IMMUNE RESPONSES TO THE *PLASMODIUM FALCIPARUM* ANTIGEN MSP2

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Contents

Declaration

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

Ackowlegements

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List of abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AMA-1	apical membrane antigen
APS	ammonium persulphate
BSA	bovine serum albumin
BTS	Scottish National Blood Transfusion Service
CS	circumsporozoite protein
CTL	cytotoxic T lymphocyte
DAPI	4'-6-diamino-2-phenylindole
dH_2O	distilled water
DNA	deoxyribonucleic acid
EBA-175	erythrocyte binding antigen
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMIF	erythrocyte membrane immunofluorescence
Exp-1	exported malaria protein
FITC	fluorescine isothiocyanate
GPI	glycophosphatidylinositol
GST	glutathione S-transferase
HLA	human leukocyte antigen
HEPES	N-2-hydroxyethypiperazine-N'-2-ethane sulphonic acid
HRP	horseradish peroxidase
IFA	immunofluorescence assay
IFN	interferon gamma
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-ß-D-thiogalactopyranoside
kDa	kilodalton
LSA-2	liver stage antigen - 2
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MRC	Medical Research Council
MSP1	major merozoite surface protein (PfMSP1)
MSP2	merozoite surface protein 2
OD	optical density

OPD	o-phenylenediamine
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDN	parasite-dependent red blood cell neoantigen
PfEMP-1	*Plasmodium falciparum*-infected erythrocyte membrane protein
PNG	Papua New Guinea
prbc	parasitised red blood cell
rAg	recombinant antigen
RAP-1	rhoptry associated protein
rbc	red blood cell
RESA	ring-infected erythrocyte surface antigen (Pf155/RESA)
RFLP	restriction fragment length polymorphism
RITC	rhodamine isothiocyanate
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSP-2	sporozoite surface protein 2
TEMED	N,N,N´,N´-tetramethylethylenediamine
TGF-ß	transforming growth factor ß
TNF	tumour necrosis factor
Tris	tris (hydroxymethyl) aminomethane
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride
WHO	World Health Organisation

Single letter amino acid codes

A	alanine	M	methionine
C	cysteine	N	asparagine
D	aspartic acid	P	proline
E	glutamic acid	Q	glutamine
F	phenylalanine	R	arginine
G	glycine	S	serine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

Abstract

The development of a vaccine to control malaria infections in humans requires the identification of antigens which are targets of protective immune responses. One potential problem for vaccine development is antigenic polymorphism of malaria parasites. Allelic polymorphisms have been demonstrated for a number of *Plasmodium falciparum* antigens, including the merozoite surface protein MSP2.

MSP2 has been classified into two allelic families (serogroups A and B), although considerable heterogeneity is known to occur within each serogroup. The protein has highly conserved N- and C- terminal sequences. There are two central regions of tandemly arranged repeats which are flanked by group-specific, dimorphic sequences. Although MSP2 is considered a candidate for inclusion in a malaria vaccine, this is based on rather limited evidence. The aim of this thesis is to examine quantitative and qualitative aspects of the serological immune response to MSP2 in a population of malaria-exposed individuals from West Africa.

Recombinant MSP2 antigens, produced using pGEX plasmid expression vectors to direct the synthesis of MSP2 in *Escherichia coli* as fusions with glutathione S-transferase, were used to determine the presence of serum antibodies by enzyme-linked immunosorbent assay. These recombinant antigens include full length proteins of serogroup A and B, and fragments representing the conserved, group-specific or repeat regions of the molecule. The antigenic integrity of the recombinant proteins was checked by mouse immunisations and testing of immune mouse serum for recognition of native MSP2 by immunofluorescence and immunoblot.

The questions addressed in this thesis are whether MSP2 is immunogenic during natural infection with *P.falciparum*, whether amino acid polymorphism of MSP2 gives rise to antigenic polymorphism, and whether the immune recognition of MSP2 is variant-specific. In addition, the dynamics of acquisition of antibody to MSP2 with age, the IgG subclass specificity of anti-MSP2 antibodies and the association of MSP2-specific antibodies with protection against malaria infection have been investigated.

MSP2 was found to be highly immunogenic, with high titre antibody present even in individuals known to have experienced only one episode of malaria. The antibody response is directed almost exclusively towards the dimorphic and polymorphic regions of MSP2 and antibodies to serogroups A and B do not cross-react. The acquisition of immunity to MSP2 is age-

dependent, with the prevalence of anti-MSP2 antibodies and recognition of MSP2 variants increasing with age. Non-responsiveness does not appear to be genetically determined. Prospective longitudinal studies indicate that antibodies to MSP2 are associated with a reduced risk of clinical malaria. Qualitative differences in the antibody response were observed between children and adults; anti-MSP2 antibodies in adults are predominantly IgG3 whilst children also possess IgG1 antibodies. The expression of the IgG3 subclass increases with age among MSP2 seropositive individuals.

1. INTRODUCTION

1.1 Malaria

Human malaria is caused by one of four species of *Plasmodium*; *P.falciparum*, *P.vivax*, *P.malariae* and *P.ovale*. *P.falciparum* and *P.vivax* are the most widely distributed and both cause a vast amount of human suffering. *P.falciparum* is the most virulent and responsible for more than 95% of malaria deaths worldwide.

Accurate information on the global incidence of malaria is difficult to obtain. However it is estimated that worldwide the disease threatens 2200 million people, approximately 40% of the world's population [355], with 300-500 million clinical cases each year [356]. Thus, malaria constitutes a major threat to health and blocks the path to economic development for individuals, communities and nations. Countries in tropical Africa are estimated to account for greater than 80% of all clinical cases and more than 90% of all parasite carriers [355]. In Africa south of the Sahara, it has been estimated that 270-480 million clinical cases may occur each year [356].

Children are particularly at risk; malaria being one of the major killers of children in tropical Africa, taking the life of one out of 20 children before the age of 5 years [355]. It afflicts the poor and underprivileged most severely, sapping productivity and causing chronic illhealth.

Social, political and economic changes all contribute to the worsening malaria problem, particularly through population movements and ecological disturbances. Construction and environmental change brought about by "development" often creates environments favourable for malaria transmission, exacerbating the existing problem and opening the way for devastating epidemics in areas which were previously malaria-free.

Yet malaria should not be considered an inevitable burden. A vast amount of knowledge about the parasite and its control has been acquired over the years. In most endemic countries, the goal of malaria control is to prevent mortality and reduce morbidity and the socio-economic losses provoked by the disease. The goal in malaria-free areas is to maintain that freedom [355].

The parasite has a complex life cycle (figure 1.1), alternating between vertebrate and arthropod hosts. Infection in man is initiated by an infected

Anopheles mosquito injecting sporozoites into the bloodstream whilst feeding. The sporozoites remain extracellular for less than 30 minutes during migration to the liver, where they invade hepatocytes. Inside the hepatocytes the parasite undergoes a phase of maturation and asexual reproduction (schizogony) to produce pre-erythrocytic schizonts. After a number of days (depending on the species), up to 30,000 merozoites are released into the blood. The merozoites rapidly enter erythrocytes and begin the erythrocytic schizogony during which the merozoite develops from the ring stage through the trophozoite to the mature schizont containing 8-24 daughter merozoites which, after the red cell ruptures will initiate a subsequent intraerythrocytic cycle. The erythrocytic cycle of schizogony is repeated, leading to a progressive parasitaemia.

The release of merozoites into the blood stream causes many of the symptoms of malaria. In the early stages of infection, fevers show no characteristic pattern. However, as the development of the parasites in erythrocytes becomes synchronous, typical febrile periodicity occurs [320].

After several generations of merozoites have been produced, a small proportion give rise to sexually differentiated forms - gametocytes. These stages are infective for the mosquito and when ingested during feeding emerge from the erythrocyte to form gametes. After fertilisation, the zygote develops into an ookinete that invades the midgut wall, maturing into an oocyst. The parasite then undergoes a process known as sporogony that produces thousands of sporozoites which, when released into the insect's haemocoel, invade the salivary glands ready for inoculation into the next host.

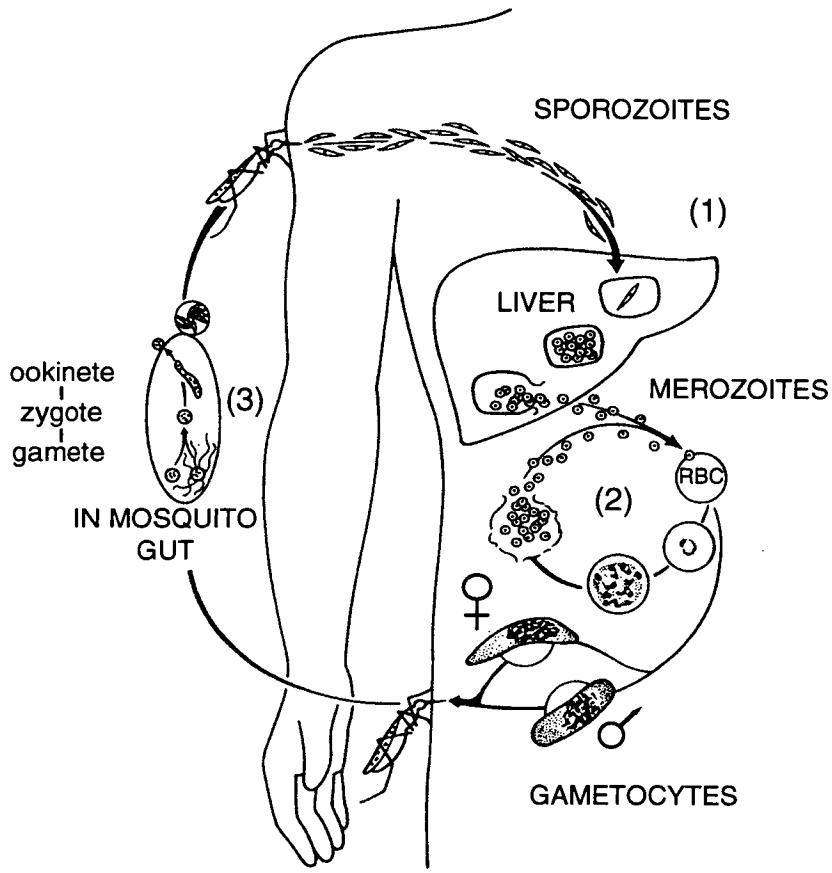


Figure 1.1: Life cycle of the malaria parasite in man. The parasite is haploid throughout the human cycle: sexual mating occurs in the mosquito. All clinical symptoms are associated with the erythrocytic stage, and fever occurs when large numbers of schizonts rupture. The duration of the erythrocytic cycle is 48 hours for all the human malaria parasites, except for *P.malariae*, which has a 72 hour cycle.
(1) - pre-erythrocytic stages; (2) - erythrocytic stages; (3) - sexual stages.

In non-immune individuals parasitaemia is accompanied by illness. Most patients exhibit some combination of fever, chills, myalgia, headache, nausea, vomiting and diarrhoea. The symptoms of falciparum malaria tend to be more severe than those caused by other *Plasmodium* species, and if left untreated, are frequently fatal. In tropical Africa, cerebral malaria and severe anaemia are responsible for most deaths from malaria [130]. Cerebral malaria may begin slowly or suddenly after the initial onset of symptoms. Headache and drowsiness are succeeded by a comatose state, due, in part, to the sequestration of parasitised erythrocytes in the capillaries of the brain. Long term residents of malaria endemic areas gradually acquire resistance to clinical symptoms, and infections in adults are usually asymptomatic or accompanied by mild symptoms of headache and malaise [268]. However, during pregnancy women become susceptible to malaria, with increased frequency and severity of infection [216].

The clinical-pathological picture of malaria infection is also complex. While the malaria parasite initiates the disease process, subsequent events depend, to some extent, on many internal and external factors, including functional cellular and humoral immune responses as well as the nutritional state of the host [37, 130].

1.2 Naturally acquired immunity to malaria

1.2.1 Epidemiological evidence for the acquisition of immunity to malaria:

Evidence for man's capacity to acquire immunity towards malaria comes from epidemiological studies which demonstrated that the clinical and parasitological manifestations of the disease decline as age advances in residents of malaria endemic areas [91, 210, 213].

Peri-natal malaria is a rare event. The persistence of erythrocytic foetal haemoglobin and deficiency of *p*-aminobenzoic acid as a consequence of breast feeding contribute, at least in part, to the resistance operating in neonates and infants [62, 213]. In studies in The Gambia, the clinical impact of malaria then increased as age progressed [128, 210]. Episodes of dense parasitaemia accompanied by severe clinical illness developed and the disease appeared to reach peak prevalence in the second year of life.

Children gradually show clinical improvement, despite the persistence of parasitaemia. Parasite density and prevalence steadily decrease with age. Complete sterile immunity is uncommon; adults show transient low levels of parasitaemia in the absence of clinical symptoms [210].
The precise timing of these events depends on local patterns of malaria transmission and endemicity [268]. Nonetheless, in highly endemic areas, it is the young child who is principally affected by malaria and in the older child an acquired immunity affords effective protection against the disease [210, 211].

During pregnancy, susceptibility to infection and the severity of the clinical manifestations is determined by the level of pre-pregnancy immunity and parity [216]. In areas of high endemicity, women acquire a significant protective immunity, therefore the effects of malaria on the mother and her foetus are, in general, less severe than in areas of low malaria transmission. Regardless of endemicity, maternal malaria causes severe anaemia, significantly contributing to maternal morbidity.

1.2.2 Specific immune responses to malaria infection:
Immunity to malaria operates at different levels - stage-specific antiparasite immunity, antitoxic immunity and transmission blocking immunity - involving both humoral and cellular mechanisms [268].

The role of antibodies in resistance to malaria is well established. The ability of antibodies to confer protection against *P.falciparum* is apparent from the protection afforded to neonates and infants by maternally derived antibodies [92]. Field studies have shown that antibodies to asexual erythrocytic parasites are high in the blood of infants born to immune mothers and that these levels fall in the weeks following birth [209, 212, 213]. Levels remain low for some time but then gradually rise throughout childhood and into adolescence. This pattern parallels clinical markers (such as the decrease in spleen and liver size, decrease in severity and frequency of clinical attacks, and diminished parasite densities) of gradually increasing resistance [210]. Furthermore, classical passive transfer experiments, in which immune serum or purified Ig reduced parasitaemias in children with acute disease [62, 63, 92, 280], lend support to the role of antibodies in protection against malaria. However, the

correlation between total anti-malarial antibody and protective immunity is poor, indicating that many antibodies may have no protective effect [200, 210, 328].

There are several mechanisms by which antibodies could exert a protective effect. The briefly extracellular forms, sporozoites, merozoites and gametes, each present an array of stage-specific surface antigens and ligands/receptors which play a crucial role in the transfer of *Plasmodium* from one intracellular environment to another. Antibody could bind to the surface of these extracellular stages blocking the receptors which function in invasion or causing the agglutination of free parasites [59-61, 223]. Cytophilic antibodies can opsonise parasites for phagocytosis [88, 195]. Antibodies against the surface of the infected cells might inhibit parasite development inside the cell or inhibit sequestration of the erythrocyte in the deep vasculature of the host, thus forcing the parasite to traverse the spleen, leading to the clearance of infected erythrocytes from the circulation.

Antibody may mediate parasite destruction through the activation of complement leading to lysis of the parasite or by the activation of NK cells or macrophages inducing the release of superoxide radicals and nitric oxide which facilitate parasite destruction. Finally, antibodies specific for the antigens that provoke the production of IL-1 and TNF could reduce the toxic effects of infection [189, 317].

A common feature of many malaria antigens is regions of repetitive sequences. It has been proposed that these may stimulate T-cell-independent immune responses through the activation of B cells by cross-linking of surface immunoglobulin [285]. However, such T-independent responses are likely to be less efficient than the response induced to antigen presented in the context of MHC.

Naturally acquired immunity to malaria is a complex interaction between the humoral and cellular arms of the immune system. Many of the antibody-dependent mechanisms require cooperation with effector cells, therefore an important aspect to consider is the isotype and subclass of the antibodies involved [34, 131]. The regulation of which depends on T cells and their cytokines to trigger the appropriate Ig class switching mechanisms [103, 301].

There is ample evidence from animal models for the role of T cells in immunity to malaria [186, 314, 328, 350]; although studies on experimental models and *in vitro* studies at the T cell clone level do support the hypothesis of a role for T cells in protection from malaria, they cannot give much idea of the real effector mechanisms acting *in vivo* during infection of humans. Murine studies have indicated that T cell-dependent immune mechanisms are crucial to the development of effective anti-malarial immunity [268, 320]. For example, thymectomised animals fail to clear parasites or become immune to reinfection whereas intact animals develop a solid, long lasting immunity [320]. Also, protection against malaria can be induced in mice following adoptive transfer of immune T cells and antigen-reactive T cell lines or clones [186, 274, 350].

T cells could act through specific or non-specific mechanisms [21, 320]: as helper cells for the activation of B cells and secretion of antibody; as cytotoxic cells having a direct effect on parasitised cells; in the activation of non-specific effector cells and in the production of cytokines that can have a direct inhibitory effect on parasite growth [21, 189, 190]. The antigen-specific effects of T lymphocytes would be predominantly directed at hepatic stages since this type of immune attack is dependent on the expression of parasite antigen on the cell surface in conjunction with class 1 MHC proteins (which are not expressed on the surface of human erythrocytes) [169].
In addition, the spleen plays a crucial role in immunity to malaria [190]. Splenectomy in humans can lead to recrudescence of a previously subpatent parasitaemia [122]. Controlled studies have shown that splenic function significantly alters with malaria infection of humans, such that in a malaria-modified spleen the clearance of red cells is enhanced [122]. In the enlarged spleen the circulation of blood through the pulp cords is increased as is the transit time of red cells [213]. Therefore, the spleen not only acts as a tissue involved in the removal of rbc containing inclusions, but also provides an intimate environment for the interaction of local immune effector cells and parasitised erythrocytes [123].

Clinical immunity against severe malaria attacks seems to depend on a complex regulation of the immune response than merely on specific

effector mechanisms, since antiparasitic factors such as TNF, IL-1, IL-6 and nitric oxide are also involved in the pathogenesis of malaria and cerebral malaria [19, 126, 189, 190, 214, 243, 245].

In conclusion, it is true to say that each transitional stage of the parasites' development in the vertebrate host presents a potential target for a multifactorial immune response (table 1.1).

Pre-erythrocytic stages:

1. Antibodies blocking hepatocyte invasion by sporozoites [169, 218, 320]
2. Direct killing of infected hepatocytes by cytotoxic T lymphocytes (CTL) [169, 218, 274, 314]
3. Killing via antibody-dependent cellular cytotoxicity (ADCC) [169, 218, 320]
4. Killing through the release of cytokines such as g IFN that induce the hepatocyte to produce nitric oxide [169, 218, 314, 320]

Erythrocytic stages:

1. Blocking of merozoite invasion by antibody [42, 60, 114, 224, 238, 320, 344]
2. Agglutination of free merozoites by antibody[59-61, 223, 224, 320]
3. Recognition of infected red blood cells (irbc) by antibody, resulting in complement-mediated lysis, or inhibition of sequestration [218, 320]
4. Opsonisation of free merozoites or irbc by cytophilic antibodies, leading to phagocytosis [88, 183, 195]
5. ADCC [4, 105]
6. Cellular cytotoxicity due to the production of toxic substances such as cytokines and reactive oxygen and nitrogen radicals [190, 218]
7. Reduction of pathology by antibodies neutralising cytokine inducers [19, 246, 317]

Sexual stages:

1. Antibodies blocking fertilisation [133, 169, 261]
2. Antibodies involved in complement-fixing reactions which destroy gametes by lysis [46, 47]
3. Gamete-specific T cells can block the transmission to the mosquito due to the toxic effects of cytokines [218]

Table 1.1: Possible immune mechanisms against different stages of the malaria life cycle

1.2.3 Immunosuppressive effects of acute malaria infection:

Acute malaria infection is thought to reduce the effectiveness of the host's immune system. This phenomenon is implied by 1) the increased susceptibility to concurrent bacterial and viral infections and 2) infected individuals are less likely to respond optimally to vaccination procedures [349]. These findings are supported by the observations that children receiving malaria prophylaxis are less susceptible to other infectious diseases than their unprotected counterparts, and also had better cellular responses to malaria antigens [268]. Moreover, the reduction in the overall childhood mortality in children receiving the prophylaxis was greater than would have been expected simply from prevention of deaths due to malaria [268]. However, a paradox exists; from a clinical perspective malaria patients appear to be immunocompromised, yet *in vitro* immunological studies show that individuals have malaria-specific cellular and serological responses. Although, it is clear that the immune response to malaria is only partially effective in controlling reinfection, many of the clinical symptoms of malaria infection are associated with the immune response to infection.

Immunologically, acute infection results in a transient decrease in the numbers of circulating T cells and decreased lymphoproliferative and cytokine responses of peripheral blood mononuclear cells (PBMC) to malaria antigens *in vitro* [268]. However, rather than this being a direct immunosuppressive effect, it has been proposed that cells recognising the malaria antigens are absent from the peripheral circulation having homed to the spleen, lymph nodes or liver [123, 125, 268].

Although acute malaria infection obviously has a profound effect on the efficiency of the immune system, the specific immunosuppressive mechanisms have not been fully elucidated. Possibilities include the suppression of T cells by activated monocytes or macrophages secreting prostaglandins or immune inhibiting cytokines like TGF-ß (transforming growth factor), or defects in early events of T-cell activation and inhibition of IL-2 function [320]. Of course, it may be necessary for the host to regulate the immune reactions which lead to harmful immunopathological effects.

1.3 Development of anti-malaria vaccines

1.3.1 The need for vaccination:

An effective vaccine against falciparum malaria would be an important step towards improved health in the tropical world, particularly in children in Africa [222]. There has been a recent resurgence in the disease due to increasing resistance to insecticides, environmental alterations that promote transmission (irrigation and timber clearing), movements of people into areas of high transmission to find work or avoid war, and the increasing inability of developing countries to afford the basic personnel and equipment required for malaria control and treatment. Drugs, for prophylaxis and treatment of malaria, are also becoming increasingly less effective [73]. Resistance to chloroquine is common in nearly all areas where *P.falciparum* is transmitted, and resistance to mefloquine, a more recently developed and very promising drug, has already been reported in many parts of the world [352, 356]. For these reasons, attention has been focused on additional means of control, particularly vaccines.

There is every reason to believe that vaccination against malaria is biologically possible, although there is debate about whether it is always desirable and the long term consequences maybe difficult to predict. The disease is most severe in young children, pregnant women and non-immune adults (such as immigrants from non-endemic areas), but with time and repeated exposure to the parasite, adults are able develop immunity to malaria which confers some degree of protection [210]. Immune individuals are generally have fewer and less dense parasitaemias and are less likely to suffer illness when parasitaemic and are significantly protected from death [210].

1.3.2 Strategies for identification of target antigens:

Immunisation experiments in animals and humans with attenuated parasites or crude antigenic preparations have demonstrated that it is possible to experimentally induce protective immunity to malaria [56, 57, 80, 133, 134, 148, 150, 151, 227, 237, 259, 260, 289-291, 351]. However such a vaccine strategy would be impractical due to the large amounts of parasite material required, the possibility of autoimmune reactions to red blood cell components, and the possibility that crude antigenic preparations may also contain immunosuppressive factors [236]. For the

further development of a vaccine against falciparum malaria it was necessary to identify those antigenic components of the parasite which induce protective immune responses in the host so that these components could be produced synthetically. The development of *in vitro* parasite culture, hybridoma technology and molecular biology has led to the identification and characterisation of many *P.falciparum* proteins. Furthermore, the advances made in understanding the mechanisms underlying naturally acquired immunity in humans and in experimental models have been, and still are, fundamental to malaria vaccine research.

Potential vaccine antigens have been identified using several strategies:
1. proteins which are recognised by malaria immune sera [33, 344];
2. proteins recognised by mAbs which inhibit parasite growth *in vitro* [102, 114, 238];
3. surface molecules exposed to the immune system;
4. proteins which are crucial for parasite function;
5. screening of parasite cDNA or genomic expression libraries with immune sera. (Anti-sera raised by immunising mice and rabbits with cloned antigens were subsequently used to characterise the *P.falciparum* proteins corresponding to the antigen-positive clone [32, 178, 208]);
6. systematic immunisation of monkeys with *P.falciparum* components eluted from SDS gel and characterisation of the molecules associated with protection [234].

1.3.3. Expression of synthetic antigens:

An important step in designing a malaria vaccine is to demonstrate that a synthetic form of the antigen (synthetic peptide or recombinant polypeptide) confers a degree of immunity similar to that obtained with native antigen. Thus it has become critical to identify appropriate methods to produce synthetic malaria antigens which are antigenically equivalent to their native counterparts. A number of options exist and have been explored for one or more malaria antigens. Synthetic oligo-peptides have been evaluated as conjugates with immunogenic carrier molecules, as components of novel, branched peptide polymers - multiple antigen peptides (MAPs) [135, 229] and as polymeric synthetic hybrid proteins [234]. However, synthetic peptides generally correspond to sequential (linear) determinants rather than conformational determinants which are characteristic of native

proteins, and a higher level of protection has been reported for native, conformational determinants [49].

A large number of malarial antigens, or antigen fragments, have been expressed as recombinant polypeptides in *E.coli*. The antigenic integrity of these recombinant polypeptides must be assessed before they are used as immunogens. The importance of conformation to protein structure is greatest for proteins in which secondary structure is stabilised by multiple intrachain disulphide bonds. Eukaryotic expression systems have theoretical advantages over ones using *E.coli*. These are the synthesis of recombinant polypeptide via the secretory pathway in a microenvironment that promotes appropriate folding and the potential for post-translational modifications including glycosylation and processing of foreign eukaryotic proteins [49]. A review of the structural features of the antigen can provide some insight into which expression systems should be tried first.

1.3.4 Assessment of potential vaccine antigens:

Having identified potential candidate antigens and expressed these as recombinant polypeptides or synthetic peptides, functional assays are required to assess the protective capacity of these molecules. Candidate proteins can be evaluated *in vitro* by the effect of antibody to these proteins on cell invasion and parasite growth and assays for cell stimulation by proliferation and cytokine production. However, it is apparent that *in vitro* results do not always correlate with *in vivo* protection [54, 105, 226]. Molecules can be assessed *in vivo* by direct immunisation of rodents and monkeys and subsequent challenge or by passive transfer of antigen-specific antibodies. In addition the correlation of development of immune responses to the antigen with the clinical status of an individual after natural exposure to malaria should be evaluated.

However, the correlation between protective immunity in experimental hosts and man has yet to be established [34]. Many of these models represent host-parasite relationships which could not be sustained under conditions of natural transmission. Furthermore, results in one host species cannot always be repeated in another. Conclusions drawn from immunological experiments with rodent malarias in rats or mice may not always be applicable to the human disease because of innate differences in host immune response. In many rodent models, unnatural host/parasite combinations are used, and depending on this combination infections

range from lethal with little or no immunity apparent, to mild, non-lethal infections where a solid sterilizing immunity is produced. Neither of these two situations is compatible with malaria under conditions of natural transmission. Nonetheless, the vast literature on the mouse immune system and the many commercially available reagents to study the cells and antibodies involved make it an attractive model. Evidence from experiments in *Aotus* monkeys suggests there may be substantial differences between *Aotus* and man since prior splenectomy is required for successful infection [34]. Furthermore, the biological properties of infection are different, for example drug cure can yield the monkey resistant to reinfection.

1.3.5 Delivery systems:

Any vaccine requires an effective delivery system to ensure optimal interaction among vaccine epitopes, antigen presenting cells and effector cells [169]. A common component of a delivery system is an adjuvant that is a non-specific immunopotentiator. This adjuvant must be safe for use in humans; at present the choices are aluminium hydroxide (alum) or liposomes, lipid spheres that self-assemble in aqueous solution [152, 169]. Another approach to the induction of immune responses is to use attenuated live vectors, such as *Salmonella* or *Mycobacterium* BCG, and these may be particularly useful for inducing CTL responses [6, 152, 169].
One of the most exciting new developments in vaccinology is the advent of nucleic acid vaccines which are attractive because of their relative simplicity [345, 346]. A gene encoding a protective antigen is cloned into a plasmid construct containing a eukaryotic promoter. This construct is usually delivered by injection into the muscle and the gene product expressed on the cell surface. The nucleic acid is taken up into the host cells, leading to the subsequent specific induction of both humoral and cell-mediated arms of the immune system. A promising DNA vaccine against murine malaria based on the circumsporozoite (CS) protein has been developed. High levels of protection were obtained in challenge experiments in mice and the vaccine was demonstrated to generate both specific antibody and CTL responses [345].

1.3.6 Vaccination strategies:

There are several vaccine strategies being developed, targeting different stages of the life cycle with the aim of either preventing blood stage infections or reducing the pathological consequences of infection (reviewed by [22, 41, 147, 152, 169, 224, 245, 274]).

Pre-erythrocytic stage vaccines

The first opportunity to stop infection is during the parasite's brief migration through the circulation to the liver. A vaccine against sporozoites would, if effective, prime the human immune system to kill sporozoites injected by the mosquito and thus prevent the development of the subsequent stages responsible for the disease and transmission of the infection to others. Such a vaccine, if successful, would be truly prophylactic [232]. However, partial immunity to sporozoites would probably have little effect on the course of infection; any sporozoites that survive may cause a fully virulent infection [224].

Sporozoite proteins -

The main antigens involved in protection against extracellular sporozoites are the CS proteins, part of a family of proteins covering the whole surface membrane of the parasite [77, 232], and the sporozoite surface-associated protein (SSP2). Passive transfer of monoclonal antibody, but not polyclonal antibody, specific for the *P.yoelii* CS protein protects mice from challenge [52]. The first synthetic malaria vaccine to be given to humans was derived from the immunodominant repeat sequence of *P.falciparum* CS protein [143], however, it was poorly immunogenic with only one out of 17 volunteers being completely protected from sporozoite challenge [143]. A second CS protein vaccine, R32tet32, was also ineffective [348]; anti-R32tet32 antibodies were not protective against naturally occurring malaria in Thailand [348].

Liver stage proteins -

Protective immunity has been achieved in hosts ranging from birds to man by repeated inoculation with irradiated sporozoites [56, 57, 134, 232, 259, 260] and this immunity probably operates mainly at the liver [314]. Induction of sporozoite-induced CTL responses may prove to be an effective strategy [274]. After immunisation with SSP2, protection from subsequent challenge was $CD8^+$ T-cell dependent in mice [169]. Immunisation with a synthetic peptide epitope containing a sequence from a *P.berghei* liver-stage

protein, LSA2, protects mice against sporozoite challenge by eliciting CTL capable of killing cultured exoerythrocytic stages [314]. Liver stages share a number of antigens with both the sporozoite and blood stages, as well as synthesising stage-specific antigens. Many more targets of protective immunity are likely to emerge and it is hoped that some of these will not suffer from the problems of variation and non-responsiveness associated with the CS protein.

It was recently discovered that naturally exposed West Africans produced HLA-restricted cytotoxic T cells that recognised a nine amino acid peptide from the liver stage-specific antigen LSA1 [132] in association with HLA Bw53 [145]. Studies in The Gambia suggest a link between the MHC class 1 molecule HLA Bw53 and protection from severe malaria [144].

If a vaccine is designed that copies natural immunity it may not be necessary to focus on lymphocyte stimulation because malaria-specific lymphocytes are present in non-exposed people at high frequency [121]; it is speculated that these may have arisen from cross-reacting microbial stimuli [121, 125], but that they may not be appropriate protective immunogens as naive people are not protected against malaria.

Erythrocytic stage vaccines

Proliferation of the asexual blood stages of the parasite life cycle is responsible for morbidity and mortality, particularly in children, and therefore is an obvious target for vaccine intervention. The aim would be to immunise the unprotected child, and, thereby reduce the pathological effects although not necessarily preventing infection. Hence, a vaccine targeting the asexual blood stages may have relatively little effect on the development of the sexual stages or transmission. Although, in evolutionary terms, there may be pressure to increase gametocyte production in vaccinees to ensure successful transmission to the mosquito.

Protection from malaria can be artificially induced in animals by immunisation with asexual blood stage parasite preparations [80, 148, 150, 151, 227, 237, 260, 289-291, 351]. Furthermore, mAbs, human Ig and monkey immune serum specific for the erythrocytic stages of the parasite can inhibit *P.falciparum* growth *in vitro* [33, 45, 238].

The blood stage malaria parasites are antigenically complex, but individual antigens have been identified [7-9, 141, 150, 155, 177, 179] and some potential vaccine candidates are described in section 1.4.

Another idea is to vaccinate against the disease rather than the parasite. Anti-disease vaccines may prevent the serious pathological complications of infection. It could be argued that antigenic variation represents the parasites adaptation to the host immune response and should therefore be a feature of those antigens that induce anti-parasitic immune responses. There may be less reason for any disease-inducing antigens to vary since this would increase the chance of host mortality, which would be counterproductive for the parasite. Disease is caused by the release into the blood stream of parasite products that induce responses in the host that cause the symptoms associated with malaria. Vaccination could induce neutralising antibodies to these parasite products [189]. In addition, vaccines directed against virulence mechanisms, in particular cytoadherence molecules, may be specifically effective in reducing the severity of disease associated with *P.falciparum* infection (see section 1.4)

Sexual stage vaccine

The aim of a transmission blocking vaccine is to arrest the sexual development of the parasite in the mosquito and to reduce or abolish malaria transmission levels. This altruistic approach would attempt to control the spread of disease within the community as a whole, but individual vaccinees would not be protected should they become infected, therefore this strategy would probably be used in combination with other types of vaccine.

During the 24 hour period of development within the lumen of the mosquito midgut, the parasites are extracellular and in continuous contact with components of the blood meal derived from their vertebrate hosts and therefore accessible to antibodies and other immunological agents [47]. Studies in avian and primate models have demonstrated that immunisation with extracellular gametes totally suppresses the infectivity to the mosquito of the subsequent blood meal [133]. It appears that inhibitory agents, ingested at the same time as the bloodmeal, react with target antigens located on the surface of gametes, blocking fertilisation or on zygotes and ookinetes, and inhibiting subsequent development in the mosquito midgut. mAbs, with transmission blocking activity, have been used to identify the antigens important for this transmission blocking immunity [46, 47, 261]: Pfs230 and Pfs48/45 expressed on gametocytes and extracellular gametes [46, 47, 170, 261] and Pfs25 expressed on the surface of zygote and

ookinete forms [17, 46, 47, 170, 171]. These antigens have been shown to have limited immunogenicity in individuals naturally exposed to malaria [127, 170, 267]. Recombinant Pfs25 protein of *P.falciparum* elicits transmission blocking immunity in experimental animals [17], but is not naturally immunogenic in humans because it is not expressed until the parasite is inside the mosquito.
An effective transmission blocking vaccine would be useful for dealing with either drug-resistant parasites or potential variants selected by partially effective pre-erythrocytic stage vaccines or asexual blood-stage vaccines [97, 222, 225].

1.3.7 Multicomponent vaccines (SPf66):

In practice, the most desirable vaccine against malaria may be one that primes both cellular and humoral immune reponses to a variety of antigens from various parasite strains and life cycle stages [191].
In 1987, Patarroyo and colleagues in Colombia, synthesised and conducted successful preliminary test of a falciparum malaria vaccine SPf66, a synthetic hybrid polymer of peptides from asexual blood stage proteins linked to sequences from the CS protein of *P.falciparum* [234]. SPf66 was tested in a randomised double-blind placebo-controlled trial in Colombia with 39% protective efficacy [233]. Since then there have been independent trials in Tanzania, Thailand and The Gambia, where malaria transmission is more intense. Results from the trial in Tanzania have recently been reported in the Lancet [5]. The estimated efficacy was 31% (95% confidence interval: 0-52%), showing a reduction in the risk of clinical malaria among children exposed to intense *P.falciparum* transmission. However, for most infectious diseases, a vaccine that cuts cases by only one third would be deemed insufficient [36, 353].

In conclusion, the discovery and characterisation of the antigens, the development of improved delivery systems and the increasingly refined understanding of the immunology of malaria, all give good reason to be optimistic about the future of malaria vaccine development [169].

1.4 Asexual blood stage antigens - vaccine candidates

Vaccines derived from asexual blood stage antigens are of special interest because they mimic the development of natural immunity in children living in endemic areas and may induce long-term immunity due to natural boosting of the immune response following periodic exposure to infection [5]. The multiplication of the asexual blood forms of plasmodia is responsible for the pathological manifestations in man. During this stage of infection the parasite develops within the host erythrocyte into three successive stages - rings, trophozoites and schizonts (figure 1.2) - antigen expression differing during the cycle. Mature schizonts rupture the erythrocyte releasing merozoites which, after a short extracellular period, invade new erythrocytes. The wide array of structures associated with merozoite invasion increases the opportunity for identifying a malaria protein(s) that will induce protective immunity. Initially any part of the merozoite attaches to the erythrocyte and reorients itself so that its apical end toward the erythrocyte membrane. Before or during invasion, the contents of the apical organelles are released onto the erythrocyte membrane forming a vacuole. Once inside the erythrocyte, the parasite is still visible to the immune system due to antigenic modifications of the infected red blood cell membrane. Since these antigens are accessible to immune recognition for a prolonged period of time (compared to merozoite surface antigens), they are also excellent targets for immune intervention.

The feasibility of a blood-stage based vaccine has been clearly demonstrated. A high degree of protection can be induced in monkeys by immunisation with *Plasmodium* schizont and merozoite preparations [227, 237, 260, 289-291, 351]. However, this is an impractical strategy.

A crucial step in the development of a synthetic asexual blood stage vaccine against malaria is to identify which of the many asexual blood stage proteins recognised by the host immune system are targets of protective immune responses (figure 1.2) [7]. The main target antigens are 1) proteins accessible at the time of schizont rupture, merozoite release and erythrocyte invasion, such as proteins on the merozoite surface, in the secretory apical complex and the parasitophorous vacuole; 2) proteins on the surface of the infected erythrocyte which may be involved in cytoadherence, rosette formation or metabolism; 3) intracellular components that are released during parasite development or destruction.

Many of these antigens are highly polymorphic and it is uncertain to what extent the diversity in these antigens will frustrate attempts to use them to induce protective immune responses.

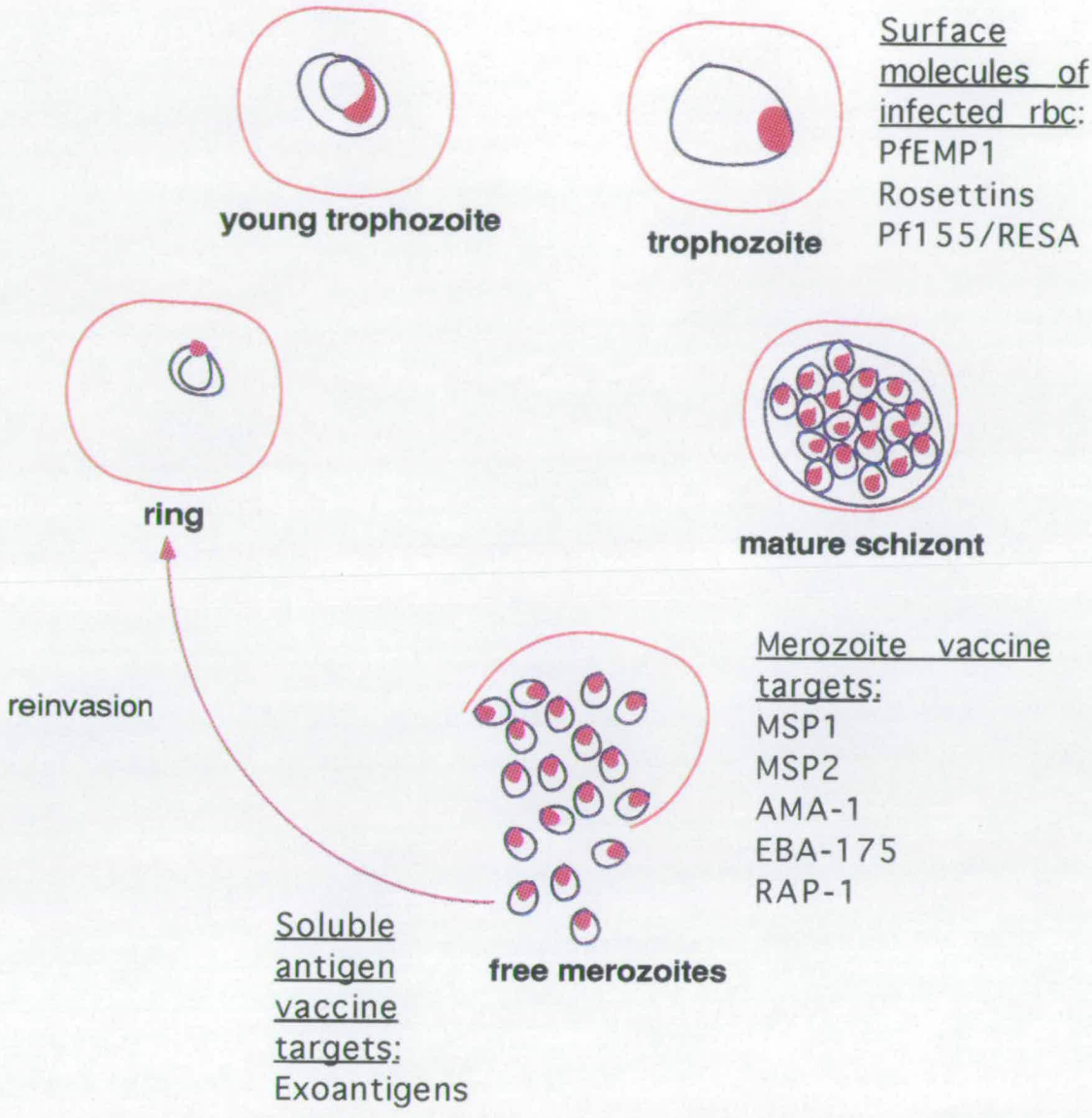


Figure 1.2: Asexual blood-stage cycle of *Plasmodium falciparum* malaria, identifying key targets for vaccine development.

1.4.1 Antigens associated with the merozoite/schizont

MSP1:

The major merozoite surface protein MSP1 (MSA1, PMMSA, gp190) is considered a leading candidate for inclusion in a vaccine directed against the asexual blood stage.

MSP1 is synthesised during schizogony and is expressed as a major component of the merozoite surface in both hepatic and erythrocytic stages [149]. The high molecular weight molecule, which ranges from 180-250kDa depending on the species and strain, has been shown in *P.falciparum* to be processed in at least two steps [28, 70, 86, 146, 149-151, 204]. First it is cleaved to several N-terminal fragments and a 42-kDa C-terminal fragment, and secondarily the 42kDa region is further cleaved into a 33-kDa and a membrane bound C-terminal 19-kDa fragment. The various N-terminal processing fragments initially remain in a complex on the cell surface and are eventually released into the plasma immediately before merozoite reinvasion [204]. The molecule is antigenically complex; the alignment of MSP1 sequences from many different isolates reveals 17 blocks of sequence that are either highly conserved, semi-conserved or variable [315] and there is marked inter-isolate antigenic diversity [206]. Additional diversity is generated by intragenic recombination [292]. The most conserved region is the 19-kDa fragment; it contains 12 cysteine residues which are completely conserved between the species with the characteristic spacing of EGF-like motifs [70, 193]. The 19kDa fragment is retained by the merozoite during erythrocyte invasion, and it is still present on the early ring stage of the next cycle of intraerythrocytic development [28]. These observation suggest that the 19kDa fragment, as well as the processing steps leading to it, may play an essential role in the reinvasion process and thus would be prime targets of vaccine induced immunity.

Partial protection against *P.falciparum* challenge in monkeys has been achieved by immunisation with recombinant MSP1 [151] and complete protection with protein purified from the parasite [290]. In addition, mono- and polyclonal antibodies to MSP1 inhibit parasite invasion *in vitro* [28, 50]. The 19kDa fragment is the target of invasion-inhibitory antibodies [28] [51]and the correct conformation of the disulphide bonds within the 2 EGF-like domains is essential for recognition by mAbs and human serum [38, 93]; this has been achieved in both eukaryotic and prokaryotic expression

systems [38, 51, 93]. Antibodies to MSP1 are prevalent in sera of individuals exposed to recurrent infection with *P.falciparum* [93, 115, 228, 266, 270, 324]. The acquisition of antibodies is age-dependent [115] and naturally acquired cellular and humoral responses to MSP1 are associated with reduced malaria morbidity [228, 266]. Taken together, these observations indicate that MSP1 is a target of protective immunity and that the conserved 19 kDa region may be particularly important.

MSP2:

A second merozoite surface antigen, MSP2, is considered a candidate for inclusion in a vaccine against falciparum malaria. It has not been studied as extensively as MSP1. The characteristics of MSP2 and its immune recognition are described in detail in section 1.5.

AMA-1:

The apical membrane antigen, Pf83/AMA-1, is an integral membrane protein that is initially associated with the apical organelles of the merozoite and then the merozoite surface [75, 230, 242]. Pf83/AMA-1 is post synthetically processed rapidly by cleavage of an N-terminal peptide to a 66kDa molecule [230]. Although AMA-1 lacks the repetitive sequences associated with other merozoite antigens of *P.falciparum*, there is limited polymorphism among the sequences of different AMA-1 alleles [242, 321]. Some regions of the molecule are well conserved; in particular, AMA-1 contains 16 cysteine residues which are conserved within the genus and the intramolecular disulphide bonds are associated with a specific structural conformation important for immunogenicity [65, 81]. Characterisation of the *P.knowlesi* AMA-1 homologue implied that the protein may have a receptor function, as Fab fragments from AMA-1 specific mAbs inhibited invasion of merozoites *in vitro* [322]. AMA-1 is considered a potential target for vaccine induced immune responses. The location of AMA-1 makes it accessible for immune recognition. Strong protective responses were induced in rhesus monkeys by immunisation with the *P.knowlesi* AMA-1 homologue in combination with exposure to the parasite [82]. Also, recombinant AMA-1 produced in baculovirus-infected insect cells has completely protected monkeys against challenge with simian parasites [65]. In addition, there is a high prevalence of antibodies to AMA-1 in individuals from malaria endemic areas and the acquisition of

these antibodies is age-dependent [323], although no correlation was observed between AMA-1 antibodies and parasite density or fever.

EBA-175:

A 175kDa erythrocyte binding antigen (EBA-175) which is thought to act as a ligand for attachment of merozoites to specific receptors on the erythrocytes [169], is another vaccine candidate antigen. The gene encoding the molecule has been sequenced, and parts of that sequence were believed to encode B cell epitopes. These were synthesised and used to immunise rabbits [292]. The hyperimmune serum reduced merozoite invasion of erythrocytes *in vitro* by 80% and inhibited binding of purified native EBA-175 to erythrocytes [292]. The protein appears to be antigenically conserved among the strains of parasites studied so far [292].

RAP-1:

Parasite molecules contained in the apical membrane complex of the merozoites are believed to play a crucial role in invasion. The major organelles that make up the apical complex are the rhoptries, membrane-bound electron dense structures which appear to discharge their contents at the time of schizont rupture and on the invasion of erythrocytes [263].
RAP-1 is a non-polymorphic multi-component antigen [262, 263]. The complex is recognised by human immune serum, mAbs specific for the *P.falciparum* RAP-1 can inhibit parasite growth *in vitro* [155, 286] and immunisation with this protein complex protects against infection [262].

1.4.2 Antigens associated with the surface of the infected erythrocytes

Erythrocytes containing mature trophozoites and schizonts adhere through knobs to endothelial cells lining the venules in deep tissues. This cytoadherence of parasitised erythrocytes prevents their passage through the spleen and their eventual destruction in this organ. Because cytoadherence requires recognition and attachment, a vaccine that induces antibodies to the components on the parasitised erythrocytes required for endothelial cell attachment could eliminate the adherence.

PfEMP1 (*Plasmodium falciparum*-infected erythrocyte membrane protein 1):

Cytoadherence can be inhibited by antisera in a strain specific manner [157, 198] but the erythrocytes also express shared determinants. Longitudinal analysis of the recognition of the parasite-dependent red blood cell neoantigens (PDN) suggested a protective effect of anti-PDN antibodies [200]. However, the cytoadherence phenotype appears to display considerable antigenic variation [273]. The main candidates for the parasite-derived ligands that mediate cytoadherence are represented by the variably sized (250-400kDa) group of proteins expressed on the surface of the *P.falciparum*-infected erythrocytes and collectively designated PfEMP1[153]. Antigenically distinct forms of PfEMP1 occur, generated by a mechanism of antigenic variation [25, 272, 273]. PfEMP1 appears to be the target antigen for naturally acquired antibodies that agglutinate infected rbc and inhibit cytoadherence [157, 198]. Isolation of the PfEMP1 gene will open the door to a molecular analysis of antigenic variation and is likely to greatly aid the development of anti-malarial vaccines.

Pf155/RESA:

A well characterised antigen Pf155/RESA (ring-infected erythrocyte surface antigen) is present in dense granule organelles of merozoites and transferred to the cytoskeleton of newly invaded red cells. RESA, which does not exhibit antigenic heterogeneity among different isolates of *P.falciparum*, contains 2 blocks of tandem repeats encoding immunodominant B cell epitopes [177]. Interestingly, the antibodies directed against these repeat regions cross-react with at least six other asexual stage components [239]. Antibodies to RESA inhibit invasion *in vitro* [23, 344] and one study has shown that animals were partially protected against blood stage challenge by immunisation with a recombinant protein expressed in *E.coli* [64]. However, more recent primate immunisation studies have been less convincing [147].

In naturally exposed populations, there is an association between increasing titres to RESA and decreasing parasitaemia [341] and with resistance to high parasitaemia [265]. Further studies confirmed this but also showed that the presence of antibodies binding to peptides containing B cell epitopes was associated with susceptibility to clinical attacks of malaria [265], however the association between antibody and disease may reflect antibody boosting due to the current infection. In a cross sectional

study of Liberian adults [240], an association was observed between Antibodies binding to the 3´ repeat region and reduced parasite density. A recent study in Papua New Guinea, found that adults having high antibody concentration to RESA were less likely to be parasitaemic [3].

Rosettins:

Distinct from cytoadherence to endothelial cells is the phenomenon of rosetting - the binding of uninfected erythrocytes to circulating erythrocytes infected with mature parasites. It has been suggested that blockage of the cerebral vessels is due to adhesion of irbc to the endothelial and concomitant rosetting [342]. In studies on children from The Gambia, parasites from cerebral malaria patients had a significantly higher rosetting rate than did those from patients with uncomplicated *P.falciparum* malaria. Anti-rosetting antibodies have been found to hinder rosette formation *in vitro* and have been implicated in protection against severe malaria [342].
The surface of the infected erythrocytes is covered with minute electron-dense excrescences called knobs. After being transported from the internal parasite, antigens involved in the binding to other cells are thought to be concentrated at the knobs and subsequently exposed to the exterior of the erythrocyte membrane. Potential rosetting ligands have been identified on the surface of irbc. These "rosettins' are of variable size (22-28 kDa) and can induce strain-specific rosette-disruptive antibodies [342].

1.4.3 Soluble antigens associated with schizont rupture

Parasitised rbc rupture allows merozoite release and invasion of new erythrocytes. Since prbc development *in vivo* is synchronous, large amounts of prbc-derived lipids, glycolipids and glycoproteins are released into the plasma within a relatively short period of time. These elicit host responses that lead to the symptoms of malaria infection, particularly fever. It has been proposed that vaccine-induced antibodies specific for these molecules could lead to immune-mediated removal of these antigens without inducing the cascade of undesired host responses [190, 245]. Heat-stable exoantigens in the supernatants of blood-stage parasite cultures induce the release of TNF *in vitro* from activated macrophages and behave like toxins *in vivo*. Mice immunised with the antigens are protected from the toxic effect and their serum specifically blocks the ability of the antigens to

stimulate the production of TNF [19, 317]. Thus vaccination with these exoantigens might provide a means of protection against the clinical effects of malaria and of generating anti-disease immunity by reducing cytokine production. Evidence suggests that the active moiety contains an inositophospholipid structure, possibly related to the glycophosphatidylinositol (GPI) anchors on the two major merozoite surface antigens and other plasmodial proteins [190]. Glycolipids induce the production of T-independent antibodies that are capable of neutralising this biological activity of TNF [19, 317].

At least seven exoantigens have been identified and it may be significant that most children in The Gambia do not develop precipitating antibody against one of them, Ag7, until they are about 4 years old [158]. This is about the time that they develop some immunity to the clinical manifestations of malaria. Riley *et al* [269] examined the association between malaria morbidity and cellular and humoral immune responses to these soluble glycoprotein exoantigens in a longitudinal prospective study of semi-immune children in The Gambia. The presence of antibody to Ag2 was more prevalent in children with asymptomatic malaria than in children with confirmed clinical malaria, suggesting that these antibodies may be able to inhibit parasite multiplication and consequently control the clinical symptoms of malaria infection.

1.5 Merozoite surface protein 2 (MSP2)

1.5.1 Characteristics of MSP2:

The *P.falciparum* merozoite surface protein, MSP2, is considered a candidate for inclusion in a vaccine against falciparum malaria. MSP2 was independently identified by several laboratories and is also known as gp56 [310], GYMSSA [254], MSA2 [282, 297], 46000 dalton antigen [220], QF122 [101], GP3 [110] and 46-53Kda antigen [55]. MSP2 is an integral membrane glycosylated protein with an observed size on electrophoretic migration varying from 35 to 56 kDa. The molecule does not exhibit conformational properties dependent on intrachain disulphide bridging - there is no significant shift in migration of the protein under reducing and non-reducing conditions [142, 220]. Unlike MSP1, there is proteolytic cleavage of the protein [297].

