration so that the titration curves for each reagent were coincident; i.e., that for a given absolute concentration of IgG1, IgG2, IgG3, or IgG4, the same OD value was obtained. The reagents used were mouse monoclonal anti-human IgG1 (code 1170317; Boehringer, Mannheim, Germany), IgG2 (code 1170309; Boehringer), IgG3 (code MCA516; Serotec, Oxford, United Kingdom), and IgG4 (code 1170287; Boehringer).

Statistical methods. Specific OD values obtained for sera from malaria-unexposed European donors were used to establish a normal range for each antigen. The OD values for these control sera tended to be normally distributed; thus, Gambian sera giving an OD value greater than the mean plus standard deviations (SDs) of the European sera were considered to contain antibody specific for the relevant recombinant protein.

The association between OD values of individual sera to the different antigens was assessed by using scatter plots and Spearman's rank correlation coefficient.

To determine whether levels of antibody to PfMSP1 proteins are associated with age, the distributions of OD values for each antigen were compared between the age groups by using the Kruskal-Wallis one-way analysis of variance. It was necessary to use a nonparametric test because the distributions of the OD values were highly skewed within each age group, and hence median OD values are presented.

RESULTS

Recognition of recombinant PfMSP1 proteins by human antibody. More than 40% of sera from 195 malaria-exposed donors contain antibodies which specifically recognize the double EGF motif of $PfMSP1_{19}$ (Table 1 and Fig. 2a). In contrast, less than 20% of the sera show reactivity with the single EGF motif proteins (Fig. 2b and c [reactivity with the first EGF motif of the Wellcome version is almost identical to the data in Fig. 2b; not shown]). Of the sera which recognized $MSP1_{19}$, approximately 50% recognized only $MSP1_{19}$ and did not recognize any of the single EGF motif proteins. This indicates that there are epitopes present in $PfMSP1_{19}$ that are not present when either EGF motif is expressed alone.

Responses to the double-motif protein produced in *E. coli* (Wellcome $MSP1_{19}$ [19-GST]) and to the same sequence produced in *S. cerevisiae* (FVO) were highly correlated (Spearman's rank correlation coefficient: r = 0.901, n = 195, P <

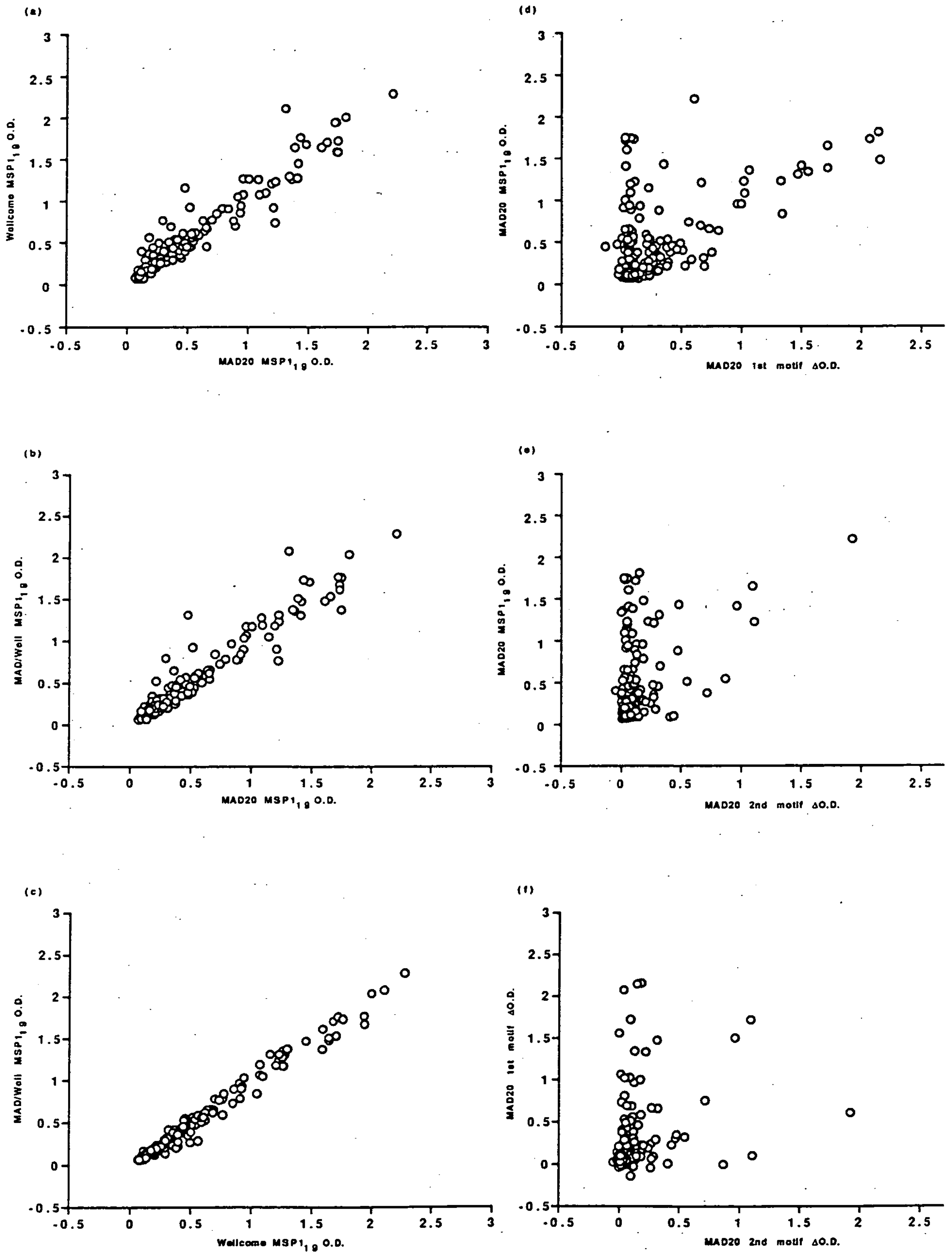


FIG. 3. Comparison of antibody recognition of different PfMSP1$_{19}$ sequences. (a) Wellcome PfMSP1$_{19}$ (FVO: Q-KNG) versus MAD20 PfMSP1$_{19}$ (3D7; E-TSR); (b) MAD20/Wellcome PfMSP1$_{19}$ (FVO/E: E-KNG) versus MAD20 PfMSP1$_{19}$ (3D7: E-TSR); (c) MAD20/Wellcome PfMSP1$_{19}$ (FVO/E; E-KNG) versus Wellcome PfMSP1$_{19}$ (FVO: Q-KNG); (d) MAD20 PfMSP1$_{19}$ (3D7) versus MAD20 first motif (1B); (e) MAD20 MSP1$_{19}$ (3D7) versus MAD20 second motif (2B); (f) MAD20 first motif (1B) versus MAD20 second motif (2B).

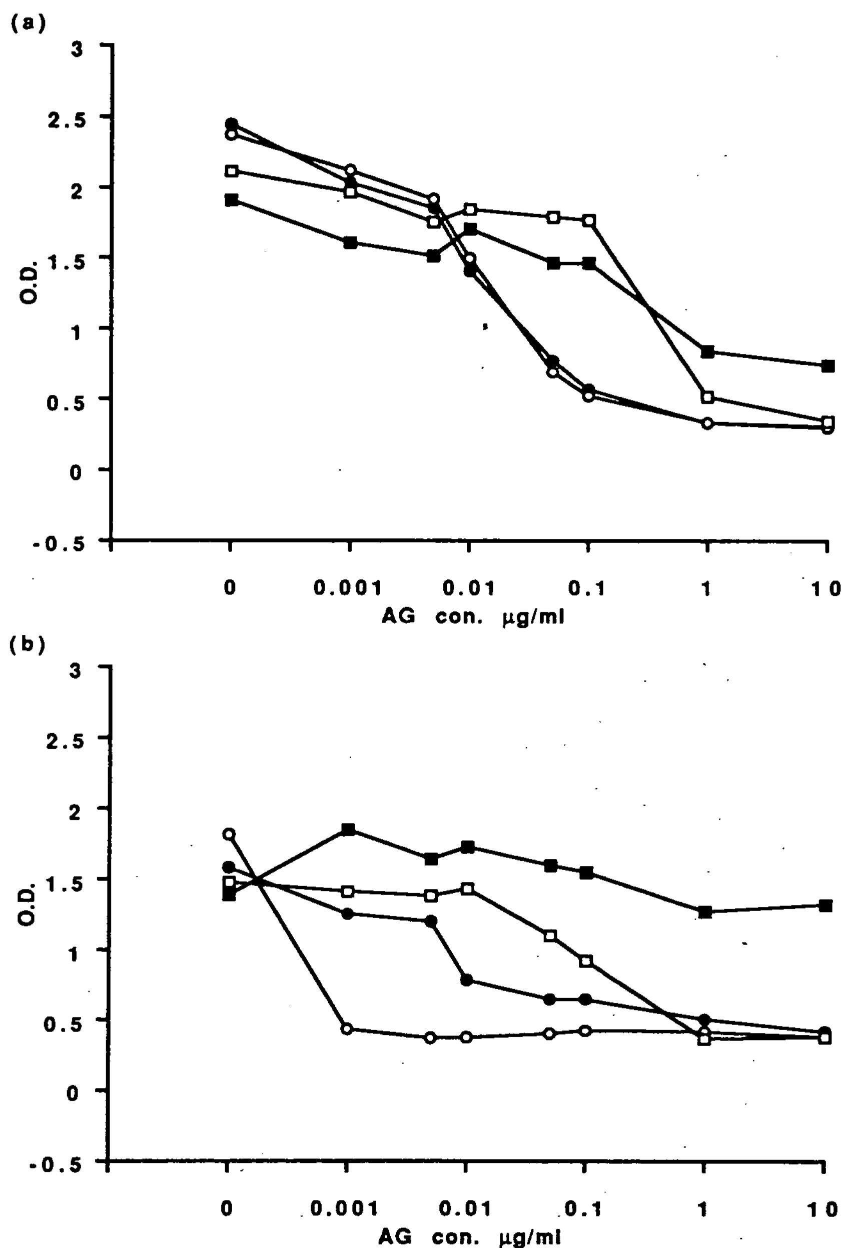


FIG. 4. Competition ELISAs demonstrate that additional epitopes are present within the double EGF motif protein (3D7). Eight sera which react with the single EGF motif (1B) and with the double EGF motif (3D7) were tested; the results for two sera are shown here. (a) Serum 1493. Preincubation of the serum with 1B reduces the level of binding to 3D7 but does not abolish it. This serum may recognize an epitope in 3D7 which overlaps with an epitope in 1B. (b) Serum 1515. Preincubation of the serum with 1B has no effect on the recognition of 3D7. This serum appears to recognize an epitope(s) in 3D7 which is distinct from the epitope(s) in 1B. ●, preincubation with 3D7, tested on 3D7; ○, preincubation with 3D7, tested on 1B; □, preincubation with 1B, tested on 1B; ■, preincubation with 1B, tested on 3D7. AG, antigen.