1.5.2 Location of MSP2:

MSP2 has been demonstrated in trophozoites, "segmenters", schizonts, and isolated merozoites by immunoblotting using either poly- or monoclonal antibodies [55, 101, 220, 254, 297, 310], by immuno-EM [55, 101, 220, 310] and by IFA [55, 101, 297]. These observations are consistent with observation that MSP2-mRNA transcription begins in young trophozoites and reaches the highest levels during the transition from trophozoites to schizonts [18]. MSP2 has been detected on the outer merozoite surface by surface-iodination [142] and mAb-labelling - giving a grape-like fluorescence pattern characteristic of antibody binding to the merozoite surface [101, 254].

After erythrocyte invasion, the protein is not detected [55, 254] or is detected in low quantities in the new ring stages [220, 297]. This indicates that the molecule is completely or partially lost during invasion, and thus possibly suggests a role for MSP2 in attachment to the erythrocyte.

1.5.3 Structural characteristics of MSP2:

A striking structural feature of MSP2 is its high level of variability. Characterisation of the protein by restriction-fragment length polymorphism (RFLP) analysis [108], hybridisation studies [201, 217, 231, 250, 304], serological analysis with mAbs [66, 67, 101, 109] and DNA sequencing [109, 201-203, 250, 256, 304] has shown that MSP2 is a highly

polymorphic protein. From this information, MSP2 has been classified into two allelic families: serogroup A (3D7/CAMP) and serogroup B (FCQ-27) [109, 202, 203, 297-299, 304, 321]. Sequencing of the gene provides an absolute means of examining the diversity of MSP2 and studies comparing information obtained on size and RFLP have shown that a considerable amount of microheterogeneity was evident once isolates were sequenced [201, 250]. Alignment of the many MSP2 sequences shows the 3´ and 5´ ends of the gene to be highly conserved, whereas a large central region is variable [109, 201-203, 250, 297-299, 304, 321] (figure 1.3). The amino- and carboxy-termini, 43 and 74 residues respectively, are conserved among all isolates; 3 amino acid substitutions have been reported in the C-terminus [321]. The central region contains two tandemly repeated sequences, R1 and R2, flanked by serogroup specific sequences. Both the repetitive and non-repetitive sequences exhibit a dimorphism; however, the non-repetitive sequences define the two basic, prototypic allelic forms and are highly conserved within each serogroup, showing limited conservative substitutions [321] and a deletion permissive area [109, 321] (figure 1.3).

The R1 repeats of serogroup A consist of 1 to 13 copies of glycine-, serine- and alanine-rich sequences containing between 4 and 10 residues. Variable repeat units can occur within one isolate and in different isolates (for examples see figure 1.3). Downstream there is a second repeat unit (R2) which consists of 3 alternative codons for the amino acid threonine [299]. Subfamilies of serogroup A exist, defined by the presence or absence of short regions within the sequences flanking the polymorphic domain [202] (figure 1.3) and reactivity with serogroup A specific mAbs [66, 67, 109].

The corresponding repeat regions in serogroup B are unrelated to those of serogroup A, consisting of 32 and 12 amino acids respectively. These repeats display less sequence polymorphism than the serogroup A repeats, but occur in varying numbers. Isolates have been sequenced which lack the 12-mers altogether [201, 250, 257]. The permissiveness for variation in size is limited; changes in repeat number are compensated for by deletions or insertions elsewhere in the variable region.

Despite the extensive differences between the serogroups, there is a striking conservation of the overall amino acid composition, net charge and hydrophobicity [299].

As in MSP1, additional diversity has been superimposed on the dimorphism by intragenic recombination [203, 231]. However, in contrast to MSP1 where homologous recombination has apparently occurred between sequences in the conserved blocks close to the 5´ end of the gene [66, 67], the recombination within MSP2 has occurred in the repeat regions which, although very different, have a short region of homologous sequence which has presumably facilitated the recombination event [8, 203].

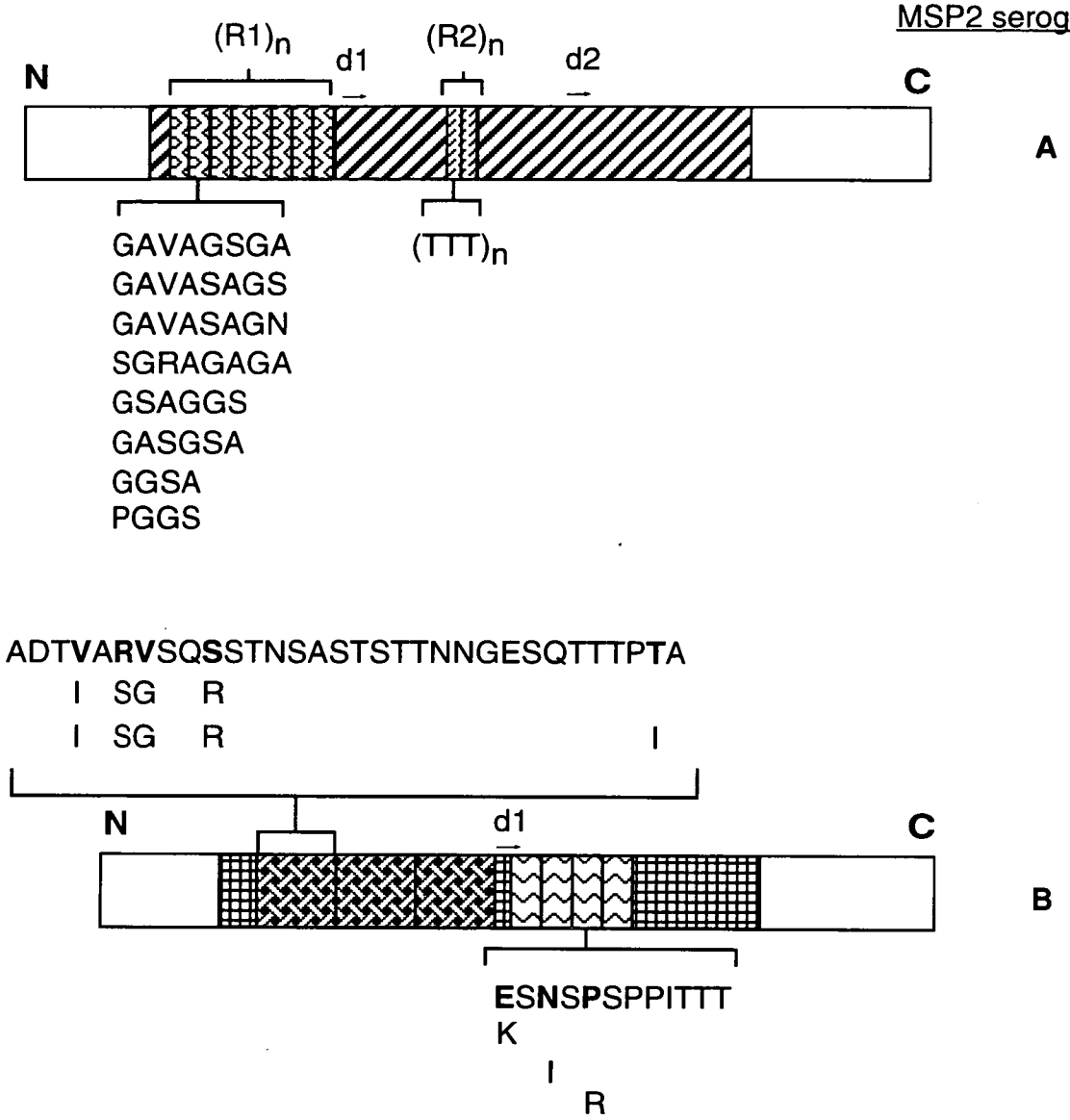
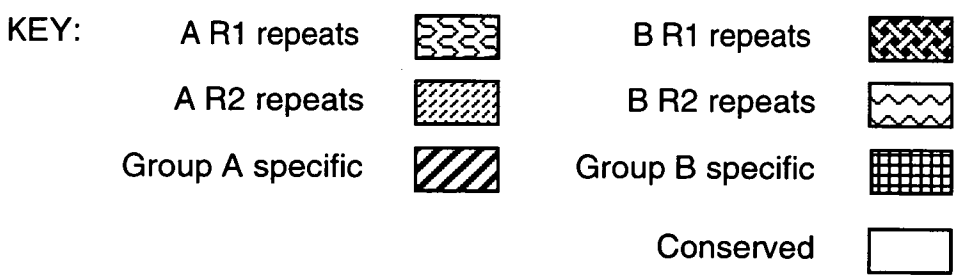


Figure 1.3: Schematic representation of MSP2 serogroups A and B. Published sequences from the R1 and R2 regions of each serogroup are shown [109, 201, 250, 298] . d1 and d2 denote the deletion permissive regions of the molecules



1.5.4 Immunological studies of MSP2:

MSP2 is a potential target of protective host immune responses due to its location on the surface of extracellular merozoites. MSP2 specific poly- and monoclonal antibodies inhibit *in vitro* parasite invasion and growth [55, 101, 220, 254, 255, 282, 299]. These antibodies define variable, linear epitopes [101, 255].

The immunogenicity of peptides representing sequences from the conserved and variable regions of MSP2 have been investigated [165-168, 276-279, 282]. Several of these peptides, when administered with adjuvant either free or conjugated to diptheria toxoid, elicit antibodies in mice that recognise the native protein [165-168, 276, 282]. In addition, some of the peptides induced proliferation of murine and human T cells [277-279]. Furthermore, mice, immunised with peptides from the conserved regions of *P.falciparum* MSP2, were protected from challenge with asexual parasites of the murine malaria *P.chabaudi* [284]. However, immunisation of *Saimiri* monkeys with a rAg representing the full length protein of serogroup B expressed in vaccinia virus failed to protect monkeys against challenge with *P.falciparum* [252].

Despite the apparent protective capacity of the conserved sequences, contradictory results have been obtained for the recognition of these regions. *Aotus* and human serum eluted from immune clusters of merozoites did not react with rAgs representing the N- and C-termini [321] and Saul *et al* [284] were unable to detect antibodies against conserved peptides in individuals from Papua New Guinea (PNG). However, antibodies have been detected to C-terminal sequences in individuals naturally exposed to malaria in Sri Lanka and another PNG study [256] [2] . Antibodies to MSP2 are prevalent in individuals from malaria endemic areas [2, 256, 279]. Antibodies to MSP2 are acquired in an age-specific manner [2, 256], with the highest prevalence of seropositivity in individuals older than 15 years in Papua New Guinea [2]. A proportion of these individuals had antibodies to only one form of MSP2 indicating that recognition may be serogroup-specific and not directed towards the conserved regions, although the authors postulated that non-responsiveness may be genetically regulated [2]. A negative association between antibody to MSP2 and fever was observed, suggesting a possible protective role of anti-MSP2 antibodies in natural infection [2].

1.6 Aims and outline of the thesis

The aim of this thesis is to examine the qualitative and quantitative aspects of the serological immune response to MSP2 in a population of malaria-exposed individuals from The Gambia, West Africa (figure 1.4).

The Gambia is a small country on the west coast of Africa; one of the smallest and most densely populated in Africa. It is an area of flat Sudan savannah with some mangrove and rice swamps near the river Gambia. The climate is characteristic of the sub-Sahel with a long dry season (mid-October to June) and a shorter rainy season with around 500-1000mm of rainfall per year [66, 67, 128]. This results in hyperendemic malaria transmission with a seasonal point parasite prevalence which may exceed 50%, and a peak number of clinical cases occurring between September and November [200].

Recombinant antigens, representing MSP2 and fragments thereof, are used to determine the presence of serum antibodies by enzyme-linked immunosorbent assay (ELISA). The antigenic integrity of the rAgs has been confirmed to ensure that they accurately reflect the antigenic characteristics of native MSP2 (Ch.3). Optimisation of the ELISA procedure was performed to ensure that antibodies specific for MSP2 are detected and the assay procedure is robust and reproducible (Ch.4).

Serum antibodies are initially determined in malaria immune adults from the village of Brefet (figure 1.4) to determine the immunogenicity of MSP2 after natural exposure to malaria and the relative antigenicity of conserved, dimorphic and polymorphic regions of the molecule (Ch.5). Cross-sectional (Ch.6) and prospective longitudinal (Ch.7) studies, using serum samples collected in villages around the town of Farafenni (figure 1.4), are used to examine the dynamics of the acquisition of anti-MSP2 antibodies with age and the association of anti-MSP2 antibodies with subsequent susceptibility to clinical malaria, respectively. The quality of MSP2-specific antibodies are investigated (Ch.8), in terms of IgG subclass specificity, and related to age and clinical status.

The effect of the extensive polymorphism of MSP2 on immune recognition is elucidated and associated with age and exposure to distinct parasite genotypes (Ch.9). A field study has been carried out in Fajara, The Gambia (figure 1.4) (Ch.10). Serum samples and infected erythrocytes will be obtained from individuals with uncomplicated clinical malaria, to determine

the relationship between the specificity of the immune response to MSP2 and the MSP2 genotype of the *P.falciparum* infection.

The possibility that the immune recognition of MSP2 may be genetically regulated is investigated by comparing the antibody responses of monozygous and dizygous twins, and by looking for associations between antibody specificity and HLA-type (Ch.11).

Each chapter is a discrete unit with its own introduction and discussion. An overall discussion (Ch.12) consolidates the findings and discusses their implication for vaccine development.

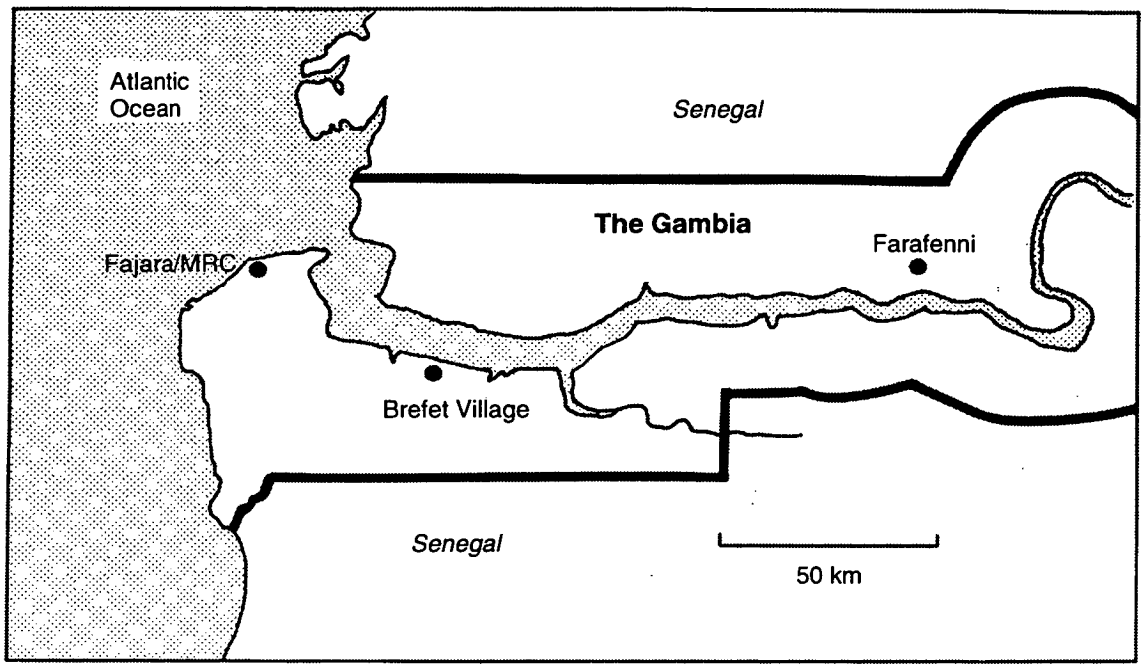


Figure 1.4: A map of the western part of The Gambia showing the locations of the villages from which samples were collected.

2. Materials and Methods

Unless otherwise stated reagents were obtained from SIGMA Chemical company Ltd., Poole, UK.

2.1Recombinant MSP2 proteins:

2.1.1 Construction of plasmids

pGEX expression vectors were used to direct the synthesis of MSP2 polypeptides in *E.coli* as fusions with the C-terminus of glutathione S-transferase (GST) of *Schistosoma japonicum* [295] (figure 2.1). Polymerase chain reaction (PCR)-amplified fragments of the MSP2 gene were ligated into pGEX vectors, after the PCR products were cut with the appropriate restriction enzymes. The pGEX vectors provide *Bam*HI, *Sma*I and *Eco*RI cloning sites in all three reading frames followed by a series of termination codons. After transformation into *E.coli*, transformants were screened for the expression of the required GST fusion protein by small scale purification. Individual transformants were picked into 2ml of L-broth containing 10µg/ml ampicillin and incubated at 37°C, with shaking, for 3-5 hours. Isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1mM to induce the expression of the fusion protein and incubation continued for 1-3 hours. Analysis of a 10µl aliquot of the culture by SDS-PAGE is used to determine the expression of a protein of the expected molecular weight. Sequencing is used to determine that the protein is in the correct orientation and reading frame and is of the predicted sequence. Appropriate transformants were stored as glycerol stocks at -70°C.

These recombinant proteins were produced by Dr Donald Smith and Mrs Jane Robinson in Dr J McBride's laboratory, Edinburgh University.

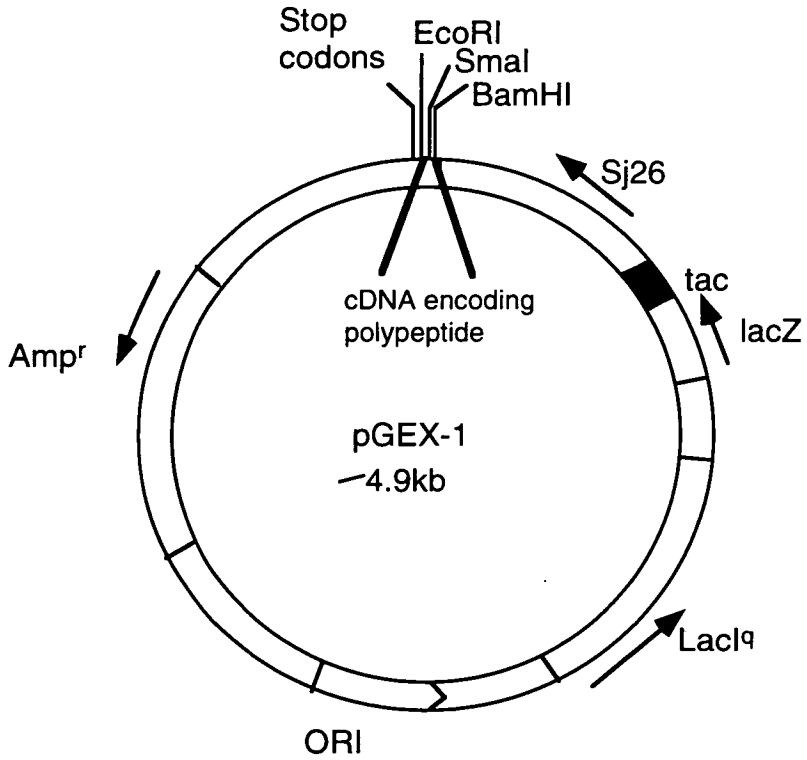


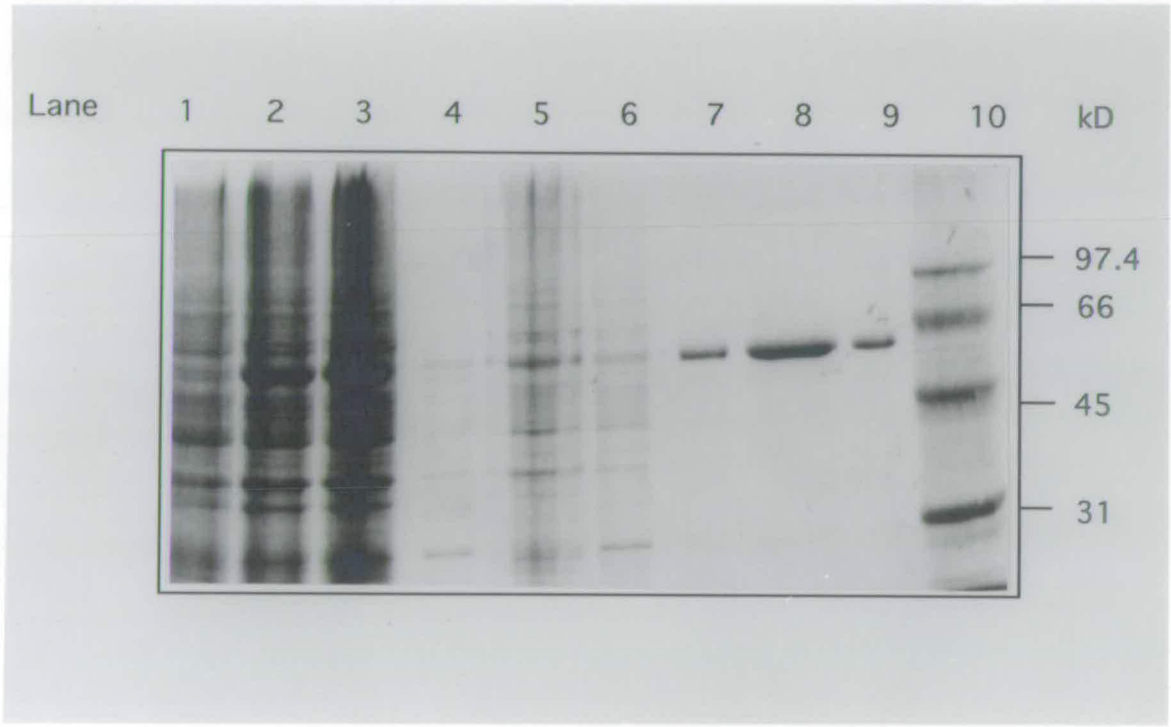
Figure 2.1: Schematic representation of the pGEX vector. The plasmid contains Amp^r encodes ampicillin resistance, ORI denotes the origin of transcription, a fragment of the lac operon containing the overexpressed lacIq allele of the lac repressor and part of *lacZ*. In the absence of inducer (IPTG), the plasmid encoded *lacI^q* allele is efficient in repressing transcription from the *tac* promoter. Sj26 cDNA encode glutathione S-transferase of *S.japonicum*; the normal termination codon is replaced by a polylinker containing unique recognition sites for *BamHI*, *SmaI* and *EcoRI* and followed by TGA translation termination codons in all three reading frames.

2.1.2 Purification of rAgs

To generate fusion proteins for immunisations and use in ELISA, colonies of transformed *E.coli* were grown on agar plates (ampicillin@50μg/ml) overnight at 37°C. 100 ml of L-broth containing 50μg/ml of ampicillin was inoculated with a single colony and grown overnight at 37°C in a shaking incubator. This culture was added to 1l of L-broth (ampicillin@50μg/ml) and incubate for 2hrs at 37°C before addition of 1ml of 100mM IPTG. After a further 2-4hr incubation, cells were pelleted by centrifugation at 10,000g at 4°C for 10 minutes. Pellets were resuspended in 20mls PBS and lysed by sonication at 4°C. A 5mm probe was used for sonication and care was taken to minimise frothing of the cell suspension by sonicating in short bursts for 1-3 minutes.

Contamination of fusion protein by *E.coli* proteins was minimised by addition of Triton X-100 (BDH Lab. Supplies, Poole, UK) to 1% and gentle mixing. In order to remove insoluble material, the mixture was transferred to eppendorfs and centrifuged for 5 minutes at 10,000g. The supernatant was transferred to 50ml tube containing 4mls of 50% (w/v) glutathione-agarose beads in PBS and incubated for 30-60 minutes at room temperature on a shaker. After centrifugation at 500g, the beads were washed three times in 50mls in PBS. Fusion protein was recovered from the beads by incubation with an equal volume of 50mM tris-HCl containing 5mM glutathione, gently mixing at room temperature for 10-30 minutes. The beads were pelleted by brief centrifugation and the supernatant containing eluted fusion protein removed. Fusion proteins were stored at -70°C.

The purity and concentration of fusion proteins were estimated by the intensity of Coomassie blue staining on acrlyamide gels (section 2.2); see figure 2.2.


Lane
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2
3
4
5
6
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8
9
10
kD
97.4
66
45
31

2.1.3 Recombinant MSP2 antigens:

Recombinant antigens (rAgs) produced and the regions of MSP2 they represent are listed in table 2.1. Recombinant proteins are schematically represented in figure 2.3 and in appendix 1A.

Serogroup	rAg	Isolate	Region
A	A1	T9/96	Full length
	A2	T9/96	R1 repeat
		Thai Tn	
		7G8	
		T9/102	
		CH12/12	Part of C-terminal gp. spf. seq. deleted
		RO33	
		T9/94 (i)	
		T9/94 (ii)	N-terminal gp. spf. seq. deleted
	A3	RO33	R2 repeat/group specific
		CH12/12	
		CH150/9 (i)	part of gp. spf.seq. deleted
		CH150/9 (ii)	
		G1	
		T9/102	R2 repeats deleted
B	B1	Dd2(13/14)	Full length
		Dd2(5/6)	Shortened conserved regions
	B2	K1(ii)	R1 repeats
		T9/105	Shortened C-terminus
		K1 (i)	Shortened N-terminus
	B3	K1	R2 repeats/group specific
		T9/105 (i)	
		T9/105 (ii)	No repeats
	N	K1	Conserved
	C	K1	Conserved

Table 2.1 Recombinant MSP2 proteins produced as fusions with GST and expressed in *E.coli*. rAgs will be referred to as A1, A2, A3 B1, B2 and B3 in subsequent chapters; to avoid confusion the isolate from which they were produced will be stated in brackets.

2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

2.2.1 Preparation of the SDS-polyacrylamide gel:

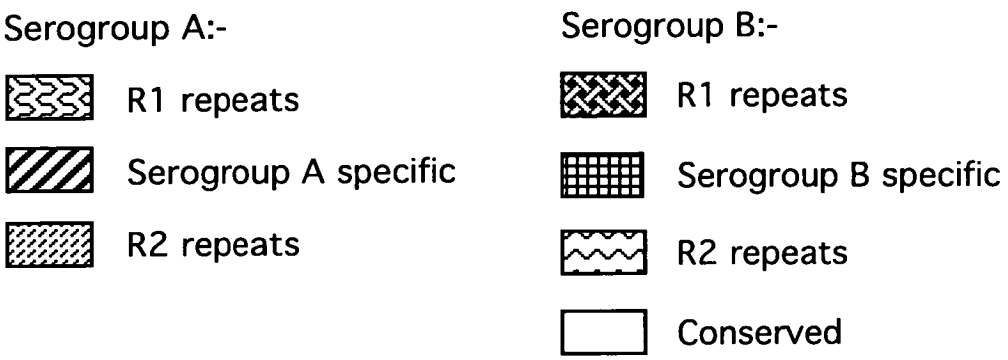
A 10% acrylamide gel solution (resolving gel solution) was prepared and poured between glass plates using the SE250-Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, USA). The meniscus of the acrylamide solution was 1.5cm below the top of the notched plate to allow room for the comb. Approximately 0.5ml of water-saturated isobutanol was carefully layered on top of each gel to create a barrier to oxygen, which would inhibit the polymerisation of the acrylamide. After the gel had set (1-2 hrs), the overlay solution was poured off and the top of the gel washed several times with distilled water (dH_2O), then drained well. The comb was inserted and the stacking gel solution was poured and left to set (30mins). The comb was removed and wells washed with dH_2O to remove unpolymerised acrylamide.

2.2.2 Running the gel:

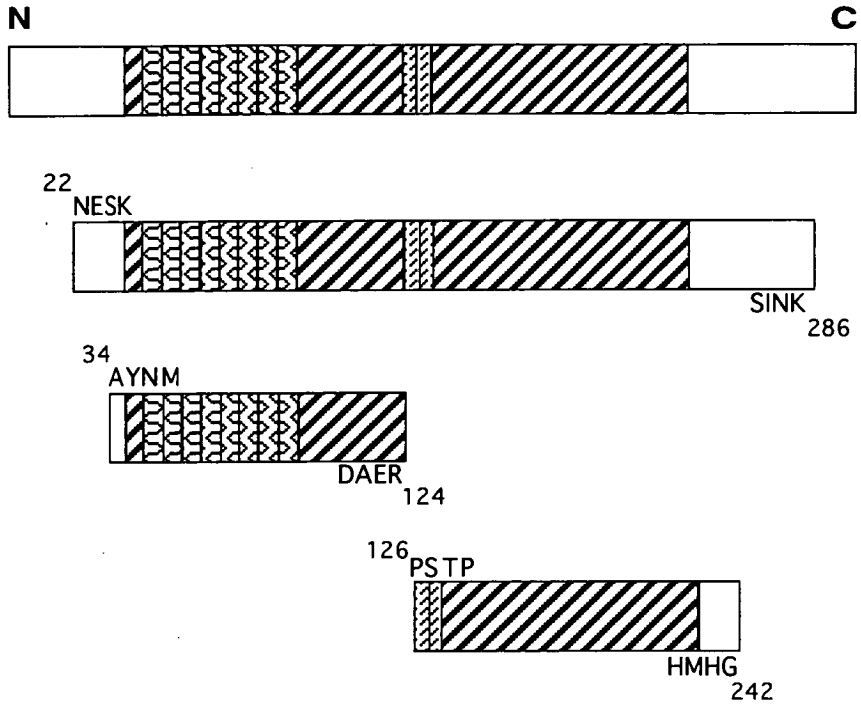
Running buffer was added to the upper and lower reservoirs of the gel box. Samples were loaded into the wells (figure 2.2). The gels were run at 20mA/gel for approximately 1.5hrs, until the dye front had reached the bottom of the gel. The apparatus was carefully dismantled and the gels were stained with Coomassie blue stain (0.1% Coomassie brilliant blue R250, 50% methanol, 10% acetic acid in dH_2O) for 30 mins and destained with destain solution (10% methanol, 10% acetic acid in dH_2O) until bands were clearly visible.

Gels were placed on Whatman 3MM filter paper, covered with Saran wrap and dried at 80°C for 1hour under vacuum, using a gel dryer.

Figure 2.3: Schematic representation of MSP2 recombinant antigens A1, A2 (T9/96), A3, B1, B2 (K1) and B3 (T9/105i). Isolates from which the rAgs were derived are shown in bold. The amino acid number refers to the position of the first and last residue of the proteins from the published sequences of T9/96 (serofroup A) and FC27 (serogroup B) [109].

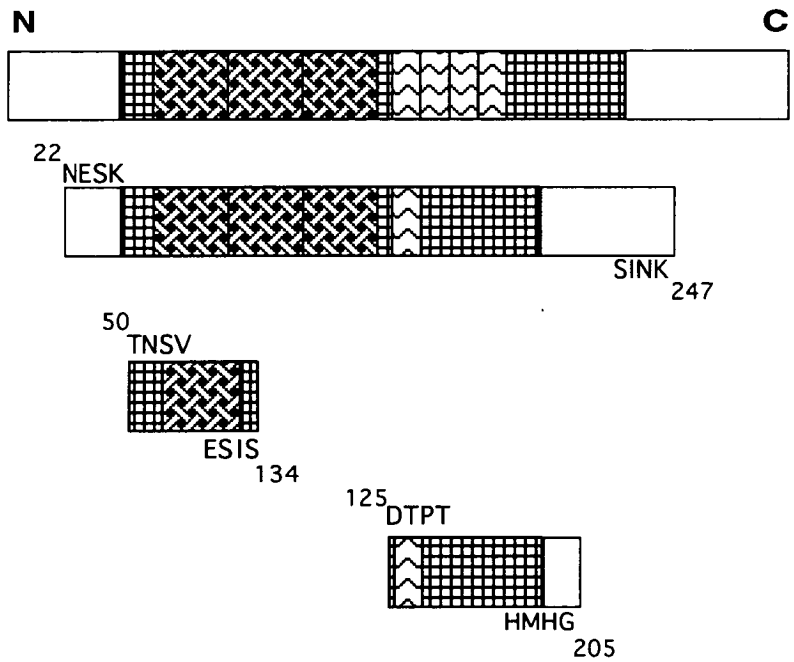


Serogroup A



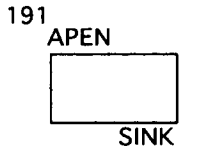
rAg	ISOLATE
A1	T9/96
A2	T9/96
A3	RO33

Serogroup B



rAg	ISOLATE
B1	Dd2
B2	K1
B3	T9/105

CONSERVED



rAg	ISOLATE
C	K1

2.2.3 Solutions for SDS-PAGE:

Resolving gel solution

Acryl	4.66ml
(30g acrylamide, 0.8g N,N'-methylene-bis-acrylamide in 100mls dH_2O)	
Resolving gel buffer	3.50ml
(19.2g Tris-HCl, 0.4g SDS in 100ml dH_2O, pH8.8)	
dH_2O	5.74ml
10% (w/v) ammonium persulphate (APS)	200µl
TEMED (N,N,N',N'-tetramethylethylenediamine)	5µl

Stacking gel solution

Acryl	0.50ml
Stacking gel buffer	1.25ml
(6.06g Tris-HCl, 0.4g SDS in 100ml dH_2O, pH6.8)	
dH_2O	3.20ml
10% APS	50µl
TEMED	7µl

Running Buffer

Tris base	15.15g
Glycine	72.05g
SDS	5g
dH_2O	5l

2.3 Immunoblotting

2.3.1 Transfer of protein from the gel onto nitrocellulose:

Transfer of proteins from gel to nitrocellulose membrane (Anderman & Co. Ltd., Kingston-upon-Thames, UK.) was achieved by electrophoretic elution [325]. The gel and nitrocellulose were sandwiched between filter paper (Whatman 3MM) and immersed in a buffer-filled tank (LKB 2005 Transphor electroblotting unit, LKB Instruments Ltd., Croyden, UK.) (electrophoresis buffer- 29.0g Tris, 14.5g glycine, 1.85g SDS, 1l methanol in 4l dH_2O), with the membrane closest to the positive electrode. Transfer is complete after 1-2hours at an electric current of 0.65mA/cm^2 of gel.

2.3.2 Labelling transferred antigenic proteins with antibodies:

When the transfer of the protein onto the nitrocellulose was complete, the nitrocellulose was separated from the SDS-polyacrylamide gel and staining with Ponceau-S was used to provide visual evidence that electrophoretic transfer of proteins had taken place. Ponceau-S is a transient stain and is washed away during the processing of the Western blot. Staining does not interfere with the subsequent detection of antigens. Ponceau-S (3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2,7-napthalene disulfonic acid) was diluted 1:20 with PBS/Tween.

After washing (washing buffer (WB)-PBS/0.05% Tween 20), the nitrocellulose was incubated in a non-cross reactive protein solution (blocking buffer (BB)-1%(w/v) nonfat dried milk in WB) for at least 1 hour at room temperature. This blocking step helps prevent the antibodies from binding nonspecifically to the nitrocellulose. The nitrocellulose was washed (x3) and probed with mouse anti-sera or mAb, diluted in BB, for 1-3 hours at room temperature. After washing, the nitrocellulose was incubated for 1-3 hours with HRP-conjugated rabbit anti-mouse Ig (DAKO, Ltd.) at a 1:400 dilution in WB.

2.3.3 Developing the Western blot:

The binding of Antibody was visualised using 4-chloro-1-napthol. Prior to developing the blot, a solution of chloronapthol was prepared (30mg chloronapthol in 10ml methanol) and added to 40ml of triethanolamine buffered saline (0.12g Tris-HCL, 0.9g NaCl in 100ml dH_2O, pH7.4). H_2O_2 (30µl) was added immediately before use.

The blot was washed in WB (x3) and TBS (x1) then developed at room temperature in the dark until bands appeared. Blots could then be photographed.

All incubation steps were carried out on a shaker.

2.4 Enzyme-linked immunosorbent assay (ELISA)

2.4.1 Basic protocol for indirect ELISA:

Microtitre plates (Immulon-4 (Dynatech)) were coated overnight at 4°C with 100µl/well of antigen in coating buffer (1.59g Na_2CO_3, 2.93g $NaHCO_3$ in 1l dH_2O; pH9.4-9.6), and blocked for 5 hours at room temperature with 200µl/well of blocking buffer (BB; 1% (w/v) nonfat dried milk in washing buffer). At the same time, sera were diluted in BB and incubated at room temperature for 5 hours. Plates were washed three times with washing buffer (PBS/0.05%Tween20 (PBS/Tween)) and 100µl/well of diluted serum was added to duplicate wells and incubated overnight at 4°C. Plates were washed and incubated with horse-radish peroxidase-conjugated rabbit anti-human IgG antibody (Dako Ltd., High Wycombe, UK) at 1/9000 for 3 hours at room temperature.

All plates were developed with 100µl/well of substrate buffer with H_2O_2 as substrate and o-phenylenediamine (OPD) as chromagen, at 4°C and the reaction was stopped after 10 minutes with 20µl/well of 2M H_2SO_4. The optical density values (OD) were measured at a wavelength of 492 nm, using a Titeretek plate reader (ICN Flow, Buckinghamshire, UK).

Data was transferred into a computerised data management system, Elisalite (Meddata, Inc., New York, USA)

Protein and antibody concentrations were selected after optimisation of the protocol, described in chapter 3.

2.4.2 Solutions for ELISA:

Substrate buffer

0.1M citric acid	6.0 ml
0.2M phosphate (Na_2HPO_4) buffer	6.4 ml
dH_2O	12.5 ml
o-phenylenediamine (OPD)	10 mg
H_2O_2	10 µl

2.5 *In vitro* cultivation of asexual stages of *Plasmodium falciparum*

2.5.1 Incomplete medium:

The medium was based on that used by Trager and Jensen [326], consisting of RPMI 1640 (Gibco BRL) supplemented with 25mM HEPES buffer (5.94g/l) and hypoxanthine (50mg/ml). The medium was filtered through a 0.22μM Nalgene filter and stored at 4°C for up to 4 weeks.

2.5.2 Complete Medium:

Freshly made and filtered sodium bicarbonate ($NaHCO_3$, 2g/l) was added to incomplete medium. The solution was adjusted to pH7.2 by the addition of 1M NaOH, and filter sterilised into 200ml sterile culture flasks. 10% (v/v) of heat inactivated human serum was added. Complete medium was used within one week and incubated at 37°C for 24hrs before use.

2.5.3 Culturing of *Plasmodium falciparum* parasites:

Plasmodium falciparum ring stage infected red blood cells (rbc) of culture adapted parasite clones (3D7 and Dd2) were obtained from existing cultures. *P.falciparum* isolates were grown at 5% haematocrit with O^+ human rbc (Edinburgh and South East Scotland Blood Transfusion Service) which had been washed free of leukocytes. These cultures were grown under sterile conditions in 25mls of complete medium in 200ml culture flasks (Corning, New York, USA). The cultures were incubated at 37°C in a 1% O_2, 3% CO_2 and 96% N_2 gas mixture. A parasitaemia of up to 10% infected rbc was permitted.

Small quantities of blood were removed with a sterile Pasteur pipette to prepare thin blood films, stained with Geimsa's stain at pH 7.2 and examined microscopically to measure the parasitaemia and health of the culture and to assess the maturity of parasites.

2.5.4 Isolation of schizont-infected red blood cells:

Isolation from culture of live schizont infected rbc was carried out using density gradient centrifugation [185].

A culture which was predominantly at the schizont stage, and at least 5% parasitaemia, was spun for 10 minutes at 2000rpm, the cells were washed in fresh incomplete medium and resuspended in 10 ml of incomplete medium. 5ml of this suspension was carefully layered onto 8ml of 60%

Percoll (60ml Percoll (Pharmacia, Uppsala, Sweden), 6ml 10xPBS, 34ml incomplete medium) in 15ml tubes, and spun at 2000rpm for 10 minutes. Several distinct layers appear; the dark brown schizont infected cells aggregate in a thin band towards the top of the gradient. These schizont infected cells were carefully removed and washed (x2) in incomplete medium. The washed schizont pellet was transferred to an eppendorf tube and stored at -20°C.
The remaining infected rbc were washed and returned to culture.

2.6 Indirect immunofluorescence assay (IFA):
Indirect immunofluorescence recognition of *P.falciparum* isolates was performed with individual mAbs or mouse serum on acetone-fixed schizonts as described by Conway *et al* [66].

2.6.1 Preparation of multispot schizont slides:
Schizont-infected erythrocytes were washed three times and resuspended at approximately 3% haematocrit in PBS. Multispot slides (C A Hendley Ltd., Essex) of schizonts were prepared with 20µl of the cell suspension per well. The slides were air-dried and stored under dessication at -20°C, in sealed polythene bags containing silica gel (BDH).

2.6.2 Preparation of antibody samples:
Working dilutions of mAbs, mouse sera and commercial antibodies in 1% (w/v) bovine serum albumin in PBS (pH 7.3-7.5) containing 0.01% sodium azide were prepared. Dilutions can be kept at 4°C for several months.

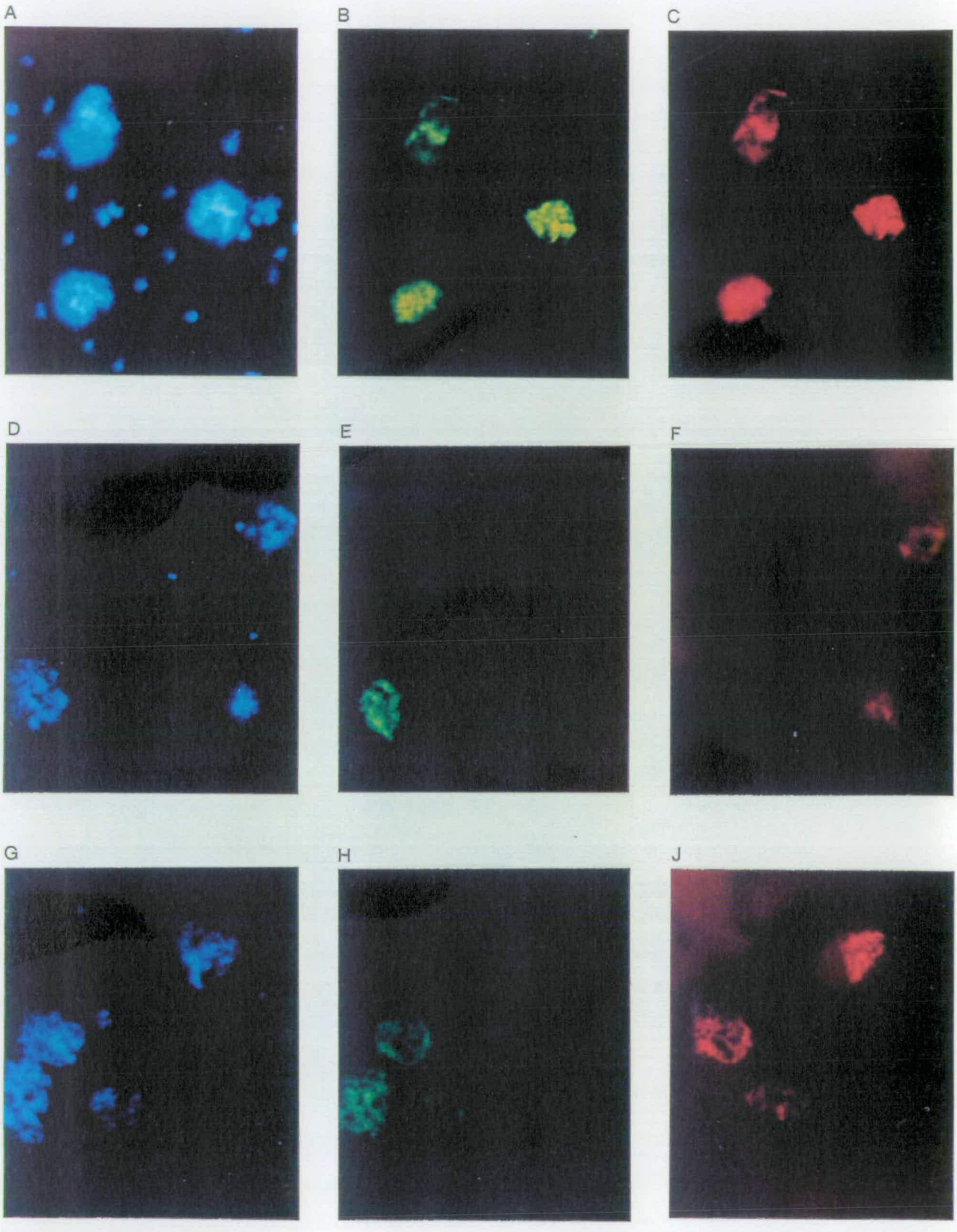
2.6.3 Single-labelled IFA:
A 25 µl volume of each mAb or mouse serum was incubated on the schizonts for at least 30 minutes at room temperature in a wet box to prevent evaporation. Care was taken to avoid cross contamination of samples on adjacent wells.
After careful removal of serum by Pasteur pipette, the slides were washed 3 times (1,5 & 5 minutes) in PBS. Slides were gently air-dried on a warm plate set at 50°C, then 12µl of a 1:50 dilution of fluoresceine isothiocyanate (FITC)-conjugated polyvalent rabbit anti-mouse Ig antibody (ICN Immunoglobulins, Lisle, Israel) was added to each well and incubated for 30 minutes in a wet box at room temperature. After 2 washes (1 & 5

minutes) in PBS; parasite nucleii were stained with DAPI (4',6-diamino-2-phenylindole; $1x10^{-6}$ (w/v) in PBS) for 1 minute. Slides were washed twice (1 & 5 minutes) in PBS and mounted under a coverslip in 2-3 drops of Citifluor (City University, London).
Parasites were visualised by FITC-fluorescence (green, serum specific) and DAPI-fluorescence (blue, DNA specific), with incident light of 450-490nm and 390-440nm respectively, at magnification x360 or x600.

2.6.4 Double-labelled IFA:

Combinations of two mAbs with different epitope specificities and different isotypes were used to test for the presence of two or more populations of parasites within an isolate or to look at coexpression of two distinct epitopes. Each pair of mAbs was incubated (12µl of each) together on a well for 30 minutes. An RITC (rhodamine isothiocyanate)-conjugated and an FITC-conjugated antibody (Southern Biotechnology Associates Inc., Birmingham, Alabama; dilution 1:50), each specific for the different isotypes of the two mAbs, were then incubated together on the well for the second stage for 30 minutes and carefully removed with a Pasteur pipette. After 2 washes (1 & 5 minutes) in PBS, parasite nuclei were stained with DAPI for 1 minute. Slides were washed twice (1 & 5 minutes) in PBS and mounted under a coverslip in 2-3 drops of Citifluor.
RITC-fluorescence (red) was visualised using incident light 515-560nm.
The proportion of schizonts showing (i) green (and blue) fluorescence only, (ii) red (and blue) fluorescence only, (iii) red and green (and blue), and (iv) neither red nor green (blue only), was recorded for each pair of mAbs tested. Slides can be photographed and examples of staining patterns are shown in figure 2.4.

Figure 2.4: Double-labelled IFA slides of *P.falciparum* isolate from The Gambia, using DNA staining and differential labelling of MSP1-specific monoclonal antibodies. a) to c) show one microscopic field stained with: a) DAPI, b) mAb 9.5 (isotype IgG2b) and FITC-conjugated anti-IgG2b, c) mAb 10-2B (isotype IgG2a) and RITC-conjugated anti-IgG2a. In this field each of the schizonts gives a positive reaction with both mAbs.
d) to f) show a second field, stained with: d) DAPI, e) mAb 12.2 (isotype IgG1) and FITC-conjugated anti-IgG1, f) mAb 10-2B and RITC-conjugated anti-IgG2a. In this field, each schizont is positive for only one of the mAbs.
g) to j) show a third field stained with: g) DAPI, h) mAb 111.4 (isotype IgG1) and FITC-conjugated anti-IgG1, j) mAb 10-2B and RITC-conjugated anti-IgG2a. In this field, one of the schizonts gives a positive reaction with both mAbs, and the others are positive with one or other of the mAbs.
This photograph was reproduced with the kind permission of Dr David Conway.


A
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2.7 Mouse immunisations:

2.7.1 Preparation of antigen:

Recombinant MSP2 proteins were mixed in an equal volume of Freunds adjuvant (either incomplete or complete) to give a concentration of 50μg of rAg per mouse. This mixture was then emulsified with an equal volume of Tween 80 solution (1% v/v Tween 80 in PBS), which promotes the formation of oil-in-water emulsions. A Sorvall Omnimixer was used for mixing.

2.7.2 Immunisation protocol:

Mice were given a primary immunisation with 0.2ml of the antigen preparation intraperitoneally. The mice were boosted approximately 4 weeks after the first immunisation. Mice were bled retro-orbitally at least 2 weeks after each immunisation. The blood was allowed to clot, then the serum was separated, aliquoted and stored at -70°C.

Immunisations were performed by a licensed animal technician.

3. Optimisation of enzyme-linked immunosorbent assays (ELISA) for use in malaria serology

" There is plenty to challenge the intellect in this apparently simple method" D.M Kemeny 1991 [172]

3.1 Introduction

The introduction of enzyme labels in immunoassays by Engvall and Perlmann in 1971 [98, 99], represented a significant technical advance in diagnostic and serological assays. Since then, the enzyme-linked immunosorbent assay (ELISA) has been widely used for the assay of infection-specific antibodies and antigens [334, 336]. The technique has been shown to have an important role in the laboratory and in field studies, however, the scope of the assay is limited. Because the assay is quick and inexpensive it is widely used, but the data it generates is not always properly interpreted. Thus, it is essential to optimise the assay for its particular purpose.

3.1.1 The indirect ELISA:

In the indirect ELISA [98-100, 173, 175, 331, 333-336], antigen is immobilised by passive adsorption onto a solid phase. The antigen solid phase is then used to bind specific antibody in the test sample. Unbound material is removed by washing and bound antibody is detected with an enzyme-labelled anti-immunoglobulin antibody (figure 3.1a). When it is not possible to label this secondary antibody directly, an anti-species enzyme-labelled immunoglobulin can be employed (figure 3.1b).

In a direct ELISA, the antibody in the test sample is conjugated to the enzyme. This type of assay is used more for antigen detection than for antibody analysis.

Inclusion of the additional steps leads to amplification of the reaction and therefore, increases sensitivity. However, there is the possible hazard that each additional step may result in a loss of specificity.

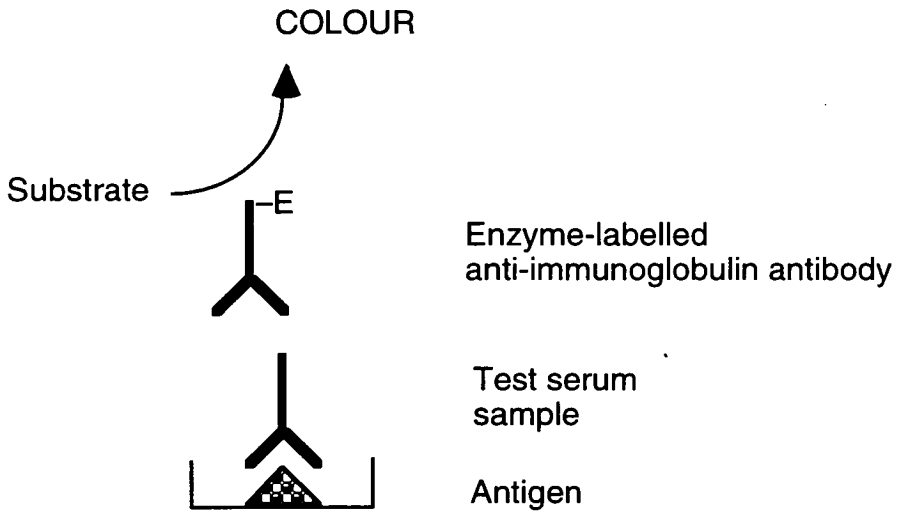


Figure 3.1a: Schematic representation of the standard indirect ELISA

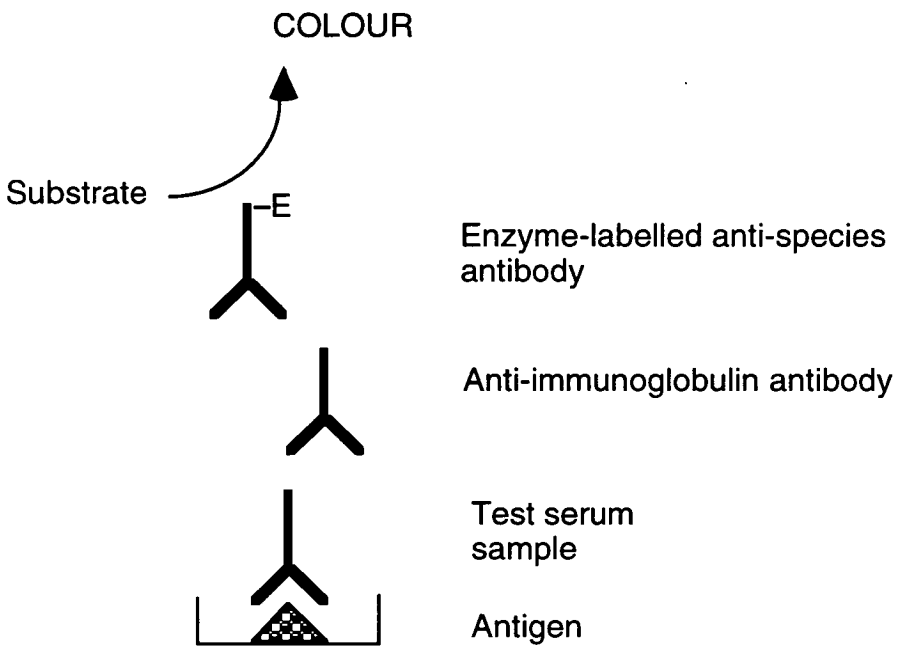


Figure 3.1b: Schematic representation of a two-step indirect ELISA

Bound antibody is visualised by the addition of an appropriate substrate and chromagen. The resulting colour change is detected using a spectrophotometer which measures the amount of light absorbed by a solution at a particular wavelength and converts this to an optical density (OD) or absorbance value. Absorbance is defined as the logarithm of the ratio of incident to transmitted light (Beer-Lambert Law) [117].