0.001), indicating that proteins produced in the two expression systems are very similar.

For the double-motif proteins, there is an apparent increase in antibody levels with age (Table 2); such a trend is not apparent for the single EGF motif proteins.

Serological cross-reactivity between Wellcome and MAD20 antigens of PfMSP1$_{19}$. (i) Comparison of antibody recognition of proteins representing different PfMSP1$_{19}$ sequences. Sera with high levels of antibody to the Wellcome proteins also tend to have high levels of antibody to the MAD20 proteins. When recognition of two double-motif proteins is directly compared, there is a high degree of correlation between them (Fig. 3a to c). However, occasional sera do react more strongly with one sequence than with the other; this seems to be due more to a differential recognition of three dimorphic amino acid residues in the second EGF motif (Fig. 3a and b) than to detection of the single-amino-acid change in the first motif (Fig. 3c). Some sera show similar levels of reactivity to both the double motif and the single first motif (Fig. 3d), whereas other sera recognize only the double motif. Similarly, occasional sera recognize both the double motif and the second single motif (Fig. 3e), and one or two sera recognize both the first and second motifs

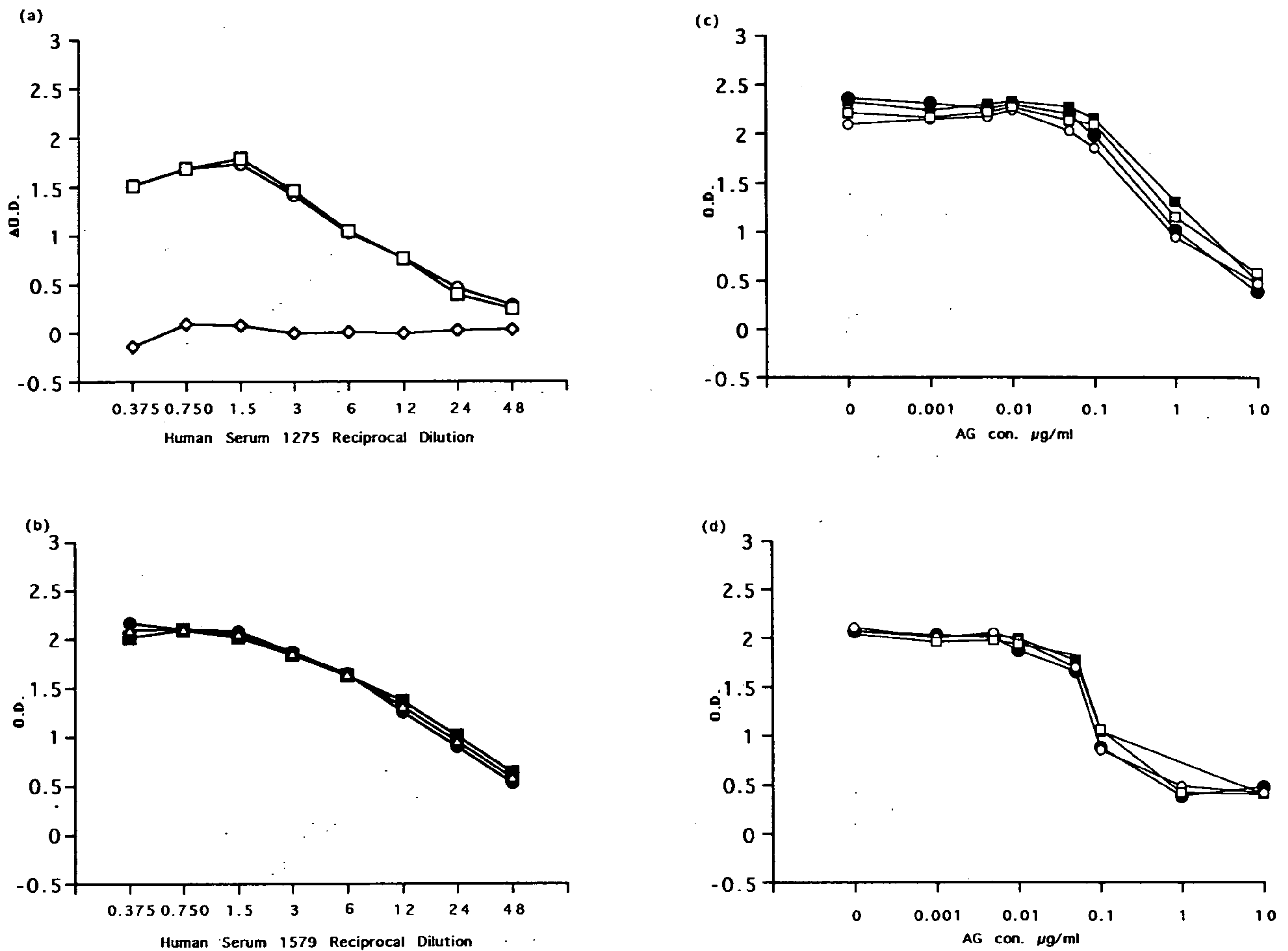


FIG. 5. Human sera recognize epitopes which are cross-reactive between MAD20 and Wellcome sequences of $PfMSP1_{19}$. (a) Human serum 1275 recognizes both sequences of the first EGF motif (1A and 1B) but not the second motif (2B). (b) Human serum 1579 recognizes all three yeast double-motif $PfMSP1_{19}$ constructs. (a and b) ○, 1A: □, 1B: ◇, 2B: ●, 3D7; △, FVO; ■, FVO/E. Dilutions are in thousands. (c and d) Preincubation of human serum 1592 (c) or 1614 (d) with 1 μg of either protein 1A or protein 1B per ml inhibits subsequent binding of that serum to plates coated with either 1A or 1B. ●, serum preincubated with 1A on 1A-coated plates; ■, serum preincubated with 1B on 1A-coated plates; ○, serum preincubated with 1A on 1B-coated plates; □, serum preincubated with 1B on 1B-coated plates.

(Fig. 3f). However, many sera (54%) which recognize the double motif do not recognize either of the single motifs. Competition ELISAs show that, of the sera which recognize both the single- and double-motif proteins, most recognize additional epitopes that are present only in the double-domain construct (two examples are shown in Fig. 4).

(ii) Titration of individual human sera against different $PfMSP1_{19}$ proteins. The above data suggested that most human antibodies do not differentiate between the variant sequences of $PfMSP1_{19}$. The two allelic forms of the first EGF motif differ in sequence by only one amino acid (glutamine in the Wellcome form and glutamate in the MAD20 form at position 14 [Fig. 1]); it is thus possible that the two sequences are immunologically cross-reactive. Six sera containing antibody to the first EGF motif were titrated against the three single EGF motif recombinant proteins; an example is shown in Fig. 5a. All six sera gave identical titration curves for the proteins representing the two allelic types of the first EGF motif, suggesting that a single population of antibodies recognized both proteins. All of these sera also gave identical titration curves for the three yeast-derived double-motif proteins (an example is shown in Fig. 5b).

(iii) Polyclonal sera contain cross-reactive populations of antibodies. To confirm that there was a single cross-reactive antibody population, competition ELISAs were performed. Individual sera which recognized MAD20 and Wellcome-derived proteins with equal avidity were selected. As shown in Fig. 5c (serum 1592), preincubation of the serum with 1 to 10 μg of either protein 1A or protein 1B per ml inhibits subsequent binding of that serum to plates coated with either 1A or 1B. For another serum (1614, Fig. 5d), inhibition was obtained by preincubation with 0.1 to 1.0 μg of protein per ml.

Human antibodies inhibit the binding of MAbs. $PfMSP1_{19}$-specific MAbs can block merozoite invasion of erythrocytes in vitro (1). To see if human sera recognized the same epitopes as inhibitory MAbs, competition ELISAs were performed. Five MAbs were tested: 111.4 recognizes the first motif of Wellcome specifically (1A), 12.8 and 14-1C recognize a conserved epitope in the first motif, and 12.10 and 111.2 recognize the double motif only (8; our unpublished data). Human sera with high titers of antibodies to $PfMSP1_{19}$ were able to block the binding of all these MAbs in a dose-dependent manner (examples in Fig. 6a and c), indicating that the epitopes recognized by these MAbs are also recognized by human sera. How-