3.2 Principles of assay optimisation:

It is necessary to ascertain the optimum conditions for each stage of the assay [173, 175, 334, 336], the objectives being robustness, reliability and specificity with maximum sensitivity [175, 188].

3.2.1. Antigen concentration:

The optimal antigen concentration is the least quantity giving near-maximal binding [175]. If the capacity of the plate is exceeded, some of the protein will be weakly bound and become detached during the assay [172-175] or it can lead to overcrowding and concealment of epitopes [331], with subsequent loss of sensitivity and poor reproducibility.

3.2.2. Antibody concentration:

In an ELISA there are two components of the binding of antibodies in serum - non-specific binding to exposed sites on the plate or to antigen [176], and specific binding to epitopes on the antigen [331]. The aim of optimising the assay is to minimise non-specific binding and maximise specific binding. Non-specific binding can be reduced by blocking vacant sites with protein such as dried milk or bovine serum albumin [175]. However, care must be taken to choose a protein which is not recognised by naturally occurring antibodies.

The choice of serum concentration depends on the assay procedure.

i) End-point titration - all sera can be titrated by serial dilution. The end-point may be defined as the titre at which the positive sample dilution equilibrates to a negative sample [331]. Alternatively, a specific absorbance value may be chosen and the dilution of serum yielding such an absorbance value is the "titre" [336]. This procedure involves more plates, reagents and time than a single dilution assay.

ii) Single-dilution assay - single-dilution assays are more economical and thus are suitable for large scale serological screening. Under carefully controlled conditions, the results may be expressed in absorbance values [336]. In choosing an appropriate single dilution, sera are titrated and a dilution on the steep or linear part of the curve is chosen, such that the background absorbance is minimal. It is possible to relate the absorbance value from a single-point dilution to a standard curve derived from a reference serum [336].

3.2.3. Antibody detection:

In optimising the use of anti-immunoglobulin antibodies, specificity and sensitivity must be considered [159, 288]. The secondary antibody should be specific for the stated isotype and should not cross-react. The selected concentration must be sufficient to detect all specific antibody binding [331]. The concentration chosen is determined by reagent economy, obtaining a steep titration curve and minimising non-specific background [173, 331].

An ideal assay for the measurement of antigen-specific IgG subclass antibodies should possess the additional performance characteristic of equipotency [87]. Equipotency must be assured if one is to compare antibody titres or optical densities (OD) measured for different IgG subclasses. The aim is that similar concentrations of antibodies of different IgG subclasses produce the same signal. This is not easily achieved in IgG subclass assays because the anti-subclass antibodies have different affinities. To compensate for this, different concentrations of anti-subclass antibody are used to ensure equal signals [87]. However, there is a need to maintain equipotency over the whole analytical range, therefore titration curves must be parallel [138, 172, 188].

3.2.4 Choice of conjugate:

The conjugated enzyme and its substrate are chosen for sensitivity and convenience [331]. The relative merits of horse-radish peroxidase (HRP), alkaline phosphatase (AP) and ß-galactosidase (ß-gal) have been compared [247]. The marker enzyme of choice is HRP as it was shown to have higher specific enzyme activity as well as immunological reactivity [247, 248]. The chromogenic hydrogen donors of choice for peroxidase depend on the system [188]; o-phenylenediamine (OPD) is one of the most satisfactory for ELISAs [175, 188].

3.2.5. Data analysis:

There is no generally accepted way of expressing ELISA results; the methods for interpreting results from ELISA are without definition and are considered to be completely arbitrary [140]. A major problem in dealing with ELISA results is overinterpretation of the data and drawing quantitative results when only semi-quantitative or qualitative results are possible [331]. Detection of specific antibodies is much easier than their quantification [248, 334] and there is insufficient understanding of absorbance values among ELISA users [188].

Qualitative information on the presence or absence of antibody can be obtained by statistical comparison of ODs from test and control sera [138, 140, 173, 331, 334, 336]. However, the determination of a baseline is complicated and there is no standard protocol. Several approaches are employed for dichotomising results [85, 136, 140, 188, 309]. The cut-off value may be set at a fixed OD, for example 0.150 or 0.200 [136], however this is purely arbitrary. Alternatively, the cut-off may be based on the OD values of a population of serologically negative sera tested in parallel, such as two or three times the mean OD for the controls, or the mean plus two or three standard deviations (SD) [85, 188]. To reduce expenditure and time involved in establishing a cut-off value, Van Loon [330] calculated the mean and SD of a large group of normal sera and, in parallel, tested a reference serum. They observed the mean plus three SD equalled 40% of the absorbance of the reference serum. This reference was included in subsequent tests as an internal standard to define the cut-off level.

Whichever method is used, a compromise is generally necessary since, irrespective of the cut-off value, false negatives or positives will occur [188].

To avoid this, it has been suggested that intermediate results be classified as "doubtful" [140].

Semi-quantitative methods include expressing results as an end-point titre. This value is equivalent to the reciprocal dilution at which the test sample gives an OD the same as the reference negative sample [188, 334, 336]. A problem in determining the end-point titre is that the discrimination value cuts the dose-response curve in the tail of the sigmoidal curve and, consequently, low accuracy is obtained [188]. The effective-dose method [188] circumvents this problem by measuring the Antibody curves in the linear region (at maximum sensitivity) and to compare these results with a positive reference serum (figure 3.2). The result is then expressed as the logarithm of the difference in dilution of the two sera at the steepest part of the dose-response curves.

Results can also be expressed as the ratio of the OD of the test sample to a group of known reference negative samples [334]. However, this can give highly variable results because low OD values are highly susceptible to variation. Similarly, results can be expressed as the percentile probability of the OD of the test sample being within the range of a predetermined "normal" population [334].

It may be that the analyst wants to know "how much" antibody is present. However, the ability of ELISAs to measure absolute concentrations is questionable. An indirect ELISA measures antibody binding which is a function of both antibody concentration and antibody affinity [248, 331]. Furthermore, the relationship between antibody binding and colour generated is not linear [173, 175, 331], and may vary from day-to day [172, 331]. It is important to realise that antibody affinity may play a critical role in determining biological activities of antibodies [85] and may significantly influence ELISA results [43, 173, 248, 313]. In a single dilution assay, a serum sample with high affinity antibody present in low concentration may give the same binding (and OD) as a serum sample with low affinity antibody present in high concentration. End point titration curves would however differentiate the two sera (figure 3.3).

Nonetheless, ELISA results are often expressed as absolute concentrations. Results are determined by reference to a standard curve prepared by plotting OD values obtained from a series of dilutions of the reference standard [100, 173]. The basic assumption underlying the use of a standard reference curve is that the samples being compared are

essentially similar, i.e. that test and reference samples produce parallel dose-response curves [138, 172, 173, 188]; these criteria are rarely tested or fulfilled. Affinity may influence the slope of the linear part of the curve; high affinity antibodies give a steep curve and low affinity antibodies give a more gradual curve (figure 3.3). The composition of the test sample may differ from that of the reference serum [175]. For polyclonal sera, the relative amounts of high and low affinity antibodies will also influence the shape of the curve [85, 173]. Thus, unless test and reference samples are highly homogeneous, a standard indirect ELISA does not give absolute quantitative information [85, 331]. The best analysis is some sort of comparative evaluation, bearing in mind that a doubling in OD value does not necessarily mean a doubling in antibody binding .

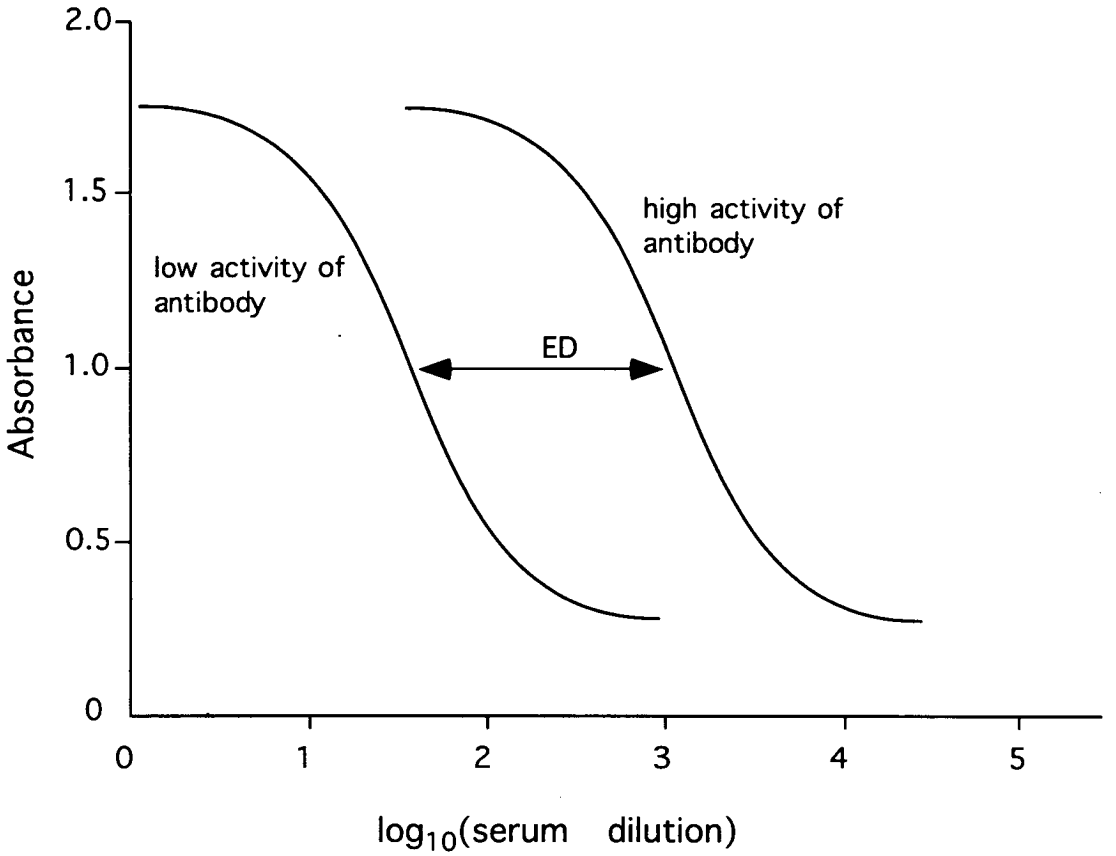


Figure 3.2: The determination of the effective dose (ED). The optical density of a test serum was measured over a range of dilutions and compared with a reference serum of high activity at the steepest part of the dose-response curves. The difference in dilution of the sera to obtain the same optical density is expressed in Brigg's logs [188].

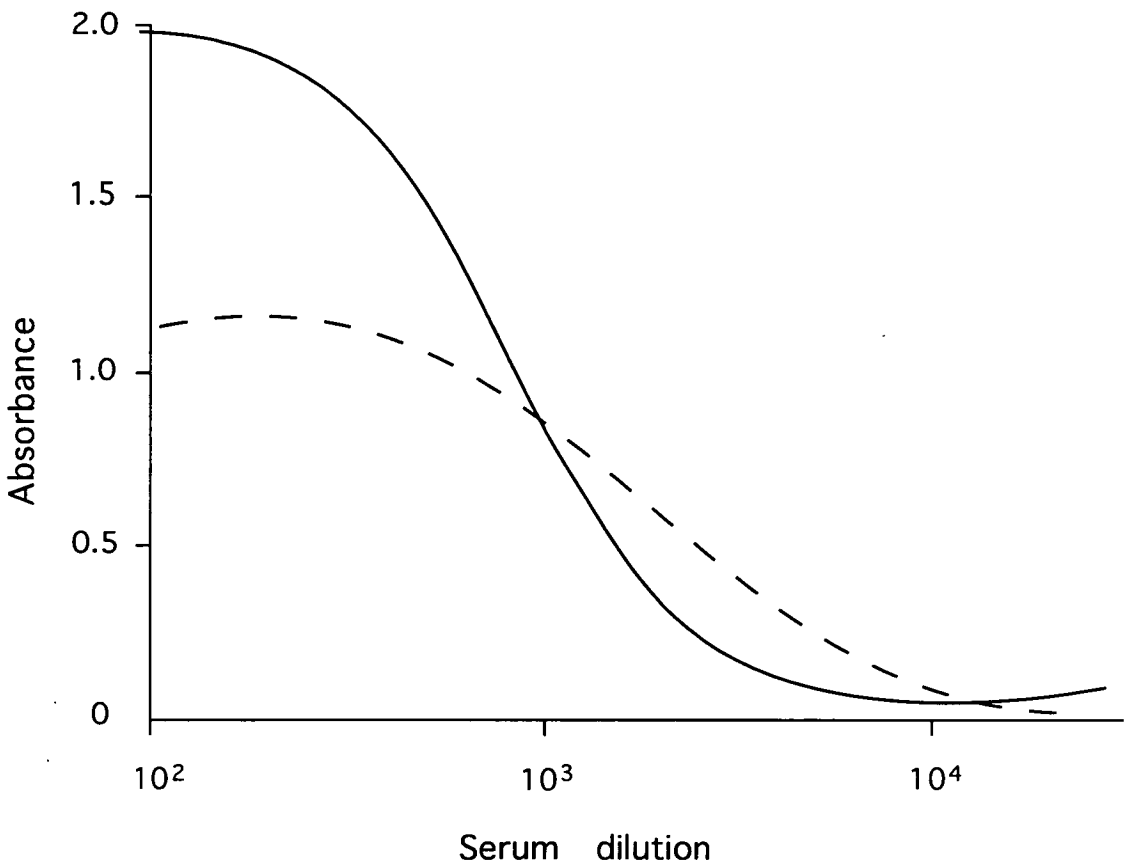


Figure 3.3: Comparison of dose-response curves obtained for antibodies of different affinities. Solid line shows the curve for a serum of high affinity; dotted line shows the curve for a serum with low affinity antibodies. At a dilution of 10^3, both sera give the same absorbance value.

3.3 Optimisation of the ELISA for assessment of human antibody responses to MSP2 using recombinant GST fusion proteins:

GST fusion proteins were coated onto solid phase, incubated with human sera and bound antibody was detected with HRP-conjugated rabbit anti-human IgG and OPD. Each step was optimised for maximum sensitivity and specificity.

3.3.1 Solid phase:

Immulon® 4 96-well microtitre plates (Dynatech Laboratories, Inc.) were selected for their ability to bind high levels of protein in a reproducible manner and provide high optical clarity. This grade of plate is specially formulated for maximum protein adsorption.

3.3.2 Reagent concentrations:

Optimal concentrations of antigen and serum were determined by chequerboard titration [175, 334, 336]. Serial four-fold dilutions of serum from malaria-exposed Gambian adults were tested for recognition of varying concentrations of recombinant antigen.

i) Antigen

Initially a wide range of antigen concentrations was tested. Overlapping titration curves were then obtained for concentrations of antigen between 5 and 0.5μg/ml (figure 3.4a). Therefore, 0.5 μg/ml was chosen to be the coating concentration of antigen.

ii) Serum

A serum dilution of 1:1000 was selected to be the optimum dilution. For positive sera, this gave an OD value on the linear part of the titration curve and non-specific binding to the GST control was low (figure 3.4b).

iii) Detecting antibodies

1. Rabbit anti-human IgG:

For the determination of specific IgG, a peroxidase-conjugated rabbit anti-human IgG specific for gamma chains (DAKO) was used. The antibody was tested at four dilutions - 1:3000, 1:6000, 1:9000 and 1:12000.

At a dilution of 1:3000, background OD values were unacceptable and OD values greater than 2.000 were produced for test samples. When the conjugate was diluted 1:12000, there was insufficient to detect all the specific antibody bound.

The results obtained for 1:6000 and 1:9000 dilutions were similar. A dilution of 1:9000 was selected for subsequent assays in order to keep the background OD values low and thus increase the sensitivity of the assay.

2. IgG subclass-specific reagents:

For the detection of IgG subclasses, murine mAbs to specific human IgG subclasses were used, followed by an HRP-conjugated rabbit anti-mouse Ig antibody. A variety of commercially available IgG subclass-specific reagents were tested; many of these were unsuitable because of cross-reactivity or low sensitivity. The subclass specificity of the mAbs finally chosen has been widely reported [161, 162, 194] and was reconfirmed prior to their use in our assay system: ELISA plates were coated with purified myeloma proteins of each human IgG subclass and then incubated with each of the murine mAbs. Each mAb was shown to be specific for the appropriate subclass. In addition, the HRP-conjugated rabbit anti-mouse Ig antibody was shown not to cross-react with the human antibodies.

In order to determine the optimal working concentration of the murine mAbs, plates were coated with four-fold serial dilutions of purified myeloma proteins from 10μg/ml and mAbs were tested over a range of concentrations.

For each mAb a working dilution was selected such that parallel titration curves were obtained for the different mAbs and these curves overlapped, such that the relationship between OD and IgG concentration was approximately equal for each subclass (figure 3.5). This was achieved for the mAb to IgG1, IgG3 and IgG4 , however, the mAb to IgG2 was known to be less sensitive [104, 162]. The mAbs finally selected, their source and working concentration are shown in table 3.1.

Subclass specificity	Clone number	Supplier	Working dilution
1	NL16/Hp6012	BDH	1:2000
2	GOM1/HP6008	BDH	1:500
3	HP6050	SEROTEC	1:1000
4	RJ4/HP6011	BDH	1:500

Table 3.1: Details of murine monoclonal antibodies to human IgG subclasses.

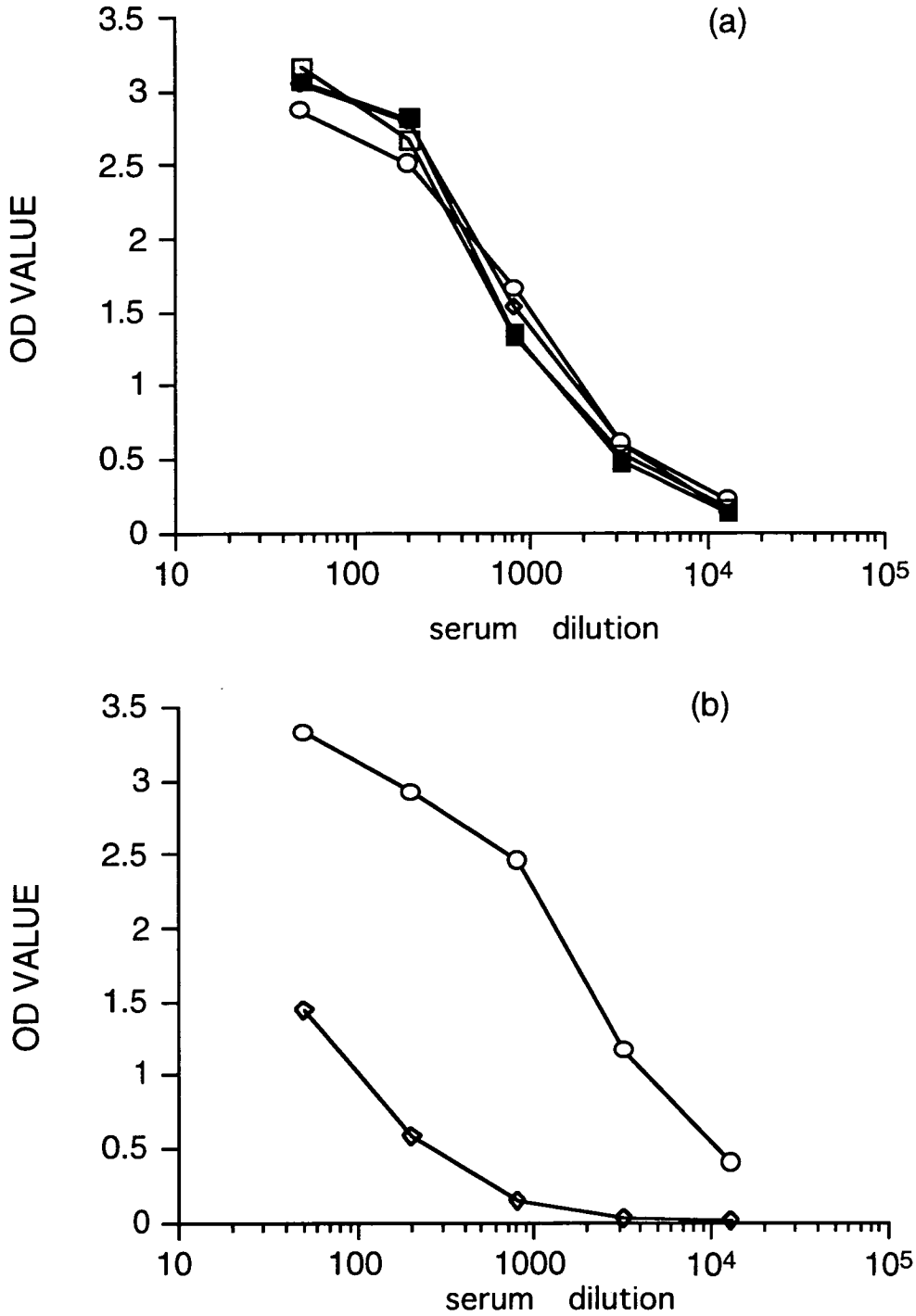


Figure 3.4a: Optimisation of A1 recombinant antigen concentration. Serum was serially diluted from 1:50 to 1:12800 against four concentrations of coating antigen.
◇ 0.5 µg/ml, ○1.5µg/ml, □3 µg/ml, ■ 5µg/ml.

Figure 3.4b: Optimisation of serum concentration. Serum was serially diluted from 1:50 to 1:12800 and tested for recognition of recombinant protein A1(○) and the GST control (◇).

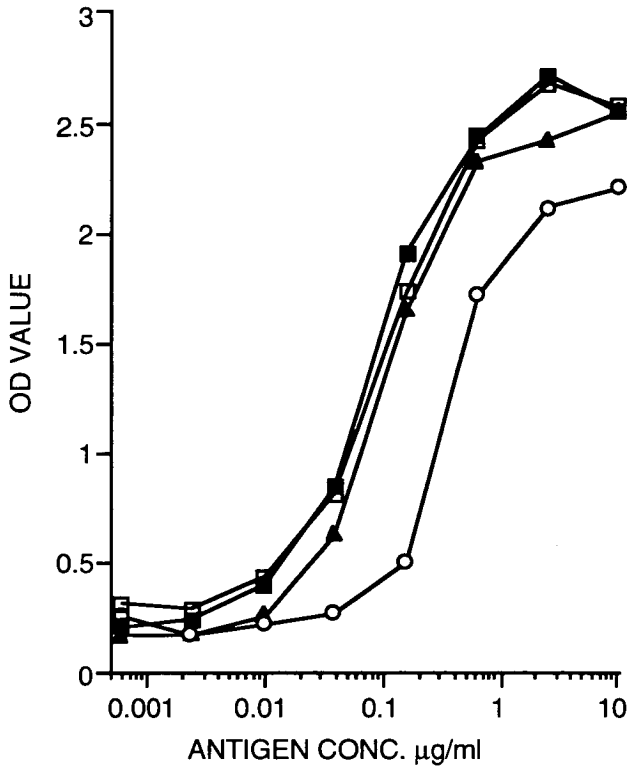


Figure 3.5: Optimisation of anti-human IgG subclass murine monoclonal antibodies. Plates were coated with purified myeloma proteins of each IgG subclass and incubated with the mAbs.

▲ IgG1, ○ IgG2, ■ IgG3, □ IgG4.

3.3.3 Data analysis:

ELISA plates were read at a wavelength of 492nm using an automated spectrophotometer (Titretek). OD values were transferred to a computer and analysed using the computer package ELISALITE.

Data is expressed as specific OD values; the OD value of the GST control antigen is subtracted from the OD of the GST fusion protein. Thus, specific OD values can be negative if the OD value for the GST control exceeds that for the fusion protein. The GST control plates are run in parallel with the MSP2 fusion protein plates.

Specific OD values obtained from malaria-unexposed European donors are used to establish a normal range for each antigen. Seropositivity is defined as a specific OD value greater than the mean plus two SD of the European control sera (95% confidence limit).

To avoid day-to-day variability of results, all the assays were designed such that a specific hypothesis was tested on one batch of plates on the same day.

3.4 ELISAs and malaria seroepidemiology

Voller *et al* [335] pioneered the application of ELISA in seroepidemiological studies of malaria and since then the method has been used extensively in studies of many parasitic diseases [332, 333]. In the first study on malaria, the tube or macro method was used with antigen prepared from *P.knowlesi*, a simian malaria parasite [338]. The microplate method was then developed [335], and *P.falciparum* antigen prepared from experimental infections of *Aotus* monkeys was used to compare seroreactivities of two Colombian populations. However, even at this early stage, the problem of standardisation was evident, particularly if comparable results were to be obtained at different times by different laboratories [335]. The source of antigen was the most variable factor [309, 335]. One result of the recent emphasis on vaccine development has been a much improved understanding of the antigenic make-up parasites, and this is having a significant impact on immunodiagnosis [332]. Expression of these antigens as recombinant proteins allows the determination of antibody binding to specific malaria antigens. Nevertheless, there is still no standardisation of assay design and data interpretation. Table 3.2 summarises the methods used for analysis of results obtained in

seroepidemiological studies of malaria using the indirect ELISA to measure IgG responses.

A major problem with some of these analyses is extrapolation to a standard curve and expression of the results in terms of concentration of antibody. This assumes parallelism of test and reference sera [188]. These studies are measuring the recognition of malaria antigens by polyclonal sera which will contain antibodies of differing affinities and are thus unlikely to produce parallel titration curves. In addition, these seroepidemiological studies are screening large numbers of sera and it is highly unlikely that each serum was tested to ensure parallelism, therefore such extrapolations are invalid. Furthermore, indirect ELISAs measure antibody binding - a function of both antibody concentration and affinity [331].
The most robust way to express the data is in a semi-quantitative manner, reporting the data in terms of OD values and defining those which are positive and negative, with minimal manipulation of the data. The choice of cut-off level depends on the relative distributions of the control and test data. If the control values represent a distinct set of values below the test samples, then a cut-off value outside the upper range of the control values would be suitable. The choice of cut-off value is particularly difficult when the test data is a continuum of values from negative to positive, and the control and test values overlap. The selection of the cut-off should be one which keeps false negatives and positives to a minimum. The upper limit of a 95% confidence interval is a common choice.

In this chapter, I have established the optimum conditions for the detection of MSP2-specific antibodies in ELISA. These conditions will be used throughout subsequent studies, unless otherwise stated.

ANTIGEN	DATA ANALYSIS	REFERENCE
Crude parasite antigen	Seropositivity - reciprocal titre of last dilution giving OD>0.3	Spencer *et al* 1979; 1981[307, 308]
	Seropositivity - OD > 0.2	Voller *et al* 1980[a] [337]
	Seropositivity - OD > 0.25	Wahlgren *et al* 1983 [340]
	Results expressed as OD values; cut-off not stated	Marsh *et al* 1988, 1989 [197, 200]
	Antibody conc. (μg/ml) determined by reference to myeloma protein SCs. Antibody concs. were corrected for amount of protein bound to plate.	Wahlgren *et al* 1983, 1986[c] [339, 343]
Pf155/RESA	% binding of high titred standard plasma. Antibody conc. of reference plasma determined from SC of purified IgG; limit of positivity - mean +2SD of control sera	Perlmann 1989 [235]; Björkmann 1990, 1991 [26, 27]; Peterson 1990 [240]
	As above; positivity determined as Antibody. conc. outside range of unexposed donors	Riley *et al* 1991[271]
	Seropositivity - OD > mean +2SD of negative controls	Beck *et al* 1995 [20]
MSP1	OD_{492} converted to units/ml by comparison with SC of high titred standard serum; seropositivity - units/ml > mean +2SD of controls	Riley *et al* 1992 [266]
	Assay was standardised using a set of positive and negative sera; cut-off = mean + 3SD of negative controls	Früh *et al* 1991 [115]
	Seropositivity - OD > mean +SD of negative controls	Egan *et al* 1995 [93]
	Seropositivity - OD > mean +2SD of negative controls	Riley *et al* 1993 [270]
	Seropositivity - OD > mean +3SD of negative controls	Tolle *et al* 1993 [324]
Pf155/RESA, CS, parasite extract	Seropositivity - OD > mean +3SD of negative controls	Quakyi *et al* 1989 [253]

MSP1, Ag5.1, aldolase MSP1, 41kDa rhoptry protein, parasite extract	Seropositivity - OD > mean +2SD or 3SD	Srivastava *et al* 1989 [309] Gabra *et al* 1986 [116]
MSP2	Extrapolation from SC of high titred serum pool. Highest conc. of positive control serum was assigned 1000 antibody units (AU); seropositivity AU > mean +2SD of AU of controls negative sera	Al-Yaman *et al* 1994 [2]
RESA	as above, except AU converted to µg/ml of IgG based on SC of known IgG standard. Positive > mean + 2SD of Antibody. conc. of controls	Al-Yaman *et al* 1995 [3]
RESA, CS (*P.f* & *P.m*)	Cut-off computed from the 95th percentile of ranked OD values for all serum tested against control antigen	Deloron *et al* 1989 [83]
GLURP	Normalised data with an OD value of 1.200 assigned to positive control serum. Cut-off determined as 99-percentile for reactivity of all sera with control antigen	Dziegel *et al* 1991[90]
PfHRP-2	Cut-off equals highest OD value of controls plus 0.05	Taylor *et al* 1993[b] [318]
Exoantigens	Adjustment of data according to a reference positive OD reading of 1.000, followed by subtraction of background value. Cut-off determined by mean + 3 SE of controls	Luty *et al* 1994[c] [196]

Table 3.2: Summary of analysis of results for the detection of IgG antibodies to malaria antigens in indirect ELISAs

a-Ig detection; b-capture ELISA; c- IgG subclass determination; SD- standard deviation; SC- standard curve; conc.- concentration

P.f and *P.m* denote *P.falciparum* and *P.malariae* respectively

4. Characterisation of the immunogenicity and antigenicity of recombinant MSP2 proteins.

4.1 Introduction:

In order to study the immune recognition of MSP2 it is necessary to isolate and purify the protein in a form suitable for serological analysis. Isolation and purification of native MSP2 is impractical as it would require substantial amounts of parasite material and MSP2-specific mAb. An alternative is to express MSP2 as a recombinant polypeptide.

Until 1987 the expression-purification systems available in *E.coli* required the use of denaturing reagents during purification, and it seemed likely that these might interfere with the immunogenicity and activity of expressed protein [294]. Smith *et al* [296] found that a glutathione S-transferase (GST) from the helminth *Schistosoma japonicum* could be expressed as COOH-terminal fusions with foreign polypeptide. It has been shown that a variety of polypeptides can be expressed in *E.coli* as soluble GST fusion proteins and could be purified by absorption of crude cell lysates to glutathione-agarose under physiological conditions. The pGEX vectors were designed such that the GST component could be removed by treatment with site-specific protease thrombin or factor Xa [295]. Since the introduction of the pGEX vector, several refinements and modifications have been made to the original protocol (reviewed by [294]) and pGEX vectors have been used to generate polypeptides for biological, structural and biochemical analyses.

Various recombinant MSP2 proteins have been produced by other groups. Thomas *et al* [321] expressed the central region of CAMP MSP2 using the vector pMG42Kn. Immune monkey serum and human serum recognised the rAg in immunoblots. Smythe *et al* [299] produced a rAg representing the full length molecule of FC27 MSP2, using the pGEX expression system. Anti-serum to the rAg, raised in rabbits, reacted in immunoblots with MSP2 of FC27 parasite extracts. Rzepczyk *et al* [279] used a recombinant MSP2 protein in ELISA to measure the immune recognition of serogroup B in malaria-exposed Melanesian donors and found that most individuals had MSP2 specific antibodies. Recently Al-Yaman *et al* [2] reported the use of three MSP2 rAgs in seroepidemiological studies in Papua New Guinea. The rAgs were produced using the plasmid vector pDS56/RBSII,6xHis and expressed in *E.coli*. Analysis of the purified proteins showed in each case a

dominant single band that reacted on Western blots with rabbit antibodies to MSP2.

These studies indicate that rMSP2 expressed in bacterial systems may be appropriate for use in epidemiological studies. Nonetheless, it is critical to ensure that our panel of MSP2 rAgs mimicked the native antigen. For example, the tertiary structure of $PfMSP1_{19}$ has been shown to be essential for immune recognition [93] [38]. $PfMSP1_{19}$ has been expressed as a GST-fusion protein in *E.coli* and studies with a panel of mAbs confirmed its structural integrity and antigenicity. The fusion protein contained epitopes that were conformation-sensitive and in this respect exhibited the same properties as the natural antigen [38].

In this chapter, I report the results of the validation of the structural integrity and antigenicity of recombinant MSP2 antigens. We have studied the immune recognition of the rAgs by a panel of MSP2-specific mAbs in ELISA. Also we have raised MSP2-specific antisera in mice and tested the specificity of these antibodies for rAg and native protein in ELISA, IFA and immunoblotting experiments.

4.2 Materials and methods:

4.2.1.Mouse immunisations:

Recombinant antigens were prepared as described in Materials and Methods (chapter 2, section 2.7.1).

Two immunisation protocols were used.

a) Groups of 5-8 female, 8 week old, Balb/C mice were immunised intraperitoneally and boosted after 4 weeks, with 50μg/mouse and 25μg/mouse respectively of fusion proteins in Freunds incomplete adjuvant. Sera for antibody determination were obtained 3-4 weeks after each immunisation.

These immunisations were performed by Dr J McBride and she kindly provided access to the sera for serological analysis.

b) Groups of 4-5 female, 8 week old, CBA and MF1 mice were immunised intraperitoneally with up to 50μg/mouse of fusion protein in Freunds complete adjuvant and challenged after 4 weeks with 25μg/mouse of fusion protein in Freunds incomplete adjuvant. Sera for antibody determination were obtained 3-4 weeks after each immunisation.

These immunisations were performed by either K Samuel or Dr E Riley.

Recombinant antigens used for these immunisations are listed in table 4.2.

4.2.2. Monoclonal antibodies

Monoclonal antibodies specific for MSP2 were used to probe recombinant MSP2 antigens in ELISA. The mAbs included; 12.3-2-2, 12.5-1-2, 12.7, and 13.4 [55, 109, 110, 207], 8-5D and 4-4F [310] specific for group A and 8G10/48 & 8F6/49 [283] specific for group B [109]. The specificities of these mAbs are shown in table 4.1 and Appendix 1B.

mAb	Serogroup	Isolate	Epitope	Reference
12.3, 12.5, 12.7	A	T9/96	Group specific	Fenton *et al* 1991 [109]
13.4	A	T9/94	$(GSAG)_n$	Clark *et al* 1989 [55]
4-4F/8-5D	A	FVO		Stanley *et al* 1985 [310]
8G10/48	B	FC27	STNS (R1 repeats)	Epping *et al* 1988 [101]
8F6/49	B	FC27	DTPTATE (gp. spf.)	Ramasamy *et al* 1990 [255]

Table 4.1: MSP2 specific monoclonal antibodies. Hybridomas were raised against schizonts of *P.falciparum* isolates. The specificity of these mAbs is indicated by MSP2 serogroup and the amino acid sequence of the epitope or the region of the molecule which is thought to contain the epitope.

4.2.3 Western blotting of *Plasmodium falciparum* schizont extracts:

Schizonts from 3D7 and Dd2 isolates of *P.falciparum* were boiled for 10 minutes in 10% SDS sample buffer and centrifuged. Samples of the soluble fraction were loaded onto SDS-PAGE under non-reducing conditions.

The samples were electrophoretically transferred to nitrocellulose as described in Materials and Methods (chapter 2, section 2.3). The nitrocellulose was probed with mouse sera (diluted 1:200 in blocking buffer) or mAb (diluted 1:100 in blocking buffer) specific for MSP2 and these antibodies were detected using HRP-conjugated anti-mouse Ig. Bands were visualised using 4-chloro-1-napthol as the substrate.

4.2.4 Immunofluorescence assay:

Sera from mice immunised with MSP2 recombinant proteins were tested using single-labelled IFA for recognition of *P.falciparum* schizonts. Sera were tested at 1:50, 1:200 and 1:800; dilutions were made in 1% BSA in PBS.

4.2.5 ELISA:

MSP2 specific antibody was measured in ELISA using HRP-conjugated rabbit anti-mouse Ig diluted 1:1000 in PBS/Tween.

Mouse sera was diluted 1:500 and serial five-fold dilutions were made. mAb was diluted 1:500 and serial four-fold dilutions were made.

Competition ELISAs:

Selected sera were preincubated with increasing concentrations of inhibiting antigen from 0 μg/ml upto 5μg/ml. Sera were diluted 1:20000.

4.2.6 Recombinant MSP2 proteins:

The rAgs used in this chapter are those described in Materials and Methods (chapter 2, section 2.1), plus variants of particular regions. The sequence differences of these variants have been described in table 4.2 and represented schematically in figure 4.1.

rAg	Isolate	Region	Repeat sequence
A1	T9/96 [a]	Full length	GAVAGSGA
A2	T9/96 [a]	R1 repeat	GAVAGSGA
	Thai Tn [a]		GASGRAGA
	7G8 [a]		GSAGGS
	T9/102		GSAGGS
	CH12/12	Part of C-terminal gp.spf. seq. deleted	GSAGGS
	RO33 [a]		GSAG
	T9/94 (i) [a]		GSAG
	T9/94 (ii)	N-terminal gp. spf. seq. deleted	GSAG
A3	RO33 [b]	R2 repeat/group specific	PSTPATPA$(T)_9$
	CH12/12		PSTPA$(T)_9$
	CH150/9 (i)	part of gp. spf. seq. deleted	PSTPA$(T)_6$
	CH150/9 (ii)		PSTPA$(T)_9$
	G1		PSTPA$(T)_6$
	T9/102	R2 repeats deleted	
B1	Dd2(13/14) [a]	Full length	3x32 mer, ?x12-mer*
	Dd2(5/6)	Shortened conserved regions	3x32-mer, 1x12-mer
B2	K1(5/3) [a]	R1 repeats	1x32-mer
	T9/105	Shortened C-terminus	1x32-mer
	K1 (13/3) [ab]	Shortened N-terminus	1x32-mer
B3	K1	R2 repeats/gp. specific	5x12-mer
	T9/105 (i) [b]		1x12-mer
	T9/105 (ii)	No repeats	
N	K1 [ab]	Conserved	
C	K1 [a]	Conserved	

Table 4.2: Recombinant MSP2 proteins. The isolates from which these proteins are derived, the region of MSP2 they represent and the sequence or number of repeats are shown.

[a] denotes mice immunised using protocol a); [b] denotes mice immunised using protocol b). * indicates that this sequence has not been fully elucidated. gp. denotes group; spf. denotes specific; seq. denotes sequence.

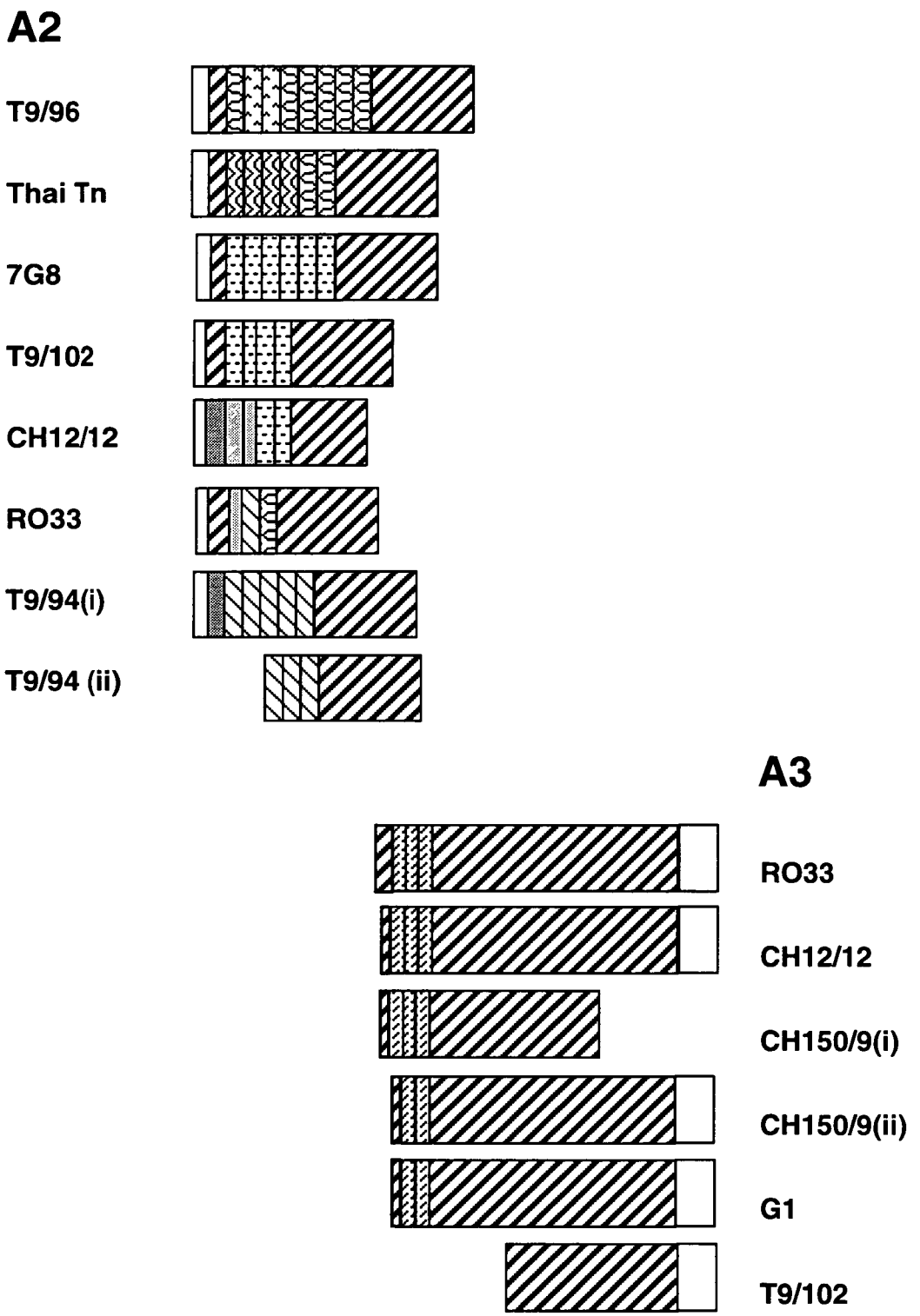


Figure 4.1a: Schematic representation of MSP2 serogroup A recombinant proteins, A2 and A3, indicating the isolates from which they were derived.

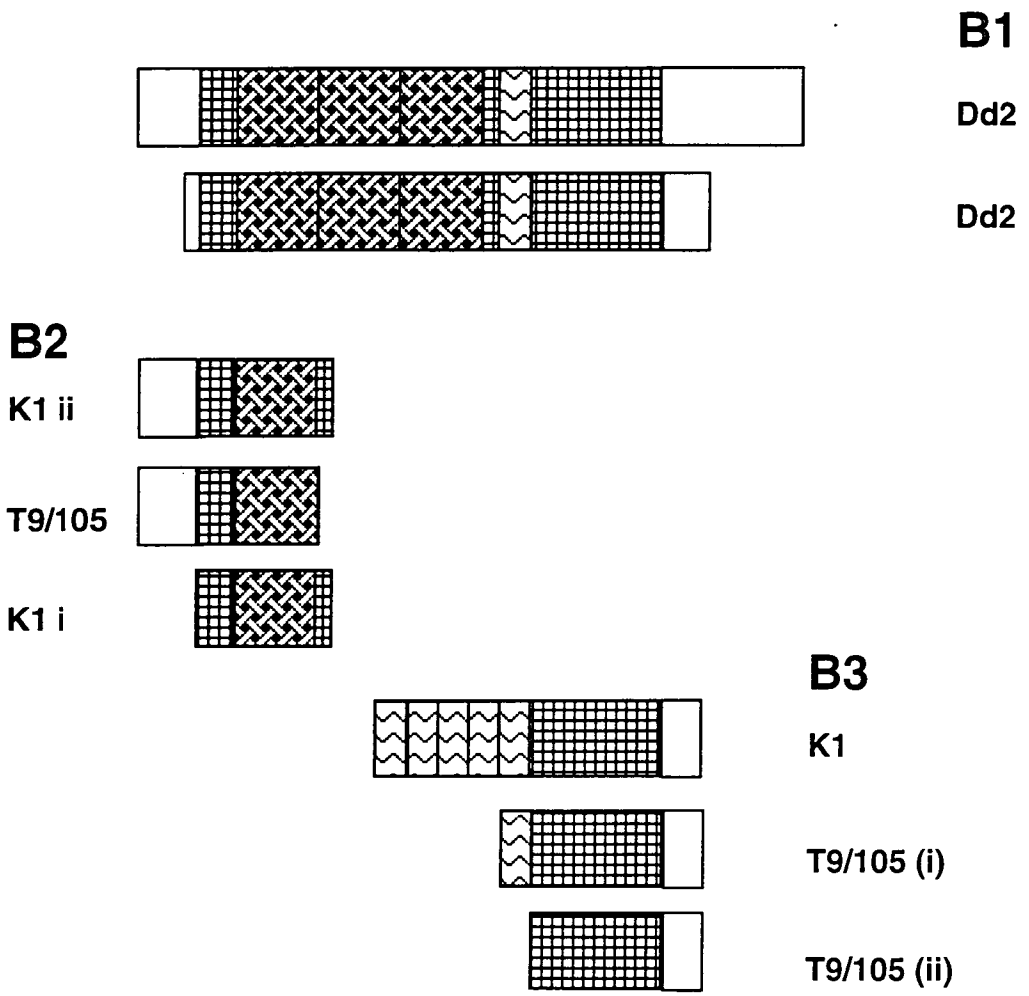


Figure 4.1b: Schematic representation of MSP2 of serogroup B recombinant proteins B2 and B3, and indicating the isolates from which they were derived.

KEY:

Serogroup A

- R1 repeats
- Serogroup A specific
- R2 repeats

Serogroup B

- R1 repeats
- Serogroup B specific
- R2 repeats

4.3 Results:

4.3.1 Recognition of recombinant MSP2 proteins by MSP2-specific monoclonal antibodies or mouse serum, in ELISA:

Monoclonal antibodies specific for MSP2 had been raised against native protein and recognised the surface of intact schizonts in IFA [55, 101, 109, 110, 207, 255, 283, 310], and for some of these mAbs epitopes have been characterised (table 4.1, appendix 1B). The mAbs were used in ELISA to probe rAgs to ensure that the rAgs contained native epitopes.

All the rAgs were recognised by the appropriate mAbs as predicted (table 4.3) with the exception of A2(CH12/12). This rAg was recognised by serogroup B-specific mAbs; there is no obvious explanation for this apparent cross-reactivity, as A2(CH12/12) does not have any sequence homology with the known epitopes of the mAbs.

In addition, we have shown differential recognition of the A3 constructs by mAbs 12.3, 12.5, and 12.7. Epitopes recognised by these mAbs are shared by group A variants and are associated with the less variable, group specific parts of the central region [109]. The use of this panel of A3 constructs has enabled us to elucidate possible epitopes of these mAbs. 12.7 seroreactivity is dependent on a group specific region which is lacking in A3(CH105/9(i)) (table 4.3). Furthermore, a natural deletion occurs in this region in some alleles, but this does not affect the reactivity of this mAb [109]. Thus, the epitope of 12.7 lies in the region between the end of the deleted sequence and the conserved C-terminus region. Whereas the epitope(s) of 12.3 and 12.5 lies in the group specific region of serogroup A, but the reactivity of these mAbs is not dependent on the R2 repeats as they recognise rAgs lacking these repeats.

Sera from mice immunised with MSP2 rAgs were tested for recognition of the immunising construct in ELISA. All the rAgs tested were shown to be immunogenic in mice, in that all sera recognised the immunising construct (table 4.4). In most mice, the antibody response was directed predominantly at the MSP2 epitopes of the rAg, rather than at the GST component (figure 4.2).

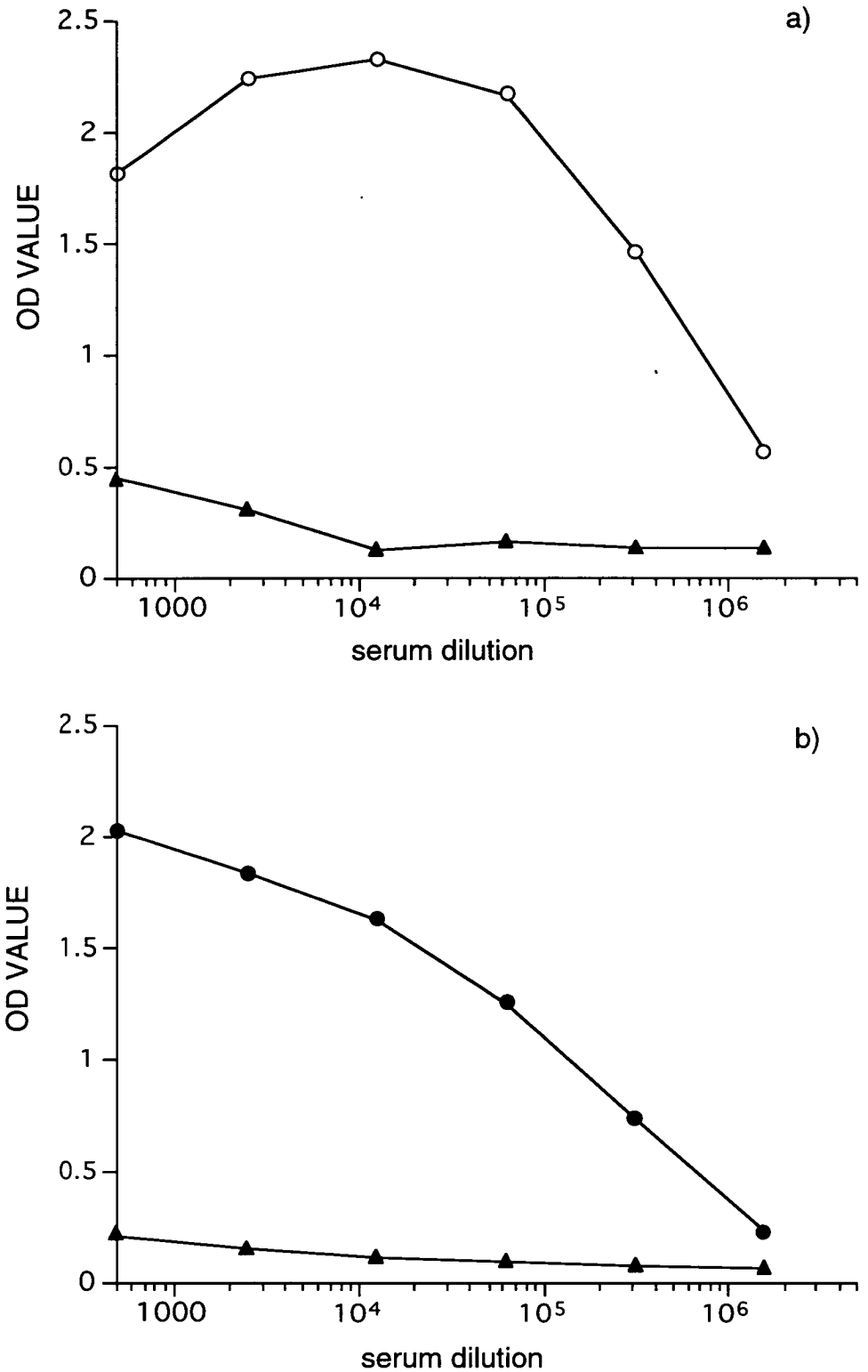


Figure 4.2: Immunisation with MSP2-GST fusion proteins induces antibodies which are MSP2-specific. Mice were immunised with recombinant proteins A2(ThaiTn) and A2(7G8), representing the R1 repeat region of MSP2 serogroup A from isolates ThaiTn (a) and 7G8 (b). The sera obtained were tested in ELISA for recognition of the immunising construct and GST. The titration curves are shown.
○- Thai Tn, ●- 7G8, ▲ - GST.

		mAb							
Antigen	**Isolate**	13.4	12.3	12.5	12.7	4-4F	8-5D	8G10/48	8F6/49
A1	T9/96	-	+++	+++	+++	-	-	-	-
A2	T9/96	-	-	-	-	-	-	-	-
	Thai Tn	-	-	-	-	-	-	-	-
	T9/94i	+++	-	-	-	+++	+++	-	-
	T9/94ii	+++	-	-	-	+++	+++	-	-
	7G8	-	-	-	-	+++	+++	-	-
	T9/102	-	-	-	-	+++	+++	-	-
	CH12/12	-	-	-	-	+++	+++	++	++
	RO33	-	-	-	-(+?)	+++	+++	-	-
A3	RO33	-	+++	+++	+++	-	-	-	-
	CH12/12	-	+++	+++	+++	-	-	-	-
	CH150/9 (i)	-	-	-	+++	-	-	-	-
	CH150/9 (ii)	-	+++	+++	+++	-	-	-	-
	G1	-	+++	+++	+++	-	-	-	-
	T9/102	-	+++	+++	+++	-	-	-	-
B1	Dd2	-	-	-	-	-	-	+++	++
	Dd2(short)	-	-	-	-	-	-	+++	++
B2	K1(5/3)	-	-	-	-	-	-	+++	++
	T9/105	-	-	-	-	-	-	+++	++
	K1 (13/3)	-	-	-	-	-	-	+++	++
B3	K1	-	-	-	-	-	-	-	++
	T9/105 (i)	-	-	-	-	-	-	-	++
	T9/105 (ii)	-	-	-	-	-	-	-	-
N	K1	-	-	-	-	-	-	-	-
C	K1	-	-	-	-	-	-	-	-

Table 4.3: Recognition of MSP2 recombinant antigens using MSP2-specific mAbs in ELISA. mAb specificities are given in table 4.1 and appendix 1B.