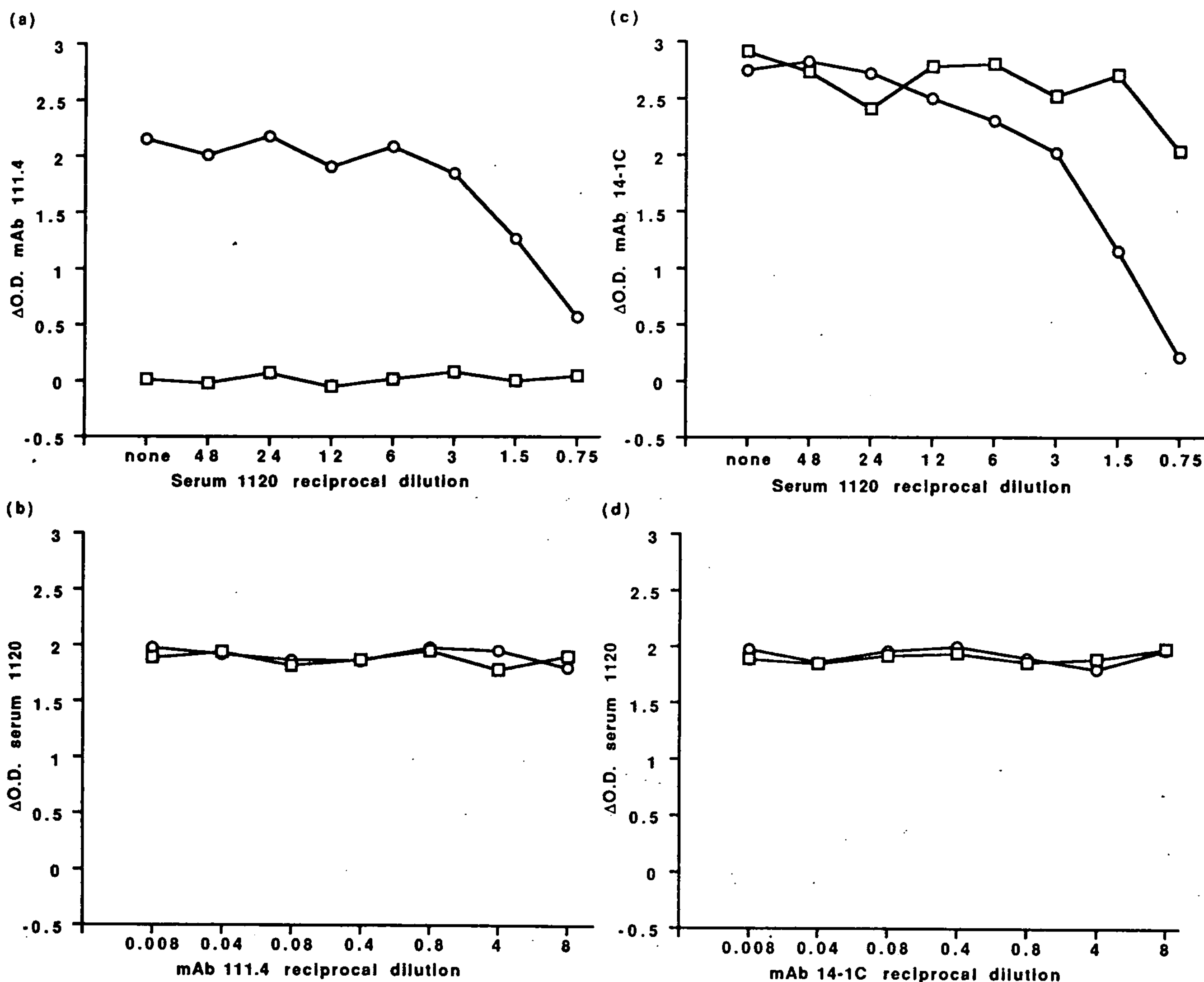


FIG. 6. Competition ELISAs. Human serum 1120 was able to inhibit the binding of MAbs in a dose-dependent manner, but MAbs were not able to inhibit binding of human antibody. (a) Serum 1120 inhibits binding of MAb 111.4 (MAb 111.4 recognizes only 1A). (b) MAb 111.4 does not inhibit binding of serum 1120. (c) Serum 1120 inhibits binding of MAb 14-1C. (d) MAb 14-1C does not inhibit binding of serum 1120. ○, 1A; □, 1B. Dilutions are in thousands.

ever, in the reciprocal experiments, MAbs were not able to completely block binding of human antibodies (Fig. 6b and d), suggesting that human sera recognize other epitopes in addition to those recognized by MAbs.

Human antibodies recognize disulfide-dependent epitopes. $PfMSP1_{19}$ is believed to contain three disulfide bonds in each of the two EGF motifs (3) (Fig. 1). To determine whether recognition of $PfMSP1_{19}$ by human antibodies is dependent on the presence of intact disulfide bonds, an ELISA comparing the reactivity of reduced and nonreduced forms of the recombinant antigens was performed (Table 3). We were able to show that epitopes recognized by human polyclonal antibodies are disulfide dependent, since reactivity with antigen was reduced or completely abolished after reduction and alkylation of the recombinant proteins. Recognition of the individual EGF motifs is entirely abolished after reduction, though some reactivity to the double-motif proteins remains. This suggests that minor, linear epitopes are also recognized by polyclonal human antibodies. In contrast, all the MAbs tested recognized only nonreduced antigen (Table 3).

Predominant antibody response is IgG1. The subclass of IgG produced in response to a given antigen determines the function of the antibody. IgG subclass was determined by subclass-specific ELISA. In most positive sera, the IgG was predominantly of the IgG1 subclass (Fig. 7). Occasional individuals produced mainly IgG3, and there was a single IgG4 positive serum for antigen 1A. The predominant IgG subclass did not vary among persons of different ages (data not shown).

DISCUSSION

Recent studies have indicated that antibodies recognizing epitopes within the $PfMSP1_{42}$ and $PfMSP1_{19}$ processing products of PfMSP1 may be involved in protective immunity to malaria (1, 6–8, 31). The amino acid sequence of the 19-kDa fragment of PfMSP1 is relatively highly conserved between parasite isolates (22), and thus $PfMSP1_{19}$ is an attractive vaccine candidate. However, murine MAbs appear to recognize this protein in a sequence-specific manner (8, 11).

We have screened sera from a population from the Gambia

TABLE 3. Comparison of OD values for ELISA with nonreduced and reduced recombinant antigens[a]

Antigen	OD											
	MAbs						Human sera					
	14-1C	7.5	111.4	6E2/53	12.10	4H4/34	1559	1572	1579	1584	1592	1599
Wellcome 1st motif												
Nonreduced	2.531	2.688	2.658	0.170	0.150	0.193	1.433	0.129	0.153	0.145	1.856	1.188
Reduced	0.117	0.199	0.172	0.102	0.137	0.113	0.089	0.127	0.112	0.121	0.151	0.403
MAD20 1st motif												
Nonreduced	2.475	0.479	0.137	0.181	0.199	0.130	1.859	0.184	0.100	0.126	1.878	1.168
Reduced	0.124	0.118	0.106	0.114	0.135	0.109	0.095	0.125	0.113	0.113	0.160	0.353
MAD20 2nd motif												
Nonreduced	0.311	0.123	0.148	1.496	0.309	0.133	1.491	0.329	0.194	1.405	0.163	2.392
Reduced	0.159	0.138	0.113	0.191	0.135	0.125	0.198	0.251	0.284	0.355	0.108	0.410
MAD20 $MSP1_{19}$												
Nonreduced	1.914	0.995	0.142	2.130	0.710	1.721	1.899	0.824	2.516	1.270	1.655	2.623
Reduced	0.157	0.114	0.086	0.099	0.104	0.093	0.295	0.220	0.282	0.222	0.612	0.813
MAD20/Wellcome $MSP1_{19}$												
Nonreduced	1.932	0.241	0.152	0.140	0.309	0.104	1.828	0.691	2.333	0.829	1.521	2.334
Reduced	0.287	0.106	0.089	0.092	0.104	0.108	0.664	0.316	0.851	0.132	1.016	1.486
Wellcome $MSP1_{19}$												
Nonreduced	1.998	1.971	2.124	0.126	1.712	0.105	1.712	0.627	2.408	0.820	1.410	2.412
Reduced	0.211	0.158	0.249	0.094	0.132	0.087	0.523	0.272	0.655	0.121	0.824	1.314
Wellcome/GST $MSP1_{19}$												
Nonreduced	2.549	2.403	2.449	0.125	2.324	0.225	2.176	0.646	2.553	1.159	1.747	2.480
Reduced	0.138	0.138	0.188	0.091	0.178	0.080	0.686	0.272	0.593	0.143	0.660	0.905
GST												
Nonreduced	0.262	0.119	0.208	0.235	0.234	0.169	0.105	0.101	0.108	0.102	0.121	0.120
Reduced	0.134	0.124	0.101	0.122	0.128	0.116	0.078	0.084	0.087	0.078	0.105	0.120

[a] Underlining is used to highlight values for positive antigen-antibody combinations. Human sera were tested at a dilution of 1:3,000, which represents the midpoint of the titration curve. MAbs were tested at a dilution of 1:1,000, which represents a saturating concentration of antibody.

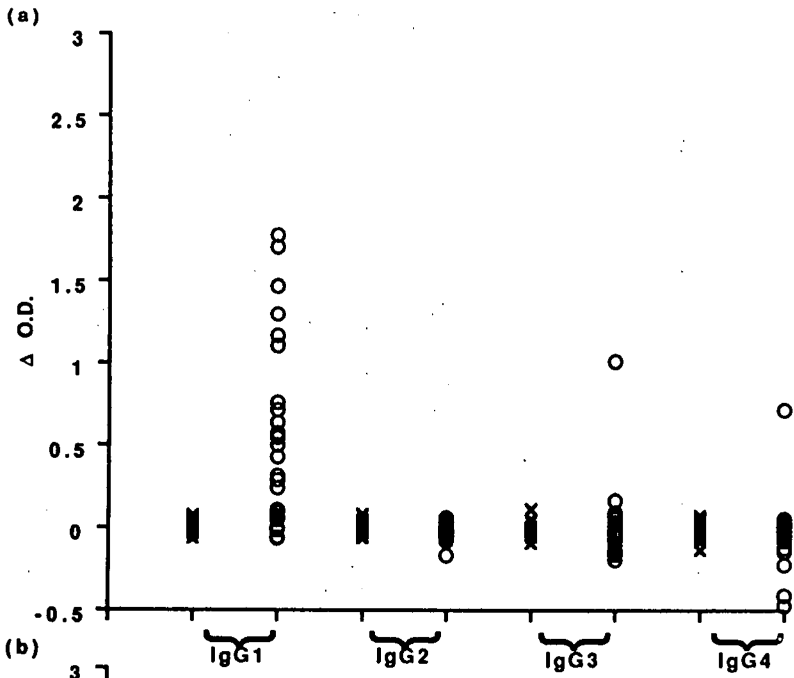
with documented previous exposure to malaria for antibodies recognizing recombinant proteins representing the two allelic forms of the single and double EGF motifs of $PfMSP1_{19}$. We find that recombinant $PfMSP1_{19}$ proteins produced either in *E. coli* or in *S. cerevisiae* are equally suitable for ELISAs. Direct comparison of antibody binding to proteins produced in the two expression systems showed that they were essentially identical. The secondary structure of $PfMSP1_{19}$, which is known to be necessary for optimal recognition by MAbs raised against the native protein, appears to be appropriately formed in recombinant proteins produced in both *S. cerevisiae* and *E. coli*. Antibodies to $PfMSP1_{19}$ in immune human sera tend to recognize disulfide-dependent epitopes; however, there is some serum reactivity with reduced double-motif proteins, suggesting that conformation-independent antibodies are also present.

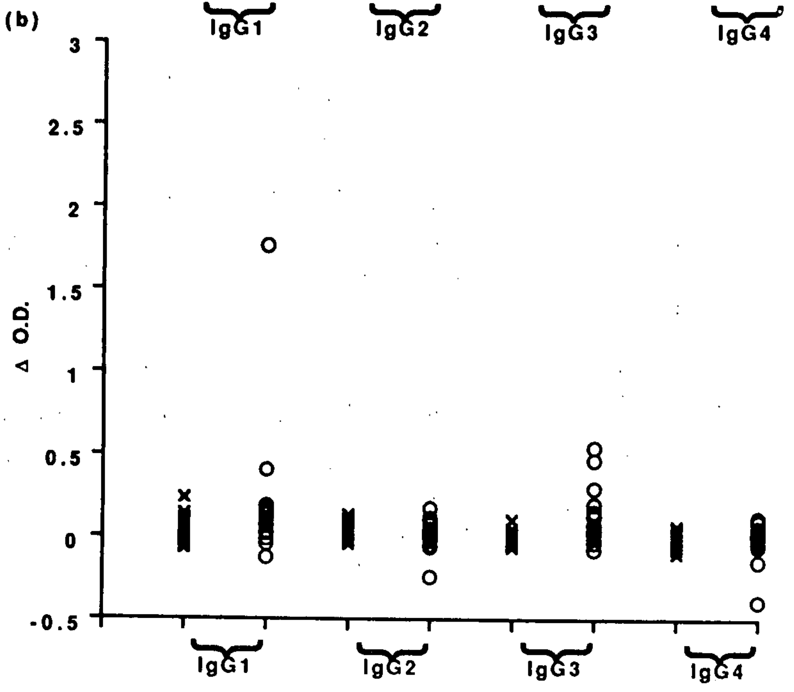
The prevalence of antibodies to the double EGF motif increases with age, and serum from up to 60% of adult donors aged 16 years and above contained antibodies which recognized these constructs (Table 1). However, only a minority of individuals (either children or adults) had antibodies recognizing either of the two EGF motifs when these were expressed singly. This indicates that a dominant epitope(s) requires the presence of both EGF motifs and is missing when either motif is expressed on its own.