- denotes negative result; ++ and +++ indicate moderately and strongly positive results, respectively.

4.3.2. Recognition of native MSP2 by mouse antibodies raised against rMSP2:

The sera raised in mice were also tested for the recognition of native MSP2 by immunofluorescence of mature, schizont infected erythrocytes and by immunoblotting of SDS-soluble extracts of *P.falciparum* schizonts. In all cases, with the exception of mice immunised with N, the murine antibodies gave specific parasite surface fluorescence of homologous isolates (figure 4.3).

Sera were used to probe Western blots of schizont extracts from two *P.falciparum* clones 3D7 (MSP2 serogroup A) and Dd2 (MSP2 serogroup B). Sera from mice immunised with rAgs representing the full length molecule and the group specific regions, and B2-specific sera recognised the schizont extract (figure 4.4). However, not all the A2-specific sera recognised the 3D7 schizont extract. Recognition may depend on sequence homology of the repeats.

All positive reactions resulted in a single band of the expected molecular weight, as determined by recognition by mAbs.

Parasite-specific staining was not seen with pre-bleeds (naive mouse serum) under the same assay conditions.

4.3.3. Cross-reactivity of antibodies to serogroup A and B:

Antibodies to A1 were shown to cross-react with MSP2 serogroup B in IFA, Western blotting and ELISA, and vice-versa (table 4.4). These sera were seropositive for the conserved C-terminus but did not recognise the conserved N-terminus, indicating that cross-reactivity may be due predominantly to epitopes within the C-terminus (figure 4.5a & b). This was supported by the results of inhibition ELISAs. Sera from mice immunised with the full length constructs, A1 and B1, were preincubated with increasing concentrations of C (the C-terminal conserved region), and tested for recognition of A1 and B1 in ELISA (figure 4.5c & d). The inhibition curves show that preincubation of anti-A1 sera with C can inhibit the recognition of B1 and C; and vice versa. Thus, the cross recognition of the serogroups seems dependent on antibodies specific for the conserved C-terminus.

These results also show that recognition of the full length proteins by the mouse sera is not solely dependent on antibodies to the conserved C-

terminus, as preincubation with C does not appear to have a significant effect on the recognition of the same full length rAg.

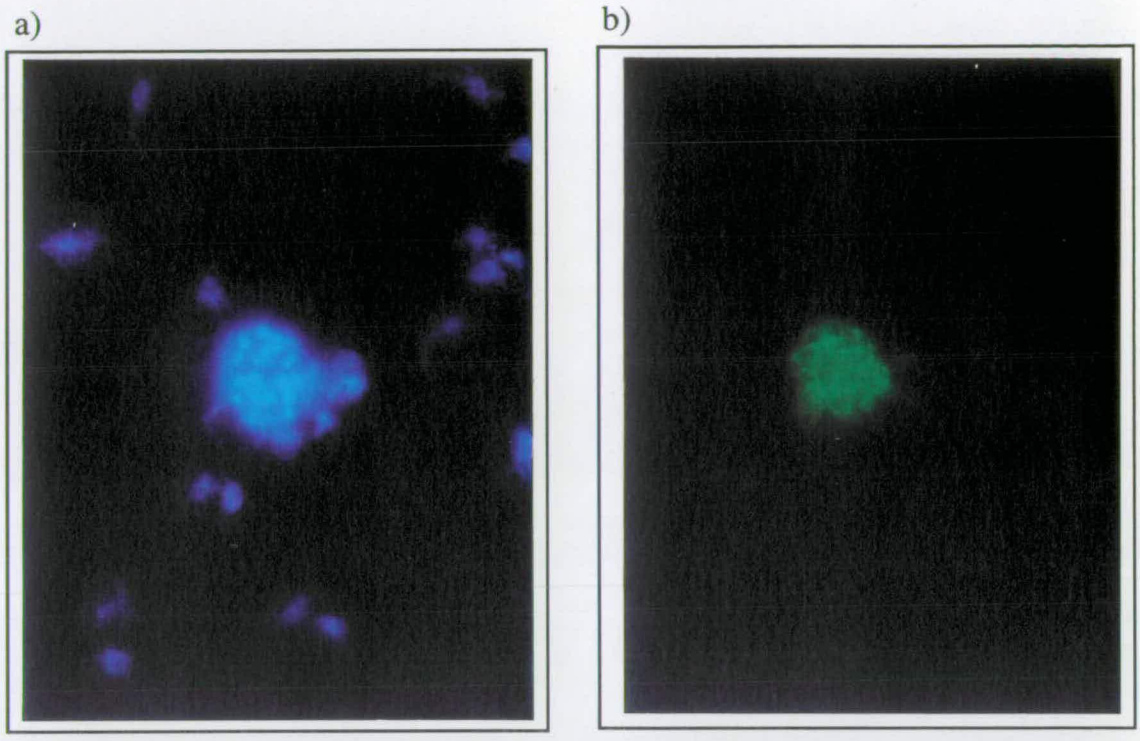
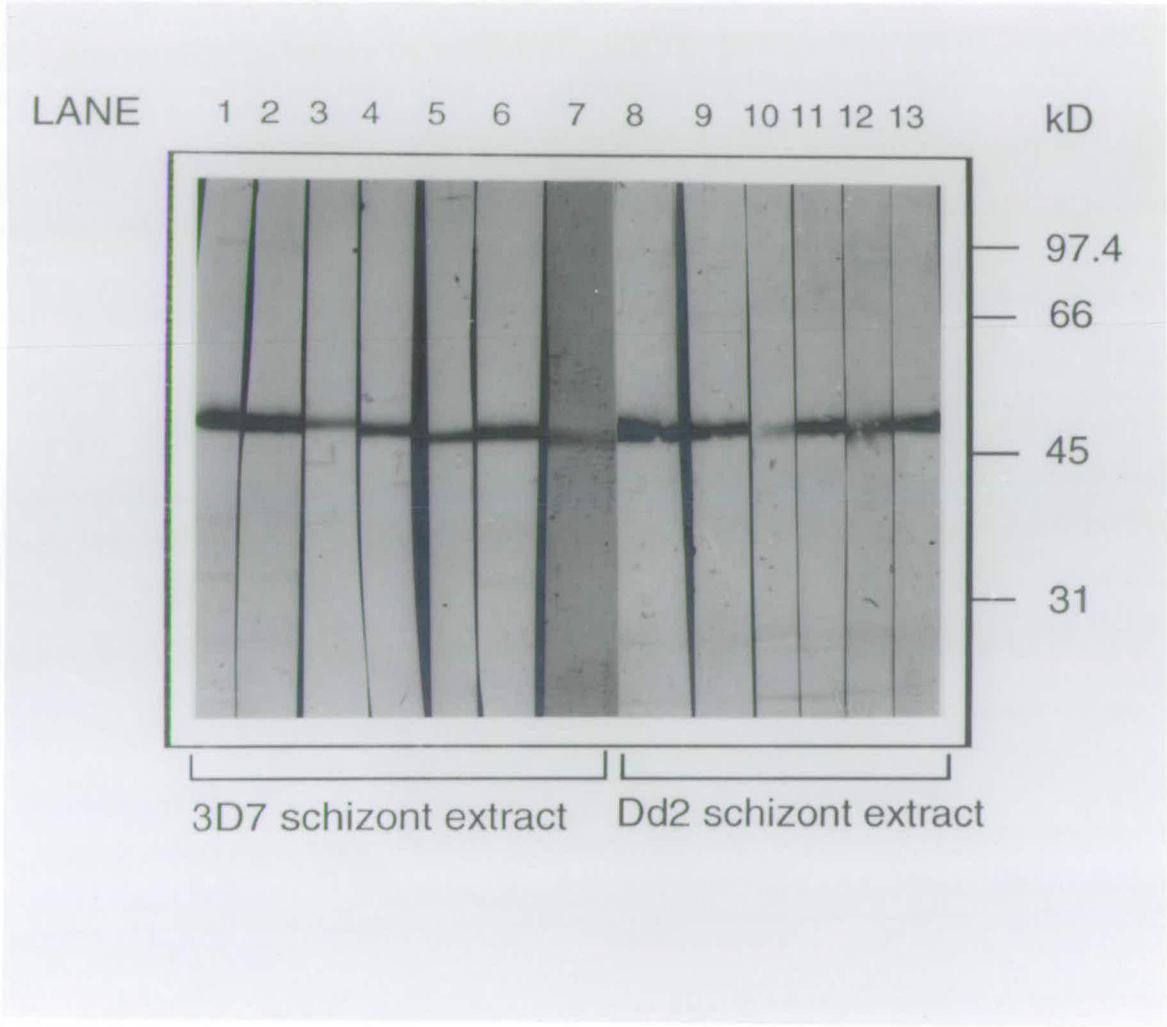


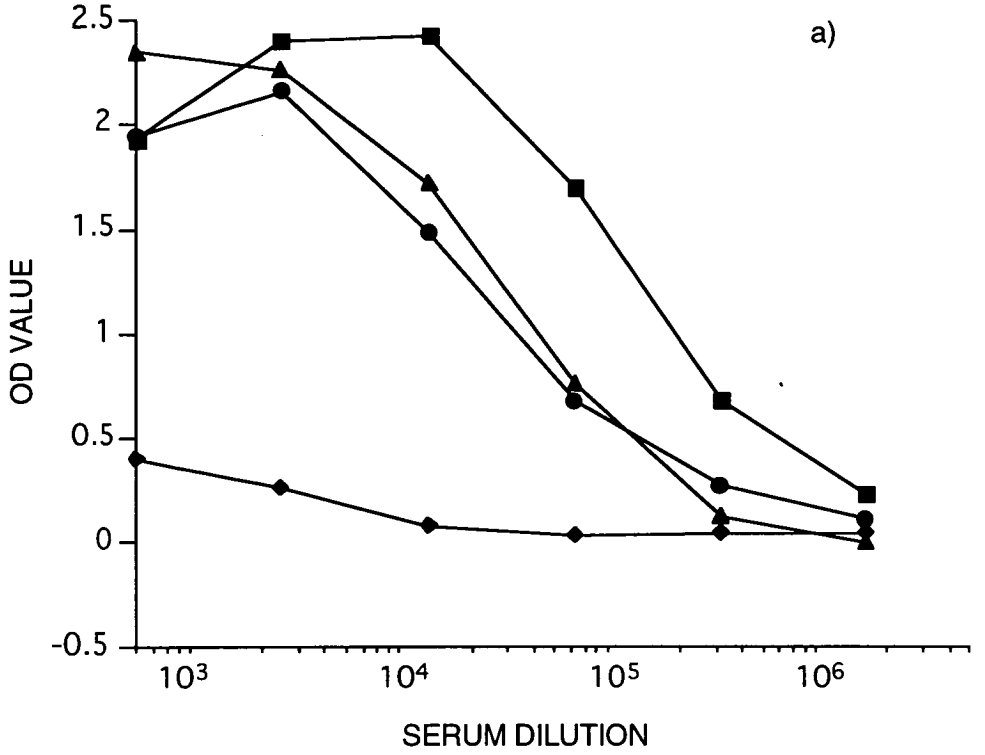
Figure 4.3: Photographs showing the double-stained fluorescence pattern of malaria parasites using serum from a mouse immunised with B2(K1i). a) DAPI stained nuclei; b) mouse anti-B2 serum plus anti-mouse Ig-FITC.

Figure 4.4: Western blot of schizont extracts from 3D7 (lanes 1-7) and Dd2 (lanes 8-13) *P.falicparum* isolates probed with sera from mice immunised with A1, B1 and C.
Lane 1,2,8,9: sera from mice immunised with A1 (full length serogroup A); lanes 3,4,10,11: sera from mice immunised with B1 (full length serogroup b); lanes 5,6,12,13: sera from mice immunised with C (conserved C terminus). Lane 7: mAb 8-5D.

Figure 4.5 (a-d): Cross-reactivity of antibodies to MSP2 serogroups A and B is due to recognition of epitopes within the C-terminal conserved region of the protein.
Mice were immunised with recombinant proteins representing the full length molecule of MSP2 serogroup A (a) or B (b). The sera produced were tested for recognition of A1, B1,N and C.
Inhibition curves obtained after preincubation of individual serum samples with the rAg representing the C-terminal conserved region are shown for the recognition of A1, B1 and C (c & d).

- A1 (full length serogroup A), ● - B1 (full length serogroup B), ◆ - N (N-terminal conserved region), ▲ - C (C-terminal conserved region)


LANE
1 2 3 4 5 6 7 8 9 10 11 12 13
kD
97.4
66
45
31
3D7 schizont extract
Dd2 schizont extract


a)
2.5
2
1.5
1
0.5
0
-0.5
OD VALUE
10^3
10^4
10^5
10^6
SERUM DILUTION

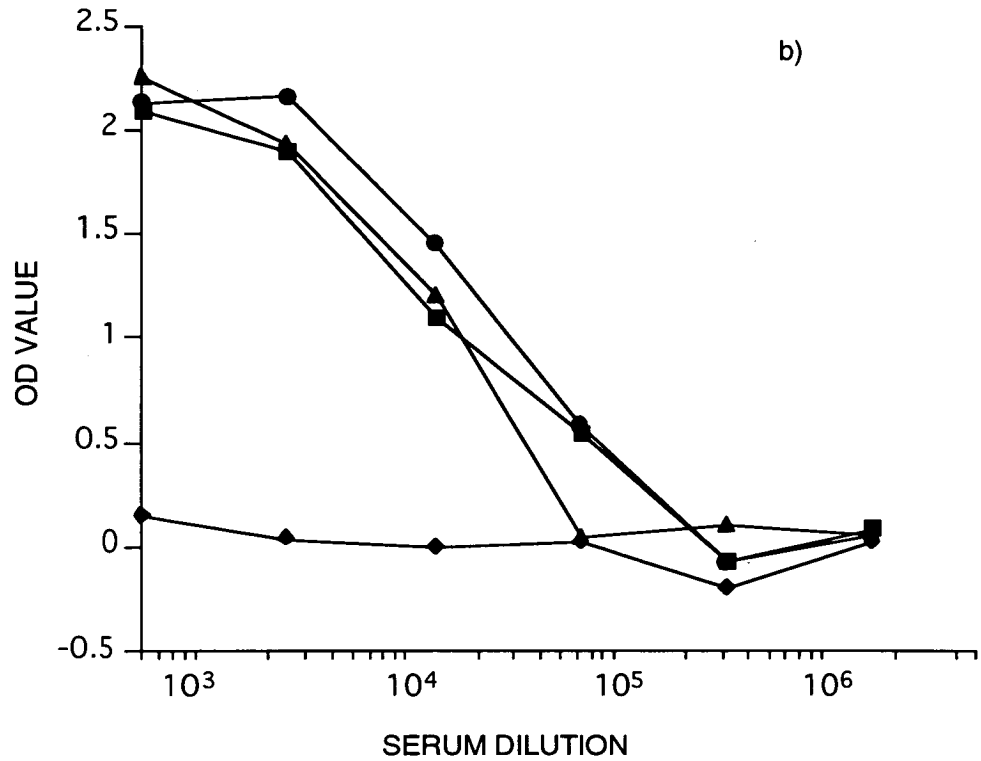
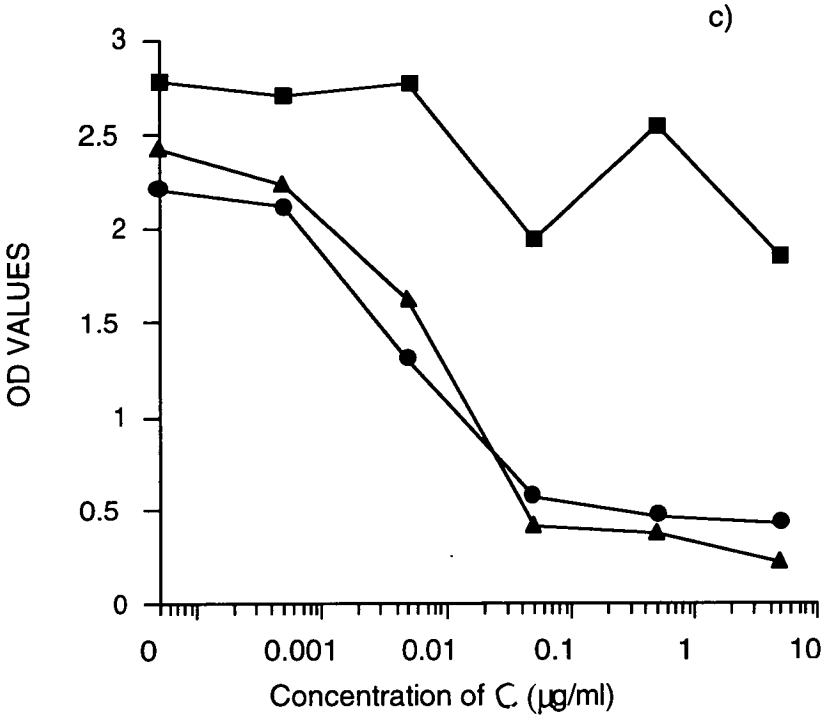

b)
2.5
2
1.5
1
0.5
0
-0.5
OD VALUE
10^3
10^4
10^5
10^6
SERUM DILUTION

Figure 4.5 a & b.


c)
OD VALUES
3
2.5
2
1.5
1
0.5
0
0
0.001
0.01
0.1
1
10
Concentration of C. (µg/ml)

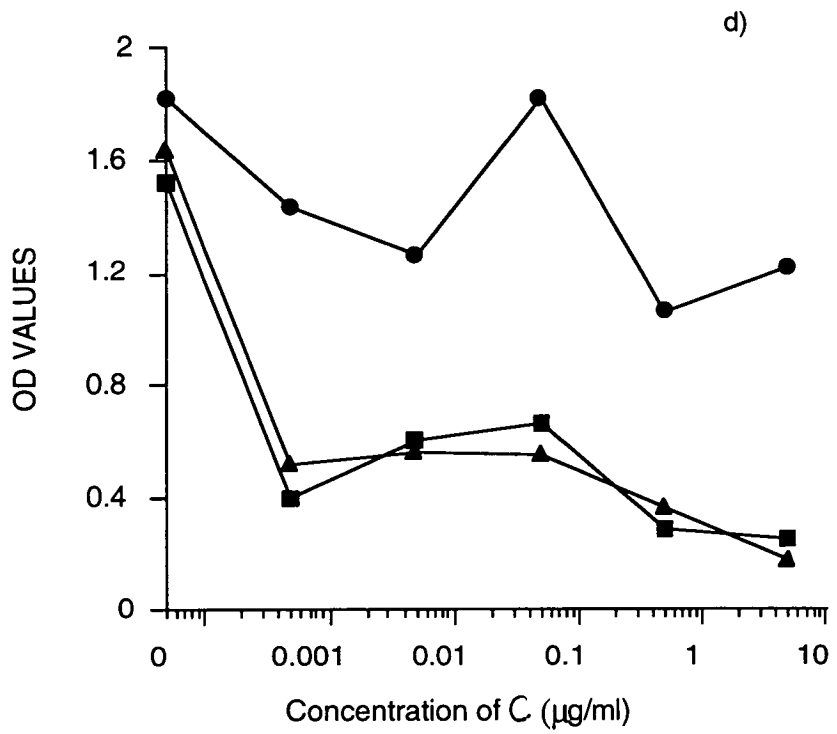

d)
OD VALUES
2
1.6
1.2
0.8
0.4
0
0
0.001
0.01
0.1
1
10
Concentration of C. (µg/ml)

Figure 4.5 c & d.

		rMSP2	Native MSP2	
Antigen	Isolate	ELISA	IFA	WESTERN
A1	T9/96	+	+	+
A2	T9/96	+	+	-
	Thai Tn	+	+	-
	7G8	+	+	-
A3	RO33	+	+	+
B1	Dd2	+	+	+
B2	K1(5/3)	+	+	n.t
	K1(13/3)	+	+	+
B3	T9/105	+	+	+
N	K1	+	-	-
C	K1	+	+	+

Table 4.4: Summary of reactivities of mice sera after immunisation with MSP2 recombinant proteins. Sera were tested for reactivity to homologous recombinant MSP2 proteins in ELISA. n.t - not tested

4.4 Discussion

Immunity to the asexual stages of malaria parasites has been induced in primate models of human malaria both with whole merozoites [227] and with purified merozoite antigens [137, 237]. These studies indicate that native malaria antigens associated with the merozoite induce significant protective immunity.

MSP2 has been identified as an important antigen on the surface of the merozoite and it is essential to determine the immune recognition of this molecule in malaria-exposed individuals. However, the use and purification of native MSP2 is impractical. Thus, it has become critical to identify appropriate methods to produce synthetic malaria antigens which are immunologically equivalent to their native counterpart.

A large number of malaria antigens, or antigen fragments, have been expressed as recombinant polypeptides in *E.coli*. The pGEX expression system has been used for the production of rMSP2 proteins. We have expressed a panel of rAgs representing the full length molecule of the two allelic forms of MSP2, plus fragments representing defined regions of the protein from various *P.falciparum* isolates/clones. It is important to determine whether the rAgs have been expressed in the correct conformation, i.e. whether the proteins accurately reflect the antigenic characteristics of native MSP2, before studies using human serum begin.

Our approach was two-fold. Firstly, mAbs specific for MSP2 were used in ELISA to determine whether the rAgs contained native epitopes. With one exception, all those rAgs which were known to contain mAb epitopes (determined from sequence data) were recognised by the appropriate mAb in ELISA. Thus, at least some of the rAgs presented native epitopes. Secondly, we immunised mice intraperitoneally with the rAgs and tested the sera for reactivity with both recombinant MSP2 (by ELISA) and native MSP2 (by IFA and Western blotting). All the rAgs were shown to be immunogenic in mice and, in most cases, high titres of MSP2-specific antibodies were induced. The GST component was rarely recognised and if it was, low titres of Ab were induced allowing the distinction between MSP2-specific Ab and GST-specific Ab.

All but two of the rAgs tested by mouse immunisation induced antibodies which were able to bind to native MSP2, indicating that the rAgs were conformationally correct. The apparently defective construct represents the conserved N-terminus. Previous studies have indicated that peptides

representing the N-terminus conserved sequence are immunogenic only when attached to a carrier protein [166, 278]. In our study, the GST fusion protein appeared to act as a suitable carrier, and the protein was immunogenic in mice, although the majority of mice produced low titres of antibody. However, the protein did not seem to mimic the structure of the native antigen. This tends to support the study of Jones *et al* [166] who reported that sera from mice immunised with synthetic peptides representing the conserved N-terminus did not recognise native MSP2 in IFA or Western blotting. There is no obvious explanation why the rAg failed to present the same epitopes as the native protein, for example there are no cysteine residues in the N-terminus sequence. One could propose, that this region of the molecule is not available for Ab binding in the native antigen.

Interestingly, antibodies from mice immunised with A1 cross-react with B1 in ELISA, IFA and Western blotting, and vice versa. Competition ELISAs have shown that the cross-reactive antibodies are specific for the C-terminal conserved region. This could also infer that the N-terminus is not available for immune recognition and antibody binding.

In conclusion, we have established that rAgs of MSP2 produced in *E.coli* resemble the natural *P.falciparum* antigen very well. For the subsequent studies reported in this thesis, we will present data for a range of the rAgs, including N, in addition to those rAgs not tested in mouse immunisations, but the possibility that these do not accurately reflect native protein will be kept in mind.

5. Recognition of recombinant MSP2 proteins by malaria-immune sera

5.1 Introduction:

Recombinant proteins representing MSP2 have been produced as fusions with glutathione S-transferase in *E.coli.* It has been established that many of these rAgs resemble the native protein and therefore are extremely useful tools for studying the naturally acquired immune response to MSP2 (chapter 4). To date, there is very little published data on naturally acquired immunity to MSP2 and no information on the relative immunogenicity of conserved, group specific (dimorphic) and repetitive (polymorphic) regions of the molecule. The epitopes of MSP2-specific mAbs are within the dimorphic and polymorphic regions of the molecule [55, 101, 109, 255]. This indicates that these regions are immunogenic. However, it is important to determine which regions are recognised during natural infection. In order to better understand the nature of protective immunity to malaria it is necessary to determine whether naturally acquired antibody responses to MSP2 are cross-reactive within and between serogroups. Such information would also be useful for determining which, if any, regions of MSP2 might usefully be included in a malaria vaccine. A study of adult, malaria exposed, Melanesians showed that 82% had antibodies against MSP2 serogroup B [279], but the specificity of these antibodies was not fully characterised. A recent study in a highly endemic area of Papua New Guinea found high antibody prevalence (≥ 90%) to recombinant antigens representing the full length proteins of both MSP2 serogroup A (3D7) and serogroup B (FC27) [2]. Using a serogroup A construct lacking the central repeats, this study also showed that a proportion of immune individuals responded only to epitopes within the repeat region. Experimental immunisation of *Aotus* monkeys with *P.falciparum* results in an anti-MSP2 antibody response directed primarily to a recombinant antigen representing repeat and group-specific regions of MSP2 in Western blot [321]. Antibodies purified from immune clusters of merozoites also recognise group specific sequences, suggesting that this region is accessible at the surface of the intact parasite and may therefore be a target for parasite-inhibitory immune responses [321]. However, immunoblotting studies using affinity purified human serum suggest that naturally immunodominant epitopes are encoded within the repetitive sequences of the molecule

[299]. None of these studies has addressed the crucial question of whether sequence polymorphism gives rise to antigenic diversity and whether epitopes in one allelic form of the protein cross-react with similar epitopes from other parasites.

The aims of this study are to determine:

1) if MSP 2 is naturally immunogenic in man in an area of seasonal malaria transmission;

2) the relative antigenicity of conserved, dimorphic and polymorphic regions of MSP2; and

3) if the naturally acquired immune response to MSP2 is cross-reactive between serogroups.

In this chapter, I report a comprehensive study of the immune recognition of MSP2 by sera from malaria-immune adults from The Gambia, and the identification of regions of the molecule which are immunogenic during malaria infections. Adults were chosen because they have had many years exposure to malaria and would be likely to have anti-MSP2 antibodies.

5.2 Materials and methods:

5.2.1.Sera

Serum samples were obtained from 70 adults (aged 15-65 years) living in rural and peri-urban areas of The Gambia.

These serum samples were collected by Dr E Riley.

Control serum samples were obtained from 15 European adults who had not been exposed to malaria.

5.2.2. Recombinant antigens:

The recombinant MSP2 antigens used in this chapter are listed in table 5.1 (see appendix 1A).

ANTIGEN	ISOLATE
A1	T9/96
A2	T9/96
A2	Thai Tn
A2	7G8
A2	T9/94 (i)
A3	RO33
B1	Dd2
B1(short)	Dd2
B2	K1(i)
B2	K1(ii)
B3	T9/105 (i)
N	K1
C	K1

MSP1 - p190.1

As a positive control for prior exposure to *Plasmodium falciparum,* sera were also tested for reactivity with a conserved sequence from another merozoite surface protein, MSP1. p190.1 represents a non-variable region of MSP1 (gp190) which has previously been shown to be recognised by more than 90% of malaria exposed individuals [266] and was produced in *E. coli* as a free polypeptide [118]. p190.1 was a kind gift of Dr J.R.L Pink, F. Hoffman La Roche, Basel, Switzerland.

5.2.3. Enzyme-linked Immunosorbent Assay (ELISA):

Sera were tested for IgG recognition of the recombinant proteins in ELISA using HRP-conjugated rabbit anti-human IgG antibody diluted 1:9000 in PBS/Tween. Sera were diluted 1:1000.

Competition ELISA:

Sera were preincubated with increasing concentrations of inhibiting antigen from 0μg/ml up to 5μg/ml. Sera were diluted 1:1000.

5.2.4. Statistical analysis

i. Determination of positivity:

The reactivity of the sera with various MSP2 fusion proteins in ELISA was calculated by subtracting the OD value for the GST control from the value obtained for the MSP2 fusion protein, to obtain specific OD values. Positive samples were defined as those giving a specific OD above the normal range for control European sera. The normal range was taken as the mean ± 2 standard deviations of 15 control sera.

ii. Spearman's Rank Correlation analysis:

The reactivity of individual sera with different antigens was compared by means of Spearman's rank correlation test [305]. This is a non-parametric measure of the association between two numerical values. The parametric equivalent is the correlation coefficient. The values of each variable are independently ranked and the measure is based on the differences between pairs of ranks of the two variables [184].

5.3 Results

5.3.1. Reactivity of human serum IgG antibodies with recombinant MSP2 proteins:

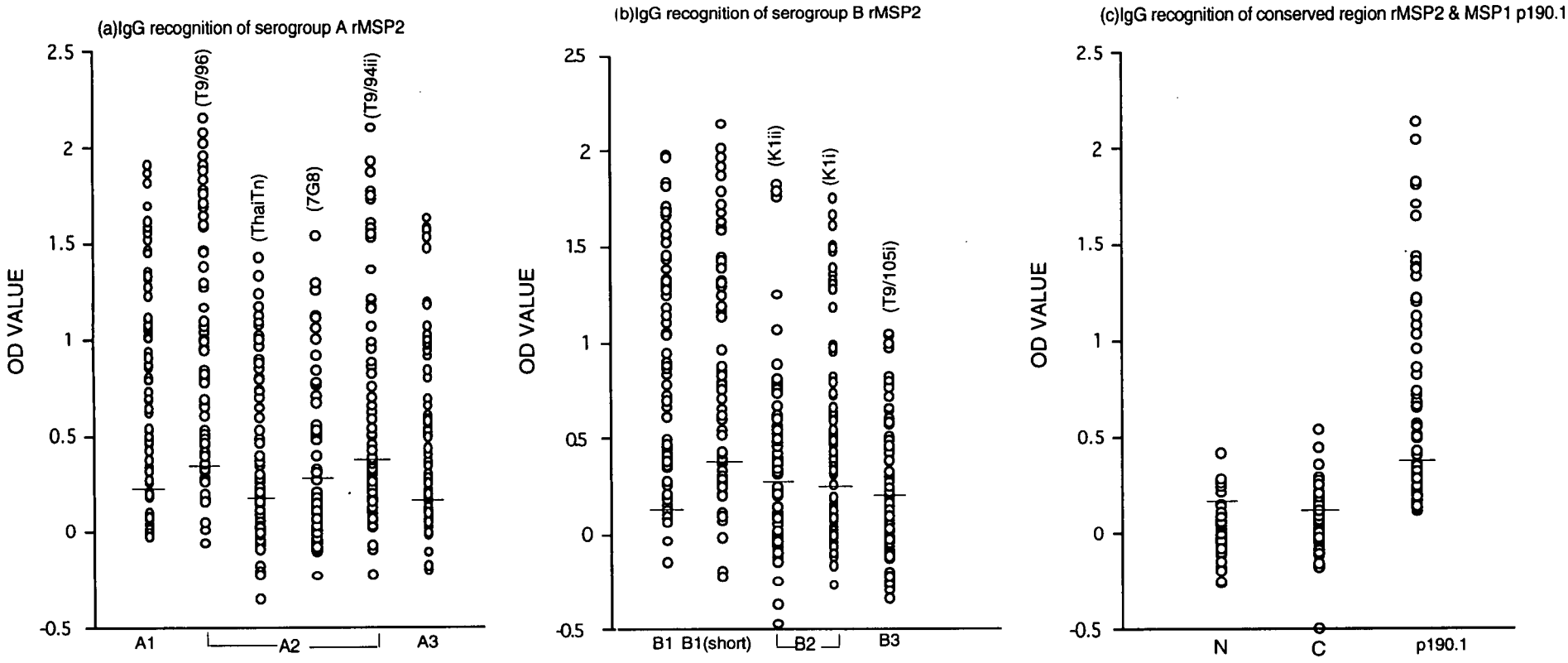
Serum IgG antibodies from Gambian and European adults were tested for the recognition of a panel of MSP2 rAgs and a recombinant MSP1 protein, p190.1. The recognition of p190.1 has been included in this study to ensure that these Gambian adults have anti-malarial antibodies and thus have been exposed to malaria.

The MSP2 rAgs were specifically recognised by sera from individuals who have been exposed to malaria (figure 5.1a-c). The cut-off levels for positivity, determined from the control sera OD values, ranged from 0.117 to 0.384 OD units.

These results show that MSP2 is well recognised (table 5.2). Serogroup A and serogroup B full length rAgs are recognised by 81% and 86% of sera, respectively, indicating that MSP2 is highly antigenic during natural infections with *P.falciparum*. The repeat regions and group specific regions are also well recognised. Of the four A2 rAgs used, the one representing the T9/96 sequence was recognised most often. However, the proportion of parasites expressing the A2(T9/96) R1 repeat sequence has not been determined, so whether this reflects the degree of exposure to this MSP2 sequence or is a result of the T9/96 sequence being more immunogenic than the others is not known. A high percentage of individuals also recognise the A3 rAg which contains predominantly serogroup-specific sequences, indicating that the repetitive sequences are not recognised preferentially over the group-specific regions of serogroup A. The B2 and B3 rAgs of serogroup B seem less well recognised than the corresponding serogroup A constructs, even though these sequences are relatively conserved. However, each contains only one copy of the repeated sequence whereas higher antibody binding would be obtained if the epitopes were repeated.

The conserved sequences at the N and C-termini seem to be poorly recognised. If the cut-off limit for positivity is strictly applied, thirty-six percent of these individuals are seropositive for the conserved C-terminus, however the OD values were very low and only 3 sera recognised this protein with an OD value > 0.300.

Figure 5.1: Dot plots showing specific IgG responses (OD 492) to MSP2 and a MSP1 recombinant proteins of serogroup A (a) and B (b) and conserved epitopes (c) in 70 Gambian sera.
The cut off level for positive sera is indicated by a horizontal line. Sera were tested at a dilution of 1/1000.



Antigen	Isolate	%responders	Range of OD values	
			minimum	maximum
A1	T9/96	81	-0.026	1.991
A2	T9/96	81	-0.053	2.157
	ThaiTn	59	-0.356	1.427
	7G8	40	-0.225	1.540
	T9/94 (i)	51	-0.220	2.107
A3	RO33	73	-0.194	1.632
B1	Dd2	86	-0.140	1.981
B1(short)	Dd2	71	-0.218	2.147
B2	K1i	64	-0.259	1.755
	K1ii	34	-0.466	1.831
B3	T9/105i	43	-0.339	1.044
N	K1	10	-0.262	0.418
C	K1	36	-0.439	0.542
p190.1		63	0.117	2.754

Table 5.2: Percentage of responders recognising MSP1 and MSP2 recombinant proteins. The range of OD values obtained is stated for each rAg. Sera from 70 Gambian adults were tested at a dliution of 1:1000 in ELISA.

5.3.2 Comparisons of the antibodies reactive with different MSP2 rAgs:

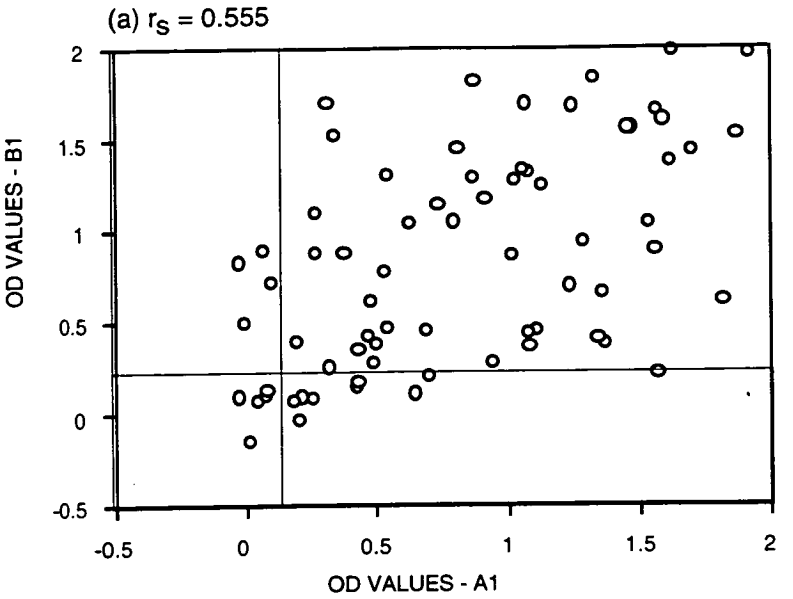
i. Lack of antigenic cross-reactivity between serogroups A and B:

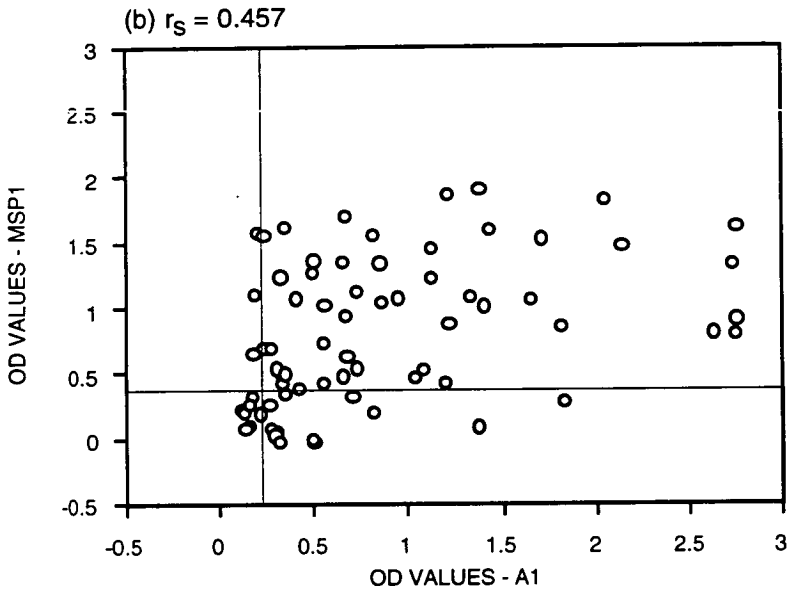
Seventy-nine percent of sera from adult Gambians recognised both the A and B serogroups of MSP2 (proteins A1 and B1, which represent the full length molecules). To determine whether this 'dual' recognition was due to a single population of antibodies which react with epitopes common to both proteins or whether the serum contained two separate, non-cross-reacting, populations of antibody, the reactivity of individual sera was compared with each of the full length proteins (A1 and B1) (figure 5.2a). It is clear that although some sera recognise the two proteins to approximately the same extent, other sera clearly recognise one protein but not the other.

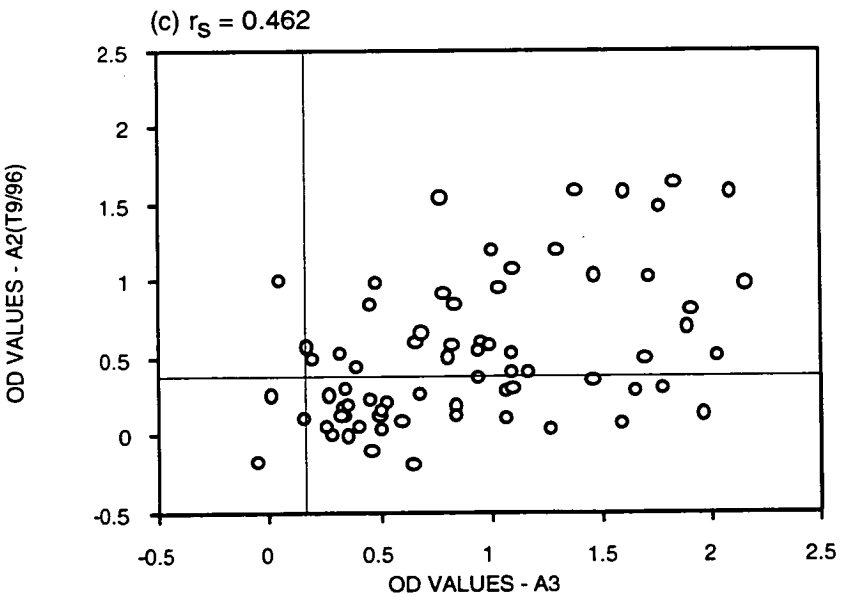
Using Spearman's rank correlation test, a positive correlation was obtained between responses to A1 and B1 ($r=0.555$; $r_S=0.307$, $p=0.005$). However,

we consider that the high level of statistical significance is the result of the large sample size and the presence of double negative sera; the association is in fact quite weak and may simply reflect independent exposure to both serogroups of *P.falciparum*. As an estimate of association due to exposure, the recognition of MSP2 proteins with the recognition of a recombinant protein representing a conserved region of an unrelated merozoite surface protein (MSP1, p190.1) was compared (figure 5.2b). Correlation coefficients of up to 0.492 were obtained. Thus the correlation coefficient obtained for A1 versus B1 was only slightly higher than that obtained for MSP2 versus MSP1, suggesting that the correlation is indeed due to exposure rather than to cross-reacting antibodies.

To confirm that antibodies to the two serogroups are not cross-reactive, individual sera were tested in competition ELISAs. Sera which were known to contain antibodies to both A1 and B1 were selected; these sera were preincubated with either A1 or B1 and tested in ELISA for recognition of the other protein. The four example shown in figure 5.3 was typical of the sera tested: whilst preincubation with increasing concentrations of A1 prevents subsequent binding of antibodies to A1-coated plates, it has no effect on binding of antibody to B1-coated plates, and vice versa. Thus, in double positive sera, there appear to be two distinct populations of antibodies, one specific for serogroup A and the other specific for serogroup B.


(a) r_S = 0.555
OD VALUES - B1
OD VALUES - A1
2
1.5
1
0.5
0
-0.5
-0.5
0
0.5
1
1.5
2


(b) r_S = 0.457
OD VALUES - MSP1
OD VALUES - A1
3
2.5
2
1.5
1
0.5
0
-0.5
-0.5
0
0.5
1
1.5
2
2.5
3


(c) r_S = 0.462
OD VALUES - A2(T9/96)
OD VALUES - A3
2.5
2
1.5
1
0.5
0
-0.5
-0.5
0
0.5
1
1.5
2
2.5

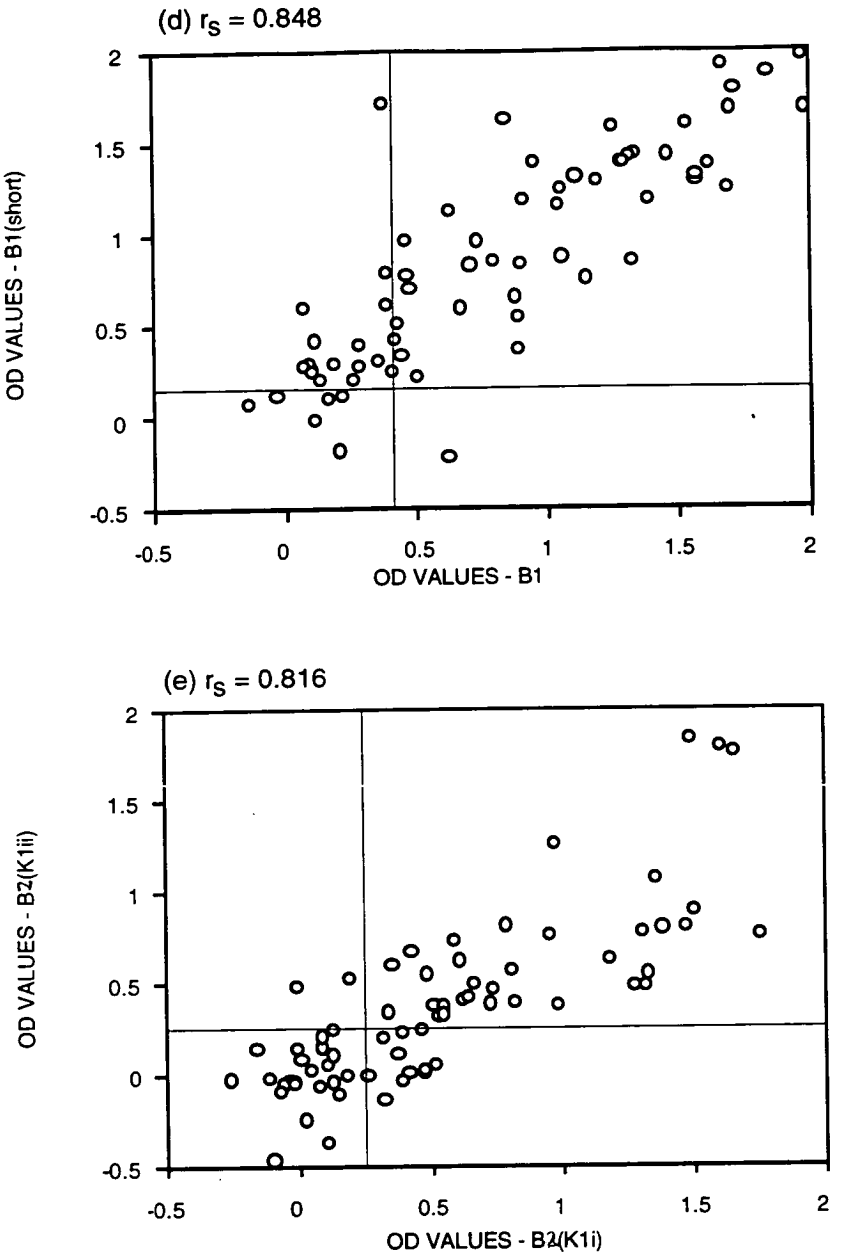
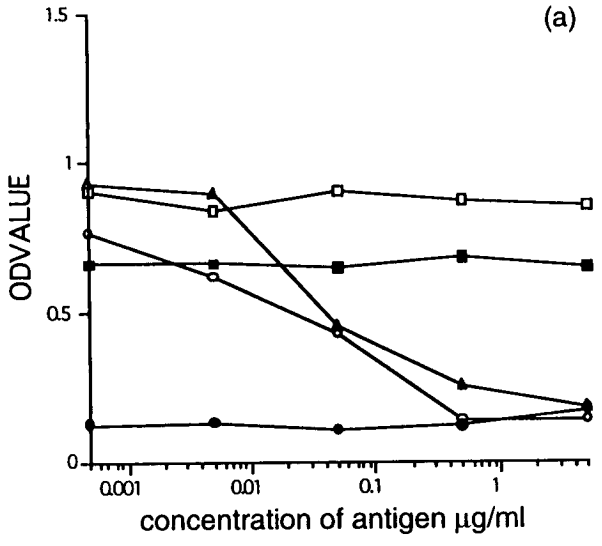


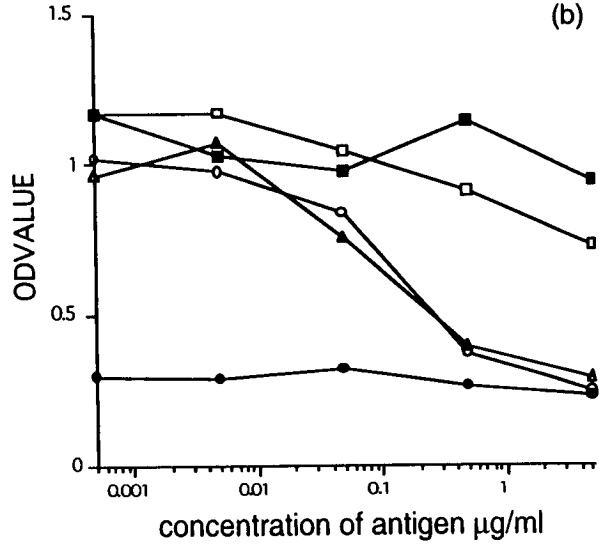
Figure 5.2 a-e: Comparison of OD values for sera from 70 Gambian adults. (a) A1 vs B1, (b) A1 vs MSP1, (c) A2(T9/96) vs A3, (d) B1 vs B1(short) and (e) B3(K1i) vs B3(K1ii). Spearman's values (r_s) are shown.

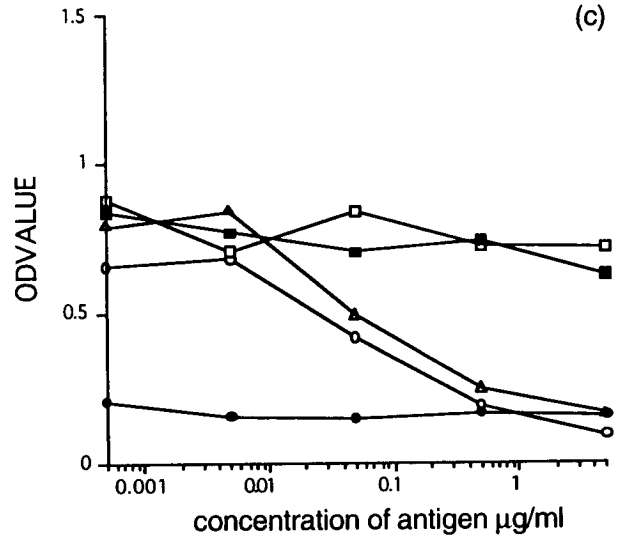
Solid lines indicate the cut-off levels for each antigen. Each circle represents a single serum tested at a dilution of 1/1000.

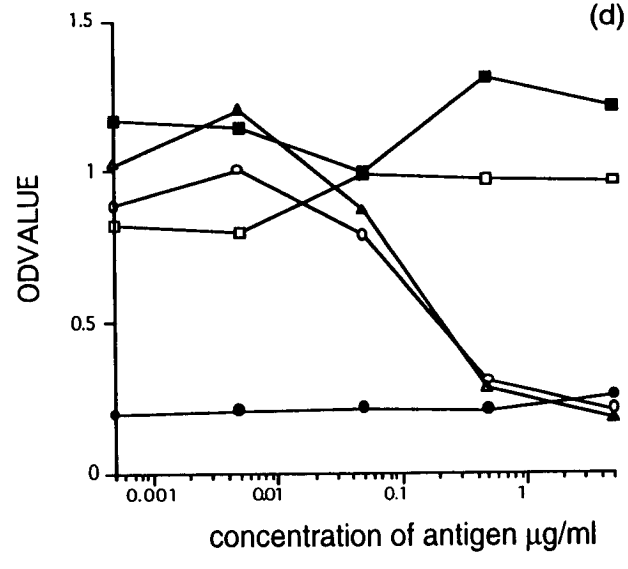
Figure 5.3: Competition assay to determine the extent of cross-reactivity between antibodies recognising A1 and B1. The results for a single, typical, serum are shown. Sera were preincubated with antigen at concentrations from 0µg/ml - 5µg/ml. Sera were tested at a dilution of 1/1000.

Key: ▲ A1(on plate) vs A1 (in serum), □ A1 vs B1, ○ B1 vs B1, ■ B1 vs A1, GST vs GST.


(a)
ODVALUE
1.5
1
0.5
0
0.001
0.01
0.1
1
concentration of antigen µg/ml


(b)
ODVALUE
1.5
1
0.5
0
0.001
0.01
0.1
1
concentration of antigen µg/ml


(c)
ODVALUE
1.5
1
0.5
0
0.001
0.01
0.1
1
concentration of antigen µg/ml


(d)
ODVALUE
1.5
1
0.5
0
0.001
0.01
0.1
1
concentration of antigen µg/ml

ii.Distinct populations of antibodies recognise different regions of MSP2 serogroup A:

Sixty-three percent of the Gambian adults were seropositive for A2(T9/96) and A3, but the dot plots show only a poor correlation between the two (figure 5.2c) and the correlation coefficient was lower than for MSP1 vs. MSP2. Inhibition ELISAs clearly demonstrate that there are two distinct populations of antibodies (figure 5.4). One population are specific for the R1 repeat region and the other for the R2 repeat/group specific region. These data fit with the demonstration that MSP2 serogroup A-specific mAbs recognise R1 repeats (13.4, 4-4F & 8-5D) or the group specific region (12.3, 12.5 & 12.7), and therefore, distinct epitopes are present in both regions.

iii. Lack of recognition of the conserved C and N-termini:

The percentage of responders and the OD values obtained for the recognition of C and N indicate that these regions are not highly antigenic/immunogenic during a natural infection. However, for the N-terminus at least, there is some question as to the antigenic integrity of this rAg (see chapter 4). Comparison of the recognition of two pairs of rAgs, B1 and B1(short), and B2(K1i) and B2(K1ii), allows further analysis of the role of the conserved regions in the recognition of MSP2. B1(short) lacks a large proportion of the conserved sequences present in B1. B2(K1i) lacks the N-terminal conserved sequence present in B2(K1ii). Despite the differences between the pairs of antigens, the recognition of the rAgs is very highly correlated (table5.3, figure 5.2 d and e). These data thus confirm the initial data and indicate that the conserved sequences do not contribute significantly towards the recognition of MSP2.