$PfMSP1_{19}$ antibodies in human sera tend to recognize epitopes which are conserved or cross-reactive between variant sequences. Although occasional sera were shown to contain two separate, non-cross-reacting populations of antibodies, the majority of positive sera clearly recognize an epitope which is conserved or cross-reactive between the two sequences. A few sera contain antibodies which appear to bind with higher avidity to one sequence of the protein than to the other sequence. This was particularly apparent when antigens which differed in the sequence of the second EGF motif (where there are three amino acid differences) were compared, suggesting that there is a minor epitope in the second EGF motif which involves the dimorphic residues. In contrast, substitution of glutamate for glutamine in the first motif has very little effect on recognition by human antibodies. In this respect, human antibodies differ substantially from murine MAbs (such as 111.4), which recognize epitopes involving dimorphic amino acid residues. This cross-reactivity bodes well for the development of a malaria vaccine which could provide protection against all variants of the parasite.

Human sera can inhibit the binding of MAbs whose epitopes map to either EGF motif and MAbs which recognize the double-motif structure. However, none of the MAbs could inhibit binding of human antibodies. Thus, human sera may recognize a number of epitopes within $PfMSP1_{19}$, some of which are also recognized by MAbs. Alternatively, binding of human Ig to other epitopes may sterically interfere with MAb binding.

The reason why 40% of adult donors from our cross-sectional study did not possess antibodies to $PfMSP1_{19}$ is not known. All donors are known to have been exposed to malaria over many years and had high titers of antibody to malaria schizonts. Preliminary analysis of the data with respect to the known human leukocyte antigen (HLA) class II genotype of the donors (12a) does not indicate that the response to $PfMSP1_{19}$ is genetically restricted. It is possible that the seronegative donors may have antibody to other regions of PfMSP1 which inhibit the formation of antibodies to $PfMSP1_{19}$. Steric interference has been shown between MAbs binding to apparently distant PfMSP1 epitopes (35), suggesting that the N-terminal region of PfMSP1 may physically obscure $PfMSP1_{19}$ in the native protein. Studies are in progress to determine


(a)
Δ O.D.
3
2.5
2
1.5
1
0.5
0
-0.5
IgG1
IgG2
IgG3
IgG4


(b)
Δ O.D.
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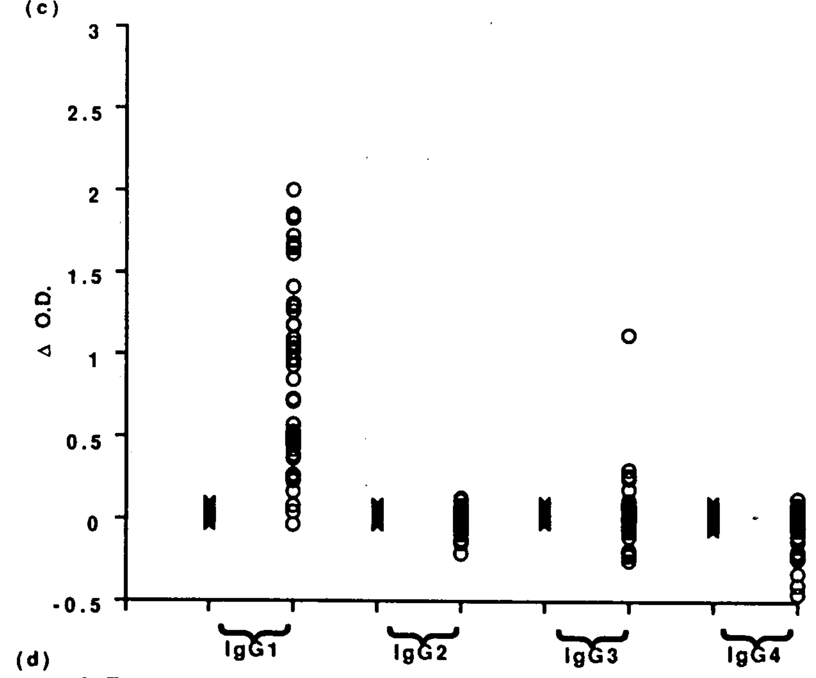
IgG3
IgG4


(c)
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IgG3
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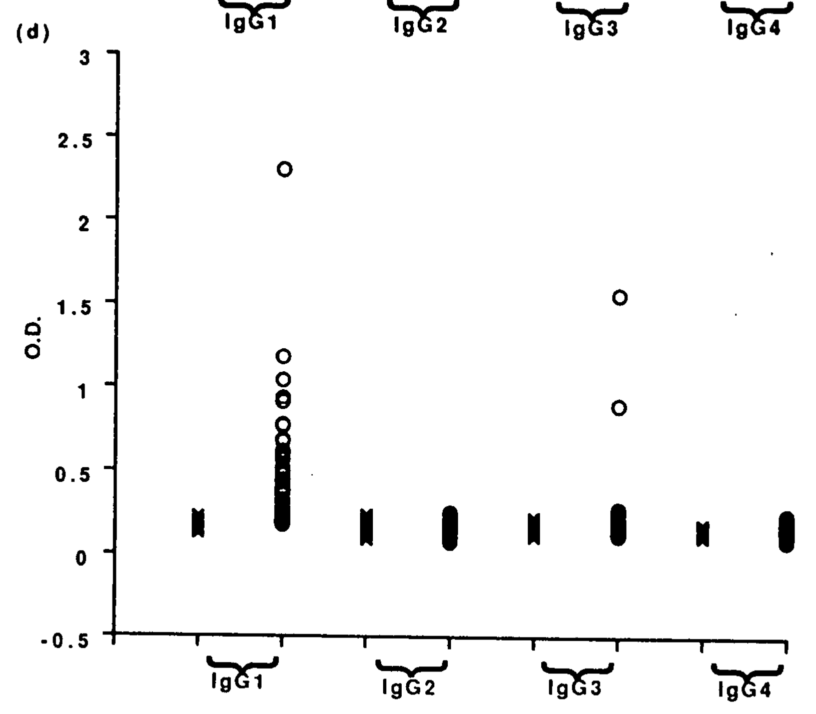
(d)
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1
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-0.5
IgG1
IgG2
IgG3
IgG4

FIG. 7. Dot plots showing IgG subclass-specific responses. Only IgG-positive Gambian sera were tested; 24 European sera are shown as controls. All sera were tested at a dilution of 1:1,000. (a) Wellcome first motif (1A); 32 Gambian sera tested. (b) MAD20 second motif (2B); 24 Gambian sera tested. (c) Wellcome PfMSP1_{19} (19/GST); 52 Gambian sera tested. (d) MAD20 PfMSP1_{19} (3D7); 54 Gambian sera tested. ×, European sera; O, Gambian sera.

patterns of antibody recognition of other regions of PfMSP1 by these sera. Alternatively, seronegative individuals may not have been recently infected with malaria, and in the absence of boosting, antibody may have fallen below detectable levels.

An important outstanding question is whether antibodies to PfMSP1_{19} are protective or not. The fact that PfMSP1 appears to induce predominant IgG1 antibodies suggests that such antibodies may play a role in opsonization or complement-mediated lysis of free merozoites. Recent experiments have shown that the membrane-bound 15-kDa fragment of MSP1 from *P. yoelii* (PyMSP15) is the target of a protective MAb (6), and vaccination of mice with a recombinant protein representing the double EGF motifs of PyMSP1_{15} protects against challenge infection with the 17XL and YM strains of *P. yoelii* (12, 25). A longitudinal immunoepidemological study suggested that antibodies to PfMSP1_{42} were associated with resistance to clinical malaria and high parasitaemia in young children (31). We have recently completed a similar longitudinal study of antimalarial antibody levels and malaria morbidity, which indicates that antibodies to PfMSP1_{19} are associated with protection from clinical malaria (unpublished data). Further studies are in progress in our laboratories to determine the functional role of human antibodies to PfMSP1_{19} and their role in protective immunity to malaria.

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Naturally Acquired Human Antibodies Which Recognize the First Epidermal Growth Factor-Like Module in the *Plasmodium falciparum* Merozoite Surface Protein 1 Do Not Inhibit Parasite Growth In Vitro

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Merozoite surface protein 1, one of the major surface proteins of the invasive blood stage of the malaria parasite, is a prime candidate for the development of a vaccine against the human disease. Previously, monoclonal antibodies which both inhibited the growth of *Plasmodium falciparum* in vitro and bound to the first of two epidermal growth factor-like modules located near the carboxy terminus of the protein had been identified. In this study, we have used affinity chromatography on a recombinant fusion protein corresponding to the first epidermal growth factor-like module in *P. falciparum* merozoite surface protein 1 to prepare antibody induced by natural infection. The antibody was purified from the total immunoglobulin G fraction of adult West African donors, shown to passively confer immunity against falciparum malaria. Such affinity-purified antibodies were shown to recognize the native protein by a number of separate criteria and to block the binding of an inhibitory monoclonal antibody, but they failed to inhibit parasite invasion in an in vitro growth assay. These results indicate that antibody alone is not sufficient to interfere with erythrocyte invasion.

There is an urgent need to develop a more effective vaccine against malaria. Much work on vaccine development has been focused on merozoite surface protein 1 (MSP-1), a high-molecular-mass protein synthesized by the intracellular schizont of the asexual blood and liver stages and expressed on the surface of merozoites released from the ruptured schizont (18, 24). MSP-1 preparations purified from a number of malaria parasites have, by immunization, induced significant levels of protection against challenge with blood-stage parasites in both rodent (26) and simian (20, 21, 41, 46) experimental model systems. Investigators have used parts of MSP-1, either expressed in *Escherichia coli* (17, 20, 23, 28, 33) or as synthetic peptides (13, 40), to induce complete or partial protection or to delay the progress of infection. A synthetic peptide polymer, which includes a sequence from the N terminus of *Plasmodium falciparum* MSP-1, produced encouraging results in a recent clinical trial (49).