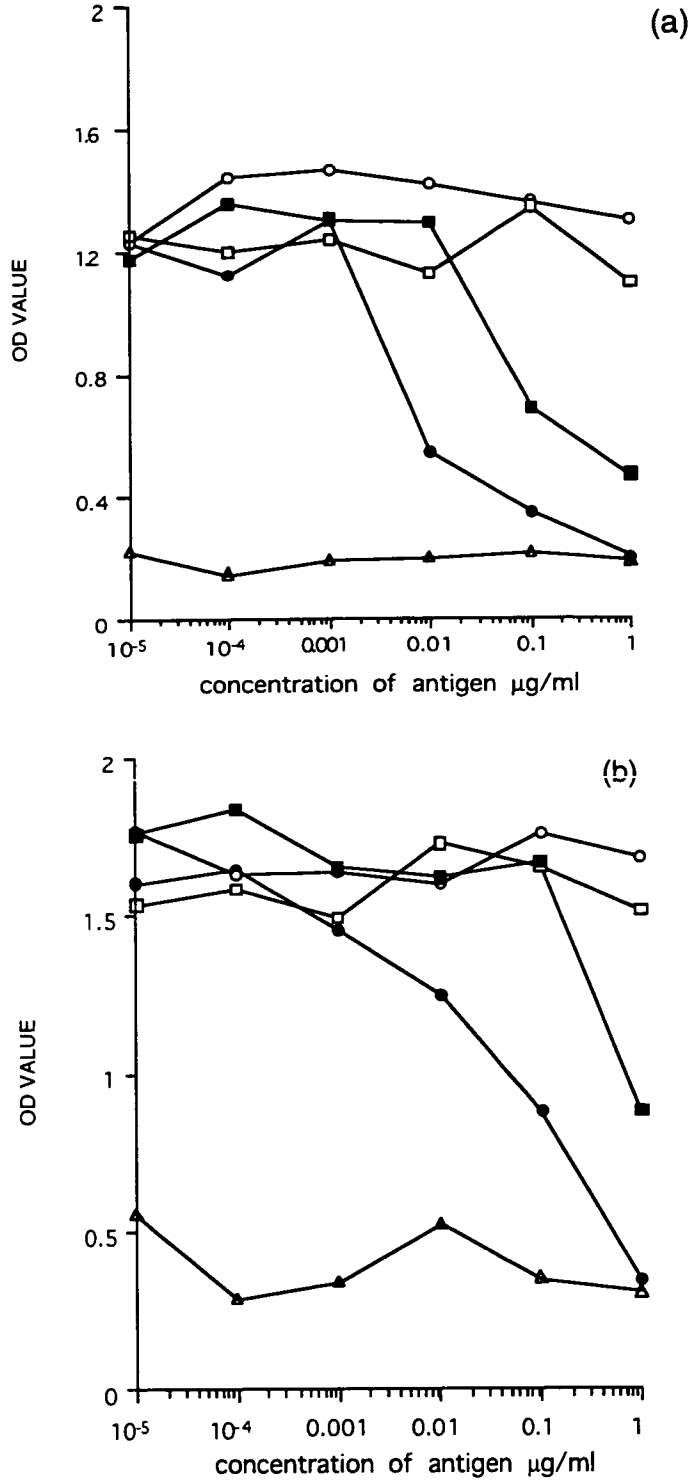


Figure 5.4 a & b: Competition assay to determine the extent of cross-reactivity between antibodies recognising A2(T9/96) and A3, for two individuals. Sera were preincubated with antigen at concentrations from 0mg/ml - 1mg/ml. Sera were tested at a dilution of 1:1000.
Key: ● A2(on plate) vs A2 (in serum), ○ A2 vs A3, ■ A3 vs A3, □ A3 vs A2, △ GST vs GST.

Antigen	MSP1	A1	A2 (T9/96)	A2 (ThaiTn)	A2 (7G8)	A2 (T9/94i)	A3	B1	B1 (short)	B2 (K1i)	B2 (K1ii)	B3 (T9/105i)	N
MSP1	1.000												
A1	0.457	1.000											
A2(T9/96)	0.071	0.620	1.000										
A2(ThaiTn)	0.270	0.558	0.733	1.000									
A2 (7G8)	0.173	0.463	0.644	0.731	1.000								
A2 (T9/94i)	0.193	0.559	0.691	0.765	0.830	1.000							
A3	0.352	0.764	0.462	0.465	0.444	0.526	1.000						
B1	0.492	0.555	0.449	0.512	0.436	0.479	0.579	1.000					
B1(short)	0.394	0.464	0.487	0.553	0.472	0.505	0.520	0.848	1.000				
B2 (K1i)	0.415	0.437	0.392	0.450	0.506	0.510	0.472	0.743	0.678	1.000			
B2(K1ii)	0.483	0.440	0.303	0.436	0.473	0.387	0.425	0.648	0.654	0.816	1.000		
B3(T9/105i)	0.226	0.350	0.367	0.249	0.166	0.215	0.339	0.527	0.498	0.380	0.344	1.000	
N	0.273	0.187	-0.041	-0.043	0.058	0.061	0.079	0.051	0.018	0.225	0.180	-0.014	1.000
C	-0.356	0.029	0.362	0.350	0.394	0.394	0.245	0.050	0.154	0.072	0.027	0.058	-0.336

Table 5.3: Comparison of the recognition of MSP1 and MSP2 rAgs using the OD values obtained from 70 Gambian adults' sera in Spearman's rank correlation tests. Critical value for n=70 is 0.307, p=0.005.

5.4 Discussion

The aim of this study was to characterise the reactivity of serum antibodies from malaria-exposed adult individuals with the merozoite surface protein, MSP2. Evaluation of MSP2 as a potential component of a subunit malaria vaccine requires an understanding of the naturally occurring immune response to MSP2 and, most importantly, the immunological significance of amino acid sequence polymorphisms. In particular, it is important to determine whether antibodies against one MSP2 serogroup will cross react with the other serogroup, or whether the Ab response is group-specific or allele-specific. Such information may also help us to understand the relative importance of allele-specific ("strain-specific") immune responses in the acquisition of clinically protective immunity to malaria by people living in malaria endemic areas.

There are two main theories to explain the slow development of protective immunity to malaria which is typically seen in individuals living in endemic areas: (i) polymorphism of antigens which are the targets of protective immune responses and, (ii) intrinsically poor immunogenicity of the target molecules. The data presented here indicate that the latter explanation is not true, at least with respect to MSP2. Sixty seven of the 70 individuals tested had clearly detectable anti-MSP2 antibodies (to either serogroup A or B or both) with end point titrations of > 1/9,000 for most sera (data not shown). This is comparable with the level of recognition of MSP2 found in the Solomon Islands [279] and Papua New Guinea [2].

Antibodies tended to recognise epitopes within the dimorphic and polymorphic regions of MSP2; the conserved C-terminus seems to be poorly antigenic. This is in agreement with Thomas *et al* [321] who reported that although intact MSP2 is recognised by monkey and human antibodies eluted from intact merozoites, such antibodies did not recognise peptides representing the N- and C-terminal regions of the molecule. Similarly, Saul *et al* [284]were unable to detect antibodies against synthetic peptides representing the conserved regions of MSP2 in sera from 18 people with high titres of anti-parasite antibodies (as determined by immunofluorescence assays).

Importantly, the sequences which are conserved within each allelic family (detected using A3 and B3 proteins) are highly immunogenic. Ninety percent of the sera tested contained antibodies which recognise either A3 or B3 (or both); thus a vaccine based on MSP2 *may* need to contain only

two different antigens - representing the group-specific sequences of each serogroup. Interestingly the group-specific A3 protein is recognised by significantly more individuals than the B3 protein. This may reflect more frequent or more recent exposure of the donors to parasites of serogroup A since approximately 60% of parasites isolated in The Gambia belong to the A serogroup [66]. Antibodies eluted from immune clusters of merozoites recognise dimorphic MSP2 sequences [321], indicating that epitopes associated with these sequences are accessible at the surface of intact merozoites, and may therefore be a target for inhibitory antibodies.

Proteins which represent the polymorphic R1 repeat regions of both serogroups (i.e. A2 and B2) were recognised by a substantial proportion of the sera, a greater proportion in fact than recognised the dimorphic regions. This finding was somewhat unexpected since, for serogroup A, the amino acid sequence of the repeats from different isolates varies extensively [201, 250]. One likely explanation of this finding is that the antibodies detected were in fact recognising epitopes within a short N-terminal segment of group specific sequence contained in the protein A2 (see chapter 4, figure 4.1). Alternative explanations include extensive immunological cross-reactivity between different repeat sequences. Whichever explanation is the case, the association between the recognition of different A2 variants is evident from the Spearman's rank correlation values (table 5.3). Immunological cross-reactivity may occur among the R1 repeats of serogroup B, where the amino acid sequence is relatively conserved. Further studies will investigate the effect, on antibody recognition, of variation in the sequence and number of repeats (see chapters 9 and 10).

Quantification of absolute amounts of Ab by ELISA is difficult since the OD value obtained is dependent on both the concentration of antibody and its affinity. It is not possible to compare the amounts of antibody in sera which react with different recombinant antigens since the number of epitopes in each assay (and thus the avidity of the reaction) is unknown [331] Therefore, in order to make comparisons between the Ab responses to different proteins, we used a nonparametric rank correlation test to compare specific OD values for individual sera, tested at a single dilution, against the rAgs. Comparison of the recognition of A1 and B1 show that the correlation between the responses to the two serogroups is weak and is probably due to exposure to parasites of both serogroups rather than to cross-reactive antibodies. Competition ELISAs demonstrate that antibodies

to serogroup A and B are not cross-reactive, since protein A1 cannot compete for binding of B1-specific antibodies, and vice versa. It should be noted that there is a short sequence of limited sequence homology within the R1 repeat region of serogroup A and B [298, 299], but this does not appear to induce cross-reactive antibodies in a natural infection.

It is apparent from studies in mice that antibodies specific for A1 will cross-react with serogroup B in ELISA, IFA and Western blot, and vice versa (chapter 4, section 4.3.3). The cross-reactivity appears to be due to recognition of the conserved C-terminus. Although this region appears to be antigenic in mice immunised with the rAg C, it is not recognised during natural malaria infections in humans and does not contribute to cross-reactivity.

Therefore we have shown here that MSP2 is naturally antigenic and that the immune response is directed primarily to the dimorphic and polymorphic regions of the molecule. Correlation analysis and competition ELISAs have indicated that, in general, there is no serological cross-reactivity between the two serogroups.

Further work is required to determine the significance of the polymorphisms within the repeat sequences in their contribution to the slow development of protective immunity to *P.falciparum* (see chapter 9).

6. Population cross-sectional studies of antibody recognition of MSP2 - the relationship between age, malaria exposure and anti-MSP2 IgG

6.1 Introduction

In areas with stable, endemic *P. falciparum* malaria transmission, parasitaemia is most common in young children, and the incidence of parasitaemia declines steadily with age. The precise timing of events depends on the local patterns of malaria transmission and levels of endemicity. In The Gambia, where transmission is seasonal but stable from year to year, parasite rates do not begin to decline until the age of 10 to 12 years, whereas the incidence of clinical disease peaks at age 6 [264]. In the same population, peak mortality from malaria occurs in children aged 4 years [128]. Thus, as protective immunity to malaria is acquired gradually, and associated with age (and exposure to malaria), the prevalence of anti-malarial antibodies would be expected to increase in parallel with increasing clinical immunity. Why does it take so long to become immune? Possible reasons for the slow development of immunity are: 1) children are unable to make appropriate antibody; adults may respond more appropriately and therefore acquire immunity more rapidly; 2) the antigens are poorly immunogenic; or 3) the antigens are polymorphic.

As early as 1965, Brown & Brown [35] proposed that children may have a constitutional inability to respond appropriately to endemic infection pressure. They had observed that young rats infected with *P.berghei* relapse more frequently and with higher parasitaemia than older animals, indicating that the young of some hosts may have an inability to respond appropriately and develop generalised immunity. Baird *et al* [15, 16] studied the effect of age on protection, independent of cumulative exposure to malaria antigens in a mixed-age population in The Philippines migrating into a malaria-endemic area from a non-endemic area. They propose that the protective acquired immune response to endemic falciparum malaria is governed by relatively brief exposure and some intrinsic immune factor(s) associated with age of the host. If this is true, antigenic polymorphisms in the parasite may only briefly influence susceptibility to infection. However, this data does not prove that adults acquire protective immunity faster than children, since the authors do not specifically address the issue of an age-specific rate of development of clinical immunity. Certainly the number of

clinical episodes that are necessary before clinical immunity develops may differ in adults and children but they report age-specific differences in prevalence of parasitaemia which does not necessarily reflect age-specific differences in clinical episodes. Baird *et al* [14-16] conclude that the degree of protection was governed by recent heavy exposure **and** age, independent of chronic heavy exposure. Clearly, immunologically naive adults suffer severe and life-threatening malaria. This fact argues against some purely physical consequence of the ageing process, i.e., people without prior exposure to malaria seem equally susceptible to the consequences of falciparum malaria, regardless of age. Furthermore, a study of epidemic malaria in Madagascar following a period of 20 years when the island was malaria free, has shown that all age groups, except those previously exposed to malaria were equally susceptible to disease [84].

Cohen & McGregor [62] suggested that the slow immunological response to malaria, which requires several years of repeated infection in order to reach a level sufficient enough to suppress the acute manifestations of the disease, is associated with the inherently poor antigenicity of the parasite. A modern interpretation of this is that immunological nonresponsiveness could be a result of the parasite antigens evolving to present limited T cell epitopes to the immune system and thus failing to give T cell help for antibody production [120].

Early observations on malaria infections therapeutically induced in neurosyphilitic patients showed that individuals convalescing from *P.vivax* infections acquire an effective immunity to the strain of the parasite that caused the infection but not to a heterologous strain [30]. These results imply that immunity may be strain- or variant-specific and targets of protective immunity are likely to be polymorphic, and importantly may depend on the immunogenicity of primary infections. Thus, susceptibility in children may mean that resistance depends on exposure to a wide, but finite range, of antigenic variants occurring in one locality [35].

The hypothesis that there is a constitutional and age-associated inability to respond appropriately to infection [14-16, 35] such that protective immunity to malaria is dependent on the age of the individuals rather than cumulative exposure to many variants over a number of years cannot be easily investigated in endemic populations.

However, the other two hypotheses can be investigated by comparing the antibody responses of relatively naive individuals with semi-immune and clinically immune individuals.

The hypothesis that malaria antigens are inherently poorly immunogenic can be tested by looking at antibody in individuals convalescing from one or two malaria infections. Such data is presented in this chapter. Finally, the theory that protection is dependent upon cumulative exposure to many variants can be investigated by comparing antibody responses of children, who have limited exposure to malaria with adults who will have experienced infection with most or all of the variants present within the local population of parasites.

Thus, in this chapter, the dynamics of the acquisition of antibodies to MSP2 in Gambian populations is described, examining the effects of age and exposure to malaria infection. We have also examined the antigenicity of MSP2 in children and individuals from non-endemic areas who are known to have experienced a limited number of malaria infections.

6.2 Materials and Methods

6.2.1 Serum samples:

1. Malaria-exposed individuals

178 serum samples were obtained from individuals (aged 1-75 years) living in rural villages around the town of Farafenni on the north bank of the Gambia river, about 100 km inland. The geographical and demographic features of this area have been described by Greenwood *et al* [129]. The majority of clinical cases of malaria occur in the 3 month period from September to November each year. The serum samples used here were collected in October 1988, during the malaria transmission season.

2. "Naive" Individuals

Sera were obtained from individuals from non-endemic areas who had been infected with *P.falciparum* whilst visiting malaria endemic countries (table 6.1). These individuals were known to have been infected either for the first time or more than twice. Some sera were collected during convalescence (CS).

Serum samples were kindly provided by Prof. G Pasvol and Dr J Carlsson, Dept. of Infectious Diseases and Tropical Medicine, Northwick Park Hospital.

3. Control serum samples were obtained from 25 European children and 25 European adults who had not been infected with malaria.

All serum samples were stored at -20°C.

SAMPLE NUMBER	EXPOSURE	COUNTRY VISITED
92:21	N & CS	Ghana
92:35	N	Nigeria
92:51	N	Ghana
93:01	N	Kenya
93:28	N & CS	Uganda
92:11	>2	Nigeria
92:30	>2	Ghana
92:33	>2	Kenya
92:41	>2	Ghana
93:33	>2	Malawi-Zimbabwe-Zaire
92:10	CS	Nigeria
92:12	CS	Nigeria
92:38	CS	Liberia

Table 6.1: Serum samples from individuals from non-endemic countries who became infected with malaria after visiting malaria endemic countries. Samples were taken after returning to Britain, on diagnosis of infection with *P.falciparum*.
N indicates naive individual; >2 indicates second or more infection; CS indicates convalescent sera.

6.2.2 Recombinant MSP2 proteins:
The following rAgs were used in this work: A1, A2(T9/96), A3, B1, B2 (K1i), B3 (T9/105i); see Appendix 1A.

6.2.3 Enzyme-linked immunosorbent assay (ELISA):
MSP2 specific IgG antibodies were measured in ELISA as described in materials and methods (chapter 2, section 2.4). For screening, all sera were diluted 1/1000 in blocking buffer and tested in duplicate.
For titration ELISAs, sera were diluted from 1/100 to 1/12800 with doubling dilutions.

6.2.3 Statistical methods:

1. Determination of positivity:

Positive samples were defined as those giving a specific OD above the normal range for control sera. The normal range was taken as the mean + 2 standard deviations of the OD values obtained for the control sera.

2. Kruskal-Wallis test:

Kruskal-Wallis test were performed using MINITAB statistical software in order to determine differences in the median OD values to each for each age group.

3: Chi-square for trend:

χ^2 tests for trend were performed using Epi Info, a statistical software package, and are used to determine whether there is a trend in proportions of responders with age.

6.3 Results

6.3.1. Age-specific recognition of MSP2:

Anti-MSP2 IgG levels were measured in 178 individuals from the area around the village of Farafenni, The Gambia, West Africa. The median OD increases markedly with age for all the rAgs tested, reaching a peak after the age of 16 years. Kruskal-Wallis tests indicate this increase is statistically significant ($p<0.01$) despite the large standard deviation (table 6.2).

Thus, these data show that the median antibody level specific for MSP2 increases with age, in a pattern which would be expected if immunity to MSP2 is associated with the acquisition of protective immunity.

6.3.2. Age-specific prevalence of antibodies to MSP2:

The large standard deviation for the analysis of variance (table 6.2) indicates a wide range of OD values in each age group. This reflects the number of non-responders in each age group. Table 6.3 and figure 6.1 shows that the number of responders, i.e. the prevalence of antibodies, increases with age. In this analysis smaller age groups were used in order to reveal the pattern of antibody prevalence, particularly in those individuals still actively acquiring immunity to malaria.

For all the rAgs, there is a gradual rise throughout childhood in the prevalence of seropositivity. The recognition of MSP2 reaches a peak in adolescence; depending on the antigen, between 71-100% of this population are eventually seropositive.

The pattern of antibody prevalence varies between the antigens (figure 6.1a & b). A1 and A3 have similar profiles with seropositivity reaching > 90% by adolescence and remaining >80% throughout adulthood. The prevalence of antibodies to A2(T9/96) is lower, reaching a maximum of 71%.

The recognition of serogroup B antigens reaches a peak of 100% responders by age 7-8 years for the full length protein B1; the prevalence of antibodies specific for B2(K1i) is >80% and for B3(T9/105i) >70% from 9-10 years onwards. The prevalence of antibodies to the R1 repeat region of serogroup B, B2(K1i), reaches levels close to those for the full length protein; this is in marked contrast to the corresponding region of serogroup A.

There is a small decline in prevalence of anti-MSP2 antibodies through adult life.

χ^2 tests for trend indicate that the antibody prevalence for the recognition each antigen is strongly age related (table 6.3)

Hence, the age trend is a reflection of the increase in prevalence of antibodies to MSP2 rather than an increase in the median OD with age. Analysis of the **positive** OD values showed that, except for B1, there was no significant difference in the median of the positive OD values with age (table 6.4). Additional analysis of the data for B1 showed that there was no difference in the median OD value between the ages of 1-15 years, but that individuals older than this tended to have higher OD values.

		Recombinant MSP2 Proteins					
AGE	n	A1	A2(T9/96)	A3	B1	B2(K1i)	B3(T9/105i)
1-5	33	0.126 (0.346)	0.037 (0.264)	0.143 (0.310)	0.208 (0.370)	0.069 (0.356)	0.111 (0.272)
6-10	36	0.343 (0.434)	0.066 (0.283)	0.266 (0.342)	0.365 (0.450)	0.191 (0.451)	0.338 (0.392)
11-15	35	0.468 (0.383)	0.320 (0.309)	0.279 (0.368)	0.535 (0.386)	0.489 (0.399)	0.482 (0.481)
16-30	35	0.528 (0.364)	0.402 (0.426)	0.526 (0.419)	0.715 (0.415)	0.615 (0.363)	0.874 (0.497)
30+	39	0.641 (0.435)	0.279 (0.441)	0.442 (0.432)	0.693 (0.420)	0.589 (0.495)	0.493 (0.512)
H		22.01	30.27	14.00	22.69	33.90	27.32
p		<0.001	<0.001	0.008	<0.001	<0.001	<0.001

Table 6.2: 178 serum samples from a cross-section of the population were tested for the recognition of a panel of rAgs representing MSP2. Median (SD) of OD values for Gambian sera (1/1000 dilution) for different age groups are shown. Differences between medians were analysed using Kruskal Wallis tests.

		Recombinant MSP2 proteins					
AGE	n	A1	A2(T9/96)	A3	B1	B2(K1i)	B3(T9/105i)
1-2	11	45 (5)	18 (2)	45 (5)	73 (8)	54.5 (6)	27 (3)
3-4	15	47 (7)	13 (2)	40 (6)	53 (8)	40 (6)	47 (7)
5-6	18	55.5 (10)	33 (6)	61 (11)	72 (13)	61 (11)	67 (12)
7-8	14	69 (9)	15 (2)	69 (9)	100 (13)	77 (10)	62 (8)
9-10	11	100 (11)	54.5 (6)	82 (9)	100 (11)	82 (9)	73 (8)
11-12	21	81 (17)	67 (14)	67 (14)	100 (9)	100 (21)	86 (18)
13-15	14	93 (13)	71 (10)	93 (13)	100 (21)	93 (13)	79 (11)
16-25	26	85 (22)	65 (24)	81 (21)	96 (25)	96 (25)	92 (24)
26-35	20	85 (17)	44 (11)	95 (19)	90 (18)	95 (19)	80 (16)
36+	28	82 (23)	46 (13)	86 (24)	93 (26)	86 (24)	75 (21)
χ^2		15.285	10.294	19.811	13.821	27.744	14.628
p		>0.001	>0.001	>0.001	0.002	>0.001	>0.001

Table 6.3: Prevalence of anti-MSP2 antibodies in sera from Gambian individuals aged 1-75 years. Percentage of positives for each age group and the number of positive samples (n) in each age group are given. Association between age and antibody prevalence for each antigen was assessed using χ^2 tests for trend.

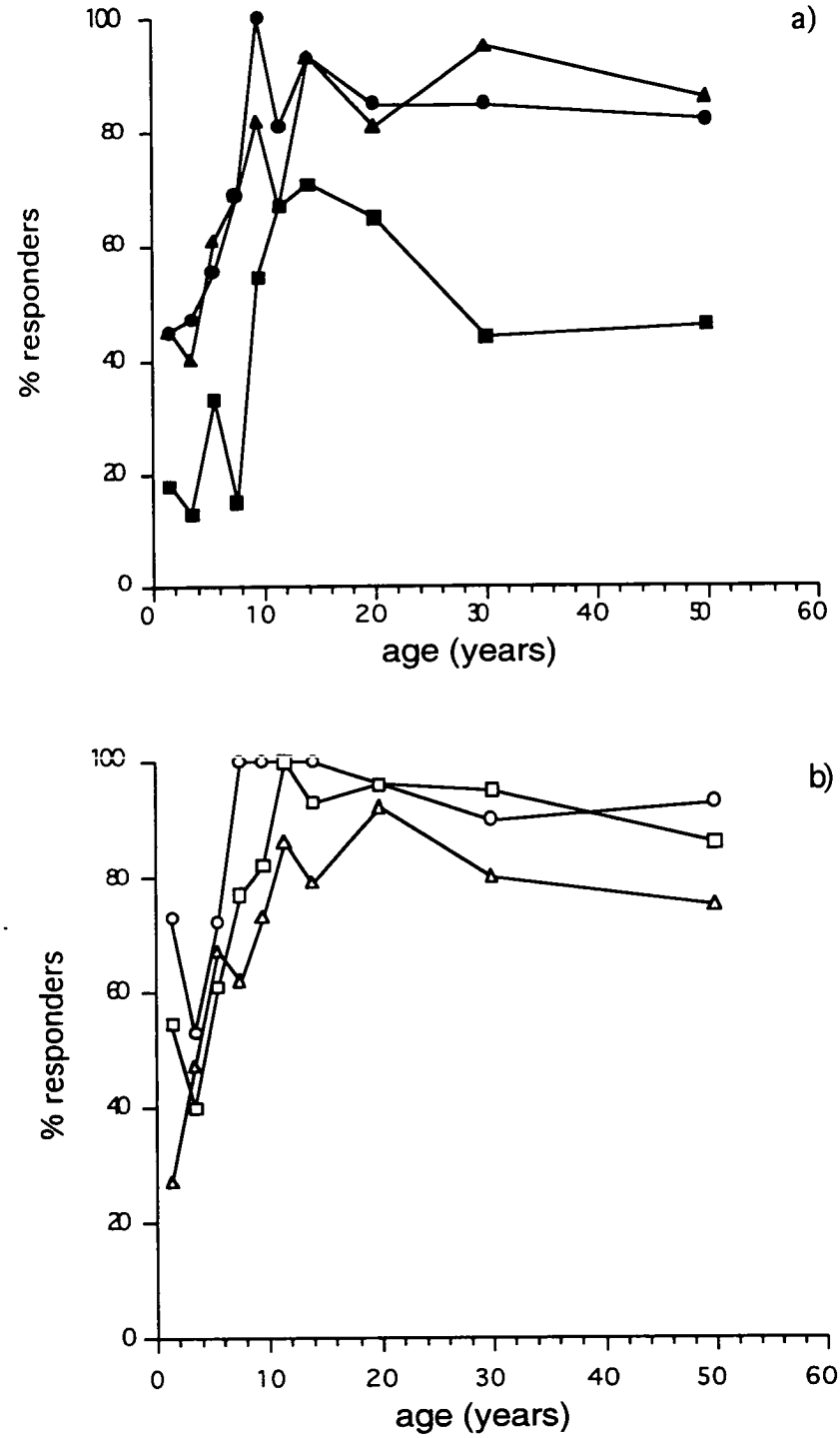


Figure 6.1: Prevalence (% responders) of IgG positive sera, by age, for MSP2 serogroup A (a) and B (b).
Age groups are 1-2, 3-4, 5-6, 7-8, 9-10, 11-12, 13-15, 16-25, 26-35, 36+ years.
(a) ● full length protein, A1, ■ R1 repeat region, A2(T9/96), ▲ R2/group-specific region A3.
(b) o full length protein, B1, □ R1 repeat region, B2(K1i), △ R2/group-specific region B3 (T9/105i)

	Recombinant MSP2 proteins					
AGE	A1	A2(T9/96)	A3	B1	B2(K1i)	B3(T9/105i)
1-5	0.541	0.455	0.555	0.581	0.402	0.523
6-10	0.718	0.573	0.611	0.414	0.290	0.609
11-15	0.629	0.400	0.403	0.535	0.500	0.525
16-30	0.560	0.533	0.583	0.764	0.633	0.894
30+	0.777	0.706	0.503	0.723	0.666	0.687
H	6.91	6.91	2.34	10.48	9.00	6.17
p	0.142	0.142	0.674	0.034	0.062	0.188

Table 6.4: Analysis of positive responses to MSP2 rAgs; median of the positive OD values per age group. Differences between medians were analysed using Kruskal Wallis tests.

6.3.3 Immunogenicity of MSP2:

It appears from the analysis above that children are capable of producing levels of antibodies equivalent to the levels in adults. However, the nature of the assay does not allow us to determine whether children produce the same quantity and/or affinity of antibody as adults when the serum is tested at a single dilution. Therefore, selected sera were titrated and comparison of the titration curves produced by these sera gives an indication of the differences in affinity and/or amount of MSP2-specific antibody (figure 6.2a & b).

A1 was strongly recognised by the three sera tested (figure 6.2a). Two donors were aged 5yrs (F1 and F2) and one was aged 42yrs (F3). The slope and the peak plateau of the titration curves were similar in all 3 cases, indicating that the concentrations and affinities of antibodies specific for A1 were similar in these individuals.

B1 was well recognised by child F2 and adult F4, and it appears from the titration curves that the affinity and concentration of these antibodies is similar (figure 6.2b). Child F3, who was 1 years old, had antibodies specific for serogroup B with similar affinity but of a lower concentration.

Therefore, in general, the MSP2-specific antibodies in children and adults are comparable in terms of concentration and affinity, indicating that MSP2 is not poorly immunogenic.

Table 6.5 summarises antibody recognition of MSP2 proteins by sera of individuals from non-endemic areas who have limited experience of *P.falciparum* infections. 6/13 of these individuals have detectable antibodies to the rAgs tested. Interestingly, all the convalescent sera were seropositive, indicating that, even after only very limited experience of *P.falciparum*, MSP2 is immunogenic.

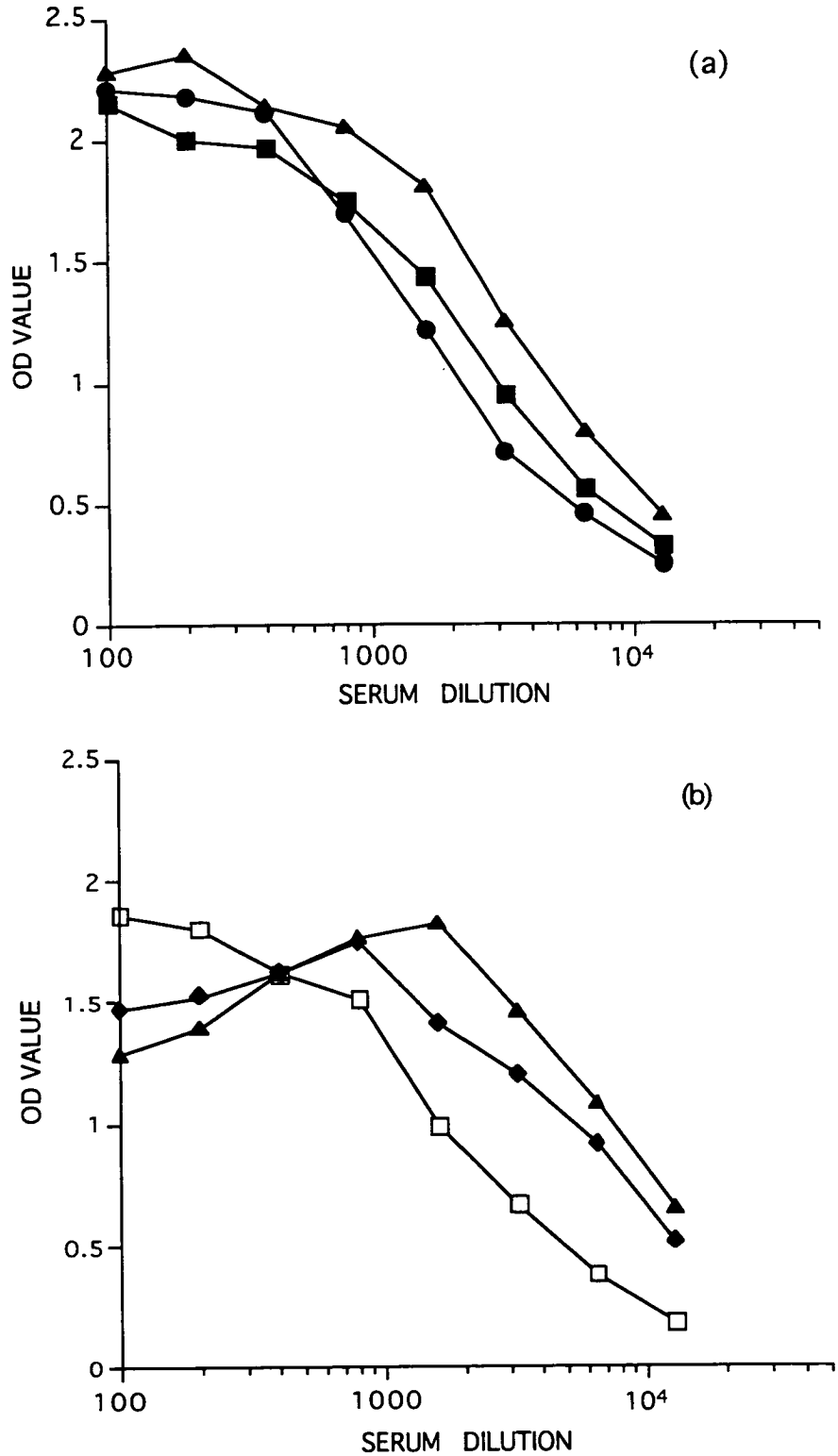


Figure 6.2 a & b: Titration curves for the recognition of A1 (a) and B1 (b), to compare the relative concentration and affinity MSP2-specific antibodies in children and adults. Sera were diluted 1/100 to 1/12800 with doubling dilutions.

(a) ● F1 (age 5 years), ▲ F2 (age 5 years), ■ F3 (age 42 years).
.(b) □ F5 (age 1 year), ▲ F2 (age 5 years), ◆ F6 (age 69 years).

		MSP2 SEROGROUP A			MSP2 SEROGROUP B		
DONOR	STATUS	T9/96 13/14	T9/96 5/7	RO33 8/6	Dd2 13/14	K1 13/3	T9/105 12/6
92:21	N	-	-	+/-	-	+	-
	CS	-	-	++	-	+/-	-
92:35	N	-	-	-	-	-	-
92:51	N	-	-	-	-	-	-
93:01	N	-	-	-	-	-	-
93:28	N	-	-	-	-	-	-
	CS	+	-	++	+/-	+/-	-
92:11	>2	-	-	-	-	-	-
92:30	>2	-	-	-	-	-	-
92:33	>2	-	+	-	+/-	-	-
92:41	>2	-	-	-	-	-	-
93:33	>2	-	-	-	-	-	-
92:10	CS	-	-	-	++	++	+
92:12	CS	+	+	+++	+/-	-	-
92:38	CS	+++	++	+++	+++	-	+

Table 6.5: Summary of results for antibody recognition of recombinant proteins of MSP2 by "naive" individuals .

- indicates an OD was measured < mean +2SD of the control sera

+/- indicates an OD was measured close to the mean + 2SD of the control sera

+ indicates an OD was measured > mean +2SD of the control sera

++ indicates an OD was measured > mean +4SD of the control sera

+++ indicates an OD was measured > mean +6SD of the control sera

6.4. Discussion

Serum samples were obtained from a cross-section of the population around the village of Farafenni, The Gambia. These individuals were aged between 1 and 75 years. The antibody recognition of recombinant MSP2 antigens was determined in order to look at the dynamics of the acquisition of antibodies to MSP2 in a malaria endemic area.

The recognition of the rAgs was shown to be markedly age dependent. The median OD value for each age group increased with age and the association with age was statistically highly significant. This age dependency could possibly reflect the acquisition of protective immunity. Considerable variability in the recognition of MSP2 between individuals in each age group. The increase in the median OD with age was due to an increase in the prevalence of antibodies with age rather than to an increase in antibody concentration or affinity (table 6.2).

The serum samples tested here have previously been tested for antibody prevalence to other malaria antigens, PfMSP1 [266] and soluble exoantigens [158]. The pattern of recognition observed for MSP2 (figures 6.1a & b) is similar to that described for the recognition PfMSP1 and certain soluble antigens by these same sera. The humoral response to PfMSP1 appeared to be acquired in an age-dependent manner, with peak prevalence occurring in late childhood or early adolescence [266]. The antibody responses to all the soluble antigens were strongly age-dependent, but two different profiles were seen in the prevalence of antibodies. The age-related patterns of recognition of asexual stage antigens and the circumsporozoite protein were also studied in this region several years earlier [197, 200]. It was found that the immunological responses to blood-stages were all strongly age-dependent. Total blood-stage antibodies (mean OD) increased with age, reaching a peak around 15 years old [200]. This pattern was closely paralleled for the recognition of Pf155/RESA. The development of anti-CS protein antibodies was also age-related; both the prevalence of antibody positivity and mean antibody levels showed a progressive rise after the age of about 10 years [197]. The response reached a plateau in adulthood, however this pattern was distinct from that shown by antibodies to asexual blood-stage antigens, which developed more rapidly, suggesting that the CS protein is less immunogenic than the blood stage antigens.

The antibody profiles described by Jakobsen [158] represented the acquisition of anti-toxic and anti-parasite immunity. In this two stage development of naturally acquired immunity to malaria, the rate of acquisition of anti-toxic and anti-parasite immunity differ. The acquisition of resistance to the clinical effects (anti-toxic immunity) is reflected by a profound fall in the death rate in early childhood to much lower levels which are subsequently sustained. This precedes anti-parasite immunity which serves to reduce the prevalence and density of parasitaemia, occurring slowly throughout childhood to reach lowest levels in adult life.
Anti-toxic immunity, also called anti-disease immunity, is an immune response which is effective against the clinical symptoms, targeting antigens such as those which induce TNF. The pattern of this anti-toxic immunity is such that the prevalence of antibodies increases very rapidly reaching its peak by age 5-8 years and remains high throughout life. Anti-parasite immunity, which controls parasite multiplication and reduces parasitaemia, results in a low antibody prevalence in children under 7 years but increases to a plateau after age 25-35 years. It appears that the data for MSP2 fits with the latter profile i.e. antibody prevalence is low in children but increases to a plateau by adulthood, although the precise timing and level of response varies between antigens. This pattern has also been described for other studies of age-dependent recognition of *P.falciparum* antigens [158, 197, 241]. Indeed, the incidence of severe disease and death declines rapidly in young children (peak mortality in The Gambia occurs in children aged over 4 years [128]). Therefore, since the peak recognition of MSP2 occurs after the age of 4 years and antibodies to MSP2 are unlikely to play a role in anti-toxic immunity.

It has been proposed that the slow immunological response to *P.falciparum* malaria may be associated with the intrinsically poor antigenicity of malaria parasites [62]. This does not appear to be the case for MSP2. Analysis of the median positive ODs (table 6.4) showed no significant difference with age, except for B1. Thus, although the **prevalence** of antibodies to MSP2 is strongly age dependent, the **level** of antibody does not depend on age. The affinity and concentration of antibody in the serum of children appeared to be comparable to the antibody of adults (figure 6.2a & b). Furthermore, convalescent sera from individuals who were known to have been exposed to malaria parasites a limited number of times, in some

cases only once, recognised MSP2 with OD values comparable to endemic sera. Therefore it can be concluded that MSP2 is naturally highly immunogenic.
A2(T9/96) represents one variant sequence of the most polymorphic region of MSP2. The median OD and prevalence of antibodies to this antigen were strongly age-dependent, but in comparison to the other antigens, the antibody prevalence was lower. However, it is likely that this antibody prevalence is an underestimate of the recognition of this region of MSP2 serogroup A reflecting the high degree of polymorphism in the R1 repeats rather than a lack of immunogenicity.

To conclude, our data indicates that immunity to MSP2 is gradually acquired with age, with a prevalence profile that suggests a role in anti-parasite rather than anti-toxic immunity. This antibody response is, in part, strain specific. The data indicate that poor immunogenicity is not the reason for the lack of MSP2 antibodies in children. Neither is constitutional nonresponsiveness in small children a problem. However, several possible explanations remain for the relatively low prevalence of antibodies to MSP2 in children:
1) seropositivity in children may be transient. When the serum samples were collected it is possible that a proportion of the children had not been infected since the previous transmission season and their immune responses to MSP2 had not been boosted, whereas adults have a more stable immune response due to the development of immunological memory;
2) the antibody in some very young childrens' sera may be predominantly IgM, which could not be detected in this ELISA;
3) immunity may be strain-specific - it may be necessary for an individual to be infected with all the isolates circulating in the local population.

From this data it is not possible to determine whether the increased prevalence with age of anti-MSP2 antibodies is associated with **protective** immunity to *P.falciparum*. The prevalence of antibodies appears to correlate, at a population level, with acquired immunity, but this may not hold up at an individual level. Therefore, it is important to determine whether there is an association between antibody to MSP2 and

clinical status of children who are actively acquiring immunity to *P.falciparum* malaria.

7. Association of the immune recognition of MSP2 with malaria morbidity

7.1 Introduction

Individuals who are clinically immune to malaria are characterised by the rarity and mildness of clinical symptoms if an attack of malaria should occur. Acquired immunity is only partial, as parasites are often found in low densities in the peripheral blood of "protected" individuals, and immunity wanes once exposure to the parasites ceases [320]. Children suffer the most from clinical malaria, with the greatest prevalence of malaria-related deaths. Malaria is responsible for approximately 25% of deaths in children aged 1-4 years in The Gambia [128]. The first evidence of acquired immunity in children is often their ability to carry a relatively high parasite burden without suffering severe clinical symptoms [211]. With increasing age (and exposure), children and adolescents suffer fewer and less acute malarious episodes. By adulthood, the level of infection is subpatent and clinical manifestations are rare [240]. The clinical symptoms of *Plasmodium* infection are associated with the erythrocytic stages of the life cycle; episodes of fever coincide with the release of merozoites from the infected rbc.

There are several pieces of evidence to suggest that anti-bloodstage antibodies play a role in protection against malaria.

In vitro assays have examined the inhibitory effect of immune monkey serum, human serum or mAbs on parasite growth. Cohen *et al* [61] demonstrated that serum from monkeys immune to *P.knowlesi* could specifically inhibit the cyclical proliferation of the parasite. This protective antibody was shown to be associated with IgG and IgM, but its activity was complement independent and could be provided by $F(ab')_2$ fragments [59]. The immune serum had little effect on the growth of the intracellular parasites but suppressed the cycle following schizogony, suggesting that merozoites were the target and antibody inhibited their invasion into rbc.

After the development of improved methods for parasite culture, the effects of immune serum on *P.falciparum* growth in continuous culture could be assessed [45, 54, 105, 223, 258]. *Aotus* monkeys could be made immune to *P.falciparum* by drug-therapy after the development of high parasitaemia [45]. Immunity to *P.falciparum* could also be induced by injecting the animals with parasite suspensions enriched in merozoites emulsified in

Freund's complete adjuvant [226]. For example, Reese and Motyl [258] found that 0.5-3.5% immune monkey serum in the culture system caused 29-56% inhibition of parasite growth after 4 days. This was increased to 75% inhibition by the use of purified Ig. Although the animals were resistant to malaria and developed antibodies capable of inhibiting growth *in vitro*, suggesting that such antibodies were protective, it was not assumed that this was an absolute correlation [54]. Early passive transfer experiments using a primate system demonstrated that, in some instances, serum taken from monkeys with chronic infection and injected into those suffering from an acute attack could prevent death or at least prolong the course of the experimental disease [58]. However, Fandeur *et al* [105] showed that the presence of inhibitory antibody, measured *in vitro*, was not correlated with the level of functional immunity or with the ability to confer protection upon *in vivo* passive transfer. It is possible that, in such cases, the effector function of antibody was dependent upon co-factors such as activated mononuclear cells to mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

The role of human immune serum, in resistance to malaria, has also been evaluated. Serum or purified Ig from individuals living in endemic areas has been tested for inhibitory effects *in vitro* on the growth of parasites originating from the same area or different geographical areas [33, 164, 360]. The results of these studies indicated that antibodies from some, but not all, individuals can inhibit parasite growth *in vitro*. Therefore, studies of immunity to malaria have demonstrated that sera collected from experimentally infected animals, or from human living in malaria endemic areas, contain factors that inhibit parasite development *in vitro*.

The role of antibody in protection against malaria *in vivo* has previously been inferred from the occurrence of neonatal immunity in the offspring of immune mothers and the presence of protective antibody in such newborn infants' sera. Edozien *et al* [92] demonstrated a close correlation between levels of gammaglobulin in blood from the umbilical cord and from the mother, concluding that increased gammaglobulin in the blood of African mothers could be transferred across the placenta to their babies. Passive transfer of cord-blood gammaglobulin [92] was shown to dramatically decrease *P.falciparum* parasitaemia in a Nigerian infant. Also, passive transfer of gammaglobulin from Nigerian adults demonstrated antiparasitic properties with high parasite densities in patients consistently being

reduced within 4 days of starting treatment [92]. This confirmed the observation of Cohen *et al* [63] who administered purified gammaglobulin from immune adult Gambians to Gambian children suffering from acute attacks of malaria; the treatment had a marked effect on parasitaemia and clinical symptoms. This was more recently demonstrated by passive transfer of African IgG antibodies to Thai patients [280].

The utilisation of hybridoma technology has permitted the description of mAbs active against different malarial parasites. In the rodent model, it has been possible to examine the *in vivo* protective capacity of such mAbs [114]. Protection was evident with the use of mAbs specific for determinants on the merozoite, thus it appears that antibody-mediated protection against malaria was acting at the level of the free merozoite, inhibiting invasion of the host erythrocytes. In the case of *P.falciparum* and *P.knowlesi*, antibody activity was measured *in vitro* and mAbs were shown to block invasion of merozoites into rbc [102, 238].

Thus, the target antigens of the protective immune response are thought to be antigens either on the surface of the merozoite [59, 102, 114, 238] or alternatively antigens expressed on the surface of infected erythrocytes containing late stage parasites. Antibody against merozoites could promote a number of possible anti-parasite effector mechanisms. *In vitro* studies have shown that immune serum or mAbs can prevent invasion of merozoites into erythrocytes. This could be mediated either by agglutination of free merozoites by immune serum [59, 102], or antibodies may bind to the surface and interfere with the parasite ligand-rbc receptor [114, 238]. Others suggest a direct interaction of protective antibodies with some cellular effectors through a mechanism like antibody-dependent cellular cytotoxicity; Cytophilic antibodies may act by stimulating blood monocytes through Fc receptors after binding to the parasite target [42, 88, 105, 195].

Several epidemiological studies have examined the relationship between anti-malarial immune responses and protection from clinical malaria [158, 197, 200, 264-266, 269]. The direct, causative relationship between antibody level to a specific antigen and malaria morbidity is difficult to prove. However, it would be expected that antibody levels would rise with age at approximately the same rate as clinical resistance, that these antibody levels would remain high throughout adulthood, and that antibodies to a particular antigen are directly associated with protection in

prospective longitudinal studies. Such studies are used to examine the relationship between naturally acquired immune response to malaria antigens and subsequent susceptibility to malaria infection and clinical disease. The serum samples which have been used in our study have previously been used to study the association of antibody recognition of CS protein [264], PfMSP1 [266], Pf155/RESA [265] and soluble exoantigens [269] with the subsequent susceptibility to malaria infection. Significant levels of anti-CS antibodies were found in a minority of Gambian children and although the proportion increased gradually with age, no association could be demonstrated between the presence of antibody and protection from malaria [264]. A study in the same area a few years earlier, also indicated that the humoral immune response to the CS protein did not play a major role in the development of immunity to clinical malaria, despite seropositivity being age-related [197]. However, antibody to blood stages was found, in some cases, to be associated with protection from malaria. The prevalence and concentration of antibodies to PfMSP1 increased with age and it was found that high titres of antibody to the N-terminal conserved region and to the C-terminal $PfMSP1_{42}$ were significantly associated with resistance to clinical malaria and high parasitaemia. Antibody responses to soluble exoantigens have been shown to be strongly age-dependent [158]. Apparent associations were seen for antibody to Ag4 [158] and antibody to Ag2 [269] and resistance to clinical malaria, however, it was concluded that although antibody to these soluble exoantigens did not protect seropositive children against fever, these antibodies did seem to be associated with resistance to high levels of parasitaemia [269].

In The Gambia an association between increasing titres of anti-Pf155/RESA antibodies and decreasing parasitaemia with age was found, but no association could be demonstrated at an individual level between the presence of erythrocyte membrane immunofluorescence (EMIF) antibodies and protection [200]. Riley *et al* [265] confirmed the temporal association between the development of clinical immunity and the acquisition of EMIF antibodies. In addition, it was shown that the presence of antibodies binding to peptides containing B cell epitopes of Pf155/RESA was associated with reduced susceptibility to clinical attacks of malaria and with resistance to high parasitaemia. Marsh *et al* [200] also reported that between the ages of 3 and 8 years the development of antibodies to parasite-dependent rbc neoantigens (PDN) was rapid and that these

antibodies were associated with protection from clinical episodes of malaria. Thus in such longitudinal prospective studies it is possible to demonstrate significant associations of malaria specific antibodies with resistance to clinical malaria.

There are several factors which suggest that antibodies to MSP2 may have a protective role in malaria infections. Antibodies to MSP2 are strongly age-dependent, reaching peak prevalence in adolescence (chapter 6). The rate of acquisition of antibodies is consistent with the development of anti-parasitic immunity. However, this is not an index of functional malaria immunity and does not mean that the increase in antibody prevalence and the decrease in malaria morbidity with age are causally related. The location of MSP2 on the merozoite surface makes it directly accessible to the immune effector mechanisms [298]. mAbs specific for MSP2 serogroups A and B have been produced which inhibit parasite replication *in vitro* [101, 255] and prevent free merozoites from invading erythrocytes [55]. Rabbit anti-serum raised against MSP2 was also shown to inhibit merozoite invasion [220]. Furthermore, Saul *et al* [284]report protective immunisation of mice with invariant peptides of MSP2: octapeptides from the conserved regions induced anti-MSP2 antibody which cross-reacted with a homologue in the rodent malaria parasite *P.chabaudi.* Vaccinated mice, challenged with an otherwise lethal inoculum of *P.chabaudi,* showed substantial resistance with most mice surviving. Recently, Al-Yaman *et al* [2] studied the relationship between the humoral response to MSP2 and malaria morbidity in Papua New Guinea. The proportion of the population with antibodies to MSP2 increased significantly with age. The authors observed a negative association between antibody prevalence/ concentration and fever episodes and a positive association between a decrease in antibody and haemoglobin levels, indicating a possible protective role of antibodies against presumptive *P.falciparum* malaria.

The aim of the study described in this chapter is to determine whether the acquisition of antibodies to MSP2 is associated with increasing resistance to malaria infection. The approach used is to examine the association between malaria morbidity and the immune recognition of MSP2 in a cohort of semi-immune children, in a prospective, longitudinal study. The study was designed to determine whether pre-existing antibodies to

defined MSP2 epitopes were correlated with the subsequent malaria history of the children.

7.2 Materials and methods

7.2.1 Study design

Plasma samples were obtained from a previous study of antimalarial immunity [264].

In May 1988 (before the malaria transmission season), 355 children aged 3-8 years from hamlets around the village of Farafenni, were recruited into the study and a blood sample collected. The children were examined by a physician; the spleen was palpated and any enlargement noted. Blood films were examined for malaria parasitaemia and all parasitaemic children received a curative dose of chloroquine and Maloprim®. Heparinised blood was centrifuged and plasma removed, aliquoted and stored at -40/-20°C.

During the following six month period each child was visited once a week by a field worker who filled in a health questionnaire and recorded the child's axillary temperature. If the temperature was 37.5°C or greater, a blood film was made and examined for malaria parasites. Infected children were treated with chloroquine. The children were re-examined in November, at the end of the malaria transmission season, and a fingerprick blood sample was obtained and examined for malaria parasites.

It was possible to allocate each child to one of four groups depending on their malaria experience during the preceding 6 months:

Group 1 - clinical episode of malaria, defined by fever > 37.5°C in the presence of *P.falciparum* parasitaemia ≥5000 asexual stage parasites/µl.

Group 2 - Indeterminate infection, defined by fever (> 37.5°C) in the presence of low parasitaemia (<5000 asexual stage parasites/µl).

Group 3 - Asymptomatic infection, defined as any child in whom an episode of fever had not been detected but who had a positive blood film at the second survey, or who had splenomegaly that had not been present in May or that had increased by ≥ 2cm.

Group 4 - no evidence of infection, defined as an individual who experienced no clinical episode of malaria, no parasitaemia and no splenomegaly.

Control sera were obtained from European donors, children (n=19) and adults (n=12). These individuals had not been exposed to malaria.

7.2.2 Recombinant proteins

The rAgs used for this study represented the R1 repeat region and the R2 repeat/group specific region of serogroups A and B - A2(T9/96), A3, B2 (K1i) and B3 (T9/105i).

7.2.3 Enzyme-linked immunosorbent assay (ELISA):

IgG antibodies were measured in ELISA as described in materials and methods. For screening, all sera were diluted 1/1000 in blocking buffer and tested in duplicate.

7.2.4 Statistical methods:

1. Determination of positivity:

Positive samples were defined as those giving a specific OD above the normal range for control sera. The normal range was taken as the mean + 2 standard deviations of the OD values obtained for the control sera.

2. Logistic regression analysis:

Logistic regression analysis determines the "relative risk" of a particular outcome (in this case susceptibility malaria) in association with a measured variable (recognition of MSP2) and tests the difference in the relative risk in different groups. The difference is represented by an odds ratio (OR) which gives an indication of the strength of the association between the factor (antibody response to MSP2) and the susceptibility to malaria. In addition possible confounding factors can be included in the analysis to give the adjusted OR. If the 95% confidence interval for the adjusted OR includes 1, then there is no significant association between the measured variable and outcome of infection.

The outcome variable in logistic regression is binary or dichotomous, in this cases responder or nonresponder.

3. Generalised linear model:

This analysis compares the mean OD of each group, to determine whether the magnitude of the response is different between groups. The significance of the particular variable (OD) is assessed by fitting two models, one including and the other excluding that variable. The test is based on the difference of the deviances of the two models.

Possible confounding variables are included in the GLM and a gamma distribution is used to directly model the skewed distribution of the outcome variable.

7.3 Results

7.3.1. Association of the immune recognition of MSP2 rAgs with malaria morbidity:

Serum samples were collected from 355 children aged 3 - 8 years during the dry season when malaria transmission is minimal. Morbidity surveys were carried out during the following transmission season and the children were allocated into one of four groups defined by their infection status.
A χ^2 test for trend showed that the prevalence of MSP2 antibodies was strongly age-related (table 7.1), as previously described (chapter 6).

rAg	Age (years)				Test for trend	
	3-4 (n=129)	5-6 (n=121)	7-8 (n=105)	Total (n=355)	χ^2	p
A2	25.6	28.1	43.8	31.8	8.42	0.004
A3	13.2	24.8	43.8	26.2	27.64	<0.001
B2	11.6	14.9	28.6	17.7	10.90	<0.001
B3	39.5	53.7	69.5	53.2	20.85	<0.001

Table 7.1: Prevalence (%) of anti-MSP2 antibodies in semi-immune children from The Gambia.
Serum was collected from 355 children during the dry season (before malaria transmission season) and tested for recognition of MSP2 rAgs. The children were divided into 3 age groups: 3-4 yrs, 5-6 yrs and 7-8 yrs. A χ^2 test for trend was performed on the % responders per age group to determine the association of anti-MSP2 antibodies with age.