MSP-1 is modified by proteolysis leading to shedding of the molecule except for a small C-terminal fragment, which can be detected on the surface of the parasite in a newly infected erythrocyte. Prior to the release of merozoites from the mature schizont, *P. falciparum* MSP-1 is processed by protease(s) to at least four major polypeptides, held together in a noncovalent complex on the merozoite surface (35). One of these, a 42-kDa membrane-bound fragment from the C terminus (designated $MSP\text{-}1_{42}$), undergoes a second proteolytic cleavage to produce $MSP\text{-}1_{33}$ and $MSP\text{-}1_{19}$ (1, 6). The membrane-bound $MSP\text{-}1_{19}$ polypeptide, comprising two cysteine-rich epidermal growth factor (EGF)-like modules (4), is carried into the new erythrocyte on the surface of the invading merozoite (2).

The EGF-like modules are found in MSP-1 in all species of malaria parasite examined (16, 25), and there is evidence to suggest that they are an important target of protective immunity. In studies using the rodent malaria models *Plasmodium yoelii* and *Plasmodium chabaudi*, MSP-1-specific monoclonal antibodies (MAbs) passively protected mice against challenge infection with homologous blood-stage parasites (8, 32, 34). Two of these antibodies bind to discontinuous disulfide-constrained epitopes within the EGF-like modules (10, 37). Mice immunized with the cysteine-rich region of *P. yoelii* MSP-1, expressed as a fusion protein in *E. coli*, were partially or completely protected against challenge infection with *P. yoelii* parasites (17, 33). A number of investigators have reported *P. falciparum* MSP1-specific MAbs which inhibit the in vitro growth of the parasite (2, 15, 42), and these antibodies react with $MSP\text{-}1_{19}$. The EGF-like modules have been expressed in a correctly folded form in insect cells (11, 39), yeast cells (30), and *E. coli* cells (9, 12). Serum from rabbits immunized with a correctly folded insect cell product representing $MSP\text{-}1_{42}$, but not with an identical but incorrectly folded yeast cell product, completely inhibited *P. falciparum* growth in vitro (11). We have reproduced the individual EGF-like modules of the *P. falciparum* MSP-1 by expression as fusion proteins in *E. coli* and have shown that a number of MSP-1-specific MAbs, including the invasion-inhibitory antibodies 5B1 (42) and 12.8 (2), bound to disulfide-constrained epitopes in the first of the EGF-like modules (MSP-1–EGF1) (12).

We show here that, in contrast to results obtained with MSP-1-specific MAbs, immunoglobulin G (IgG) affinity selected on MSP-1–EGF1 from the naturally acquired repertoire

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of adults with immunity to malaria did not prevent the invasion of erythrocytes by malarial merozoites in vitro. These antibodies did recognize the native protein and competed for antigen binding with a protective MAb.

MATERIALS AND METHODS

Expression construct and protein purification. The first EGF-like module in the Wellcome\T9-94 MSP-1 (4, 29), designated MSP-1–EGF1, was expressed as a fusion protein with glutathione *S*-transferase (GST) in *E. coli*. The production and characterization of the expression construct and the purification of the protein by chromatography on glutathione-agarose (47) have been described in detail elsewhere (12). The protein was dialyzed against 50 mM ammonium bicarbonate (pH 7.4) and quantified (the A_{280} of 1 mg of protein solution ml^{-1} was estimated at 1.9). Alternatively, the soluble EGF-like polypeptide was cleaved from the immobilized GST fusion partner by proteolysis with bovine factor Xa (Boehringer) (47).

Human IgG preparations. IgG derived from 178 adult inhabitants of Ivory Coast, West Africa (designated total-IgG), was used; the source and purification of this IgG and its clinical effect upon transfer to *P. falciparum*-infected recipients have been described previously in detail (43). Control IgG (designated control-IgG) was purified from heat-inactivated, pooled sera of healthy European A+ blood donors never exposed to malaria, by chromatography on protein A bound to glass beads (Prosep-A; Bioprocessing), according to the manufacturers' instructions. IgG purity was estimated by analysis of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and quantified on the assumption that 1 mg of IgG ml^{-1} has an A_{280} of 1.4 (22). IgG preparations were concentrated to 5 to 10 mg ml^{-1} by ultrafiltration (Amicon) and stored at 4°C, supplemented with 0.02% (wt/vol) sodium azide.

Affinity purification of MSP-1-specific IgG. IgG was affinity purified by chromatography on fusion protein representing MSP-1–EGF1 covalently linked to an insoluble matrix (22). Briefly, 35 mg of purified fusion protein (32,274 Da) was coupled to 7 ml (swollen gel volume) of cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions. This affinity matrix was estimated to be of sufficient capacity to bind approximately 16 mg of IgG (on the assumption of only 1 IgG molecule bound per 10 fusion protein molecules). Nine hundred mg of total IgG was passed over the affinity matrix; IgG which did not bind (designated void-IgG) was collected. After being extensively washed with 100 mM and then 10 mM Tris-HCl (pH 8.0), bound IgG was eluted into 100 mM glycine-HCl (pH 2.5) and neutralized with 1/10 volume of 1 M Tris-HCl (pH 8.0). IgG reactive with the GST fusion partner or with native *E. coli* polypeptides was then removed by use of a bacterial acetone powder (22). Briefly, *E. coli* transformed with plasmid pGex-3X was induced to express GST and then lysed and clarified by centrifugation. Bacterial proteins in the supernatant were precipitated with acetone, harvested, and then dried. This powder was added at a ratio of 20:1 (wt/wt) over IgG, and then, after incubation at 4°C for 1 h, insoluble immunocomplexes were removed by centrifugation at 39,000 × *g* for 15 min. Affinity-purified IgG specific for MSP-1 (designated AP-IgG) was extracted from the supernatant by use of the immobilized fusion protein, concentrated by ultrafiltration, and stored as described earlier.

Culture and metabolic radiolabelling of *P. falciparum*. Clone T9/94 (48) was maintained in culture and synchronized when required, essentially as described previously (27); growth medium was supplemented with 0.5% (wt/vol) AlbuMAX (Life Technologies) instead of serum (34a). Synchronous cultures containing predominantly schizont-stage parasites with 2 to 4 nuclei were washed once in methionine-free RPMI 1640 and resuspended at a 10% hematocrit in the same medium supplemented with AlbuMAX plus 1.85 MBq of [^{35}S]methionine (Amersham) ml^{-1}. After 3 to 4 h of growth, cells were pelleted, washed three times in RPMI 1640, and then frozen at −70°C until required.

Immunoprecipitation and Western blotting methods. IgG specificities were analyzed by immunoprecipitation followed by SDS-PAGE and fluorography of the radiolabelled parasite proteins, by previously described procedures (27).

For analysis by Western blotting (immunoblotting), identical samples of the MSP-1–EGF1 polypeptide without the GST fusion partner were subjected to nonreducing SDS-PAGE by a Tricine buffer system (45) and then electrophoretically transferred to nitrocellulose, essentially as described previously (6). Primary antibody binding was detected with appropriate anti-IgG alkaline phosphatase conjugates and a chromogenic substrate. In both immunoprecipitation and Western blotting procedures, the murine MAb 111.4 (29) was used as a positive control; this antibody binds to a disulfide-constrained epitope of MSP-1–EGF1 (12).

Molecular mass markers used were obtained from Gibco BRL (high molecular mass, prestained; 14.3 to 200 kDa) or BDH, Poole, United Kingdom (CNBr-cleaved horse heart myoglobin; 2,512 to 16,949 Da).

ELISA to measure anti-MSP-1 antibodies. The titer of IgG specific for MSP-1 was measured by enzyme-linked immunosorbent assay (ELISA), essentially as described previously (3). Briefly, ELISA plates were coated with S42ΔA (39), a recombinant insect cell product which includes the carboxy-terminal 271 amino acids (D-1433 to S-1723 [38]) of the Wellcome\T9-94 MSP-1 and includes both EGF-like modules. Plates were washed, and twofold serial dilutions of each IgG (2×10^{-4} to 4.9×10^{-8} g ml^{-1}) were applied to duplicate wells. Following incubation with enzyme-conjugated anti-human IgG, plates were developed with a chromogenic substrate and A_{492} values were measured.

Competition ELISA. To determine whether the human antibodies could inhibit the binding of MAbs, the plates were first incubated with serial dilutions of each human IgG fraction. The plates were washed, and an optimal dilution of MAb determined by titration was added to the plate. The binding of MAb was measured by use of an enzyme-conjugated anti-mouse IgG and chromogenic substrate.

In vitro parasite invasion inhibition assay. Mature schizonts, purified from highly synchronous cultures by centrifugation onto a cushion of 63% isotonic Percoll (Pharmacia), were added to erythrocytes to give a parasitemia of 0.5 to 1%. Each test IgG was dialyzed extensively against RPMI 1640 and then added to four identical microcultures of *P. falciparum*, at a final concentration of 1 mg ml^{-1} (the final hematocrit was 2%). Control cultures (six of each) containing either no added IgG or 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] were also included; EGTA at this concentration effectively inhibits merozoites' invasion of erythrocytes (36, 50). After 24 h of growth at 37°C, duplicate blood smears were made from each well and then fixed with methanol and stained with Giemsa stain. Parasitemia was determined by counting of the number of young parasites within 8,500 or more erythrocytes. Multiple parasites within a single erythrocyte were recorded as one count.

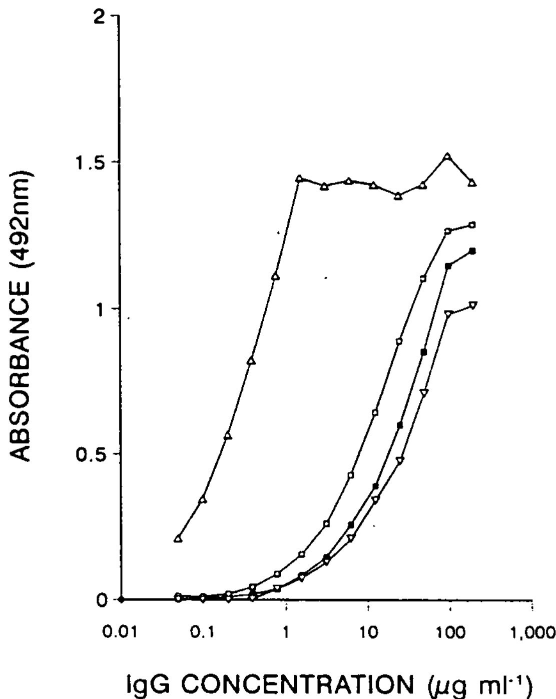

FIG. 1. The titers of MSP-1-specific Ig within four serially diluted antibody preparations (defined in Materials and Methods) were compared by ELISA utilizing the recombinant protein S42ΔA; a representative experiment is shown. The ordinate represents A_{492}, versus the IgG concentration plotted on a semilogarithmic scale for clarity. Serial dilutions of AP-IgG (△), total-IgG (□), void-IgG (■), and control-IgG (▽) were used.