To determine whether antibody positivity was an indicator of protection, the antibody recognition of MSP2 by asymptomatic individuals (group 3) and by individuals who developed clinical malaria (group 1) was compared.
Since it is not certain that children with no evidence of infection (group 4) had in fact been exposed to infected mosquitoes and since the status of children with fever and low parasitaemia (group 2) is also unclear as fever could be due to a different intercurrent infection, comparisons of immune responses and malaria morbidity were made between groups 1 and 3 only.
Two analyses were used; the first compares the proportion of responders and nonresponders to each antigen between individuals with clinical

malaria and those with asymptomatic malaria, the second compares the mean OD to each antigen for groups 1 and 3.
Both analyses are multivariate, thus age, sex, ethnic group, bednet status and sickle cell carriage can be included, as it is essential to adjust for their possible effects.
The following analyses were performed by D McGuiness, ICAPB, Edinburgh University.
1. Multiple logistic regression:
The relationship between antibody recognition of rMSP2 and subsequent malaria morbidity was modelled by logistic regression to give an odds ratio (table 7.2). An adjusted OR was calculated to allow for the possible effect of the confounding variables.

rAg	Asymptomatic %responders n=87	Clinical %responders n=108	Crude OR (95% CI)	Adjusted OR * (95% CI)
A2(T9/96)	34 (39.1)	36 (33.3)	0.78 (0.43-1.40) ns	0.97 (0.50-1.88) ns
A3	30 (34.5)	26 (24.1)	0.60 (0.32-1.12) ns	0.89 (0.43-1.84) ns
B2(K1i)	22 (25.3)	11 (10.2)	0.34 (0.15-0.74) p<0.005	0.42 (0.17-1.02) ns
B3(T9/105)	58 (66.7)	49 (45.4)	0.42 (0.23-0.75) p<0.005	0.43 (0.22-0.84) p<0.005

Table 7.2: Multiple logistic regression analysis to determine the relationship between recognition of MSP2 and subsequent susceptibility to malaria. *An adjusted OR was calculated to account for any possible effects of confounding by age, sex, ethnic group, bednet status and sickle-cell carriage.

The analysis indicates that the presence of antibodies to B3 is significantly associated with asymptomatic infection i.e the proportion of responders to B3 is higher in asymptomatic malaria cases than in clinical cases. The association between malaria morbidity and antibodies to B2 approaches

significance; the influence of the confounding variables accounting for the association observed with the crude OR.
No significant association is apparent for the recognition of the serogroup A rAgs, A2 and A3.

2. Generalised linear model:
For each antigen the mean OD for the asymptomatic group was compared with the mean OD for the clinical malaria group by means of a generalised linear model. The data is positively skewed (figure 7.1), with many OD values being close to zero. Therefore, a standard linear regression of the OD values would be inappropriate, as this assumes normal distribution of data. Instead the generalised linear model was fitted to the data using a gamma distribution to directly model the distribution of the OD values. Results from this linear regression assuming a gamma distribution are shown in table 7.3, with and without including the possible confounding variables.

rAg	mean OD		δ.dv [a]	est.β [a] (s.e)	δ.dv [b]	est.β [b] (s.e)
	Asymp. n=86	Clinical n=107				
A2(T9/96)	0.354	0.286	+1.55	-0.083 (0.067)	+0.002	0.003 (0.070)
A3	0.292	0.195	+4.98§	-0.130 (0.058)	+1.426	-0.066 (0.061)
B2(K1i)	0.204	0.103	+4.64§	-0.111 (0.052)	+0.308	-0.028 (0.054)
B3(T9/105)	0.532	0.348	+7.14§	-0.196 (0.074)	+5.21§	-0.156 (0.077)

Table 7.3: Association of mean OD to rMSP2 with malaria status. Results were analysed using linear regression assuming a gamma distribution; δ.dv is the change in deviance when 'group' (i.e either asymptomatic/clinical) is removed from the model; est.b is the estimated regression parameter.
§ indicates d.dv>3.84, which is significant.
[a] indicates analysis without allowing for confounding variables; [b] indicates analysis including confounding variables.

Inclusion of the confounding variables in this analysis clearly indicates that they are exerting an effect on the outcome of infection. Overall, the results are essentially the same as for the multiple logistic regression. No significant association of antibody to A2, A3 and B2 with subsequent susceptibility to malaria morbidity was observed, whereas the mean OD to B3 was significantly higher in individuals who were asymptomatic.

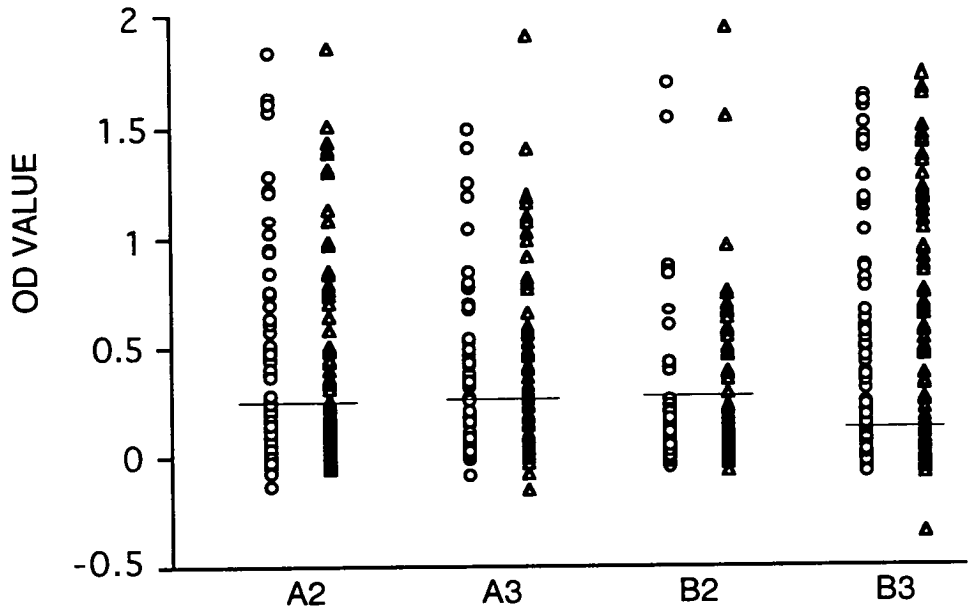


Figure 7.1: Dot plot of the antibody recognition of MSP2 rAgs. Serum samples were collected from a cohort of semi-immune children in May (pre-transmission) and individuals monitored during the malaria transmission season and subsequently classified as having clinical o or asymptomatic ▲ infections.

Horizontal lines represent the cut-off level determined from OD values of control sera

7.4 Discussion

Antibodies to MSP2 are strongly age-dependent, reaching a peak prevalence in adolescence (chapter 6). This rate of acquisition of antibodies reflected a pattern of anti-parasitic immunity rather than anti-disease immunity. However, immunity resulting in decreased parasite densities would also lead to less severe symptoms. Many epidemiological studies show that antibody prevalence to a particular antigen increases with age, but it is unlikely that all of these are involved in protective immunity. An increase in antibody with age may be associated with protection or may just be a measure of exposure. Therefore, to determine if the age-dependent pattern of MSP2 antibody prevalence reflects a role for MSP2-specific antibodies in effective protective immunity, the immune recognition of MSP2 by a cohort of semi-immune children was studied and related to their subsequent susceptibility to malaria infection and disease. The antibody recognition of rAgs representing the R1 repeat region (A2 and A3) and the R2 repeat/group specific region (A3 and B3) of each serogroup was measured during the dry season, before the malaria transmission season. These children had already begun to develop immunity to malaria and the aim was to relate existing anti-MSP2 antibodies to their subsequent infection status. Care must be taken in the interpretation of the results as any apparent associations between antibody recognition and infection status do not prove a direct, causative relationship but do identify areas worthy of further investigation.

In terms of estimating protective immunity against disease, it is appropriate to compare those children who were infected but did not become ill during the transmission season (group 3) with those who experienced at least one febrile episode associated with *Plasmodium* infection (group 1). Comparison of the proportion of responders in each group using logistic regression analysis allowing for confounders, showed a significant association between the presence of antibody to B3 and reduced risk of clinical symptoms. No such association occurred for the other antigens. A second analysis using a general linear model, taking into account the skewed nature of the data, showed that the mean OD of B3-specific antibodies was higher for individuals with asymptomatic malaria, whereas the mean OD to A2, A3 and B2 was not significantly different between the two groups.

The confounding variables were shown in both analyses to influence the outcome of infection, and therefore it is essential that such variables are included in analyses of this kind.
Thus, in both analyses, the recognition of B3 is associated with protection from clinical malaria in this cohort of children. However no association was found for the other antigens tested. Possible explanations for this are: i) that under natural conditions, individuals do not have a focalised immune response, i.e. a response directed exclusively to one particular region or epitope of an antigen, thus antibody responses to different regions of MSP2 (and possibly to other antigens) are closely associated preventing the demonstration of a protective effect to any one antigen or epitope i.e. MSP2 may be necessary but not sufficient for protection; ii) antibody to the other regions of MSP2 may be associated with protection but that the sequence polymorphism precludes the demonstration of this with any one representative sequence.

In this chapter the data suggest that the antibodies to epitopes in the 5´ repeat/group specific region of MSP2 serogroup B (B3) appear to confer resistance to clinical malaria. In previous studies of blood stage antigens [158, 200, 264, 269], few associations with protection have been found therefore, in terms of vaccine development, the results presented here are encouraging. However, additional aspects of the immune recognition of MSP2 need to be studied. Since the antibody-dependent killing of malaria parasites is, at least in part, cell-mediated, an important aspect in studying the induction of protective immunity is to assess the IgG subclass of the antibodies involved. Such studies are reported in chapter 8.

8. IgG subclass specificity of anti-MSP2 antibodies

8.1 Introduction

Human IgG subclasses are known to have a variety of biological effector functions [306, 361]. They have been designated IgG 1-4 based on their relative abundance in normal serum. The structure and function of each human IgG subclass protein has been studied extensively [39, 361], and they have been shown to differ in their abilities to fix complement and bind to cell surface Fc receptors [40]. The four subclasses of murine IgG, listed in order of serum concentration, are IgG2a, IgG1, IgG2b and IgG3 [306].

The subclass induced during an infection can be dependent on the nature of the antigen being presented. In humans, IgG1 and IgG3 are secreted in response to proteins [160], IgG2 is preferentially induced by polysaccharides [139] and IgG4 would be induced after chronic exposure to proteins [139]. Since the biological effector functions of different IgG subclasses vary greatly, the serum concentrations of antibodies belonging to a given subclass may indicate their clinical role in the course of an infection.

The study of IgG subclass responses in parasitic infections is limited; however, it is apparent that certain organisms preferentially induce antibodies of particular subclasses. These subclasses can be associated with effective, protective immune responses or immunopathology. This has been shown for human filariasis. Cabrera *et al* [44] established a correlation between a particular clinical condition of onchocerciasis (Sowda) and a predominantly IgG3 antibody response to a low molecular weight antigen of *Onchocerca volvulus*. The IgG3 antibody appears to mediated a type III hypersensitivity reaction. During infection with *Wuchereria bancrofti*, IgG4 antibody responses are associated with asymptomatic microfilaraemia, whereas IgG1 and IgG3 antibodies are associated with the clinical presentation of chronic pathology and elephantiasis [156]. Similarly, IgG4 antibodies are predominant in asymptomatic microfilaraemics during infection with *Brugia malayi* [187] whereas increased levels of IgG1-3 and IgE are associated with chronic disease. Therefore it appears that IgG4 may act as a blocking antibody, preventing the chronic outcome of infection.

Protozoal infections predominantly induce antibodies that have cytophilic and complement fixing properties in mice and humans. Parasite-specific

IgG1 and IgG3 are the predominant subclasses in visceral *Leishmania* infections [94, 95], and in Chagas' disease patients [287]. In the mouse model, IgG2a has been implicated as important in resistance to *Trypanosoma cruzi* infection [249].

A predominance of cytophilic antibodies has also been found in murine malaria models. White *et al* [354] studied the response in mice to *P.yoelii* infection to determine if protective antibody was equally distributed among all the isotypes, and if a single isotype was capable of modulating infection. The data suggested that anti-malarial antibody of the cytophilic IgG2a subclass was predominantly responsible for protection in passive transfer experiments. Also, IgG2a mediated protection in *P.berghei* infected mice [1].

The role of malaria specific IgG antibody in protection from clinical malaria in humans has been described in chapter 7. Several mechanisms of parasite clearance were proposed and these could be dependent on the induction of the appropriate IgG subclasses. For example, an antibody-dependent cellular cytotoxicity mechanism requires Cytophilic antibodies to stimulate effector cells through cross-linking of Fc receptors after binding to the parasite target. In fact, several studies have shown that *in vivo* protection from malaria correlates with an *in vitro* inhibition of parasite growth by immune IgG only in the presence of blood monocytes [183, 195].Therefore, it is likely that if ancillary cells are required for biological function, then the isotype of the relevant antibodies is an important variable. Thus, the authors suggested that, in light of the monocyte-dependent effect of antibodies functional differences may exist among antibodies of the same specificity and this may contribute to the slow development of anti-malarial immunity. This has been continued in studies which show that IgG1 and IgG3 antibodies, specific for mature schizonts, predominate in individuals protected from clinical malaria, and that IgG2 and IgM from non-protected individuals could block the activity of immunoglobulins in immune sera [29]. In these studies the antigenic specificity of the antibodies was not established, however, it was speculated that the antibodies should recognise accessible antigen on the merozoite surface [88].

Groux and Gysin [131] proposed opsonisation as an effector mechanism in protection against blood stages of malaria parasites. In humans, IgG1 and IgG3 are the most effective. opsonising subclasses. The results of their study suggested a correlation between immune protection, the ability of

serum to mediate opsonisation of infected erythrocytes and the predominance of IgG1 and IgG3 over IgG2 and IgG4 directed against the surface of infected erythrocytes.

The levels of human IgG subclasses to crude or defined malarial antigens have been investigated in several studies. The first report measured the response in three heavily parasitised *P.falciparum* patients from The Gambia to heat-stable (S) antigens of the red cell stages [357]. All 3 patients had highest antibody titres in the IgG1 subclass. Wahlgren *et al* [20] used a trophozoite/schizont-enriched culture antigen preparation of *P.falciparum* in ELISA studies of IgG subclass responses of infected Swedish patients and of African adults living in a malaria endemic region of Liberia. It was shown that antibodies occurred in all 4 subclasses with IgG1 titres being highest. However, higher IgG3 levels were found in most Liberian sera than in the European sera. A preliminary study of antibody levels to a soluble blood stage antigen in children and adults in Pakistan found that in most sera the malaria specific antibody exists as IgG1, sometimes to the total exclusion of other subclasses. Malaria-specific IgG3 antibody was present in about half the group, and IgG2 and IgG4 only rarely [48]. Using defined Pf155/RESA peptides in ELISA, Dubois *et al* [89] found that IgG1 and IgG3 subclasses were the most frequent and, when present, were coexpressed in almost all serum samples. However, in that study, IgG1 levels were inversely correlated with protection [89]. In Papua New Guinea, Beck *et al* [20] found that humoral responses directed against RESA were frequent in all IgG subclasses tested (IgG1, IgG2 and IgG3). RESA-specific IgG3 responses were shown to be age-dependent and cytophilic antibodies were associated with reduced *P.falciparum* prevalence [20]. Most recently, antibodies to a defined region of PfMSP1 (PfMSP119) were shown to be predominantly IgG1; this study involved essentially the same Gambian population as the present MSP2 study, and the results are directly comparable [93].

Therefore, it is important to determine not only the antigen specificity of anti-malarial antibodies, but also the isotype of these antibodies to ascertain their possible protective role against clinical malaria.

I have already shown that anti-MSP2 antibodies are present in serum from individuals exposed to malaria. In this chapter I aim to determine 1) the IgG isotypic distribution of these antibodies in malaria-immune adults; 2)

whether the distribution of IgG isotypes changes with age (and might therefore be related to the acquisition of protective immunity); and 3) whether there is any association between isotype distribution and clinical outcome of infection.

8.2 Materials and methods:

8.2.1 Serum samples:

Sera which and been shown to have anti-MSP2 IgG antibodies were selected from 1) Brefet adults (chapter 5), 2) Farafenni cross-sectional survey (chapter 6), and 3) Farafenni longitudinal survey (chapter 7).

8.2.2 ELISA:

IgG subclass ELISAs were carried out as described in chapter 3, section 3.3.2. Sera were diluted 1:1000, in duplicate, and tested for the presence of each of the IgG subclasses using anti-subclass reagents.

IgG subclass standard curves were included as a positive control; purified myeloma proteins were diluted in four-fold dilutions from 10μg/ml.

8.2.3 MSP2 recombinant proteins:

The rAgs used in this chapter were A1, A2(T9/96), A3, B1, B2(K1i) and B3(T9/105i).

8.2.4 Statistical analysis:

i) Determination of positivity

Control sera, from individuals with no history of malaria, were tested at the same time. The cut-off level for positivity was taken to be greater that the mean + 2SD of the control sera OD values for each subclass.

ii) Kruskal-Wallis one-way analysis of variance

This is the non-parametric equivalent of the ANOVA test, allowing comparisons of the median of a variable to be made between several groups.

iii) Mann-Whitney test:

This test is the non-parametric equivalent of the t-test and is used to test the null hypothesis that the median of a variable is the same in two groups.

iv) χ^2 for trend test:

χ^2 tests for trend were performed using Epi Info, and are used to determine whether there is a trend in proportions of responders with age.

8.3 RESULTS:

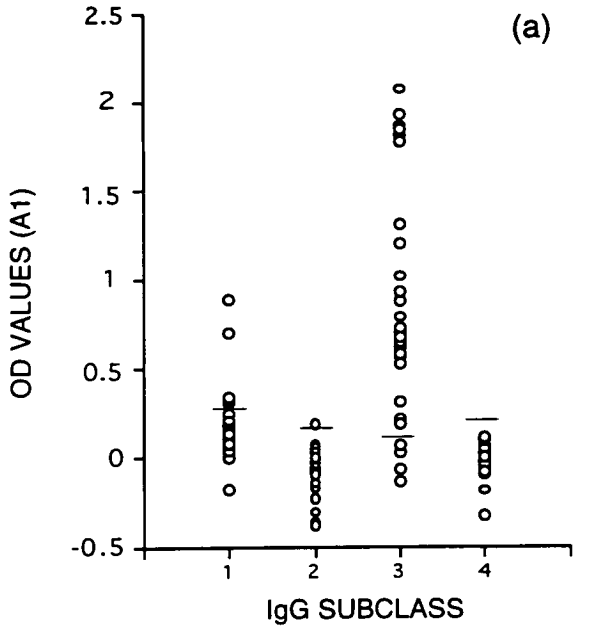
8.3.1 Isotypic distribution of anti-MSP2 antibodies in malaria-immune adults:

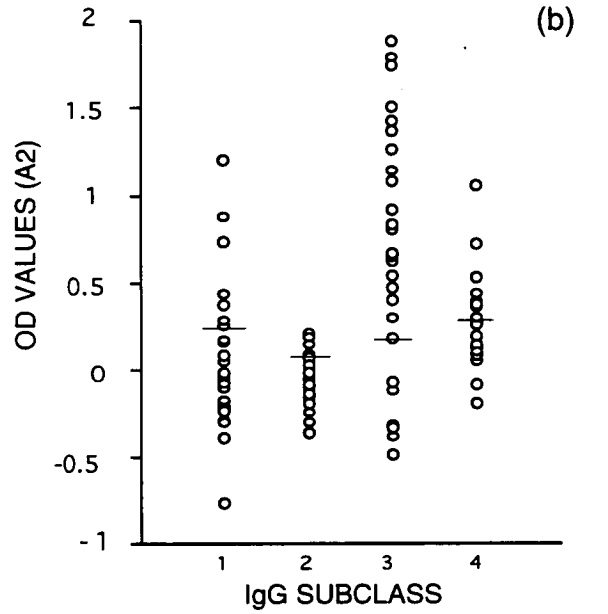
The IgG subclass specificity of anti-MSP2 antibodies was determined for sera from adults that has been shown to contain MSP2-specific IgG.

Figures 8.1, 8.2 and table 8.1 show the IgG subclass distribution of antibodies specific to each of the rAgs. Although the subclass distribution differs slightly for antibodies to the different rAgs, the predominant MSP2-specific antibody subclass is IgG3.

77% and 80%, respectively, of A1- and A3-positive sera contained IgG3 only, whereas 28% and 38% of IgG3 positive A2-specific sera contained IgG2 and IgG4 antibodies, respectively. A2 is a fragment of A1, therefore one would have expected some IgG4 containing A1-positive sera. However, the majority of the A2-positive results were close to the cut-off, and gave values below the cut-off for A1-IgG4. A1-positive sera which do not contain IgG3 antibodies, are IgG1 positive.

The pattern of recognition is similar for the serogroup B proteins, in that IgG3 is the predominant subclass. However, 59% and 47% of B1- and B2-specific sera were IgG1 positive; in the majority of cases this was coexpressed with IgG3. Although only a small number of sera were tested for the IgG subclass recognition of B3, the majority of sera expressed IgG3 antibodies.





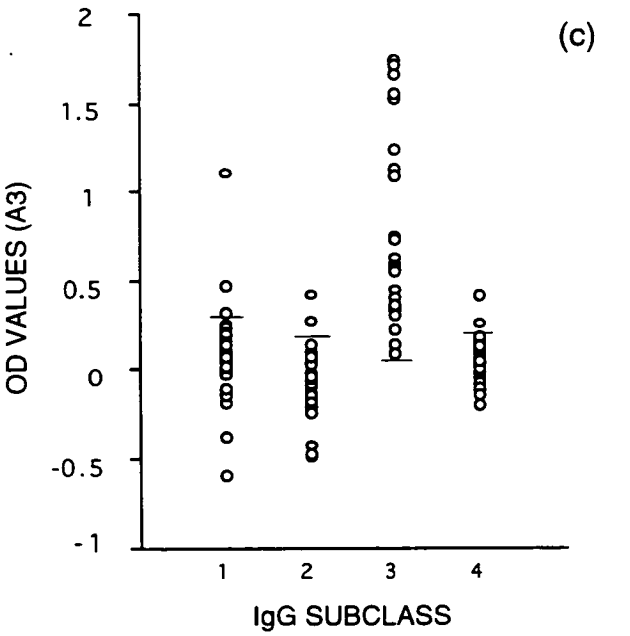
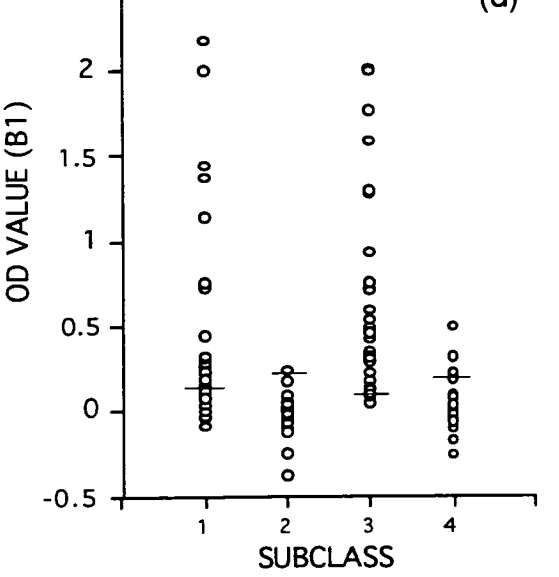
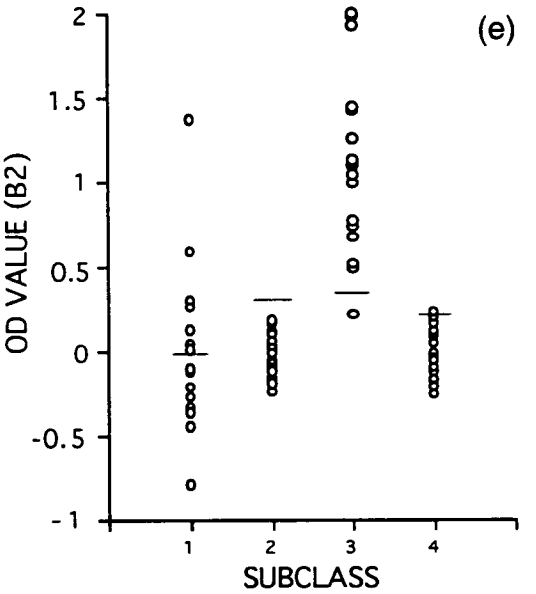


Figure 8.1: Dot plots of the IgG subclass recognition of MSP2 rAgs by sera from Gambian adults; a) A1 (n=30), b) A2(T9/96) (n=25), c) A3 (n=30), d) B1 (n=29), e) B2(K1i) (n=17) and f) B3 (T9/105i) (n=11).

The horizontal line represents the mean+2SD of European control sera.


OD VALUE (B1)
2
1.5
1
0.5
0
-0.5
1
2
3
4
SUBCLASS

(d)


OD VALUE (B2)
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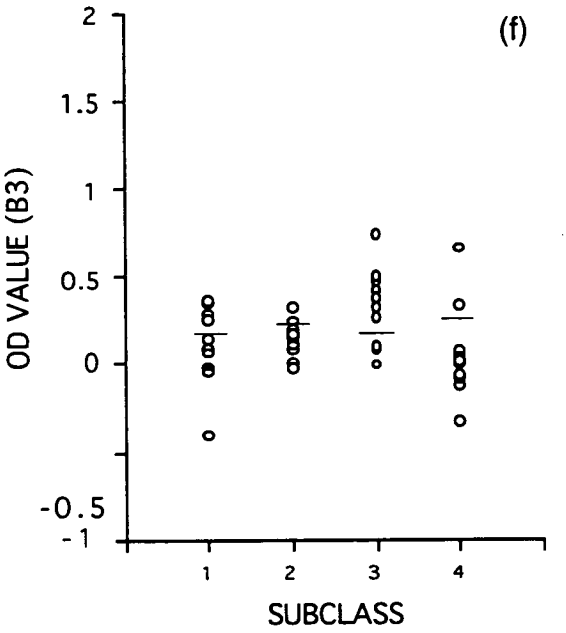
1.5
1
0.5
0
-0.5
-1
1
2
3
4
SUBCLASS

(e)


OD VALUE (B3)
2
1.5
1
0.5
0
-0.5
-1
1
2
3
4
SUBCLASS

(f)

rAg	Number	Antibody subclass Number of responders (%) IgG1	IgG2	IgG3	IgG4
A1	30	6 (20)	1 (3)	26 (87)	0 (0)
A2	25	8 (32)	3 (12)	21 (84)	11 (44)
A3	30	3 (10)	2 (7)	30 (100)	2 (7)
B1	29	17 (59)	1 (3)	28 (97)	3 (10)
B2	17	8 (47)	0 (0)	16 (94)	2 (12)
B3	11	4 (36)	2 (18)	8 (72)	2 (18)

Table 8.1: Prevalence of anti-MSP2 IgG subclasses in Gambian adult donors

8.3.2 Age-related distribution of anti-MSP2 IgG subclass antibodies in malaria exposed individuals:

In all age groups, there is a predominance of antibodies which belong to the cytophilic and complement-fixing subclasses.

In the adult sera from the age cross sectional study, the distribution of IgG subclasses was essentially similar to that shown for the Brefet adults sera, with a predominance of anti-MSP2 IgG3 antibodies.

Table 8.2 shows how the distribution of MSP2-specific IgG subclass antibodies changes with age. There is an apparent decrease in frequency of IgG1 expression and an increase in IgG3 expression with increasing age. In general the number of IgG2- and IgG4-positive sera are too small for any valid conclusions to be drawn about their association with age. However, χ^2 for trend analysis indicates that the increase in IgG3 expression with is statistically significant for A2-, A3-, B1-, and B3-specific antibodies, and the decrease in prevalence of IgG1 antibodies is significant for the recognition of A2, B1 and B2. It seems that no trend was apparent for

the IgG3 recognition of A1 and B2 because even in the youngest age group a large percentage of individuals expressed anti-MSP2 of this subclass. The mean OD for each subclass has been plotted against age (figure 8.3) . There is no obvious trend for IgG1, IgG2 and IgG4. However, the mean OD of IgG3 antibodies clearly increases with age. To determine whether any of these observations were statistically significant, data were analysed using a non-parametric Kruskal-Wallis test to compare the OD values of each age group, for each rAg (table 8.3). With one exception, recognition of B2, the level of anti-MSP2 IgG3 antibodies was shown to be age-related. Whereas anti-MSP2 IgG1 antibodies were shown to be age-related only for rAgs A2, B1 and B2.

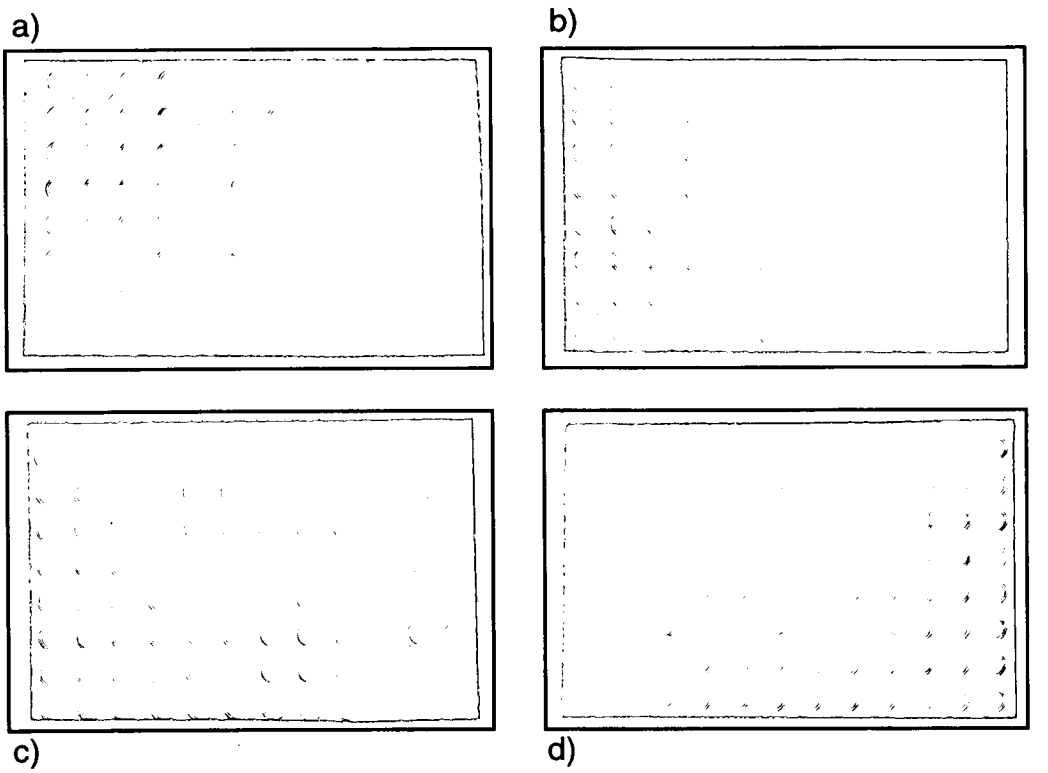


Figure 8.2: Photograph showing the distribution of anti-A1 IgG subclass antibodies in ELISA. 48 samples were tested in duplicate at a dilution of 1:1000. a) IgG1, b) IgG2, c) IgG3, d) IgG4.

rAg	Age group	Number	Antibody subclass Number of responders (%)			
			IgG1	**IgG2**	**IgG3**	**IgG4**
A1	≤ 10	20	12 (60)	3 (15)	11 (55)	0 (0)
	11-20	25	15 (60)	1 (4)	17 (68)	0 (0)
	21+	18	7 (39)	0 (0)	13 (72)	0 (0)
		χ^2 for trend	1.605	3.601	1.244	N/A
		p	0.205	0.058	0.265	
A2	≤ 10	14	12 (86)	1 (7)	4 (29)	4 (29)
	11-20	16	11 (67)	2 (13)	13 (81)	2 (13)
	21+	17	6 (35)	1 (6)	13 (82)	4 (24)
		χ^2 for trend	8.287	0.027	9.103	0.077
		p	0.004	0.870	0.003	0.781
A3	≤ 10	15	8 (53)	3 (20)	7 (47)	1 (7)
	11-20	18	10 (56)	2 (11)	12 (67)	4 (22)
	21+	13	6 (46)	1 (8)	12 (92)	1 (8)
		χ^2 for trend	0.129	0.935	6.603	0.858
		p	0.720	0.334	0.010	0.354
B1	≤ 10	14	8 (57)	0 (0)	8 (57)	5 (36)
	11-20	19	5 (26)	1 (5)	15 (79)	3 (16)
	21+	14	2 (14)	1 (7)	14 (100)	1 (7)
		χ^2 for trend	7.513	0.858	7.513	3.612
		p	0.006	0.354	0.006	0.058
B2	≤ 10	13	10 (77)	2 (15)	9 (69)	0 (0)
	11-20	26	11 (42)	0 (0)	21 (81)	0 (0)
	21+	14	5 (36)	0 (0)	12 (86)	0 (0)
		χ^2 for trend	4.386	4.158	1.077	N/A
		p	0.036	0.041	0.299	
B3	≤ 10	20	14 (70)	0 (0)	12 (60)	0 (0)
	11-20	22	8 (36)	2 (9)	19 (86)	1 (5)
	21+	21	11 (52)	3 (14)	20 (95)	1 (5)
		χ^2 for trend	1.195	2.803	8.052	0.732
		p	0.274	0.095	0.005	0.392

Table 8.2: Prevalence (%) of anti-MSP2 IgG subclass antibodies in different age groups. χ^2 for trend values are given.

rAg	Age group	Number	Antibody subclass Median OD			
			IgG1	IgG2	IgG3	IgG4
A1	≤ 10	20	0.267	0.014	0.378	0.005
	11-20	25	0.403	-0.025	0.771	0.006
	21+	18	0.308	-0.017	1.068	0.021
		H	0.34	3.13	12.15	1.41
		p	0.842	0.210	0.002	0.494
A2	≤ 10	14	0.357	-0.030	0.134	0.024
	11-20	16	0.329	0.033	0.739	-0.009
	21+	17	0.145	0.054	1.157	0.011
		H	7.12	4.96	19.13	2.20
		p	0.029	0.084	0.000	0.334
A3	≤ 10	15	0.223	0.044	0.291	0.025
	11-20	18	0.247	0.022	0.434	0.068
	21+	13	0.192	0.047	1.259	0.004
		H	0.92	0.69	8.13	3.62
		p	0.633	0.710	0.017	0.164
B1	≤ 10	14	0.336	0.003	0.371	0.110
	11-20	19	0.091	0.049	0.529	0.012
	21+	14	0.071	0.040	0.936	0.028
		H	6.69	0.80	10.77	3.32
		p	0.036	0.671	0.005	0.191
B2	≤ 10	13	0.459	-0.133	0.515	-0.030
	11-20	26	0.159	-0.103	0.665	0.005
	21+	14	0.191	-0.037	0.977	0.028
		H	7.61	2.88	3.26	9.27
		p	0.023	0.237	0.196	0.010
B3	≤ 10	20	0.197	0.013	0.106	-0.009
	11-20	22	0.086	0.009	0.275	0.005
	21+	21	0.155	0.032	0.936	-0.010
		H	2.16	1.29	20.94	1.61
		p	0.341	0.525	0.000	0.447

Table 8.3: Median OD of anti-MSP2 IgG subclass antibodies. Kruskal Wallis tests were used to determine the association of median OD with age for each antigen.

Figure 8.3: Plots of the median OD of anti-MSP2 IgG subclass antibodies in different age groups.
Age group 1: ≤ 10 years, age group 2: 11-20 years, and age group 3: 21+ years.

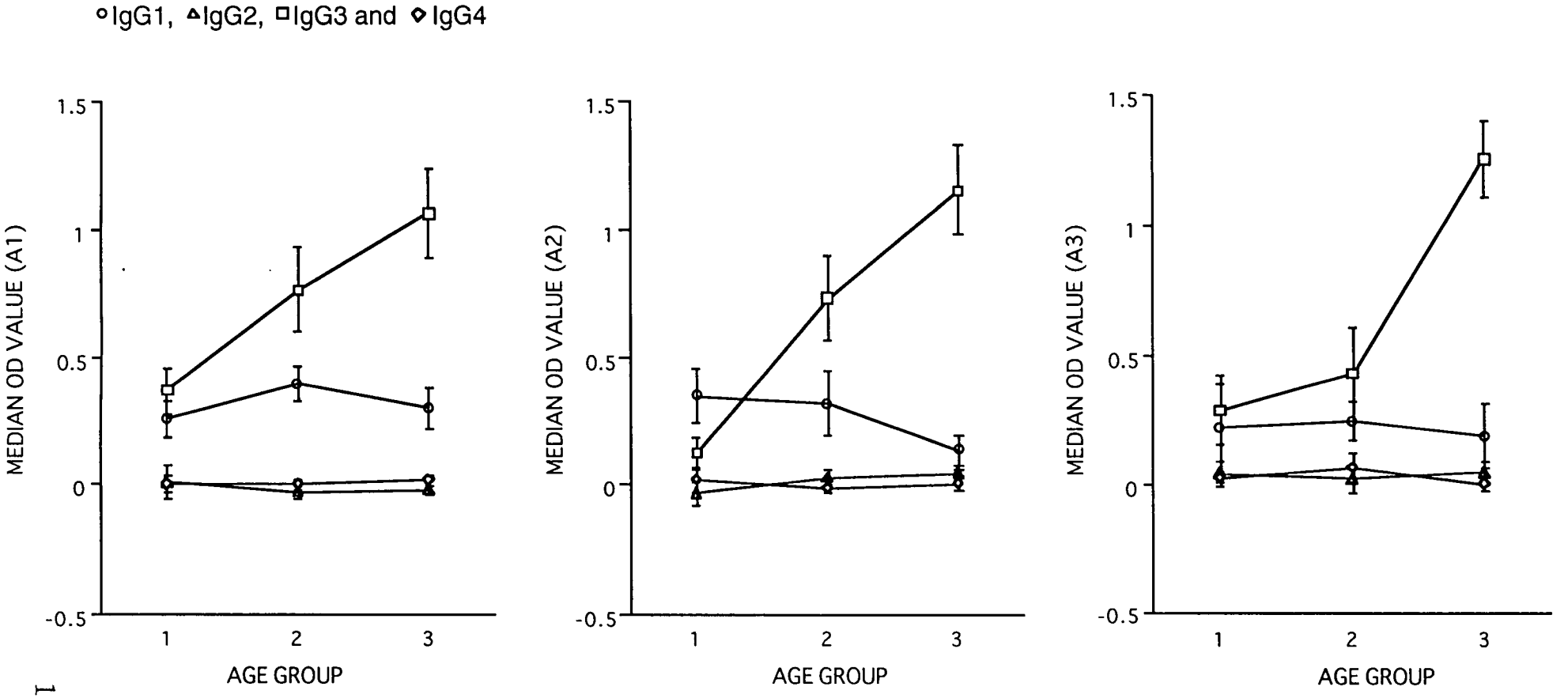
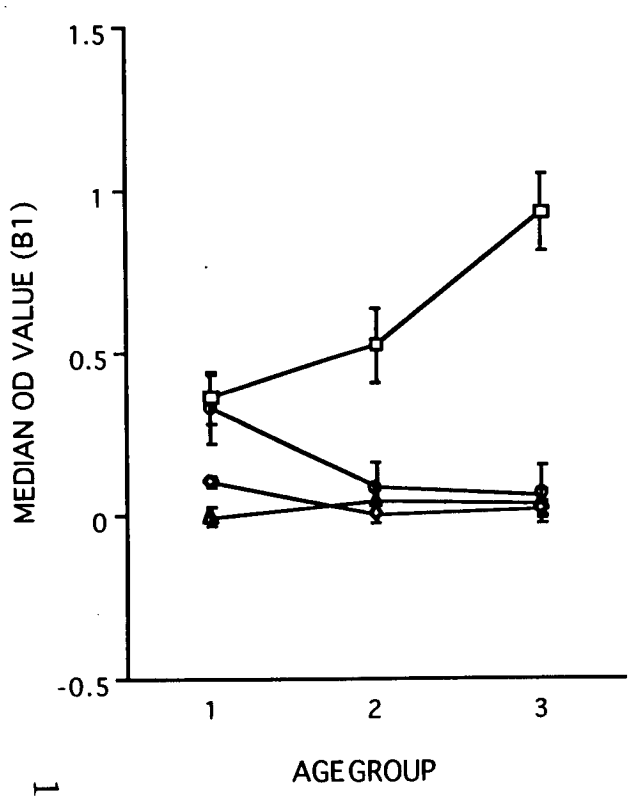
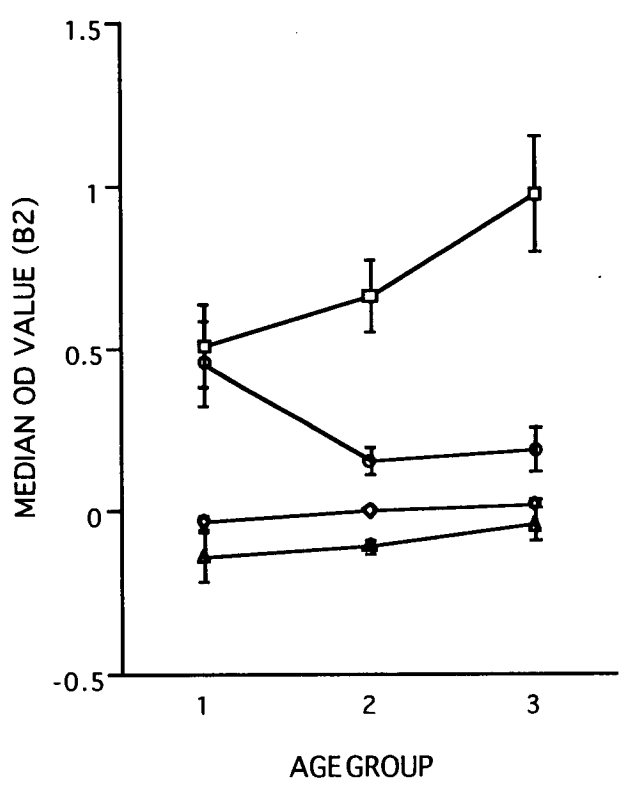
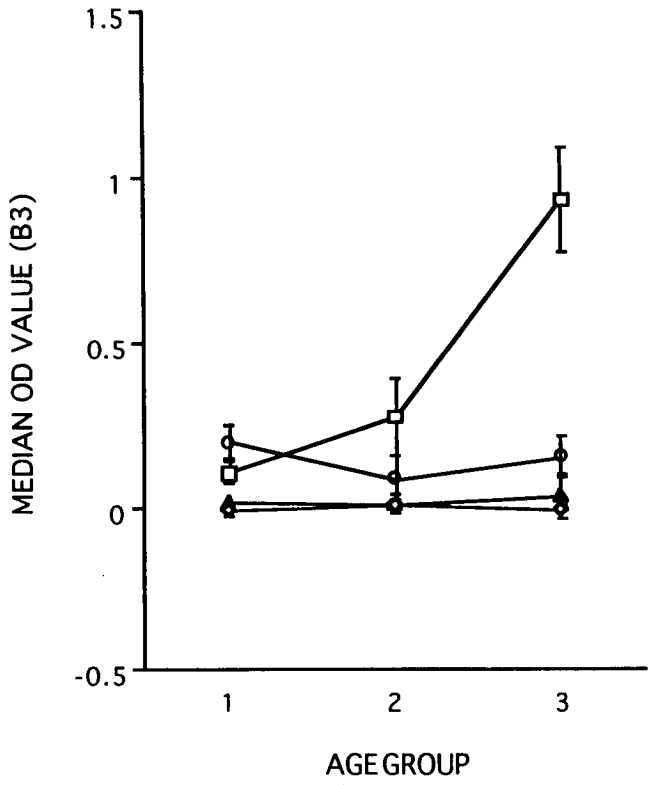


Figure 8.3 cont'd.







8.3.3 Distribution of anti-MSP2 IgG subclass antibodies in relation to subsequent susceptibility to clinical malaria:

The IgG subclass distributions of MSP2-specific antibodies were compared, using Mann Whitney tests, for individuals who had asymptomatic malaria infections and those with clinical malaria, for the recognition of A1 and B1. No significant differences between the groups were evident for the median OD values for each of the antibody subclasses. However, on comparing the proportion of responders (table 8.4), two significant results occurred. For the recognition of A1, IgG3 antibodies were associated with asymptomatic infection and for the recognition of B1, IgG1 antibodies were associated with a clinical outcome of infection.

rAg	IgG subclass	Number of responders (%)		χ^2 value	p
		Asymptomatic	Clinical		
A1		n=25	n=28		
	1	24 (86)	19 (76)	0.81	0.367
	2	12 (43)	9 (36)	0.26	0.610
	3	26 (93)	17 (68)	5.33	0.021
	4	3 (11)	1 (4)	0.85	0.356
B1		n=19	n=32		
	1	25 (78)	19 (100)	4.82	0.028
	2	4 (12.5)	2 (10.5)	0.04	0.832
	3	24 (75)	13 (68)	0.26	0.611
	4	1 (3)	0 (0)	0.61	0.436

Table 8.4: Proportion of responders with IgG subclasses specific for rAgs A1 and B1. χ^2 tests were used to determine if there were significant differences in the prevalence of A1- and B1-specific antibodies of each IgG subclass between individuals who developed clinical malaria and those who had asymptomatic infections.

These ELISAs were kindly done by Dr E Riley.

8.4 Discussion

It has previously been shown that malaria exposed individuals have MSP2-specific antibodies and that the prevalence of these antibodies is age-dependent (chapter 5 & 6). The acquisition of antibodies to MSP2 parallels the overall slow development of immunity to malaria. It has been proposed that this slow development of immunity also depends on the acquisition of the appropriate IgG subclass(es) of antibody [29]. Effective anti-malarial immunity may require a predominance of antibodies with particular functions [131].

In theory, antibodies specific for MSP2 could inhibit merozoite invasion of erythrocytes by a number of mechanisms including merozoite agglutination, complement-mediated lysis, opsonisation or blocking of receptors involved in the adherence of merozoites to erythrocytes. These various mechanisms would be mediated by antibodies of different IgG subclasses - only IgG1 and IgG3 are opsonising and complement-fixing but all four subclasses could mediate agglutination or receptor blocking. Since functional differences may exist among antibodies of the same specificity, the subclass of MSP2-specific IgG in sera from adults naturally immune to malaria has been determined. The antibodies which recognise MSP2 are predominantly of the IgG3 subclass. This is surprising in view of the fact that this subclass accounts for less than 10% of normal serum IgG [306]. It has been suggested that the ratio of IgG1 and IgG3 to IgG2 and IgG4 may be important in immunity to asexual blood stages of *P. falciparum* [29, 131] since, whilst IgG1 and IgG3 can mediate opsonisation and phagocytosis of parasitised erythrocytes or free merozoites, IgG2 and IgG4 antibodies (of the same epitope specificity) may block the binding of the protective subclasses. IgG3 is considered to be the most effective subclass for activating the complement pathway [40], and it is known to mediate cell lysis by monocytes or Fc receptor bearing lymphocytes, so called antibody-dependent cell-mediated cytotoxicity (ADCC) [306].

The distribution of IgG subclasses was measured in relation to age, to determine whether there was a change in the IgG subclass profile with age which might explain the changes in susceptibility to clinical malaria with age. Overall, IgG3 was the predominant subclass in adolescents and adults but IgG1 was more common in children under the age of 10 years. Both the prevalence and median OD of IgG3 antibodies increased with age and there was a corresponding decrease in the prevalence of IgG1 antibodies.

The shift in IgG subclass distribution with age, and the predominance of the IgG3 response in adults - who tend to have a higher degree of protective immunity to malaria - suggested that IgG3 antibodies to MSP2 may be able to mediate protective immune effector mechanisms. Therefore, the IgG subclass of anti-MSP2 antibodies (in a cohort of Gambian children) was compared with their subsequent susceptibility to clinical malaria infection. Two significant differences were detected between individuals who subsequently developed asymptomatic malaria and those with clinical malaria; these were a higher prevalence of A1-specific IgG1 in individuals with clinical malaria, and of B1-specific IgG3 in individuals with asymptomatic malaria. Although the result is not consistent between the two antigens tested, it does indicate that IgG subclass differences occur and may influence the outcome of infection.

The predominance of the IgG3 response to MSP2 is unusual and is noticeably different from the response to MSP1[93]. The only other examples, of which we are aware, of an antibody response which is significantly skewed towards IgG3, are the responses to the streptolysin M protein where IgG3 predominated in the response of more than half the individuals tested [104], and the response to the outer membrane protein of *Branhamella cattarhalis* where IgG3 antibodies represent approximately 70% of the total response in children over 4 years old [119]. Interestingly, in the case of *B.cattarhalis*, the switch to IgG3 production was age related, specific IgG3 being essentially absent in children under 4 years of age. The reason for the absence of IgG3 during the first few years of life and the subsequent "switch" to significant levels of IgG3 synthesis is unclear since synthesis of IgG3 is already approaching adult levels by the age of 2 years [192].

IgG1 and IgG3 are typically produced in response to protein antigens [160] with IgG1 present in significantly greater amounts than IgG3 but, as yet, little is known about the factors which may preferentially induce the production of IgG3. Ig class switching is the process whereby B cells that initially express either IgM and/or IgD switch, on immunisation, to the expression of IgE, IgA or one of the 4 IgG subclasses [301]. In the mouse, specific switch factors have been described for different IgG subclasses. In particular, T cells can play a pivotal role in regulating the Ig isotype switch by their ability to secrete cytokines such as, IL-4, IFN-g and TGF-ß [76, 111,

244, 251, 275, 300, 302, 303, 312, 319]. For example, IL-4 released by Th2 cells upon activation is known to induce B cell proliferation in resting B cells, to enhance MHC class-II expression on B cells, and to mediate the Ig class switch to IgG1 and IgE [104, 244]. Current evidence suggests that cytokines act as switch factors by selectively inducing transcriptional activation of the constant heavy chain (C_H) genes that encode the Ig class that is subsequently expressed [303]. C_H gene activation is believed to make the DNA accessible to a switch recombinase [311]. However, cytokines are probably insufficient in themselves to effect class switching but must act in concert with a B cell activator such as bacterial lipopolysaccharide (LPS) [303, 311], an antigen receptor [31, 275], an activated T cell or another cytokine [251].
Much less is known about the trigger factors for specific class switching in humans, however, cytokines are presumed to play a critical role. Isotype switching to both IgG1 and IgG3 appears to be controlled by similar processes which may be regulated by the T-cell derived cytokine IL-10 [31]. Falconer *et al* proposed that the preferential induction of IgG3 may be a result of the mode of antigen presentation which could have consequences for the quality of the T-cell response to the antigen i.e. in the nature and amounts of the cytokines produced, which in turn could influence IgG subclass production [104]. Similarly, Goldblatt *et al* suggested that the membrane bound nature of *B.cattarhalis* proteins, and their mitogenic activity for B cells, may be partly responsible for the IgG3 antibody response [119]. The fact that MSP2 is a membrane bound protein, and contains tandemly repeated amino acid sequences which are believed to activate B cells independently of T cells [285] may therefore account for its propensity for induction of IgG3. Antigens such as MSP2 are a useful tools for investigating subclass specific switch mechanisms in human B cells.

Thus it has been shown that in individuals who are likely to be immune to malaria i.e. adults from a malaria endemic area, the predominant anti-MSP2 subclass is IgG3, the prevalence of which tends to increase with age. Furthermore, IgG subclass responses may differ between individuals who develop clinical malaria and those who have asymptomatic infections. It is obviously important to determine if the subclass of anti-MSP2 antibodies correlates with an inhibitory effect, *in vitro,* on *P.falciparum* growth and whether this is dependent on the presence of blood monocytes.

9. Antigenic polymorphism of MSP2 and immune recognition

9.1 Introduction

The existence of diversity among *Plasmodium* strains has been evident for many years. Early observations on malaria infections therapeutically induced in neurosyphilitic patients showed that individuals convalescing from *P.vivax* infections acquire a potent immunity to the strain of parasite that caused the infection but not to a heterologous strain [30]. Jeffery [163] also demonstrated that individuals were significantly more protected after reinoculation with a homologous strain of parasite, but that reinoculation with a heterologous strain gave variable results ranging from no apparent effect to a fairly substantial modification.

Antigenic differences among malaria parasites in humans were first demonstrated in studies of soluble antigens detected in the serum of infected individuals or extracts of infected placental blood [358]. Further characterisation of malaria antigens has revealed extensive heterogeneity of malaria antigens (reviewed in [7, 9, 10, 177]), including soluble parasite antigens [24, 154, 281, 359], surface antigens - CSP [77, 79, 96], MSP1 and MSP2 [55, 108, 201-203, 205, 217, 219, 221, 250, 298, 299, 304, 315, 321]- and infected erythrocyte surface components [71, 72, 106, 157, 199].
The diversity of *Plasmodium* in the natural parasite population has been clearly demonstrated. Many patients are infected with mixtures of genetically distinct parasite clones which differ in characters such as antigens, response to drugs, and other biochemical markers [11-13, 74]. For example, Creasey *et al* [74] found that extensive polymorphism in 20 genetic markers occurs in isolates from Thailand, Zimbabwe and Brazil, and multiple infections with >1 parasite phenotype were common. Babiker *et al* [12] characterised 29 isolates from Sudan, for variation in 18 different genetically controlled characters, each of the patients contained parasites of different genotypes.
The extent of diversity in *Plasmodium* is thought to contribute to the slow development of immunity, such that immunity only develops after repeated infections over a number of years and exposure to the repertoire of circulating genotypes is necessary for protection; this assumes that different sequences are antigenically distinct with little or no cross-reactivity.