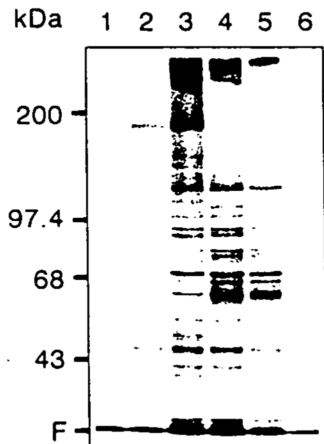

FIG. 2. Immunoprecipitation of radiolabelled parasite proteins by a selection of antibodies. Separate immunoprecipitation reactions were set up, containing equal amounts of parasite material plus 5 μg of one of the following: AP-IgG (lane 3), total-IgG (lane 4), and void-IgG (lane 5). Control reactions were set up with either no added IgG (protein A-Sepharose only, lane 1), 5 μl of MAb 111.4 ascites (lane 2), or 5 μg of control-IgG (lane 6). Immune complexes were subjected to SDS-PAGE under reducing conditions on a 7.5% polyacrylamide gel, and radiolabelled proteins were visualized by fluorography. Molecular mass marker proteins indicated are myosin heavy chain (200 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). F indicates the gel dye front.

RESULTS

Affinity purification of MSP-1-specific human IgG. IgG was purified from pooled IgG of West African adults (total-IgG) by affinity chromatography on MSP-1–EGF1 fusion protein representing the first EGF-like module in the *P. falciparum* Wellcome\T9-94 type MSP-1. A two-stage affinity selection protocol, utilizing elution by acid at pH 2.5, yielded 2.9 mg of IgG (AP-IgG), 0.3% of the 900 mg of total-IgG starting material. Analysis of each IgG fraction by SDS-PAGE under reducing conditions revealed essentially only two polypeptides, corresponding to the IgG heavy and light chains (data not shown). A significant quantity of IgG remained bound to the affinity matrix following sequential washes at pH 2.5 and subsequent washes with buffers at neutral pH containing 8 M urea or 1% SDS; such IgG was only eluted by boiling in SDS-PAGE sample buffer (data not shown), and further analysis of this IgG was not attempted.

Characterization of affinity-purified human antibodies. ELISA, immunoprecipitation, and Western blotting experiments were carried out to demonstrate the specificity and to quantify the amount of MSP-1-specific antibody in AP-IgG in comparison with other Ig preparations.

The titers of MSP1-specific antibodies in the four Ig samples were compared by use of an ELISA based on the insect cell product S42ΔA, as shown in Fig. 1. Differences in anti-S42ΔA antibody titers between samples were estimated by comparison of IgG concentrations at fixed absorbance values; such approximations are valid in regions where plotted data lines remain parallel (31). By this method, the AP-IgG preparation was estimated to contain 100-fold more S42ΔA-specific Ig than the void-IgG. Note that the level of S42ΔA-specific Ig present in the void-IgG sample was reduced from that present in the total-IgG to approximately that in control-IgG, which was assumed to represent the background level. This result suggested that a substantial proportion of the S42ΔA-specific Ig originally present in the total-IgG preparation had been removed by the affinity purification procedure.

Immunoprecipitation experiments were conducted to demonstrate that the affinity purification procedure selected IgG which could recognize and bind to the native MSP-1. Figure 2 shows that the AP-IgG preparation (lane 3) and the control MAb 111.4 (lane 2) both immunoprecipitated a 190-kDa species from radiolabelled parasite material, corresponding in size to the intact MSP-1. No equivalent species was observed in immunoprecipitation reactions with either the total-IgG (Fig. 2, lane 4), void-IgG (lane 5), or control-IgG (lane 6) preparation or with protein A-Sepharose alone (lane 1). Other faster-migrating species were immunoprecipitated by all three polyclonal IgG preparations, and most of these were common among the samples (Fig. 2, compare lanes 3, 4, and 5); such species were not characterized further.

Western blotting was used to demonstrate that the AP-IgG preparation had enhanced levels of antibodies against the first MSP-1 EGF-like module compared with the other antibody preparations. Digestion of the GST fusion protein with factor Xa produced a 52-amino-acid polypeptide comprising MSP-1–EGF1 (residues 1631 to 1678 of MSP-1) preceded by four additional residues (GIQM), with a calculated molecular mass of 6,022 Da. Identical samples of this polypeptide were subjected to Tricine-SDS-PAGE under nonreducing conditions and transferred to nitrocellulose. Figure 3 shows that both MAb 111.4 (lane 1) and the AP-IgG preparation (lane 3) clearly detected the MSP-1–EGF1 polypeptide; this species was not detectable in the conditions of this assay with the total-IgG (lane 2), and neither the void-IgG nor the control-IgG preparation reacted with it (data not shown).

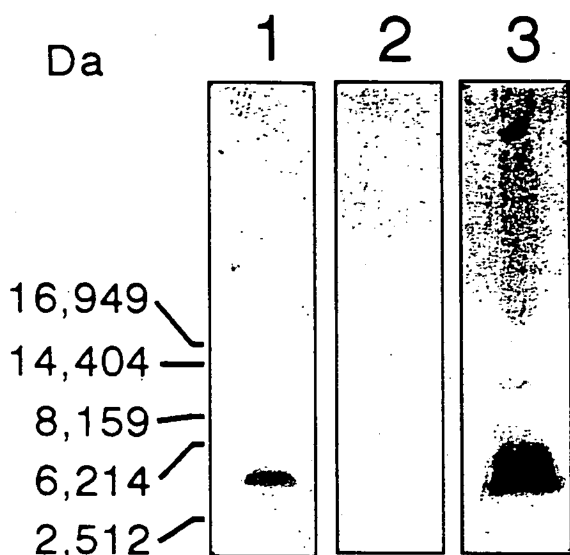

FIG. 3. The titers of IgG specific for MSP-1–EGF1 within two polyclonal antibody preparations were compared by Western blotting. Identical amounts of the 52-residue (6,022 Da) polypeptide representing MSP-1–EGF1, derived from an *E. coli*-expressed fusion protein by site-specific proteolysis, were subjected to Tricine-SDS-PAGE and then transferred to nitrocellulose. Blots were probed with either MAb 111.4 (lane 1), total-IgG (lane 2), or AP-IgG (lane 3). Color development reactions were conducted in parallel and stopped at the same time. Protein molecular mass markers indicated were derived from CNBr-cleaved horse heart myoglobin, at 16,949 Da, 14,404 Da, 8,159 Da, 6,214 Da, and 2,512 Da.

These experiments indicate that compared with the void-IgG sample, a significantly greater proportion (approximately 100-fold by ELISA) of the antibodies within the AP-IgG preparation were directed against determinants present in the first EGF-like module of MSP-1.

Affinity-purified antibodies compete with a protective MAb for antigen binding. The different IgG preparations were assayed for their abilities to block the binding of two inhibitory MAbs to S42ΔA (Fig. 4). The AP-IgG fraction was able to compete with MAb 12.8 at a concentration of 2 μg ml^{-1}, but none of the other IgG was effective in this range. None of the antibodies inhibited the binding of MAb 12.10 at 10 μg ml^{-1} (data not shown).

Parasite in vitro invasion assay. Highly synchronous microcultures of *P. falciparum* containing predominantly schizonts were incubated in the presence of various IgG preparations. After 24 h, encompassing schizont rupture-merozoite release and reinvasion of new erythrocytes, parasitemias were determined. The final parasitemia in cultures supplemented with African IgG preparations did not differ significantly from that of the control (Fig. 5), although EGTA was very effective at blocking invasion. The morphology of the parasites was normal on Giemsa-stained smears for all cultures, incubated with or without antibodies, and no agglutinated clusters of merozoites were observed in any sample.

DISCUSSION

We have investigated whether antibodies to the first EGF-like module of MSP-1 induced by natural infection are inhibitory to parasite growth in vitro. This biological property is manifested by some but not all MAbs specific for the C-terminal cysteine-rich region of MSP-1. Four murine MAbs which are specific for MSP-1 and which inhibit the growth of *P. falciparum* in vitro (2, 15, 42) have been described previously. Two of these antibodies, 12.8 (2) and 5B1 (42), bind to the first of two EGF-like modules in MSP-1 (12); another, 12.10, binds only if the two EGF-like modules are expressed together (9, 12). The present study was aimed at investigating whether or not naturally occurring antibodies with similar specificities

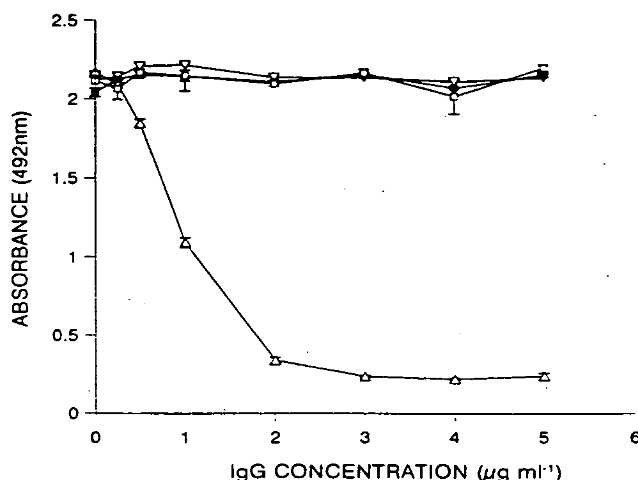

FIG. 4. Affinity-purified antibodies block the binding of an inhibitory MAb. ELISA plates coated in S42ΔA were preincubated with various concentrations of human IgG. Serial dilutions of AP-IgG (△), total-IgG (□), void-IgG (■), and control-IgG (▽) were used in triplicate. An optimal concentration of MAb 12.8 was then added, and the amount bound was determined. The ordinate represents A_{492} as a measure of bound MAb, and the abscissa represents the IgG concentration.

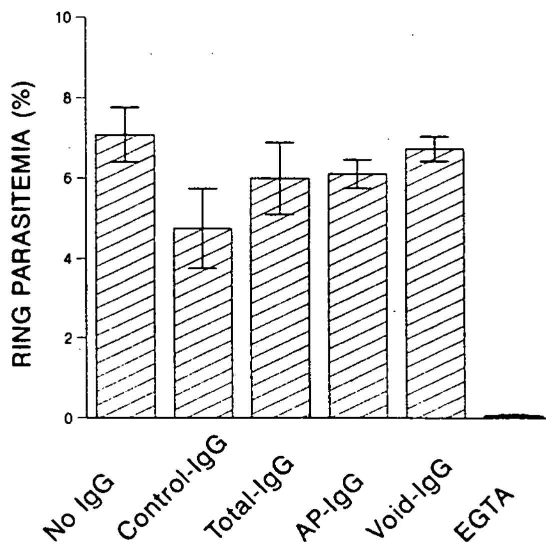

FIG. 5. Invasion inhibition assay. Various IgG preparations were added to individual microcultures of *P. falciparum* to test for their abilities to inhibit parasite invasion of erythrocytes. Growth was also monitored in cultures containing either no added IgG or 5 mM EGTA, previously shown to effectively inhibit merozoite entry into erythrocytes. Bars on the chart represent mean final percentage parasitemias; error bars indicate standard distribution within each sample.

could manifest the same biological activity and therefore be responsible (at least in part) for the protection observed in immune individuals and in nonimmune individuals protected by passive transfer.