However, Baird *et al* [14-16, 250, 257] contest this idea, proposing that a protective immune response to endemic malaria is governed by relatively brief heavy exposure plus some intrinsic immune factors associated with the age of the host. If this were true, antigenic polymorphism may only briefly govern susceptibility.

In general, antigenic diversity is generated in two ways [8]:
1) antigenic variation, whereby a clonal population of parasites periodically changes its antigenic profile. Antigenic variation has been shown to be a feature of antigens expressed on the surface of infected red blood cells [25, 35, 273].
2) antigenic polymorphism which occurs in allelic genes giving rise to the expression of structurally and antigenically distinct forms of a particular protein in different strains of parasite.
Diversity among the antigens of asexual blood stages predominantly occurs through antigenic polymorphism. Further diversity is generated through recombination, reassortment and mutation of these genes during meiosis [182, 347]. As a result, there is the potential for a very large number of different genotypes within one species. Many of the polymorphic asexual stage antigens have been shown to contain regions of tandem repeats and polymorphic residues tend to be more common in repetitive regions rather than in non-repetitive regions e.g. S-Ags, MSP1 and MSP2. However, not all repetitive sequences are polymorphic. CS protein and RESA have sequence repeats which are conserved within the species, and SPAM (secreted polymorphic antigen associated with merozoites) has repeat sequences which clearly encodes a structural motif, with the alanine residues being highly conserved in positions 1 and 4 of the heptad repeats [8]. The overall picture is one of great tolerance by the parasite for diverse repetitive structures of different amino acid sequences and sizes in a wide range of proteins.
Repeat sequences may reflect some adaptation of the parasite to its host. Schofield [285] proposed that repetitiveness causes T-cell-independent B cell activation by cross-linking hapten-specific surface Ig. Schofield undertook an investigation into the regulation of the antibody response to the repetitive domain of the CS protein on intact sporozoites, and observed that the parasite induced a thymus-independent B-cell response to the repeats [285]. This is beneficial to the parasite since lack of T cell help

leads to poor immunological memory and inefficient class switching to protective isotypes. It is a common belief that repeats also provide the parasite with a mechanism for immune evasion. Epitopes with repeats may be immunodominant, but antibody responses to them may have no serious effects on parasite survival. Furthermore, the repeats present the immune system with an extensive network of cross-reactive epitopes which promote polyclonal B and T cell activation and prevent affinity maturation of the response to protective epitopes [7, 177, 179].

It has been proposed that some repetitive sequences may have evolved as efficient ligands, specialised to mediate interactions of the parasite with host components [179]. The binding units are repeated because this allows multimeric high-avidity interactions between parasite and receptor [285]. However, the proposal that repeats are immunodominant does not reconcile with this concept of ligand-receptor interactions since ligand-receptor interactions are likely to be highly sensitive to the presence of blocking antibodies and it is thus likely that such epitopes would be concealed from the immune system in some way. In addition, radically different repeat sequences occur in the same region of allelic variants of a protein which presumably have the same function. It may be that the repeats in some gene products may have no function other than immune evasion, whereas other repetitive genes may retain critical functions. Polymorphism in the repeats may therefore be a marker for immunologically irrelevant sequences; conserved repeats on the other hand may be functional.

Diversity in MSP2 has been demonstrated in many studies both at the protein level using mAbs [12, 55, 69, 74, 109, 299], and at the genetic level by hybridisation analysis [201, 219, 298] and RFLP techniques [108, 217, 250]. Several alleles of MSP2 have been sequenced from different isolates/clones, revealing extensive polymorphism [109, 201, 202, 250, 298, 299, 321]. The molecule has been classified into two allelic prototypes - serogroup A or CAMP allelic family and serogroup B or FC27 allelic family - defined by regions of nonvariable, group-specific sequence. Within each group a number of variants have been described and these are characterised by tandem repeats which vary in number, length and sequence. Further diversity is created by intragenic recombination ([203, 231]; unpublished data); with crossover appearing to occur within the repetitive variable regions.

Polymorphism is most extensive among the serogroup A alleles; the R1 repeat region is glycine-, serine- and alanine-rich with repetitive sequences of 4-8 amino acids occurring up to 13 times [201] (see chapter 1, figure 1.3 & figure 9.1). Although the repeats can vary dramatically from isolate to isolate, some underlying patterns can be seen [298]. Indeed, Fenton *et al* [109] suggested that the repeat sequence of serogroup A could be derived from some common ancestor. Point mutations may have arisen in this postulated ancestral gene and these mutations accumulate until a new repeat spreads through the sequence [109]. Although there is a considerable microheterogeneity in MSP2 [201, 250] there must be some constraints which limit the diversity as the same repeat sequences occur in isolates from different geographical areas and there is a striking conservation of the overall amino acid composition, net charge and hydrophobicity of the molecule [299]. In addition the permisiveness for variation in size of MSP2 is much less than for S-antigens; changes in repeat number are often compensated for by deletions or insertions elsewhere in the non-variable region [109, 299, 321].

The repeat sequences of MSP2 have been shown to be immunogenic - mAbs and naturally acquired antibodies recognise this region ([2, 55, 101, 299, 321]; Chapters 5 & 6). However, the repeats are not necessarily immunodominant, unlike the repeats of the S-antigens which can encode upto 90% of the molecule and against which the entire antibody response is directed [7]. Al-Yaman *et al* [2] compared the recognition of a recombinant antigen representing the full length molecule of 3D7 (serogroup A) and a recombinant antigen of 3D7 which lacked the R1 repeats (d3D7). The majority of individuals were seropositive for both of the constructs, however, there was a small proportion of individuals among all age groups who responded only to the variable region. Monoclonal antibodies are known to recognise group-specific sequences as well as repetitive sequences [109].

The function of MSP2 repeats remains to be elucidated. One could propose that MSP2 may be involved in merozoite invasion of the erythrocyte due to its location on the merozoite surface and the ability of MSP2 specific mAbs to inhibit parasite growth *in vitro* [55, 220, 283]. However, the repeat region of the molecule may not be essential for parasite survival as isolates have been found which lack some of the repetitive sequences [250, 257, 321]. For example, Thomas *et al* [321] reported the absence of tandemly

repeated sequences within the GSA-rich region of CAMP isolate. The authors suggest that this may occur as a result of continual *in vitro* cultivation, however, others have reported wild isolates which lack the 12-mer repeats of serogroup B [250, 257].

In this chapter the aim was to determine whether sequence diversity within MSP2 serogroup A results in antigenic diversity. A significant association between the recognition of four variant sequences of serogroup A by malaria immune adults was observed (chapter 6). In this chapter I will investigate whether this association is due to cumulative exposure or cross-reactivity by looking at the recognition by a number of immune sera of a panel of A2 rAgs representing the R1 repeats (plus short group-specific regions flanking the repetitive sequences) of MSP2 serogroup A. Nine rAgs were produced from 8 different isolates. Two of these rAgs were shown to have deletions in the expected sequence - A2(CH12/12) lacks 8 amino acids in the C-terminal group specific region and A2 (T9/94ii) lacks the N-terminal sequence (figure 9.1).
Sera from mice immunised with single constructs were tested in ELISA for the recognition of the panel of A2 variants. Also, sera from individuals with limited exposure to *P.falciparum* and from children and adults from a malaria endemic area were tested for their seroreactivity with this panel of rAgs.

9.2 Materials and methods

9.2.1 Serum samples

i. MSP2-specific mouse serum:

Mice were immunised with A2(T9/94i), A2(TTn), A2(7G8), A2(RO33) or A2(T9/96) as described in chapter 4. The sera produced were tested in ELISA for recognition of the panel of A2 rAgs.

ii. Naive sera:

Sera was obtained from individuals from non-endemic areas who had been infected with *P.falciparum* whilst visiting malaria endemic countries; details are provided in chapter 6.

iii. Malaria-exposed sera:

Sera was obtained from individuals aged 1-75 years living in rural villages around the town of Farafenni on the north bank of the Gambia river (as described in chapter 6).

9.2.2 Recombinant proteins:

The recombinant antigens used in this study represent the R1 repeat region of MSP2 serogroup A from eight different isolates (figure 9.1). Nine constructs were produced; two of the proteins have deletions in the group specific sequences flanking the repeat region A2(T9/94 ii) and A2(CH12/12).

9.2.3 Enzyme-linked immunosorbent assay:

MSP2-specific antibodies were detected in the mouse serum and human serum using HRP-conjugated anti-mouse Ig or HRP-conjugated anti-human IgG antibodies, respectively.

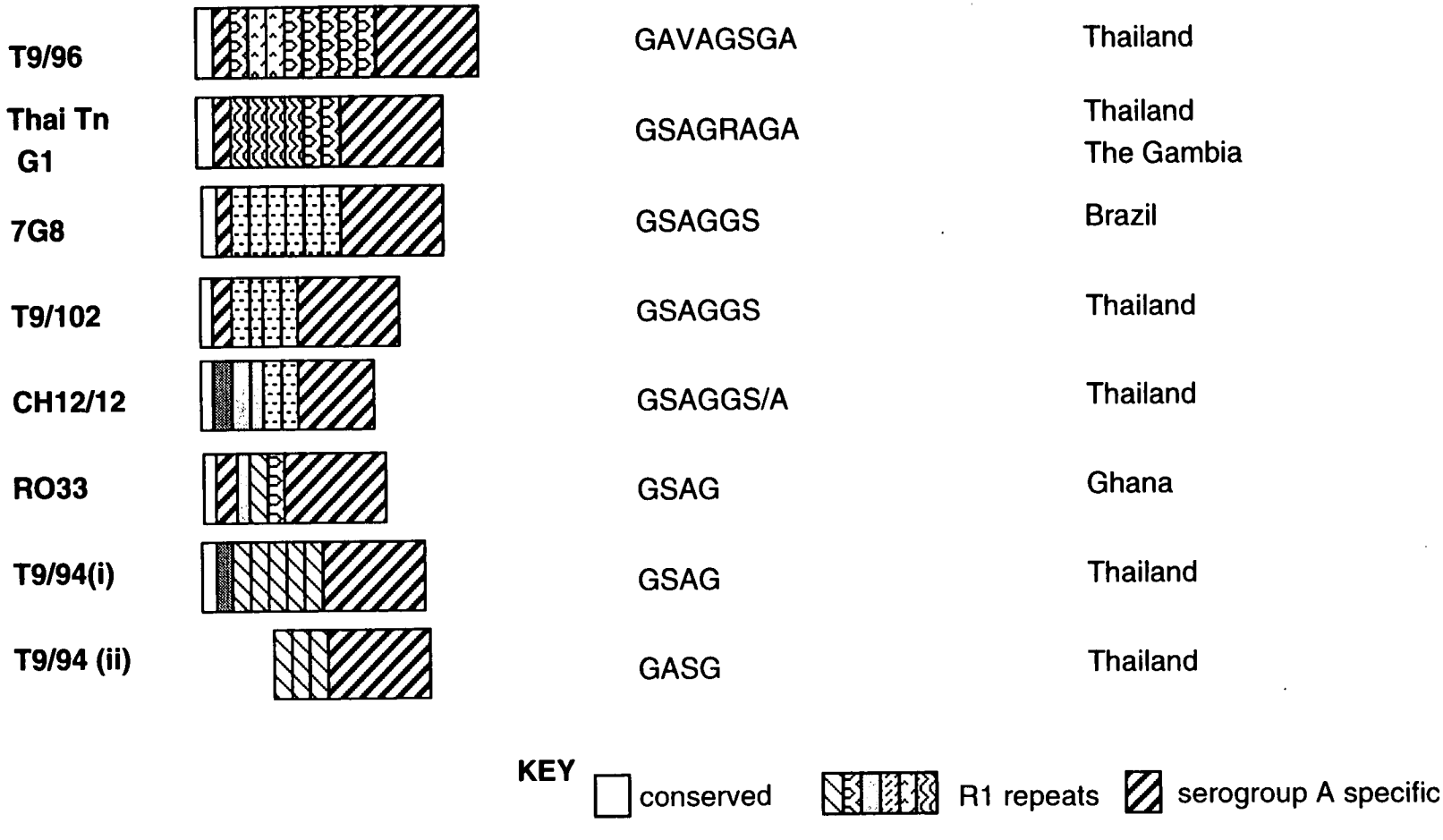
Human serum was diluted 1:1000 or serial two-fold dilutions from 1:250. Mouse sera were titrated with serial ten-fold dilutions from 1:500.

9.2.4 Statistical methods:

i. Kruskal-Wallis one way analysis of variance:

This non-parametric one way analysis of variance was performed using MINITAB statistical software in order to determine the differences in seroreactivity with age.

Figure 9.1: Schematic representation of A2 recombinant proteins of MSP2 derived from different isolates.



9.3 Results

9.3.1 Recognition of serogroup A R1 repeats by anti-A2 antibodies induced in mice:

Mice were immunised with a selection of A2 rAgs (as described in chapter 4) and their serum was tested for the recognition of the panel of A2 rAgs in ELISA. In each case, the anti-serum produced recognised all the rAgs. Thus, after immunisation with a particular A2 rAg, the antibodies induced recognised epitopes which were cross-reactive between variants.

In the majority of cases, the titration curves were overlapping indicating that the antibodies produced had the same affinity for all the constructs (figure 9.2a). In some cases, the affinity of the antibodies varied, with the recognition of A2(T9/94ii) being the poorest (figure 9.2b).

It appears that mice immunised with one particular sequence produced antibodies which cross-react with the panel of A2 rAgs which have varying sequences. The group-specific sequences are not essential for this cross-reactivity since the constructs which lack these regions (A2(CH12/12) and A2 (T9/94ii)) are also recognised. Although in some cases, A2(T9/94ii) is slightly less well recognised suggesting that the group specific region may be involved.

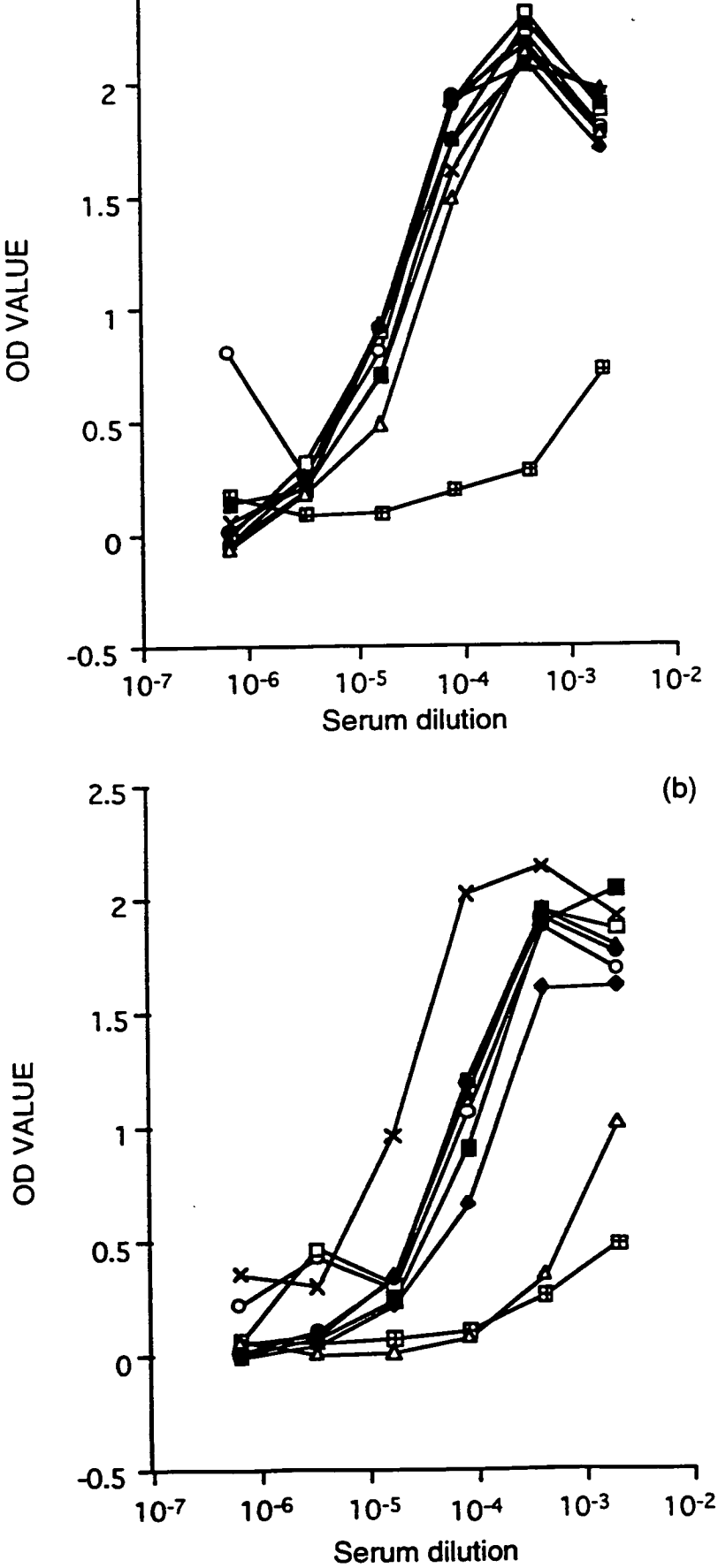


Figure 9.2: Recognition of A2 proteins by sera from mice immunised with (a) A2(T9/96) and (b) A2 (RO33). Sera were titrated with ten-fold dilutions from 1:500.

9.3.2 Recognition of A2 rAgs after limited exposure to malaria:

Sera from individuals known to have had only one malaria infection or a limited number of infections, were tested in ELISA for recognition of A2 rAgs in ELISA (table 9.1).

Sera collected at the time of infection were all seronegative, with the exception of donor 92:33. This individual recognised one rAg, A2 (T9/96). These antibodies appear to be of low affinity and concentration, indicated by the low OD value, and this may limit the ability of the antibodies to cross-react with other sequences.

In four out of five individuals, convalescent sera were seropositive for this region. Two of these individuals recognised only one rAg - donor 93:28CS recognised A2 (T9/102) with an OD value very close to the cut-off level, and donor 92:21 CS was seropositive for A2(T9/94ii). Donor 92:12 had antibodies which recognised A2(T9/94i) and A2(T9/96). There is no sequence similarity between T9/96 and T9/94 suggesting that this individual was exposed to a mixed genotype infection or two separate infections.

Sera from donor 92:38 recognised eight of the rAgs, being seronegative for A2(CH12/12). This construct is known to have a deletion in the group-specific sequence at the C-terminus. Therefore, it appears that in this case antibodies are directed to an epitope in the group-specific region rather than the repetitive sequences.

		MSP2 recombinant proteins							
	Isolate	T9/94ii	T9/94i	T9/102	7G8	CH12/12	RO33	TTn	T9/96
	Sequence	GSAG	GSAG	GSAGGS	GSAGGS	GSAGGS/A	GSAG	GSAGRAGA	GAGSGA
							GAVASA	GAVASA	GAVAGSGA
Donor	**Status**								
92:21	N	-	-	-	-	-	-	-	-
	CS	-	+	-	-	-	-	-	-
92:35	N	-	-	-	-	-	-	-	-
92:51	N	-	-	-	-	-	-	-	-
93:01	N	-	-	-	-	-	-	-	-
93:28	N	-	-	-	-	-	-	-	-
	CS	-	-	-	-	-	-	-	+
92:11	>2	-	-	-	-	-	-	-	-
92:30	>2	-	-	-	-	-	-	-	-
92:33	>2	-	-	-	-	-	-	-	+
92:41	>2	-	-	-	-	-	-	-	-
93:33	>2	-	-	-	-	-	-	-	-
92:10	CS	-	-	-	-	-	-	-	-
92:12	CS	-	++	-	-	-	-	-	++
92:38	CS	+++	+++	+++	+++	-	+++	+++	+++

Table 9.1: Summary of results for antibody recognition of MSP2 recombinant A2 proteins by sera from individuals from non endemic countries. N indicates one infection; >2 indicates more than two infections; CS indicates convalescent serum.

- indicates an OD value less than the mean + 2SD of the control sera; + indicates an OD value greater than the mean + 2SD of the control sera; ++ indicates an OD value greater than the mean + 4SD of the control sera; +++ indicates an OD value greater than the mean + 6SD of the control sera.

9.3.3 Recognition of serogroup A R1 repeats by malaria-exposed children and adults:

Forty-four individuals, aged 1-75 years, were tested for recognition of A2 rAgs. The individuals selected had previously been tested for recognition of A2(T9/96) in ELISA (chapter 6) and seroreactivity ranged from low responders to high responders. The association in recognition of these A2 sequences was determined using a non-parametric Spearman's rank correlation test (table 9.2). In all cases, the recognition of the A2 rAgs was significantly associated.

ISOLATE	T9/94ii	T9/94i	T9/102	7G8	CH12/12	RO33	G1	TTN
T9/94ii	1.000							
T9/94i	0.725	1.000						
T9/102	0.639	0.907	1.000					
7G8	0.720	0.926	0.971	1.000				
CH12/12	0.311	0.644	0.644	0.616	1.000			
RO33	0.624	0.607	0.617	0.631	0.630	1.000		
G1	0.352	0.595	0.677	0.634	0.794	0.767	1.000	
TTN	0.407	0.582	0.647	0.604	0.774	0.781	0.970	1.000
T9/96	0.395	0.340	0.402	0.434	0.318	0.662	0.597	0.535

Table 9.2: Spearman's rank correlation analysis of the recognition of MSP2 serogroup A R1 repeat regions from different isolates.
Forty-four individuals, aged 1-75 years, were tested for the recognition of this region in ELISA using recombinant proteins.
Critical Spearman's rank correlation value = 0.251, p=0.05.

The recognition of certain rAgs was very strongly associated and this appeared to reflect sequence similarities. A2(TTn) and A2(G1) are known to be identical in sequence and a correlation value of 0.970 ($p<0.005$) was obtained; this also confirms the robustness and reliability of the assay. A2(7G8) and A2(T9/102) have the same sequence repeated 5 and 3 times, respectively; the recognition of these rAgs was strongly associated ($rs=0.971$, $p<0.005$). Strong associations were also shown between A2(T9/94i) and A2(T9/102) or A2 (7G8), each of which contains the sequence GSAG.

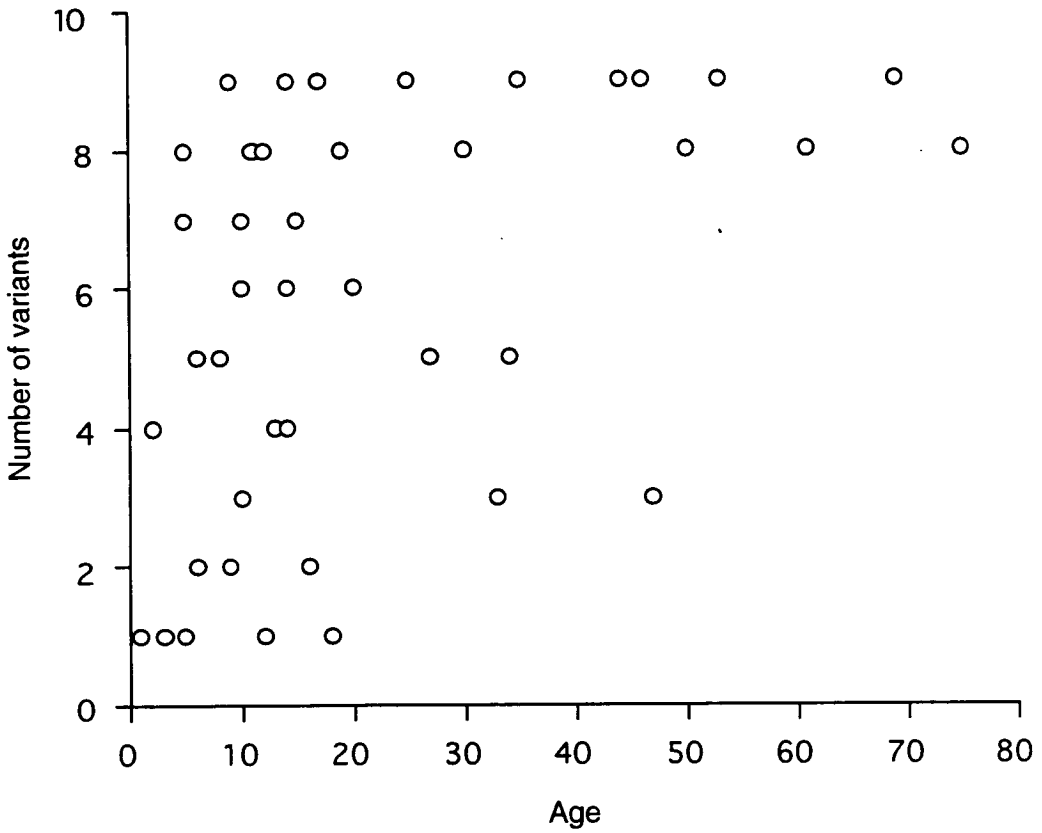


Figure 9.3: Number of variants of MSP2 A2 proteins recognised by sera (n=44) from children and adults from The Gambia. Recognition of nine rAgs was tested for each individual.

The number of variants recognised by each individual was determined and related to age (figure 9.3). The number of variants recognised was shown to be age dependent. Despite this, some older individuals (e.g. donor 1617, 47 years old) only recognised 3 variants, and some children recognised the majority of A2 rAgs.

Nine of the 44 individuals apparently recognised group-specific sequences rather than the tandem repeats since seropositivity was dependent on sequences which are absent in either A2(T9/94ii) or A2 (CH12/12) (table 9.3). A further three individuals , who were seropositive for all the rAgs, had the lowest titres for either A2(T9/94ii) or A2 (CH12/12), indicating that recognition may be partly dependent on these group-specific sequences, for example epitopes overlapped with the group specific sequences. This data makes it clear that humans see antigens individually and do not preferentially recognise the short group specific regions, i.e. humans do develop sequence-specific immune responses.

For certain individuals it is apparent that the rAgs recognised have sequence similarities in the repeats (table 9.3). For example, donor 1574 is seropositive for rAgs from the isolates T9/96, G1, TTn and RO33, which all contain the sequence GAVASA.

	MSP2 A2 rAg								
Donor	T9/94ii	T9/94i	T9/102	7G8	CH12/12	RO33	G1	ThaiTn	T9/96
1574	0.004	0.217	-0.006	-0.020	-0.011	0.607	0.832	0.731	1.028
1649	0.034	0.161	0.103	0.143	0.157	0.634	0.528	0.474	0.740
1610	0.749	1.342	1.122	1.158	0.084	0.063	0.093	0.024	1.043
1568	1.102	1.193	1.106	0.969	0.194	1.204	1.185	1.051	1.492
1528	0.203	0.924	0.823	0.645	0.618	0.722	0.659	0.374	0.722

Table 9.3: Examples of recognition of MSP2 A2 rAgs by individuals from The Gambia. Underlined OD values are greater than the mean + 2SD of the control sera.

9.3.4 Cross-reactive antibodies

To investigate the hypothesis that antibodies to closely related sequences are cross-reactive, competition ELISAs were performed. Sera were preincubated with increasing concentrations of rAg and tested for recognition of the other rAgs.

1) Donor 1535 (aged 5 years): This individual recognised all the A2 rAgs with approximately the same affinity, except A2(CH12/12) (which lacks the C terminal group specific region) (figure 9.4a). Therefore, it was hypothesised that this serum recognised an epitope in the C terminal group specific region. The serum was preincubated with A2(T9/94ii), A2(TTn), A2(7G8) or A2(T9/96) and tested against the A2 antigen panel. Recognition of all antigens was inhibited (example for incubation with A2(TTn) is shown in figure 9.4b) confirming our hypothesis.

2) Donor 1638 (aged 12 years): This serum recognised all the A2 rAgs, except A2 (T9/94ii), but the affinity of the reaction varies from antigen to antigen. When the serum was preincubated with A2(T9/94i), A2(T9/94ii), A2(CH12/12), A2(TTn) or A2(T9/96) antigen recognition was inhibited, but the degree of inhibition varied (figure 9.5).

The titration curves (figure 9.5a) and inhibition ELISAs indicate that there may be two populations of antibodies with different specificities, but both are dependent on the presence of the N-terminal group specific sequences. One group of antibodies appears to recognise T9/102, 7G8 and CH12/12, and the other group recognises RO33, ThaiTn, T9/94i and T9/96. In fact, competition assays indicate that the two groups of antibodies recognise sequences in A2(7G8), A2(T9/102), A2(T9/94i) and A2(CH12/12) or sequences present in A2(T9/96) and A2(RO33). An example is shown in figure 9.5b. A2(TTn) inhibits the recognition of all the rAgs to some extent; this may be due to the partial homology of the repeat sequence GRAG and GSAG, the latter is present in A2(7G8), A2(T9/102), A2(T9/94i) and A2(CH12/12), and to the sequence GAVASA which is present in A2(T9/96) and A2(RO33).

3) Donor 1610 (aged 27 years): This individual was shown to recognise 5 of the A2 proteins (figure 9.6a). In the inhibition ELISAs, preincubation with either A2(T9/94i), A2(T9/94ii), A2(7G8) or A2(T9/102) inhibited the recognition of the other rAgs, whereas preincubation with A2(T9/96)

inhibited the recognition of A2(T9/94ii) only (table 9.4, figure 9.6b). The common sequence in A2(T9/94i), A2(T9/94ii), A2(7G8) and A2(T9/102) is GSAG, however, this occurs once in A2(CH12/12) but this construct is not recognised. There is no obvious explanation, in terms of sequence similarities, for the inhibition of recognition of A2(T9/96).

	Antigen on plate				
Antigen in serum	T9/94i	T9/94ii	T9/102	7G8	T9/96
T9/94i	+	+	+/-	+/-	+
T9/94ii	+/-	+	+/-	+/-	+
7G8	+/-	+	+	+	+/-
T9/102	+/-	+	+	+	+/-
T9/96	-	+	-	-	+

Table 9.4: Inhibition of recognition of A2 rAgs in ELISA, by donor 1610, after preincubation with either A2(T9/94i), A2(T9/94ii), A2(7G8), A2(T9/102) or A2(T9/96) in increasing concentrations from 0-5 µg/ml. - indicates no effect; +/- indicates partial inhibition; + indicates inhibition.

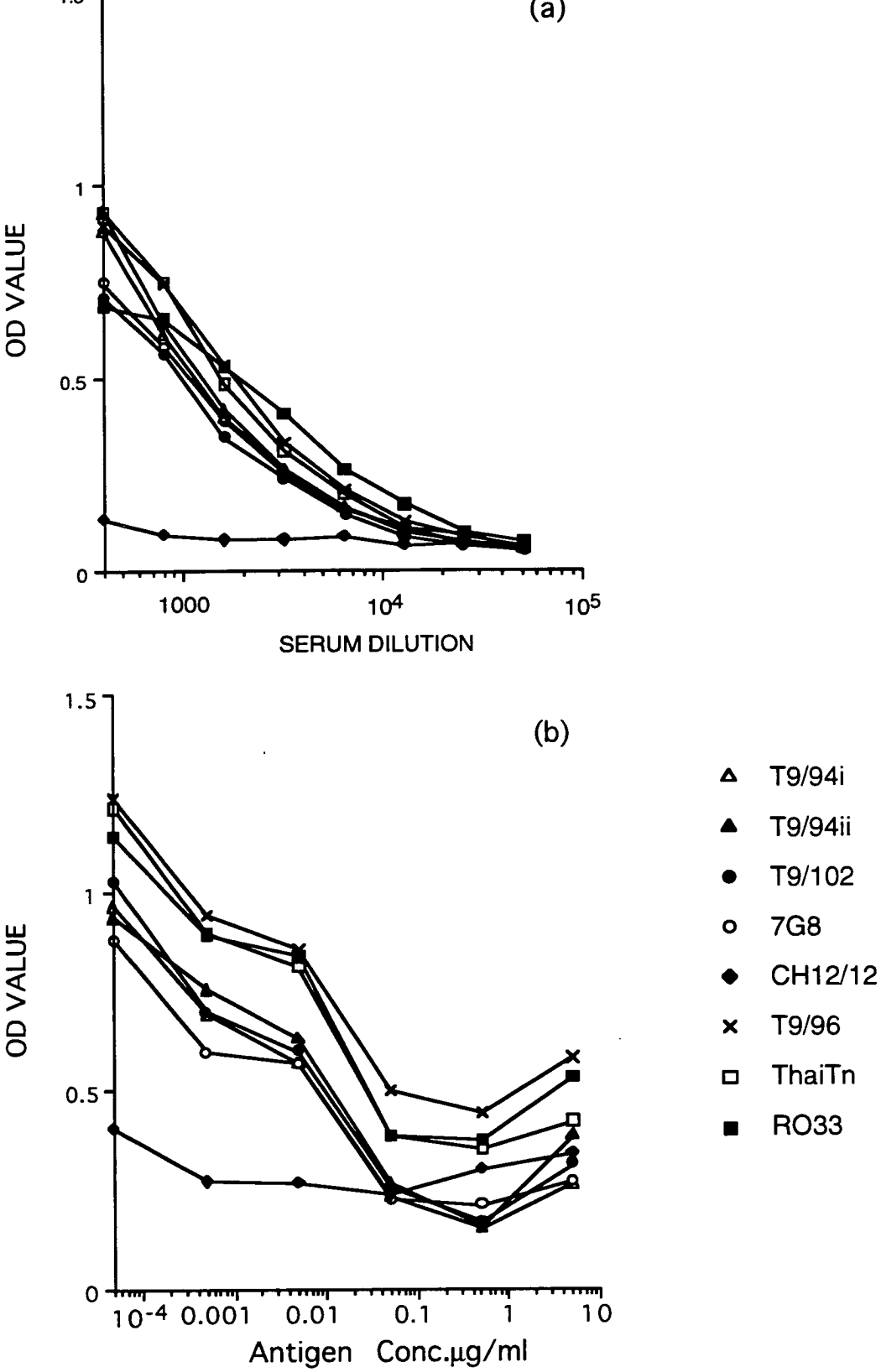


Figure 9.4: Recognition of A2 proteins by donor 1535, aged 5 years.
(a) Titration curves showing the recognition of A2 rAgs. Sera was titrated by four-fold dilutions from 1:400;
(b) Example of inhibition ELISA curves, after preincubation of sera with increasing concentrations of A2(ThaiTn) from 0-5 µg/ml.

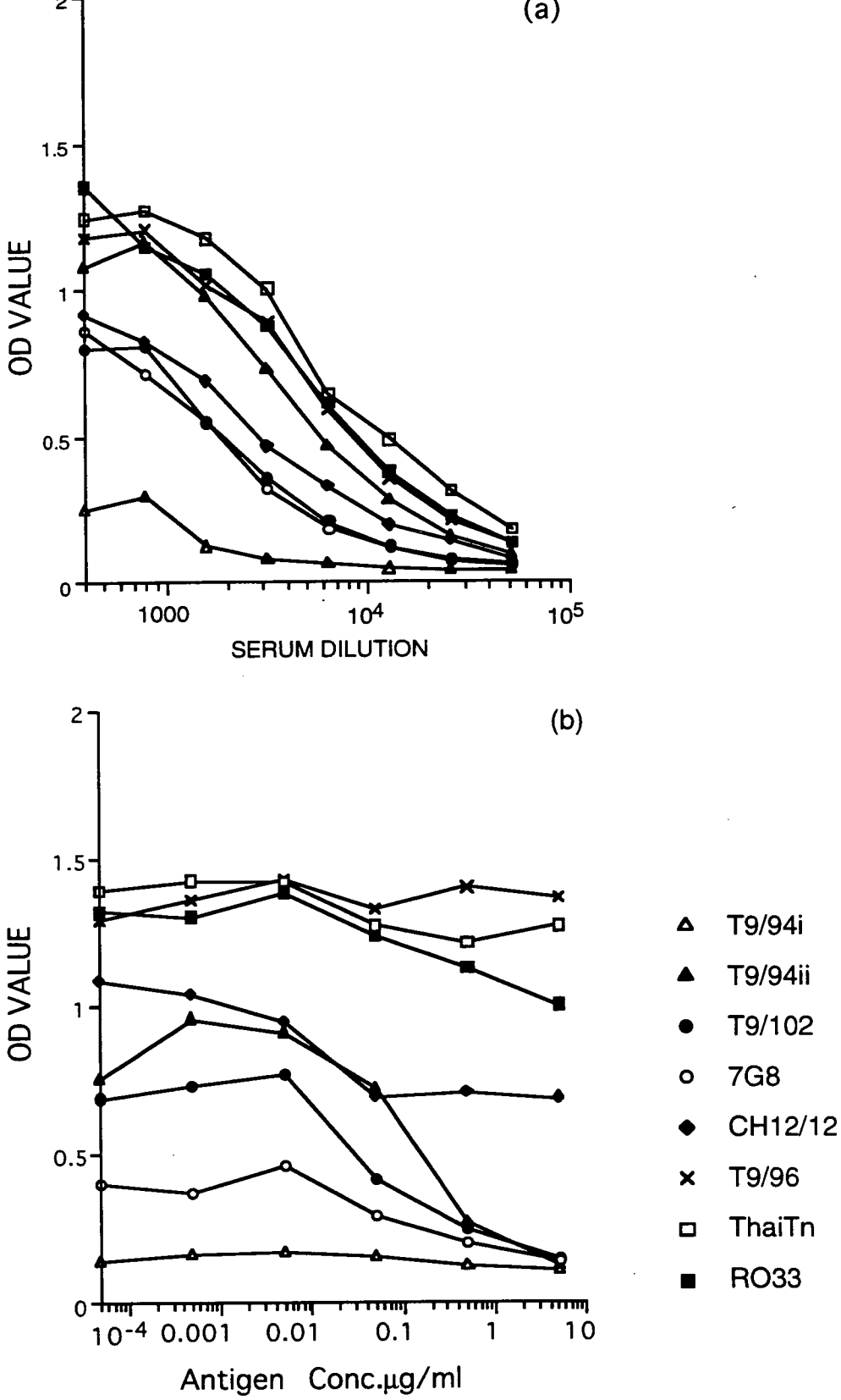


Figure 9.5: Recognition of A2 proteins by donor 1638, aged 12 years.
(a) Titration curves showing the recognition of A2 rAgs. Sera was titrated by four-fold dilutions from 1:400;
(b) Example of inhibition ELISA curves, after preincubation of sera with increasing concentrations of A2(T9/94i) from 0-5 µg/ml.

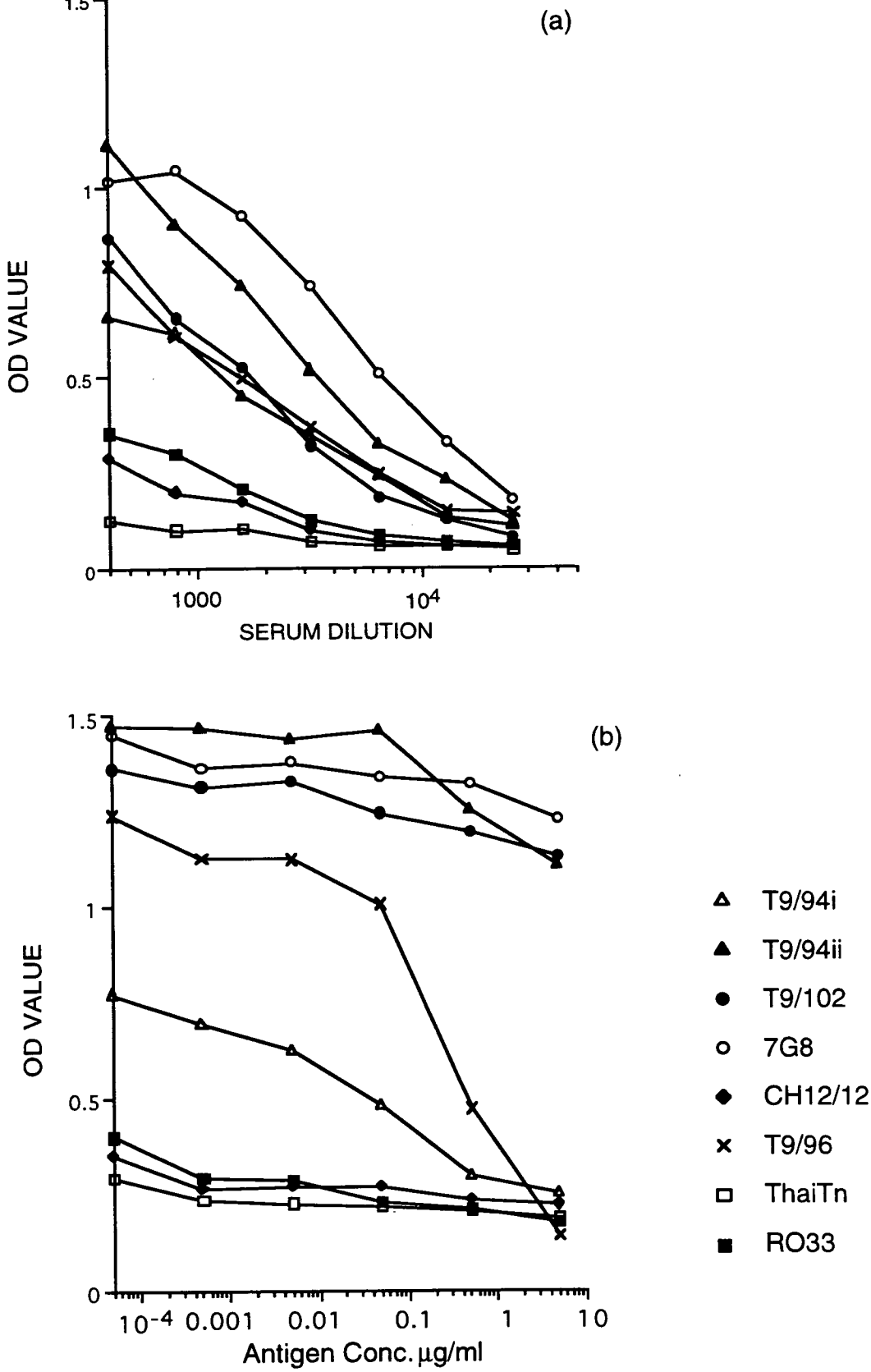


Figure 9.6 Recognition of A2 proteins by donor 1610, aged 27 years.
(a) Titration curves showing the recognition of A2 rAgs. Sera was titrated by four-fold dilutions from 1:400;
(b) Example of inhibition ELISA curves, after preincubation of sera with increasing concentrations of A2(T9/96) from 0-5 µg/ml.

9.4 Discussion

In chapter 5 we observed that the recognition of 4 different A2 proteins was significantly associated within individuals, but were not able to tell whether this was due to the presence of multiple, non overlapping, antibody specificities or to the presence of a single, cross-reactive antibody. Here, I have studied the recognition of MSP2 serogroup A2 rAg repeat sequences from different isolates to determine whether amino acid sequence polymorphism reflected antigenic polymorphism and whether associations in recognition are a reflection of cumulative exposure or cross-reactive epitopes. Although the isolates present in The Gambia and the other areas of Africa where these individuals were infected, may not be fully represented in our panel of rAgs, these rAgs do represent a worldwide distribution of isolates (figure 9.1).

Anti-sera from mice immunised with the rAgs were tested in ELISA against the panel of A2 rAgs. In all cases, sera from mice immunised with any one of the rAgs, recognised all of the other A2 rAgs, irrespective of sequence. This indicates that these sequences have the potential to encode cross-reactive epitopes and that such epitopes may be immunodominant in mice. However, we also determined the reactivity of malaria immune human sera and observed considerable diversity in antigen recognition after natural malaria infections.

The overall associations in recognition were confirmed (reported in chapter 5) and the strongest correlations were shown to be between rAgs with the most sequence homology. In other words, the degree of cross-reactivity could, in some cases, be related to the degree of sequence similarity. However, it is difficult to clearly differentiate the effects of cumulative exposure and possible variant-specific immune recognition just by looking at statistical corrrelations. The data indicate that sera from individuals with infrequent malaria-exposure (i.e. individuals from non-endemic areas and children from endemic areas) recognise fewer variants than do individuals with life long malaria exposure. The data from semi-immunes confirm that MSP2 A2 sequences are recognised in a sequence specific manner; data from the adults confirm that the number of variants recognised is cumulative and related to the diversity of parasite exposure. Although immune recognition of MSP2 A2 repeats is strain specific, some cross-reactive antibodies are present. Several individuals were shown to recognise

several A2 proteins. For one of these individuals, preincubation of the serum with A2 proteins was shown to inhibit the recognition of the other rAgs in ELISA, indicating that these antibodies were recognising cross-reactive epitope(s). A proportion of individuals were shown to have antibodies specific for epitopes in the group specific regions flanking the repeats rather than for the repetitive sequences, indicating that the repeats of MSP2 are not necessarily immunodominant. Therefore, "cross-recognition" can occur due to recognition of either the group-specific sequences or due to cross-reactive antibodies recognising truly variable sequences.

Thus, the correlation in recognition of different R1 repeat sequences, and the increase in number of variants recognised with age, could arise as a consequence both of cumulative exposure to different genotypes and limited cross-reactivity of repeat sequences. Protective immunity may therefore depend upon exposure to a number of parasite isolates which, between them, induce antibodies which cross-react with the whole range of sequence diversities.

10. Strain-specific immune recognition of MSP2

10.1 Introduction

In areas with endemic malaria transmission, immunity to malaria is acquired gradually with increasing age and is reflected by a decline in both parasite density and prevalence and the incidence of clinical disease. The long period required to develop immunity to malaria has been interpreted, by some, as a consequence of antigenic diversity of the parasite, such that exposure to the local repertoire of circulating "strains"[1] in the parasite population is necessary for effective immunity. This assumes that immunity to malaria is essentially strain-specific. However, direct evidence for strain-specific immunity is limited and rather speculative.

Jeffery [163] examined the significance of repeated experimental human infections with homologous and heterologous "strains" of *P.falciparum*. In general, the homologous reinfections were considerably milder than those where heterologous strains were used. The asexual parasite and gametocyte densities were considerably lower in those given homologous infections than in those who received heterologous reinoculations. The author interpreted the data as an indication of the presence of strain-specific antibodies.

To dissect the relative importance of serotype-specific immune responses to different polymorphic antigens, an obvious strategy is to relate the prevalence/incidence of a particular serotype of *P.falciparum* to the prevalence of immunity to that serotype. This can be done both at the population level and at an individual level. In the last decade the characterisation of polymorphic antigen genes and their expression as recombinant polypeptides has allowed more detailed study of strain-specific immune recognition of malaria parasites.

Naturally acquired antibodies to MSP1 appear to be serotype-specific, in that the antibody prevalence against the dimorphic regions of the molecule

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[1]The genes encoding different polymorphic antigens of *P.falciparum* lie on different chromosomes and will undergo genetic recombinantion independently of each other during meiosis [180, 181]. The term **strain** which implies a stable clonal lineage, is not a particularly useful epidemiological description of parasites with non-linked polymorphic antigen loci that may be rapidly interbreeding [13, 78]. The terms **genotype** and **serotype** are more useful, where genotype refers to the presence of a particular allele of a polymorphic locus and serotype refers to the presence of a serological reactivity to a gene product of a particular allele [78].

appears to correlate with the prevalence of the corresponding allelic type in the infecting parasite population [115, 228, 324]. Similarly, the transmission dynamics of the FC27 S-antigen serotype in village communities in Madang, Papua New Guinea, have been related to the prevalence of antibodies to that serotype [78, 112, 113]. Measurement of IgG antibody responses to repeat sequences of the FC27 S-antigen showed a marked age-dependent increase in prevalence. Recent transmission of the serotype in one village induced specific IgG, particularly in children under 15 years of age. Spatial and temporal variation in the transmission of the FC27 S-antigen serotype was considered to be consistent with the hypothesis that serotype-specific immunity occurs and contributes to the changes in frequencies over time [78, 112, 113], although it has been argued that random fluctuations in allelic frequencies are inevitable in *P.falciparum* populations in villages due to genetic drift imposed by a restricted population size [66, 69]. Furthermore, as genes for other polymorphic asexual blood stage antigens are on different chromosomes, parasites of a particular S-antigen serotype will undoubtedly be heterogeneous with respect to some of these antigens, and, if immune responses to these antigens have any protective effect, transmission of a particular S-antigen serotype will vary independently of anti-S-antigen responses [112, 113].

Marsh and Howard [199] demonstrated isolate-specific antibody responses to determinants on the surface of malaria-infected erythrocytes. Antibody specificities of Gambian children recovering from acute malaria infections were related to the antigen phenotypes on the surface of their own *P.falciparum*-infected erythrocytes. Children developed isolate-specific antibody responses against the parasites with which they had recently been infected, but in general these antibodies did not react with infected cells from other children. However, sera from uninfected Gambian adults contained antibodies that cross-reacted with the surface antigen(s) of many isolates. The authors proposed that the differences in reactivity between sera from children and adults could be due to the adults having experienced previous infections with many different malaria "strains". Although the surface antigens were not characterised, it was concluded that they were extremely diverse and that this diversity may represent a strategy by the parasite for evasion of the host's immune response. According to this concept of immune evasion, the susceptibility of the

acutely infected children to *P.falciparum* was due, at least in part, to the fact that they had not previously experienced infection with the same "strain".
Evidence for serotype-specific immune recognition of MSP2 is limited. Specific murine mAbs differentiate variants of MSP2, giving isolate specific reactions [109]. The pattern of recognition by different mAbs allows the characterisation of isolates into MSP2 serotypes [66-69, 109]. Thus, the mAb reactivities can delineate variant forms of MSP2, some more serologically related than others. The extent of MSP2 polymorphism and the frequency of different serotypes has previously been determined by indirect immunofluorescence analysis with a panel of mAbs to identify allelic variants of the 3 polymorphic blood stage antigens, MSP1, MSP2 and Exp-1 [66-69]. The frequencies of polymorphic epitopes and serotypes of these antigens were shown to remain stable for several years (1982-1989) in The Gambia; epitope variants remaining either rare or common, suggesting that immunity to these antigens does not lead to detectable frequency-dependent selection, even though naturally acquired antibodies are serogroup specific (chapter 5).
The recognition of MSP2 serogroup A variants was investigated in chapter 9, to determine whether serotype-specific antibodies were present in children and adults from The Gambia. Non-immunes and semi-immunes were shown to recognised a limited number of variants but the number of variant sequences recognised increased with age, indicating that an individual may need to experience the local repertoire of parasites in order to develop antibodies capable of recognising all MSP2 serotypes.
In this study, variant-specific immune recognition of MSP2 is investigated further at an individual level. To study the relationship between MSP2 polymorphism and naturally acquired immunity to malaria, the humoral immune response to MSP2 variants is examined in sera obtained from children with acute, uncomplicated malaria and compared with the antigenic characteristics of the infecting parasites. The antigenic profile of the infecting parasites is determined by IFA using a panel of mAbs specific for the major merozoite surface protein, MSP1, MSP2 and an exported protein Exp-1. These antigens are each encoded by a single locus within the haploid genome, and the loci have been mapped to different chromosomes [180, 181]. The antibody profile of the infected patients is determined in ELISA using MSP2 rAgs.

10.2 Materials and methods

10.2.1 Study design:

Children with acute, uncomplicated malaria[2] who attended the outpatients department at the Medical Research Council clinic, Fajara, The Gambia, over a six week period from October 1992, were recruited for our study, with parental consent. A sample of blood was obtained from each patient by fingerprick or as part of a venous sample obtained for other studies.

74 complete sample sets (serum and multispot parasite slides) were collected from children attending the clinic, after identification of *P.falciparum* infection and successful *in vitro* culture of the isolated parasites. Of these, 51 children were re-examined 4 weeks after treatment and follow-up serum samples obtained.

After centrifugation of the blood sample, the serum and buffy coat were removed and stored at -20°C. The rbc were retained for parasite culture.

10.2.2 Parasite culture

Erythrocytes were washed and parasites were grown in approximately 3ml culture volumes at a 5% haematocrit (section 2.5.3.), in covered petri dishes. They were cultured for 24-48 hours until the majority had matured to schizonts (as determined by periodic microscopic examination of Geimsa stained thin smears).

Multispot slides were prepared (section 2.6.1.) for immunofluorescence typing.

10.2.3 Indirect immunofluorescence assay (IFA):

All mAbs were first used individually in indirect IFA (section 2.6.3.) to determine the MSP1, MSP2 and Exp-1 variants expressed by the schizonts of each isolate. The epitope site of MSP1 mAbs are shown in figure 10.1, and the putative epitopes sites of MSP2 mAbs have been described in chapter 4, table 4.1 and figure 10.1 and appendix 1B. Details of monoclonal antibodies are given in appendix 2.

Those isolates which appeared to contain more than one genotype were subsequently tested using pairs of mAbs (with different isotypes and

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[2]Individuals who have a temperature >37.5°C and a parasitaemia of >5000 parasites/µl. These patients show no signs of cerebral involvement or severe anaemia and do not require hospitalisation.

epitope specificities) in a double-labelled IFA (section 2.6.4.) Allelic serotypes for each of the antigens was distinguished according to their different profiles of reactivity with the mAbs [66, 67, 109]. Each allelic serotype was assigned a number according to a classification scheme based on combinations of individual mAb specificities (tables 10.1 & 10.2).

10.2.4 Enzyme-linked immunosorbent assay (ELISA):

Sera were tested for IgG recognition of MSP2 rAgs in ELISA, at a dilution of 1:1000. Acute sera were tested for recognition of A1 and B1; acute and convalescent sera were tested, in parallel, against A1, B1, A2 variants and B3 variants.