By affinity chromatography on the first EGF-like module of MSP-1 expressed as a fusion protein in *E. coli*, we selected a subset of IgG from a polyspecific IgG pool derived from West African adults and shown to passively confer immunity to malaria. We have previously concluded that MSP-1–EGF1 expressed in bacteria adopts the correct disulfide structure: it is reactive with MSP-1-specific MAbs, and this binding is diminished or abolished by prior treatment with thiol reagents (12). From 900 mg of total-IgG, 2.9 mg of IgG was selected by the affinity chromatography procedure. On the basis of these figures, and on the assumption that the AP-IgG is wholly specific for MSP-1, approximately 0.3% of the total-IgG population was recovered. Taking into consideration the fact that higher-affinity antibodies remained bound to the column during the elution process, the results suggest that large amounts of IgG are directed against the first EGF-like structure in MSP-1. No similar quantitative estimates for the proportion of MSP-1-specific antibodies in human serum have been previously reported.

The AP-IgG preparation was shown by ELISA to contain approximately 100-fold-enhanced levels of S42ΔA-specific antibodies compared with the void-IgG preparation. S42ΔA includes both EGF-like modules and parts of MSP-1 N terminal to this region; antibodies to these regions will increase absorbance values measured in the ELISA for the nonselected samples.

We found that only the AP-IgG preparation contained enough antibodies of the correct specificity to generate an MSP-1-specific signal in Western blotting and immunoprecipitation experiments. Successful immunoprecipitation of radiolabelled MSP-1 demonstrates that AP-IgG can bind to the native protein. These data support the ELISA results and suggest that a substantial proportion of antibodies within the AP-IgG preparation are specific for MSP-1–EGF1. In addition, the selected antibodies inhibited the binding of MAb 12.8, which binds specifically to the first domain, but had no effect on the binding of MAb 12.10, which is known to be directed to a conformational epitope distinct from that of 12.8, when S42ΔA was used as the antigen.

Immunity against malaria, which may be acquired by long-term residents in regions where malaria is endemic, can be passively transferred to young children who have not yet developed an effective immunity by the administration of IgG from immune adults (14, 19, 43). The mechanism for this protection remains unclear. Our findings that a concentration of 1 mg of total-IgG ml^{-1} did not significantly affect parasite multiplication were not surprising. In previous experiments, identical IgGs had no inhibitory effect on either parasite growth or invasion in vitro at concentrations of up to 5 mg ml^{-1}, indeed sometimes stimulating parasite growth, at least of some strains (7). Nevertheless, this antibody preparation exerted a profound reduction in asexual parasitemia when administered to human malaria patients by intravenous inoculation (43), and it is perhaps significant that it was shown to inhibit parasite growth in vitro in cooperation with monocytes (7). Although the invasion inhibition assay used in the present study was similar to that used previously (2), in which merozoite invasion was significantly inhibited by MAb at a concentration of only 100 μg ml^{-1}, a 10-fold-higher concentration of the polyspecific AP-IgG preparation of human origin did not hinder merozoite entry into erythrocytes. This discrepancy suggests that minor differences in the target epitopes may be critical in the function of the corresponding antibodies.

It has been demonstrated that $MSP\text{-}1_{19}$ displays multiple distinct Ig-binding sites, and competition assays suggest that these are clustered or overlapping (12, 51). Fine-structure mapping of these epitopes will be difficult to achieve, in view of their conformational nature. Expression of the constituent EGF-like domains individually does not reproduce all antigenic determinants formed when they are expressed together, such as the binding site for the invasion-inhibitory MAb 12.10 (12). Idiotypes of the 12.8-like specificity should be present in the AP-IgG, and this is confirmed by our results, but these antibodies do not have inhibitory activity. The method of affinity purification used here would not be expected to select antibodies with 12.10-like specificities. The antibodies which bound to the affinity matrix and could only be eluted after denaturation may have had a very high affinity for MSP-1–EGF1; although it was not possible to assay them, this subset of affinity-selected IgG may have possessed invasion-inhibiting activity.

Natural infection or immunization with MSP-1–EGF1 may stimulate the production of several different anti-MSP-1–EGF1 idiotypes of which only a subset exert the required biological effect(s). Hence, it may be difficult to achieve the requirement for relatively high concentrations of inhibitory Ig suggested both by a kinetic model of merozoite invasion (44) and by experiments using MAbs (2, 15, 42). In addition, the binding of inhibitory MAbs such as 12.8 may be blocked by the binding of other antibodies which are themselves not inhibitory in vitro (5, 51). It is encouraging that polyclonal Ig in serum from rabbits immunized with an insect cell product representing the carboxyl-terminal processing product $MSP1_{42}$ completely inhibited the multiplication of the malaria parasite in vitro (11). Further experiments in primates and human volunteers will be critical to determine whether or not the artificial antigen can induce blocking antibodies whereas natural infection does not, or alternatively whether or not it is the host species which determines the type of antibody produced. At least in a mouse malaria, immunization with the two EGF-like modules of *P. yoelii* has provided very significant protection against challenge infection (17, 31). In these experiments, it is possible that epitopes in the second EGF module or formed from the interaction of the two domains are most important as targets of inhibitory antibodies.

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Running title: Serum antibodies to $PfMSP1_{19}$

CLINICAL IMMUNITY TO Plasmodium falciparum MALARIA IS ASSOCIATED WITH SERUM ANTIBODIES TO THE 19kDa C-TERMINAL FRAGMENT OF THE MEROZOITE SURFACE ANTIGEN, PfMSP-1.

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Footnotes:

1. This work has been presented, in part, at the 12th European Immunology Meeting, June 1994, Barcelona, Spain (abstract no. W44/10) and the VIII International Congress of Parasitology, October 1994, Izmir, Turkey (abstract no. O50.6/850).

2. Blood samples were obtained with the informed consent of the patients or their parents/guardians. Ethical permission for this study was obtained from the MRC/Gambia Government Medical Ethics Committee and the Ministry of Health, Sierra Leone.

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Abstract.

The development of an effective malaria vaccine depends upon identification of antigens that are targets of protective immune responses. An immuno-epidemiological approach has been used to investigate the relationship between antibody responses to a defined region of the major merozoite surface protein of Plasmodium falciparum ($PfMSP\text{-}1_{19}$) and resistance to clinical malaria in two populations of children from West Africa. After allowing for the confounding effects of age, antibodies to $PfMSP\text{-}1_{19}$ were shown to provide approximately 40% protection against clinical malaria in Sierra Leonean children and antibodies to one of the EGF-like motifs of $PfMSP\text{-}1_{19}$ were shown to be strongly associated with resistance to both clinical malaria and high levels of parasitemia in Gambian children.

Introduction.

The Plasmodium falciparum merozoite surface protein, PfMSP-1, is a prime candidate for a vaccine against the erythrocytic stage of the malaria parasite, which is responsible for the pathology of malaria infection. Antibodies to PfMSP-1 can block parasite invasion of red blood cells in vitro and primates immunised with native PfMSP-1 are partially or completely protected against homologous challenge [1-3]. PfMSP-1 is synthesised during schizogony; post-synthetic processing gives rise to multiple fragments including a C-terminal 19kDa polypeptide (PfMSP-1_{19}) which is made up of two epidermal growth factor (EGF)-like motifs and which may be involved in attachment of the parasite to specific red cell surface receptors [3]. Immunization of mice with recombinant proteins representing the corresponding membrane-bound component of the major merozoite surface protein from Plasmodium yoelii has been shown to induce high levels of protection against challenge with a lethal P. yoelii strain [4]. In Gambian children, IgG antibodies recognising the 42kDa C-terminal fragment of PfMSP-1 (PfMSP-1_{42}) are significantly associated with resistance to clinical malaria [5]. In this study we have investigated the hypothesis that it is antibody directed against the PfMSP-1_{19} component of PfMSP-1_{42} that is associated with protective immunity.

Materials and Methods.

Study population and study design.

The study concentrated on children who were actively acquiring immunity to malaria (327 children aged 3-8 years in the Gambia and 645 children aged 0-8 years in Sierra Leone).

Malaria in The Gambia is seasonally endemic [6]. Children are bitten by between 1 and 5 inféctive mosquitoes each year and become clinically immune to malaria by the age of 8-10 years [6]. In Sierra Leone, malaria is perennial with an average of 30 infective bites/adult/year; clinical cases are uncommon in children over the age of 5 years [7].

Serum samples were collected immediately before the commencement of the annual rainy season. The children were monitored weekly for the presence of fever (T > 37.5°C); a blood film was made from all febrile children and examined for malaria parasites. Morbidity analysis continued until the end of the malaria transmission season in The Gambia (November 1988) and for one complete year in Sierra Leone, at which time children were classified into one of four morbidity groups on the basis of their clinical experience of malaria. Clinical malaria was defined as at least one episode of malaria parasitemia (> 5,000 parasites per μL blood) together with fever (T > 37.5°C). Asymptomatic malaria was defined as malaria parasitemia (of any level) in the absence of fever. Fever (T > 37.5°C) in the presence of low parasitemia (< 5,000/μL) was classified as an indeterminate infection, since the fever may not have been directly attributable to the presence of parasites. Children in

whom there was no evidence of parasitemia were classified as "no infection". This may be due to lack of exposure to infected mosquitoes or immune clearance of parasites in the absence of clinical symptoms.

Recombinant antigens.

PfMSP-1 is a dimorphic protein encoded by genes belonging to two major allelic families (MAD20/3D7 and Wellcome/K1) [8]. The MAD20 family accounts for more than 95% of clinical infections in The Gambia [9]. The sequence of PfMSP-1_{19} is highly conserved (four amino acid differences, out of 95 residues) between the two allelic sequences.

The first and second EGF motifs of the MAD20 allele were expressed as glutathione S-transferase (GST) fusion proteins in E. coli transformed with recombinant pGEX3 plasmids [10]. As a control, unfused GST was prepared from bacteria transformed with PGEX3. The complete MAD20 PfMSP-1_{19} protein and a protein representing a recombinant allele which has the MAD20 sequence of the first EGF motif and the Wellcome sequence of the second motif (FVO/E), were expressed in recombinant S. cerevisiae [11].

ELISA assays.

Sera were tested for reactivity with the PfMSP-1 proteins by enzyme-linked immunosorbent assay (ELISA) as described previously [12]. Briefly, microtiter plates (Dynatech, Billingshurst, UK) were coated with recombinant protein; serum was added to duplicate wells and incubated overnight at 4°C. Plates were developed with peroxidase-conjugated rabbit anti-human IgG (Dako

Ltd, High Wycombe, UK) and developed with H_2O_2 and o-phenylenediamine (both Sigma, Poole, UK). Plates were read at 492nm. For GST fusion proteins the optical density (OD) values for binding to the GST control protein were subtracted from the OD values obtained for the PfMSP-1-GST fusion protein to obtain the PfMSP-1-specific OD. Antibody positive sera ("responders") are defined as those giving an OD > normal range (mean plus 2 SD of OD's of 42 European sera tested in parallel with the malaria-exposed samples).

Statistical Methods.

Only the clinical and asymptomatic malaria categories were compared since the status of the other two morbidity groups was not certain. The χ^2 test for trend assessed age-related trends in the prevalence of positive antibody responses and clinical malaria. The association between malaria morbidity and antibody response was assessed using odds ratios (OR); an OR of less than 1.0 would indicate a protective antibody effect. To allow for potential confounders of the association between morbidity and antibody response, multiple logistic regression was used to obtain adjusted OR's and significance assessed by the likelihood ratio test.

Results.

1. Relationship between age, clinical symptoms of malaria infection and anti-PfMSP1_{19} antibody levels.

The prevalence of clinical malaria declined with age in both cohorts and the incidence of asymptomatic infections increased with age (Table 1). This confirms that the age groups being studied were those in which clinical immunity was developing rapidly.

Within the Sierra Leonean cohort, children below one year of age were less likely to become infected than children aged 2 to 5 years. The resistance of infants to malaria infection is due to a combination of the protective effects of maternal immunoglobulin and fetal hemoglobin and lower exposure to infective mosquitoes. However, infants who did become infected were more likely to be symptomatic than were older children (79% of infected 0-1 year olds were symptomatic compared with 61% of 2-3 year olds and 34% of 4-5 year olds).

The geometric mean level of parasitemia was significantly lower in children with asymptomatic infections than in children with clinical infections (Table 2; for The Gambia, $t = 12.99$, $df = 198$, $P < .001$; for Sierra Leone, $t = 24.2$, $df = 506$, $P < .001$), confirming that children with asymptomatic infections were able to control parasite growth.

Both the prevalence (Table 1) and mean concentration (data not shown) of IgG antibodies to PfMSP-1_{19} increased with age. The prevalence of antibody responses to some of the PfMSP-1_{19} constructs was higher in Sierra Leonean children than in Gambian

children; this may reflect the less seasonal pattern of malaria transmission in Sierra Leone.

2. Relationship between anti-PfMSP1$_{19}$ antibodies and malaria morbidity.

Antibodies measured in the children's sera at the beginning of the malaria transmission season were compared with their subsequent malaria morbidity. Neither the sex of the child nor carriage of the sickle cell gene (data not shown) had any confounding effect on PfMSP-1$_{19}$ antibody prevalence or OD value. However, age appeared to have a major confounding effect on the association between malaria morbidity and antibody responses, and was allowed for using multiple logistic regression analysis. For the Gambian children, ethnic group was also allowed for since there were differences in antibody prevalence between ethnic groups (data not shown).

The prevalence of antibodies to PfMSP-1$_{19}$ tended to be higher in children who experienced only asymptomatic infections (and who are thus deemed to have acquired a significant degree of immunity to malaria) than in children who experienced clinical infections (Table 2). In Gambian children the prevalence of antibodies to the second EGF-like domain was significantly higher in asymptomatic children than in those experiencing clinical malaria. This association remained highly significant ($P < .001$) after allowing for age and ethnic group. For the Sierra Leonean children, response to the MAD/Well$_{19}$ antigen offered protection against clinical malaria, reducing the risk of clinical malaria by an estimated 40% and there was a suggestion of a reduced risk

of clinical malaria associated with positive responses to the $MAD20_{19}$ and MAD20-second EGF motif, although these were not statistically significant.

The subclass specificity of the $PfMSP-1_{19}$ antibodies was determined by a subclass specific ELISA [12]. There was no detectable association between IgG subclass and age or morbidity (data not shown). The vast majority of positive sera (> 95%) contained IgG1 (and only IgG1) antibodies for $PfMSP-1_{19}$.

Discussion.

This study establishes that there is a significant association between antibody responses to epitopes within the EGF motifs of the 19kDa, membrane-bound fragment of PfMSP-1 and resistance to clinical malaria infection and provides evidence that a vaccine which induces an appropriate antibody response to PfMSP-1_{19} might protect children from high levels of asexual stage parasitaemia and clinical malaria. The protective effect of antibodies to PfMSP-1_{19} is in marked contrast with the lack of such an effect for antibodies to other malaria antigens tested in similar studies [13,14]. Clearly, however, possession of PfMSP-1_{19}-specific antibodies is not the only means of protection as many antibody-negative children appeared to be protected. These children presumably possess antibodies, or specific cellular responses, to other erythrocytic stage antigens.

The prevalence and mean OD of antibody responses were higher to constructs representing the entire PfMSP-1_{19} protein (i.e.both EGF motifs) than to constructs representing either of the single EGF motifs; this is consistent with our previous studies with these constructs [12] and indicates that immunodominant B cell epitopes are dependent upon the presence of sequences from both EGF motifs. However, the highest degree of protection was associated with antibodies to the second EGF-like domain: all ten Gambian children who were seropositive to this antigen experienced only asymptomatic malaria infections indicating that they were able to control parasite growth to below levels at which clinical symptoms become manifest.

A similar trend was seen for the Sierra Leonean children, but 4

children who were seropositive to the second EGF-like domain did experience clinical infections. It is possible that the non-protected Sierra Leonean children possessed antibodies which recognise the MAD20 sequence of the second EGF-like motif but were infected with parasites expressing the alternate (Wellcome) sequence. (The prevalence of parasites carrying the Wellcome allele of PfMSP-1 is less than 5% in The Gambia, but may be higher in Sierra Leone).

The identification here of a short and relatively conserved antigen which is the target of protective anti-malarial immune responses marks a significant step forward in the search for a subunit malaria vaccine which could be manufactured using recombinant DNA technology. One potential problem is the apparently poor immunogenicity of PfMSP-1_{19} and particularly of the second EGF-like motif. Despite multiple infections, no more than 20% of Sierra Leonean children possessed antibodies to PfMSP-1_{19} and only 3-4% possessed antibodies to the second EGF-like motif. Only 60% and 14% respectively of adult Gambians are seropositive to PfMSP-1_{19} and the second EGF-like motif [12], indicating that even lifelong exposure to this essentially conserved protein may be insufficient to induce an antibody response.

Poor natural immunogenicity may be a consequence of the way in which the protein is presented to the immune system during malaria infection. For example, secondary processing of PfMSP-1_{42} is a pre-requisite for erythrocyte invasion [3,15] but may substantially affect the antigenic integrity of the resulting PfMSP1_{19} polypeptide. In addition, recent data from our

laboratory indicate that few malaria-immune donors show *in vitro* T cell proliferative or cytokine responses to PfMSP-1_{19}, suggesting that lack of T cell help for antibody production may be a problem (A. Egan and E. Riley, unpublished data). If so, immunization with a polypeptide incorporating additional T cell epitopes, from other regions of PfMSP-1 or from heterologous proteins, may induce a significantly better antibody response than that induced by the native protein during natural infection.

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Table 1: The relationship between age and malaria morbidity, and between age and anti-PfMSP-1_{19} antibody reactivity. The number (percentage) of positive children in each age group is shown.

	The Gambia					Sierra Leone					
	Age (years)			χ^2 trend	P	Age (years)				χ^2 trend	P
	3-4	5-6	7-8			0-1	2-3	4-5	6-8		
n	120	110	97			167	162	157	159		
Morbidity:											
Clinical	42 (35)	42 (38)	25 (26)	1.8	.18	103 (62)	90 (56)	51 (33)	25 (16)	86.0	< .001
Asymptomatic	29 (24)	27 (25)	35 (36)	3.5	.06	19 (11)	51 (32)	83 (54)	86 (54)	78.2	< .001
Indeterminate	8 (7)	13 (12)	15 (16)	4.3	.04	9 (5)	6 (4)	15 (10)	12 (8)	2.0	.16
No Infection	41 (34)	28 (26)	22 (23)	3.7	.06	36 (22)	15 (9)	8 (5)	36 (23)	0.02	.89
Antibody:											
MAD20_{19}	6 (5)	8 (7)	14 (15)	5.9	.01	11 (7)	31 (19)	25 (16)	43 (27)	19.6	< .001
MAD/$Well_{19}$	9 (8)	15 (14)	19 (20)	6.9	.01	17 (10)	36 (22)	30 (19)	49 (31)	17.4	< .001
EGF-1/Well	5 (4)	6 (5)	7 (7)	0.9	.33	15 (9)	20 (12)	10 (7)	21 (13)	0.5	.49
EGF-1/MAD20	6 (5)	7 (6)	7 (7)	0.5	.49	15 (9)	28 (17)	15 (10)	25 (16)	1.2	.28
EGF-2/MAD20	1 (1)	3 (3)	7 (7)	6.5	.01	1 (1)	3 (2)	7 (5)	11 (7)	11.4	< .001

Table 2: The relationship between anti-PfMSP-1_{19} antibodies and malaria morbidity. The number (percentage) of responders to each antigen, in each morbidity group, is shown.

* Odds Ratio for clinical malaria adjusted for age and ethnic group
** Odds Ratio for clinical malaria adjusted for age + Confidence Interval
P value represents significance of likelihood ratio test, obtained using logistic regression.

	The Gambia				Sierra Leone			
	Malaria morbidity		OR* 95%CI+	P	Malaria morbidity		OR** 95%CI+	P
	Asymptomatic	Clinical			Asymptomatic	Clinical		
n	91	109			239	269		
MAD20$_{19}$	11 (12)	8 (7)	0.7 0.3-2.0	.52	53 (22)	34 (13)	0.7 0.4-1.2	.16
MAD20/Well$_{19}$	16 (18)	12 (11)	0.7 0.3-1.6	.35	65 (27)	40 (15)	0.6 0.4-1.0	.06
EGF-1/Well	5 (6)	8 (7)	1.7 0.5-5.7	.38	26 (11)	24 (9)	0.9 0.5-1.8	.79
EGF-1/MAD20	5 (6)	9 (8)	1.8 0.6-5.9	.31	32 (13)	32 (12)	1.0 0.5-1.7	.90
EGF-2/MAD20	10 (11)	0 (0)	0.0 0.0-0.5	<.001	12 (5)	4 (1.5)	0.4 0.1-1.4	.14
Parasitemia: geometric mean parasites/μL (range)	412 (206-821)	32,112 (26,871-38,376)			831 (667-1043)	40,644 (37,008 - 44,606)		