10.2.4 Data analysis:

i. Determination of seropositivity

Positive serum samples were defined as those giving a specific OD above the normal range for control European sera. The normal range was taken as the mean ± 2 standard deviations of European control sera.

ii. Mann-Whitney test

A non-parametric two sample rank test was performed to test the difference between the median OD values of two groups.

iii. χ^2-test

This analysis was used to determine if an observed distribution differed from the expected distribution.

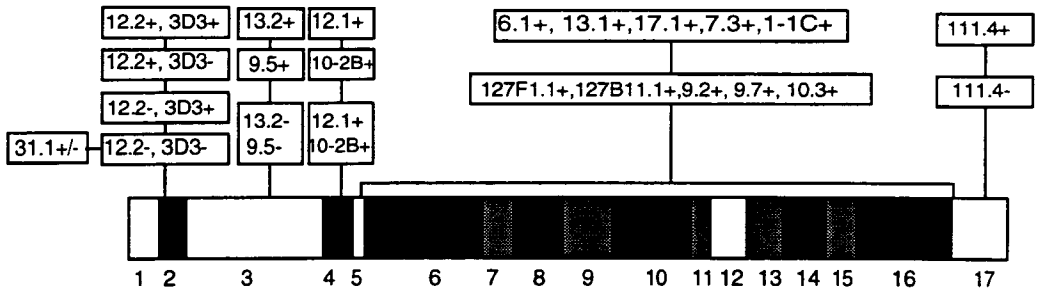


Figure 10.1a: Alternative epitope specificities at different domains of MSP1 are shown in boxes.
Polymorphism in MSP1 is also shown schematically, indicated by the numbered blocks, according to Tanabe *et al* (1987).

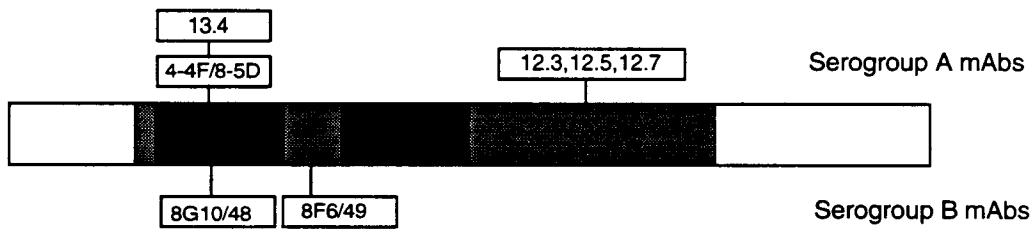


Figure 10.1b: Epitope specificties of MSP2 mAbs for serogroup A and B dimorphic and polymorphic domains.

denotes conserved sequences

denotes semi-conserved/dimorphic sequences

denotes polymorphic sequences

10.3 Results

10.3.1 Parasite serotyping:

Blood-stage parasites from patients with acute, uncomplicated malaria were cultured *in vitro* until they matured to schizonts. Multispot slides were prepared of each isolate. At least 200 schizonts from each isolate were scored for reactions with the mAbs and mouse sera by indirect IFA. All schizonts in every isolate gave specific reactions with the control mAb 9.8, a conserved epitope of MSP1 [204]. Each of the other mAbs identified some isolates within which all schizonts were positive, some isolates within which all parasites were negative, and some isolates within which only a proportion of schizonts gave specific fluorescence (i.e multiple-clone infections).

74 isolates were serotyped and a total of 111 clones were identified, more than half of the isolates being apparently single genotype infections.

i. MSP1 serotypes

Clones were assigned a serotype on the basis of reactivity with MSP1-specific mAbs and their frequency recorded (table 10.1). For the sake of comparability between studies, serotypes which had been described previously were given the same number as in the published studies [66, 67]. 6 clones remain unclassified due to difficulty in interpretation of reactivity with particular mAbs. 29 different MSP1 serotypes were identified; individual serotypes were found in up to 9 different isolates.

The frequency of these serotypes can be compared with previous studies carried out in the area by Conway *et al* [66, 67, 69] from parasite isolates from patients presenting to the outpatients department of the MRC, Fajara and the Royal Victoria Hospital, Banjul. Overall, the frequency of serotypes was comparable, particularly the strong bias towards the MAD20-type block 6-16 (reactivity with mAbs 127F1.1, 127B11, 9.2, 9.7 and 10.3).

Two serotypes, 22 and 30, which had not been observed previously in The Gambia [66, 67], occurred at low frequency in our study population. However, χ^2 tests indicate that there are no significant differences in the frequencies of MSP1 serotypes observed in this study and previous studies [66, 67, 69].

Eight serotypes (53-60) are based on lack of seroreactivity with mAbs specific for block 4 (12.1 and 10-2B). These two mAbs are mutually

exclusive in their reactivity, however, parasites negative for both have previously been observed [67]. The single example that has been sequenced has a third alternative sequence corresponding to this region. In this study, 4 of these additional serotypes were observed and designated 53, 56, 58 and 59.

Block 2 is the most polymorphic region of MSP1. mAb 12.2 defines the 3D7-type sequence, 3D3 defines the CAMP-type sequence and 31.1 is specific for the RO33-type sequence. mAb 31.1 was included in our study, but reactivity with this mAb was not used in the definition of the MSP1 serotypes. Reactivity with 31.1 appears to be mutually exclusive of reactivity with 122.2 and/or 3D3; of those clones which were negative with 12.2 and/or 3D3, 32 out of 49 were positive for 31.1. Previous studies in The Gambia have not used this mAb, therefore no comparisons in frequency of reactivity can be made.

	Monoclonal antibody										
	12.2	3D3	13.2	9.5	10-2B	12.1	127F1	1-1C	111.4		
Block	2	2	3	3	4	4	6-16	6-16	16-17		
Isolate	3D7	CAMP	K1	MAD	K1	MAD	MAD	K1	K1	n	%
Serotype										N=111	
3	+	-	+	-	-	+	+	-	+	1	0.9
4	-	-	+	-	-	+	+	-	+	2	1.8
5	+	+	+	-	-	+	+	-	-	3	2.7
6	-	+	+	-	-	+	+	-	-	1	0.9
7	+	-	+	-	-	+	+	-	-	3	2.7
8	-	-	+	-	-	+	+	-	-	4	3.6
9	+	+	+	-	+	-	+	-	+	9	8.1
10	-	+	+	-	+	-	+	-	+	3	2.7
11	+	-	+	-	+	-	+	-	+	9	8.1
12	-	-	+	-	+	-	+	-	+	5	4.5
13	+	+	+	-	+	-	+	-	-	6	5.4
14	-	+	+	-	+	-	+	-	-	2	1.9
15	+	-	+	-	+	-	+	-	-	3	2.7
16	-	-	+	-	+	-	+	-	-	5	4.5
20	-	-	-	+	-	+	+	-	+	9	8.1
22	-	+	-	+	-	+	+	-	-	1	0.9
24	-	-	-	+	-	+	+	-	-	7	6.3
28	-	-	-	+	+	-	+	-	+	6	5.4
30	-	+	-	+	+	-	+	-	-	1	0.9
32	-	-	-	+	+	-	+	-	-	7	6.3
33	+	+	-	-	-	+	+	-	+	3	2.7
36	-	-	-	-	-	+	+	-	+	2	1.9
43	+	-	-	-	+	-	+	-	+	2	1.9
44	-	-	-	-	+	-	+	-	+	1	0.9
52	-	-	+	-	-	+	-	+	+	1	0.9
53*	+	+	+	-	-	-	+	-	+	2	1.9
56*	-	-	+	-	-	-	+	-	+	1	0.9
58*	-	+	+	-	-	-	+	-	-	4	3.6
59*	+	-	+	-	-	-	+	-	-	2	1.9

Table 10.1: Serotypes, and their frequencies, of MSP1 defined by combinations of reactivities with individual mAbs [11, 16]. * denotes those serotypes not previously defined by Conway *et al* [11, 16].

Reactivities with mAbs 127B11.1, 9.2, 9.7, 10.3 and 127F1.1 all give the same pattern of recognition; to save space serotypes are defined as + or - in reactivity to 127F1.1 only.

Reactivities with 1-1C, 6.1, 13.1 and 17.1 are the same for each serotype; 1-1C reactivities only are recorded here.

ii. MSP2

MSP2 serotype frequencies are given in table 10.2. The results appear consistent with the frequencies in 1988 and 1989 [66, 67], in that serotypes 3 and 9 have not been detected. However, fluctuations in the frequencies of the other serotypes are apparent (table 10.2). In particular, there is an increase in the frequency of serotype 7 from less than 15% (in 1989) to 33% (in 1992), which is statistically significant (χ^2=89.7, d.f=1, p<0.001). This increase in serotype 7 (serogroup B) is compensated for by a decrease in the frequency of several other serotypes. The decrease in frequency of serotype 6 from 7.7% in 1989 to 0.9% in 1992 (χ^2=6.26, d.f=1, p<0.025).

	Monoclonal antibody							
	13.4	8-5D	12.3	8G10/48	8F6/49	Characteristic isolate*	n N=111	%
Serogroup	A	A	A	B	B			
Serotype								
1	+	+	+	-	-	T9/94	4	3.6
2	-	+	+	-	-	CH12/12, RO33	30	27
3	+	-	+	-	-		0	0
4	-	-	+	-	-	T9/96	19	17.1
5	-	+	-	+	+		7	6.3
6	-	+	-	+	-	K29	1	0.9
7	-	-	-	+	+	K1, FC27	37	33.3
8	-	-	-	+	-		9	9.1
9	+	+	-	-	-		0	0
10	-	+	-	-	-		2	1.8

Table 10.2: MSP2 serotypes, and their frequencies, defined by combinations of reactivity with individual mAbs [66, 67] collected from patients with acute, uncomplicated malaria in The Gambia.

* determined from Fenton *et al* [109]

10.3.2 Antibody recognition of serogroup A and B by **acute** sera and the relationship with the antigenic profile of the infecting parasites:

74 sera from children with acute, uncomplicated malaria were tested for recognition of MSP2 rAgs A1 and B1. The frequency of antibodies to these proteins is given in table 10.3. Table 10.4 gives the frequency of parasites expressing serogroup A and B, including mixed infections.
To examine the relationship between the presence of parasites expressing a particular serogroup of MSP2 and the presence of antibodies to that serogroup χ^2 tests were performed (table 10.5). Individuals infected with parasites of MSP2 serogroup A were divided into those with antibody to A1 and those without (and similarly for serogroup B). Antibodies to B1 were significantly associated with infection with malaria parasites expressing MSP2 serogroup B. No such association was observed for anti-A1 antibodies and serogroup A parasites. However, a greater than expected number of individuals who had antibodies to A1 were **not** infected with serogroup B parasites .
A Mann-Whitney test was performed to compare the median OD values to each rAg of individuals infected with the corresponding, or alternative, serogroup (table 10.6). The median OD to B1 was greater if an individual was infected with serogroup B parasites, and no association was observed between the median OD to A1 and serogroup A parasites.

Taken together, the data indicates that there is evidence for boosting of the antibody response to serogroup B, after infection with parasites expressing MSP2 serogroup B. The results for serogroup A are inconclusive. Importantly, there was no evidence that children with high titres of antibody to one serogroup were less likely to be infected by parasites expressing that serogroup - as one would expect if "strain-specific" immunity was operating at the level of MSP2.

Antibody specificity	Acute Number (N=74)	Acute frequency %	Acute Number (N=51)	Acute frequency %	Convalescent Number (N=51)	Convalescent frequency %
A1 only	11	14.9	8	15.7	4	7.8
B1 only	19	25.7	9	17.6	16	31.4
A1 and B1	20	27.0	20	39.2	25	49.0
neither	24	32.4	14	27.5	6	11.8

Table 10.3: Frequency of antibodies to rAgs A1 and B1 in acute samples (N=74) and acute and convalescent samples (N=51).

MSP2 serogroup	Acute only		Acute and Convalescent	
	Number (N=74)	Frequency	Number (N=51)	Frequency
A only	22	29.7	15	29.4
B only	26	35.1	19	37.3
A mix	5	6.8	3	5.9
B Mix	5	6.8	1	2.0
A/B mix	16	21.6	13	25.4

Table 10.4: Frequency of parasite serogroups and mixed infections in acute samples only, and paired acute and convalescent samples.

Parasite MSP2 serogroup	Antibody specificity	n Ab+	n Ab-	χ^2	p
A	A1	21	22	2.03	0.154
A	B1	15	28	13.07	>0.001
B	A1	15	32	5.26	0.022
B	B1	30	17	6.40	0.011

Table 10.5: χ^2 analysis to determine the association between antibody specificity of an individual and serogroup of parasites infecting that individual, for 74 acute samples collected from children with acute, uncomplicated malaria in The Gambia.

		Median OD			
Antibody specificity	Parasite serogroup	Parasite positive	Parasite negative	U	p (n=74)
A1	A	0.130	0.221	0.045	0.355
B1	B	0.549	0.117	0.239	0.018
A1	B	0.109	0.316	0.132	0.027
B1	A	0.117	0.639	0.414	0.0005

Table 10.6: Mann-Whitney analysis to determine the association between median OD to A1 or B1 and infection with parasites expressing serogroup A and/or B, for 74 acute samples collected from malaria patients attending the MRC outpatients clinic, Fajara.

10.3.3 Antibody recognition of MSP2 by **acute** and **convalescent** sera and the relationship with the parasite serotype:

The recognition of MSP2 was studied for paired **acute** and **convalescent** serum samples. Table 10.3 shows the pattern of antibody recognition of A1 and B1 in these individuals and the characteristics of the infecting parasites (table 10.4). The antibody profile of acute and convalescent sera was

significantly correlated, as determined by Spearman's rank correlation tests (data not shown).

Mann-Whitney analysis of both acute and convalescent samples again showed no association between the median OD to A1 and the presence of serogroup A parasites, whereas the median OD to A1 was greater if an individual was infected with parasites that were **not** serogroup B. The latter association implies that the median OD to A1 should be greater if the parasites are expressing serogroup A. This apparent contradiction could arise due to the extensive polymorphism in the R1 repeat region of MSP2 serogroup A. Therefore we tested the sera for recognition of the panel of A2 rAgs. 8 individuals who were seronegative for A1, but infected with serogroup A parasites, were seropositive to some of the A2 variants (table 10.7).

Some individuals were seronegative for A1(T9/96) but recognised A2(T9/96). A possible explanation for this is that A2-specific antibodies were in low concentration (indicated by a low OD) and the epitope density when the plate is coated with the larger A1 molecule is lower, therefore these antibodies are not detected.

			Antibody recognition				
			A1		B1		A2 variants recognised
Donor	Age	MSP2 serotype of infecting parasites	a[1)]	c[1)]	a	c	
227	1	7,2	-	-	-	+/-	RO33(c), CH12/12(c)
230	6	4,2,1	+	+/-	-	-	T9/96(a), 7G8(a)
237	1	7	+/-	+	+	+	
238	1	4,2	-	-	-	-	T9/96(c), RO33(c), CH12/12(c), T9/94i(c)
245	2	2,5,7	-	+/-	-	+	T9/96(a&c), RO33(c), T9/94i(c), ThaiTn(c)
248	7	7	-	+/-	-	++	T9/96(a&c)
249	6	7	-	-	+	++	
251	4	7,2*,10	++	+	++	++	T9/96(a&c)
262	4	4	-	+/-	-	+	T9/96(a&c)
266	6	7	-	+/-	++	++	
284	1	7	++	+	+	+	RO33(a&c), CH12/12(a&c), T9/94i(a&c), ThaiTn(a&c)
292	4	8	-	-	++	++	
306	5	4	+	-	+	+	
310	1	2	-	-	-	-	T9/96(a&c)
311	1	4	++	+/-	-	-	
342	2	7	++	+	++	++	
345	8	4	++	++	+	+/-	T9/96(a&c), 7G8(a&c), ThaiTn(a&c)
353	1	2	-	-	-	-	RO33(c), CH12/12(c), T9/94 i(c)
354	9	7	+	-	++	++	
355	5	7,10	++	+	++	++	
356	1	8	+	+	++	++	
367	2	7	-	-	-	-	
369	2	7,2	-	-	+/-	++	
375	8	5	+	-	-	+	
377	4	7,1	-	+	-	+	T9/94(c)
380	1	7	-	-	-	+/-	
386[b]	4	8	+/-	-	++	++	T9/96(a&c)
387	6	7	+/-	+/-	-	++	T9/96(a&c)
389	3	5,7	+	+	++	++	T9/96(c)
390	9	5	-	-	++	++	

391	6	2 ,5	+	++	-	++	ThaiTn(c)
393	1	2	-	++	-	+	RO33(a&c), ThaiTn(c)
394	4	2	+/-	+	+	+	T9/96(c)
400	6	8,2∞	-	+	-	+	RO33(a&c), T9/102(c), T9/94(a&c)
401	3	2	+	+	-	++	
405	3	7,2	++	++	++	+	T9/96(a), RO33(a&c), T9/102(a&c), ThaiTn(a&c), T9/94(a&c), 7G8(a&c)
407	1	2,4	++	+	+	-	ThaiTn(a)
414	3	8	-	-	+/-	+/-	
415	3	2∞,8	-	-	+	++	T9/96(c)
416	5	2	+/-	-	+/-	-	
421	2	7	++	+	++	+/-	T9/96(a&c), RO33(a), CH12/12(a), T9/94i(a)
424	4	2	-	+/-	-	+/-	
426	0	2	++	-	++	+	
429	4	7	+/-	-	++	++	
430	6	4,8	-	-	+/-	+	T9/96(a&c)
435	7	4	++	+	-	-	
441	1	2	-	+/-	++	++	
455	9	7	+/-	-	+	++	RO33(a)
457	4	2∞,5	+	-	+	-	
458	6	2	+	-	-	-	CH12/12(a&c), T9/102(a&c), T9/94i(a&c),7G8(a&c), ThaiTn(a&c)
460	3	7,4	-	-	-	++	

Table 10.7: Details of parasite serotype (major serotype first) and antibody specificity for 51 children aged 10 months -10 years from the villages around Fajara, The Gambia. Samples were collected in October - November 1992.

* denotes positive reactivity with anti-ThaiTn mouse serum

∞ denotes positive reaction of parasites in IFA with anti-T9/96 mouse serum

b denotes recombinant allele determined by DNA sequencing

1) a & c denote acute and convalescent samples, respectively.

Antibody recognition is denoted by - (negative), +/- (OD value close to cut-off), + (seropositive), ++ (strongly positive).

10.3.4 Recognition of A2 rAgs and the relationship with serogroup A parasites:

The variant-specific antibody profiles of individual sera could be compared with the serotype of the infecting parasites by measuring antibodies to A2 rAgs (table 10.7). Several examples are given below:

Donor 227 was seropositive for A2(RO33) and A2(CH12/12) 4 weeks after infection. The majority of infecting parasites expressed serogroup B, however a subpopulation of the parasites (approximately 7%) were shown to express epitopes 12.3 and 8-5D (serotype 2). This combination of epitopes is characteristic of MSP2 alleles in RO33 and CH12/12 strains [109] (table 10.2). Thus this child shows clear evidence of boosting of the immune response to the parasites with which it was infected.

Donor 238 was seropositive in convalescence for T9/96, RO33, CH12/12 and T9/94. Approximately 97% of the infecting parasites were MSP2 serotype 4, the sequenced expressed by T9/96, despite this the OD for A2(T9/96) was very low. Approximately 3% of the parasites were serotype 2 and strong antibody responses were detected for the corresponding A2 proteins - RO33 and CH12/12. In addition, a high OD was measured for A2(T9/94i), although no parasites of this particular serotype were detected. However, partial sequence homology does occur between RO33 and T9/94. Thus, a small proportion of parasites boosted the antibody response to a construct with similar sequences to those predicted by the mAb reactivity pattern of the parasites infecting this child.

Donor 353 was seropositive, in the convalescent sample, for A2(RO33), A2(CH12/12) and A2 (T9/94i) although the OD values were low. The parasites were an apparently single genotype infection of serotype 2 (RO33/CH12/12), which appears to have boosted the antibody response.

Donor 377 was seronegative at the time of infection, however became seropositive to T9/94 4 weeks later. This individual was infected with a mixed isolate; 35% of the parasites were serotype 1, characteristic of T9/94. The majority of parasites were serotype 7 (serogroup B) and antibodies to B1 were detected during convalescence.

Donor 430 was seropositive for A2(T9/96) in the acute and convalescent samples. Approximately 80% of the infecting parasites were serotype 4 (T9/96-type). The remainder of the parasites were serotype 8 and this child was weakly seropositive to B1 in the acute sample and clearly seropositive in the convalescent sample. Thus the antibody profile of this child is clearly related to the MSP2 serotypes expressed on the parasites with which it was infected.

Donor 458 was seropositive for A2 variants CH12/12, T9/102, T9/94i, 7G8 and ThaiTn, with OD values very similar for acute and convalescent samples. This individual had an apparently homologous infection with serotype 2, characteristic of CH12/12. Isolates CH12/12, T9/102, T9/94 and 7G8 all contain the sequence GSAG and there is partial homology with the sequence GRAG of ThaiTn. There is no obvious boosting of the immune response during convalescence. However, boosting may have already occurred by the time the acute sample was obtained if this child did not attend the clinic early during infection.

There were individuals who had antibodies to serogroup A variants but were <u>not</u> infected with serogroup A parasites. For example, donor 284 is seropositive for A2(RO33), A2(CH12/12), A2(T9/94i) and A2(ThaiTn), however only serogroup B parasites were detected. There was a decrease in the OD values to these constructs after 4 weeks, which may indicate that this individual had made a response to a previous infection with serogroup A parasites and the antibody levels were beginning to decline. This individual did have anti-B1 antibodies.

<u>10.3.5 Recognition of B3 variants by acute and convalescent sera:</u>

Four of the serogroup B parasites collected during this study were subsequently shown to lack the 12-mer R2 repeat sequence (sequencing carried out by Dr. P. Roberts and C. Dobano). Because of this observation, I investigated the recognition of this region by the childrens' sera. Three rAgs were used - B3(K1), B3(T9/105i) and B3(T9/105ii) which are serogroup B proteins with 5, 1 and 0 12-mer sequences, respectively.

The construct with 5 12-mers was recognised more often by acute and convalescent sera than were the constructs with 1 or 0 12-mers (figure 10.2). Thus, it seems as though a dominant epitope in this construct is dependent on the presence of more than 3 12-mer repeats.

Ten sera, which were positive for at least one of the B3 rAgs, were selected and titrated against each of the B3 constructs to compare the relative affinities and concentrations of antibodies to this region. Examples of three such sera are shown in figures 10.2 & 10.3. Varying patterns were seen. Some individuals appeared to recognise epitopes outwith the 12-mer sequence as titrations curves were virtually overlapping for each of the rAgs including the one with no 12-mers (figure 10.3a). In others, the recognition of the 12-mers occurred in a "dose-dependent" manner, with the greatest antibody binding to B3(K1) (figure 10.3b). In others, the epitope recognised was completely dependent on the presence of more than 1 12-mers.

Interestingly, donors 248, 249, 354 and 387 were strong responders to B3(K1) but low or non responders to B3(T9/105i) and B3(T9/105ii) (figure 10.2 - closed circles; for example figure 10.3c) and yet were infected with parasites which lacked the 12-mer sequence which perhaps enables the parasites to evade the existing antibody response.

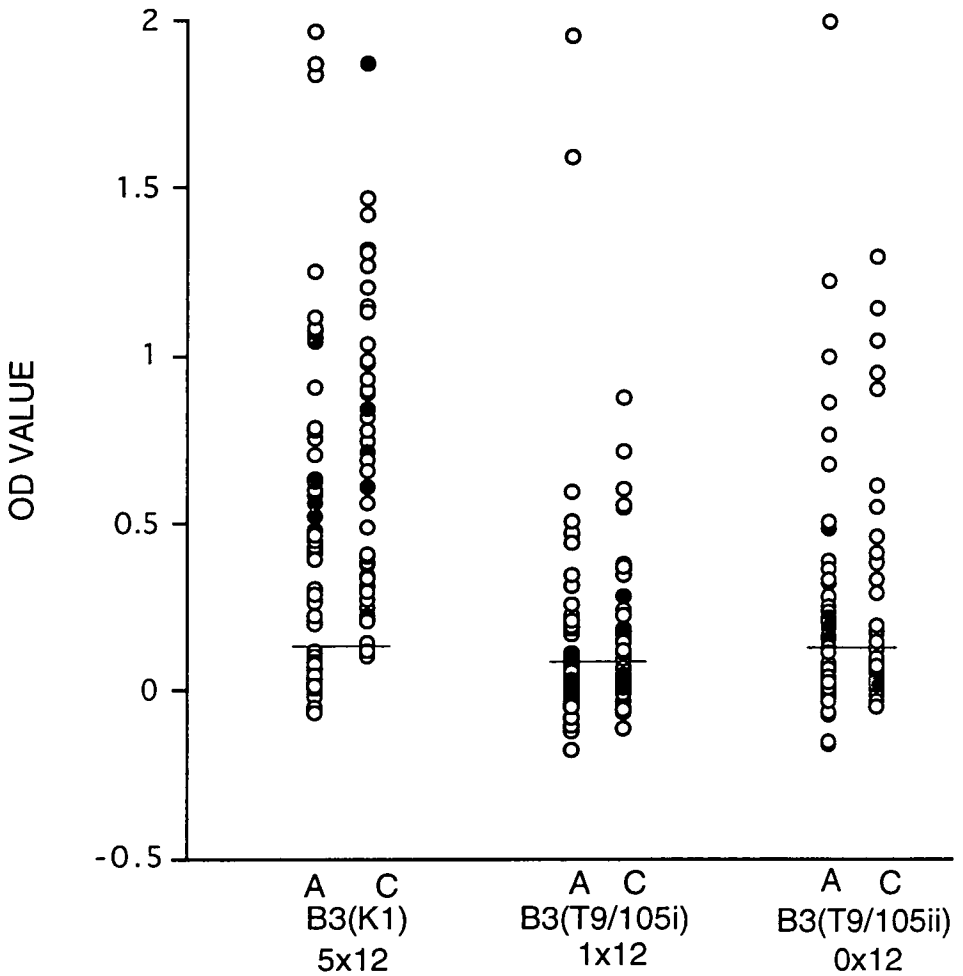


Figure 10.2: Recognition of B3 rAgs by acute (A) and convalescent (C) sera from 51 mild malaria patients. Each circle represent one individual. Closed symbols represent donors who were infected with parasites which lacked the 12 mer repeat. Horizontal lines indicate the mean + 2SD of the OD values for the control sera.

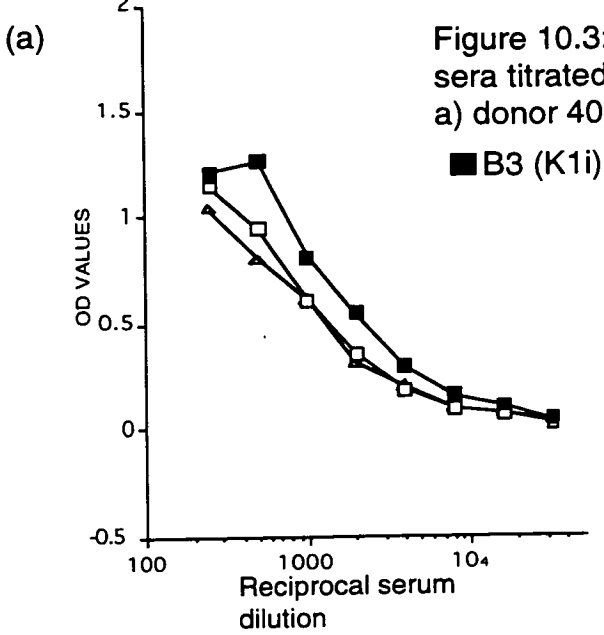
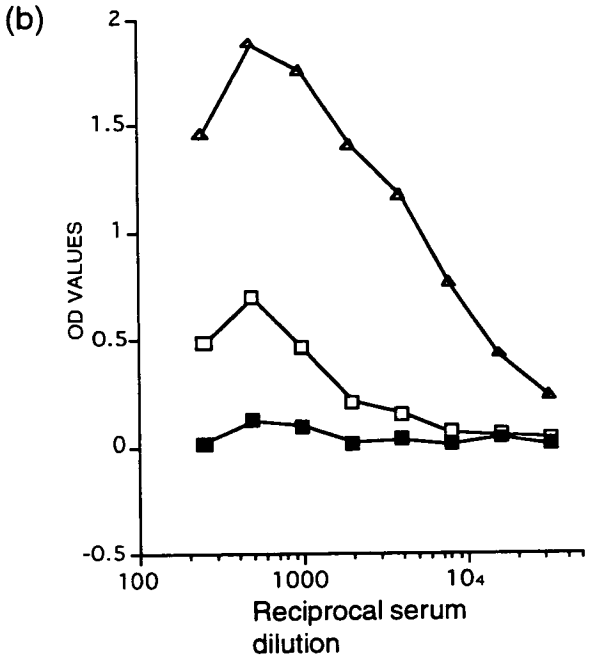
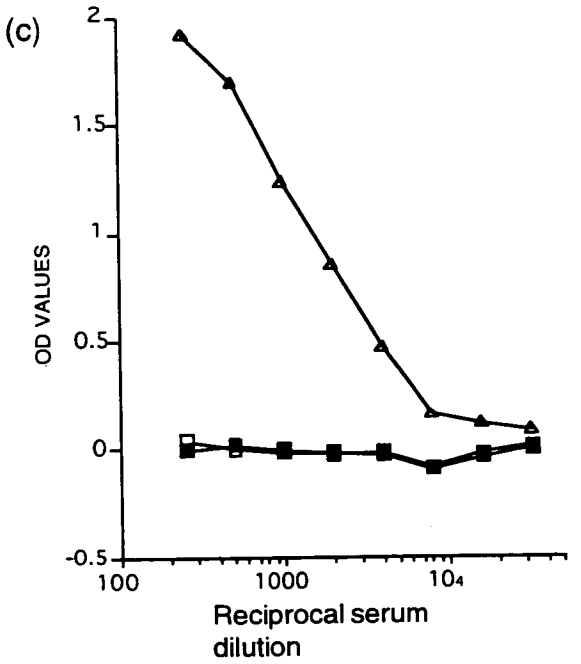


Figure 10.3: Recognition of B3 rAgs by acute sera titrated from 1:250 in doubling dilutions. a) donor 405, b) donor 389 and c) donor 248.

■B3 (K1i) □B3 (T9/105i) △B3 (T9/105ii)





10.4 DISCUSSION

Immune recognition of MSP2 is acquired in an age-dependent manner (chapter 6). Data from chapter 9 demonstrate variant-restricted immune recognition of MSP2 polymorphic sequences in non-immune individuals and children, in particular. The data is consistent with the hypothesis that the gradual development of immunity to MSP2 requires that an individual experiences a range of MSP2 serotypes and that clinical immunity develops once an individual has experienced a representative sample of parasites and possesses antibodies which cross-react with all potential sequences.

In this study the objective was to look for correlations between such variant-specific antibodies in children and the antigenic profiles of the parasites responsible for the clinical episode to see, firstly, whether boosting of the antibody response to specific MSP2 sequences could be observed and secondly, whether pre-existing antibody protected against subsequent infections with parasites carrying that sequence. Parasites were typed for MSP1 and MSP2 [66, 67]. Each of these antigens exhibits allelic polymorphisms. The frequencies of the serotypes were comparable to the frequencies observed by Conway *et al* [66, 67] in the same region in 1988 and 1989. Some fluctuations were observed for certain serotypes of MSP1 and MSP2. We are aware that considerable microheterogeneity occurs in MSP2 [201, 250] and that typing with mAbs will tend to underestimate the extent of this diversity. However, within the limits of the assays used, we do not believe that major shifts in allelic frequencies are occurring over time, in this population.

The antibody profile of each individual at the time of infection and during convalescence was determined by ELISA, primarily using rAgs A1 and B1, the full length recombinant proteins of each serogroup. We compared the antibody profile and the serogroup of MSP2 expressed by the parasites with which that child was infected.

Interpretation of serogroup B associations were straightforward, whereas the serogroup A data were more complicated - probably because MSP2 serogroup A sequences are more heterogeneous. A significant correlation was observed between the presence of antibody to B1 (during the acute and convalescent stages) and infection with serogroup B parasites. However, there was no direct association between presence of anti-A1 antibodies and presence of serogroup A parasites. However, individuals

were shown to have a higher median OD to A1 if they were not infected with serogroup B parasites. Since, if the parasites are not serogroup B, they must be serogroup A MSP2 (excluding recombinant forms). The data indicate that antibodies to serogroup A are associated with infection with serogroup A parasites.

A panel of rAgs representing the R1 repeat region of MSP2 serogroup A were used to investigate the recognition of polymorphic repeat sequences. Several individuals who were seronegative for A1(T9/96), but were infected with serogroup A parasites, were shown to have antibodies specific for other serogroup A variants. In many cases, the antibody profiles of the children, particularly in convalescence, corresponded to the antigenic profiles of the parasites with which they had been infected.

Therefore, overall, the data show a strong relationship between the antibody profile of an individual and the MSP2 serogroup expressed by the malaria parasite, with the antibody response to a particular serotype being boosted by the infection.

A few children were seronegative to all proteins tested, however these children may have antibodies specific for sequences which are not represented by our panel of proteins. Some individuals were seropositive for either A1 or B1, but were not infected with the corresponding serogroup of parasite. These antibodies had obviously persisted from a previous infection, but it is not known whether these antibodies protected against infection with parasites expressing that serogroup of MSP2 or whether these children had simply not been exposed to those parasites in the interim.

It is not known whether polymorphisms in MSP2 enable the parasite to evade variant-specific immune responses. If so, such polymorphisms might be subject to frequency-dependent selection. In this context, it is of interest that MSP2 alleles that lack the 12-mer repeat sequence of serogroup B were identified. Such variants have been reported elsewhere [201, 250] and these parasites appear to be viable (similar have been sequenced in oocysts [257]). The individuals harbouring these novel variants were shown to have high antibody responses to a rAg containing 5 copies of the repeat sequence. It is therefore tempting to conclude that parasites lacking the 12-mer sequence were able to evade the immune responses in these individuals, and were therefore at a selective advantage. In serogroup B

polymorphism within the R2 repeats is minimal with isolates varying in repeat number rather than in sequence, therefore not having the repeats may be very advantageous.

More extensive longitudinal studies are now required to investigate the dynamics of individual serotypes of *P.falciparum* in parallel with a description of the dynamics of the immune response to these serotypes, to determine whether polymorphisms arise as a result of immune selection pressure or due to random genetic drift, and to see whether pre-existing antibody protects against infection.

11: Longitudinal studies of antibody responses to PfMSP1 and MSP2

11.1 Introduction

In chapter 6, describing the age profiles of anti-MSP2 antibodies, I found that there was a significant increase in the prevalence of anti-MSP2 antibodies with age, whereas the overall mean OD for positive samples did not increase. Many children were essentially negative for one or both MSP2 serogroups; the proportion of seronegative adults was lower than in children, but a significant proportion of adults do appear to be nonresponsive to MSP2 antigens. In children this could be explained by a lack of exposure to malaria, whereas in adults this is unlikely.

The antibody prevalence data was collected by cross-sectional analysis of a population at a single point in time, in this case during the malaria transmission season in 1988. Malaria transmission in The Gambia is seasonal and it is possible that antibody levels fluctuate over time depending on the frequency of re-exposure to infection. In order to obtain a realistic evaluation of responders and non-responders to an antigen or epitope, it is necessary to conduct longitudinal studies collecting serum samples from the same individual over a period of months or years.

Several such studies have been carried out for *P.falciparum* antigens from various stages of the life cycle [26, 27, 83, 115, 228, 235, 241, 267, 270, 348]. In general, it appears that variation in antibody levels within one individual over time is less common in individuals who are clinically immune to malaria, than in those who are still actively acquiring immunity or who have only recently been exposed to malaria; in such individuals, antibody levels do fluctuate over time [26, 53, 253, 348]. Furthermore, among those individuals who are clinically immune, a proportion are consistently seronegative to particular antigens or epitopes within an antigen, whilst being stable responders to other antigens. Riley *et al* [270] found that at any one time, up to 70% of clinically immune adults were seronegative to $PfMSP1_{42}$ and around 40% of the donors tested in a longitudinal study were persistent non-responders. This phenomenon of non-responsiveness has been observed for other antigens of *P.falciparum*. The ability of an individual to produce, or not to produce, antibodies to the 230kD gamete surface antigen was a consistent phenotype which appeared early in life [267]. Quakyi *et al* [253]observed widespread

restricted immunogenicity to antigens from the sporozoite and blood stages. Responses to peptides representing different repeat sequences of Pf155/RESA show highly consistent differences between individual Liberian donors [235].

Several explanations have been proposed for this selective non-responsiveness to certain antigens or epitopes. The detection of MHC-dependent genetic restriction of immune responses to defined peptide epitopes of malaria antigens in mouse models has led to the fear that genetically determined non-responsiveness to malaria antigens may be widespread in human populations [120, 124a]. However, in outbred human populations, polymorphism of HLA antigens is extensive compared to inbred laboratory mice. Genetic regulation of antibody responses to Pf155/RESA has previously been inferred from longitudinal studies which showed that, under conditions of continual year-round malaria transmission, heterogeneous antibody profiles were observed [27, 235, 253]. Serological data [27] indicated a higher degree of variability in seroreactivity between individuals, the more specific the antigen tested (i.e. crude parasite antigen versus synthetic oligopeptides of Pf155RESA). In addition, the seroreactivity remained consistent within an individual even though the parasitaemia, and hence the antigenic load, varied between surveys. Thus, the restricted but consistent recognition indicated that genetic regulation might be involved. A study on the recognition of candidate vaccine antigens by malaria exposed sera concluded that their observations of differential non-responsiveness were consistent with the concept that poor responsiveness was the result of immune response (Ir) genes mapping within the human MHC, but no direct evidence of MHC restriction was presented [253].

In an effort to determine the practical importance of genetic constraints in the development of anti-malarial immunity in endemic human populations, Riley *et al* [270a] examined the naturally acquired cellular and humoral immune responses to defined *P.falciparum* antigens in individuals of differing HLA class II genotypes. They concluded that non-responsiveness is not primarily due to HLA class II-mediated genetic restriction. However, it has not been excluded that non-MHC genes may influence immune responses to malaria Ags. For example, variation could be due to allotype restriction of the donors' antibody repertoire, genetic regulation of

lymphokine production, or antigen processing or at the B- or T-cell level [27, 235, 271].

An alternative approach to determine the importance of genetic versus environmental factors in the immune recognition of malaria antigens is to compare responses in identical and non-identical twin pairs. Sjöberg *et al* [293] investigated twins for possible genetic restriction of T-cell and B-cell responses to defined, repetitive epitopes of Pf155/RESA. They found that anti-peptide responses were more concordant within monozygous twin pairs than within either dizygous twins or sibling pairs who were matched for age, sex and exposure to malaria. The data implied that the antibody response to Pf155/RESA came under genetic regulation but further analysis showed no associations with HLA class II type. They conclude that regulation of antibody response reflects the impact of factors encoded by genes outside the HLA class II region.

Non-responsiveness could also be attributed to such factors as antigenic polymorphism, immunosuppression, temporal variation in antibody levels and/or inadequate exposure to malaria. Antigenic polymorphism is a common feature of malaria antigens. Since memory T cells primed by exposure to one parasite strain will not respond to a variant strain bearing a non-cross-reacting epitope, such polymorphism may account for this apparent non-responsiveness and presumably represents a parasite adaptation for immune evasion [271]. Temporal variation in antibody levels could be due to low transmission rates during long dry periods in some endemic areas and poor T cell memory. Specific serum antibody titres may fall below detectable levels, particularly in children and in areas of low endemicity [115, 200, 348]. Thus cross-sectional surveys are likely to miss a proportion of responders since sampling occurs at one point in time.

Down-regulation of the immune response due to repeated infection and chronic exposure of the individual could result in apparent non-responsiveness to particular antigens. Riley *et al* [267] have observed that a stable responsiveness phenotype is fixed early in life and is apparent in children as young as 3-5 years of age for Pfs230. Thus tolerance might be induced following prenatal or perinatal exposure to malaria antigens [246a]. It is well recognised that specific unresponsiveness to immunisation can be induced by prolonged exposure to antigenic proteins [286a]. It would be expected that exposure of the foetus or new-born to malaria antigen, at a time when the immune system is immature, would have an influence on

subsequent malaria immunity and may play a partial role in the lack of childhood immunity to malaria [246a].
A novel explanation for the selective recogniton of malaria antigens is a mechanism, originally proposed to explain the restricted antibody response to influenza viruses, known as Original Antigenic Sin [107] or clonal imprinting [118a]. Clonal imprinting has been proposed as part of the explanation for the limited antibody repertoire seen in patients with HIV-1 infections [118a]. Clonal imprinting is a phenomenon in which a primary infection with a particular variant leads to the clonal expansion of B-cells specific for certain antigen epitopes. During a secondary infection, antigens which cross-react with those presented in the original infection are recognised by memory B-cells and antibody of the original specificity is produced. Epitopes which were not present in the primary infection are effectively ignored because naive B-cells cannot compete for Ag with higher affinity memory cells. As a result certain antigens and epitopes are persistently recognised and others are not recognised.

The questions which need to be addressed concerning the recognition of MSP2 are:-
1) is non-responsiveness to particular epitopes of MSP2 a stable phenotype in adults and children who have been exposed to endemic malaria?
2) is non-responsiveness to MSP2 genetically determined?

In this chapter, I report the results of two longitudinal serological studies conducted in The Gambia. Samples were collected at six month intervals from adults and children from the villages of Brefet and Farafenni, respectively. Surveys were made at the end of the dry season (when malaria transmission is minimal) and at the end of the wet season (when transmission is at its maximum) over several transmission seasons. Antibody responses to the two major *P.falciparum* merozoite surface proteins, PfMSP1 and MSP2, were measured in ELISA. The rAgs used represented regions of the proteins that were conserved, dimorphic or polymorphic. In this way we examined the temporal variation of antibody responses in individuals who are clinically immune and in individuals who are still actively acquiring their immunity to malaria. Furthermore, we examine whether the regulation of immune recognition of MSP2 is

genetically determined by comparing antibody recognition of pairs by monozygous and dizygous twins and examining the relationship between human leukocyte antigen (HLA) class II genotype and anti-MSP2 immune responses.

11.2 Materials and methods

11.2.1 Subjects

1. Malaria exposed adults

Sera were collected from 27 adults (aged 16-65) living in the village of Brefet, The Gambia. Samples were collected at 6 month intervals (dry season and wet season) from November 1990 to November 1993. For each individual at least 4 serum samples were obtained. The age, sex and number of samples obtained for each individual is detailed in table 11.1.

2. Malaria exposed children

Sera were collected from children (aged 3-8 years in 1988) living in the villages near the town of Farafenni, The Gambia. Sera were collected at 6 month intervals from June 1988 to November 1991. For each individual at least 4 samples were collected. The age, sex and number of samples obtained for each individual is detailed in table 11.2.
These samples were collected by Dr S Allen.

3. Malaria exposed twins

Serum samples were obtained from 36 pairs of adult twins living all over the Eastern region of The Gambia. 15 pairs of twins were shown to be monozygous. Zygosity was determined using cloned human DNA minisatellites as locus-specific hybridisation probes [362].
Samples and zygosity data were kindly provided by Dr. Annette Jepson.

4 Controls

Control serum samples were obtained from European adults (n=12) and children (n=12). These individuals have not been exposed to malaria.

All serum samples were stored at -20°C.

Donor	No. of samples	Age (in 1990)	Sex
1	5	59	m
4	6	34	f
7	6	36	f
12	6	64	m
13	5	65	m
15	4	45	m
28	6	57	f
31	6	44	m
35	6	37	f
45	5	57	m
50	5	38	m
53	5	59	m
69	5	49	m
79	5	50	m
95	5	41	f
104	4	16	f
105	5	24	f
109	5	16	f
110	5	17	f
112	6	16	f
114	5	16	f
116	6	28	m
117	6	17	f
120	6	65	f
125	6	28	f
127	4	41	m
167	5	16	f

Table 11.1: Longitudinal recognition of merozoite surface proteins - adult serum samples. Donor details giving the number of samples collected for each individual, their age (at beginning of study) and sex.

Donor	No. of samples	Age (in 1988)	Sex
E07008	6	5	m
E08018	7	7	m
E09009	5	3	m
E17004	7	3	m
E18003	6	7	f
E20015	5	5	m
E24008	7	6	m
E24009	7	4	m
E25007	6	3	m
E25009	7	6	f
P06008	5	7	m
P06009	6	5	m
P10016	6	7	m
P10017	5	4	m
P11005	4	6	m
P12009	5	5	f
Q01008	4	7	m
Q03005	5	4	m
N05034	4	4	m

Table 11.2: Longitudinal recognition of merozoite surface proteins - childrens serum samples. Donor details giving the number of samples collected for each individual, their age (at beginning of study) and sex.

11.2.2 Recombinant proteins:

The following rAgs were used for this work: MSP2 - A2(T9/96), A3, B2(K1i) and B3(T9/105i); MSP1 - see table11.3.

rAg	Serogroup	Region
EGF-1A	Wellcome	19kDa (1st EGF-like motif) *
EGF-1B	Mad-20	19kDa (1st EGF-like motif) *
19/GST	Wellcome	19kDa (both motifs)*

Table 11.3: Summary of recombinant proteins MSP1 used in this chapter. Proteins kindly provided by A.Egan; for further details see Egan *et al* [93] and figure 11.1.

11.2.3 Enzyme-linked immunosorbent assay (ELISA):

IgG antibodies were measured in ELISA as described in materials and methods. For screening, all sera were diluted 1/1000 in blocking buffer and tested in duplicate.

11.2.4 Statistical methods:

1. Determination of positivity:

Positive samples were defined as those giving a specific OD above the normal range for control sera. The normal range was taken as the mean + 2 standard deviations of the OD values obtained for the control sera.

2. HLA analysis:

The overall association of HLA class II genotype with antibody prevalence was determined by logistic regression allowing for age and sex.

This analysis was performed by D. McGuiness, ICAPB, Edinburgh University.

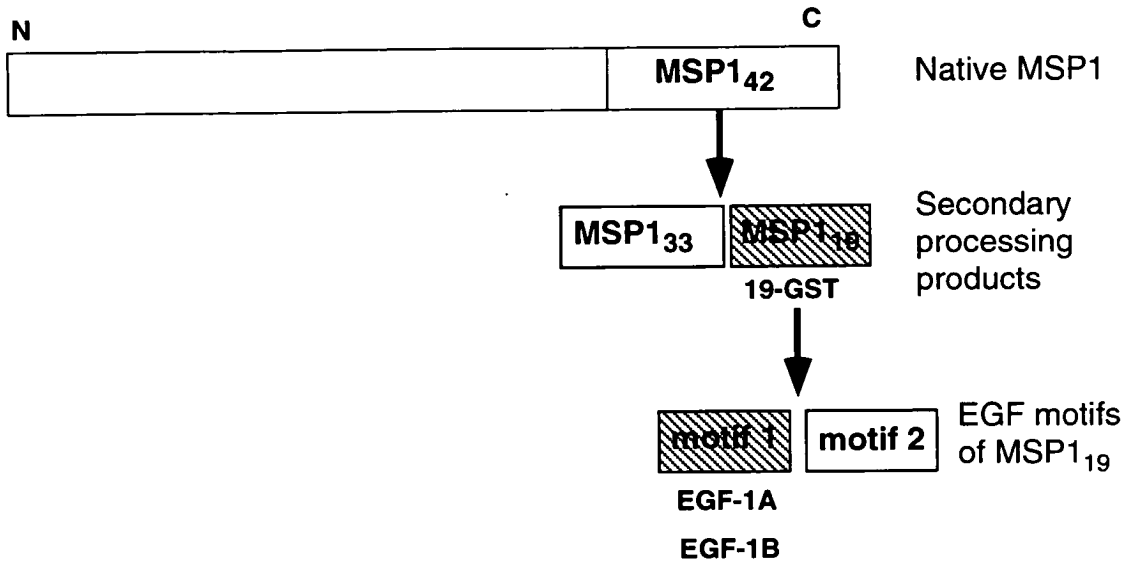


Figure 11.1: Schematic representation of PfMSP1 recombinant proteins.
Hatched boxes indicate rAgs used.

11.3 Results

11.3.1 Longitudinal study of antibody responses to *P.falciparum* merozoite surface antigens by Gambian adults:

Sera collected over a period of 3 years from adults from the village of Brefet, were tested for recognition of the two major merozoite surface antigens, MSP1 and MSP2, in ELISA. The rAgs of MSP1 represented the relatively conserved 19kDa C-terminal fragment of PfMSP1 (which contains two EGF-like motifs) and two rAgs representing the alternative allelic sequences of the first of the two EGF-like motifs (see figure 11.1).

In order to minimise intra- and inter-assay variation, all samples from a single individual were tested against all the proteins on one plate.

Table 11.4 and figures 11.2a-c show a representative sample of results; a complete set of results is given in appendix 3, table 1. There is very little seasonal variation in Ab concentration in adults despite the varying transmission conditions during the year. Where seasonal variation is observed, it tends to be for variant rather than conserved antigens and is more comonly seen in the younger donors.

The most striking finding is that, despite lifelong exposure to malaria, there are a number of individuals who are consistently non-responders to some of the rAgs, whilst consistently recognising other rAgs. In other words, these individuals show differential recognition of antigens and epitopes within those antigens. For example, donor 45JK differentiates between MSP1 and MSP2, being consistently seronegative for PfMSP1 and consistently seropositive for all of the MSP2 proteins (table 11.4, figure 11.2a). In this donor, there is some evidence of boosting during the transmission season in 1993 to MSP2 serogroup B epitopes. The Ab recognition of MSP2(B) increases but there is still no response to $MSP1_{19}$. Other donors, for example 12TS and 50OS, also differentiate between the recognition of $MSP1_{19}$ and MSP2 antigens, being consistently seronegative for $MSP1_{19}$. However, donor 79AJ (figure 11.2b) recognises all the rAgs strongly, and remains seropositive throughout the period of the study, although the titre decreases suggesting that there has been no boosting for some time. Thus, all the rAgs are immunogenic and non-recognition of any of the rAgs is not due to poor immunogenicity.

Donor 110BS differentiates between epitopes within MSP2 (table 11.4). She is seronegative for the A2(T9/96), but seropositive for the group

specific region of the same protein. She also shows differential recognition of the serogroup B rAgs and there appears to be a boosting of the response to B3(T9/105i); this is likely to have occurred in November 1992, however no serum sample was available from that collection time. This donor is unusual in that she differentiates between the slightly different allellic sequences of MSP1-EGF1 and MSP1-EGF2 (figure 11.2c); Egan *et al* [93] have shown that the recognition of these two rAgs is usually highly correlated within individual donors.

DONOR	DATE	EGF-1A	EGF-1B	19/GST	A2 (T9/96)	A3	B2 (K1i)	B3 (T9/105i)
12 TS	11/90	0.090	-0.054	-0.031	0.020	0.063	-0.056	-0.058
	11/91	-0.350	-0.352	-0.250	0.305	0.538	1.113	0.573
	4/92	-0.187	-0.199	-0.213	0.171	0.520	0.238	0.188
	11/92	-0.242	-0.173	-0.279	-0.060	0.526	0.435	0.689
	4/93	-0.168	-0.027	-0.121	0.061	0.487	1.048	0.842
	11/93	-0.088	-0.113	0.125	0.125	0.399	0.996	0.952
45 JK	11/91	0.171	0.017	0.159	1.042	0.912	0.490	1.114
	4/92	-0.032	-0.008	0.100	0.791	0.775	0.521	1.127
	11/92	0.103	0.132	0.311	0.677	0.635	0.451	0.955
	4/93	0.089	0.009	0.072	0.821	0.734	0.387	0.880
	11/93	0.040	0.036	0.037	0.640	0.737	0.826	1.300
50 OS	11/90	0.140	-0.053	-0.067	1.122	1.170	0.590	0.139
	11/91	-0.268	-0.277	-0.294	0.660	0.447	0.391	0.121
	4/92	-0.154	-0.157	-0.110	1.004	0.957	0.508	0.351
	4/93	-0.208	-0.196	-0.210	1.184	1.173	0.549	-0.017
	11/93	-0.261	-0.267	-0.256	0.936	1.082	0.463	0.101
79 AJ	11/91	0.740	0.625	0.971	1.355	1.286	1.437	1.259
	4/92	0.810	0.806	1.035	1.071	1.170	0.890	1.340
	11/92	0.909	0.886	0.971	0.800	1.212	1.129	1.654
	4/93	0.589	0.536	0.919	0.803	0.792	0.873	1.281
	11/93	0.253	0.296	0.757	0.418	0.453	0.565	1.194
110 BS	11/90	0.121	0.218	0.482	-0.140	0.990	0.786	0.321
	11/91	0.072	0.438	0.501	-0.159	1.025	1.261	0.286
	4/92	-0.012	0.406	0.504	0.105	0.793	0.665	0.195
	4/93	0.111	0.360	0.500	-0.007	0.677	0.701	1.043
	11/93	-0.017	0.338	0.442	0.180	0.824	0.848	0.630

Table 11.4: Longitudinal recognition of merozoite surface proteins by adults from Brefet, The Gambia. Serum samples were collected at 6 month intervals during the dry season (April) or the wet, transmission season (November). Sera was diluted 1/1000 and IgG recognition of rAgs measured by ELISA. Positivity is determined as OD values above the mean+2SD of the European control sera. EGF-1A = 0.249, EGF-1B = 0.229, 19/GST = 0.196, A2(T9/96) = 0.290, A3 = 0.139, B2(K1i) = 0.111 and B3(T9/105i) = 0.146. Positive responses are shaded.

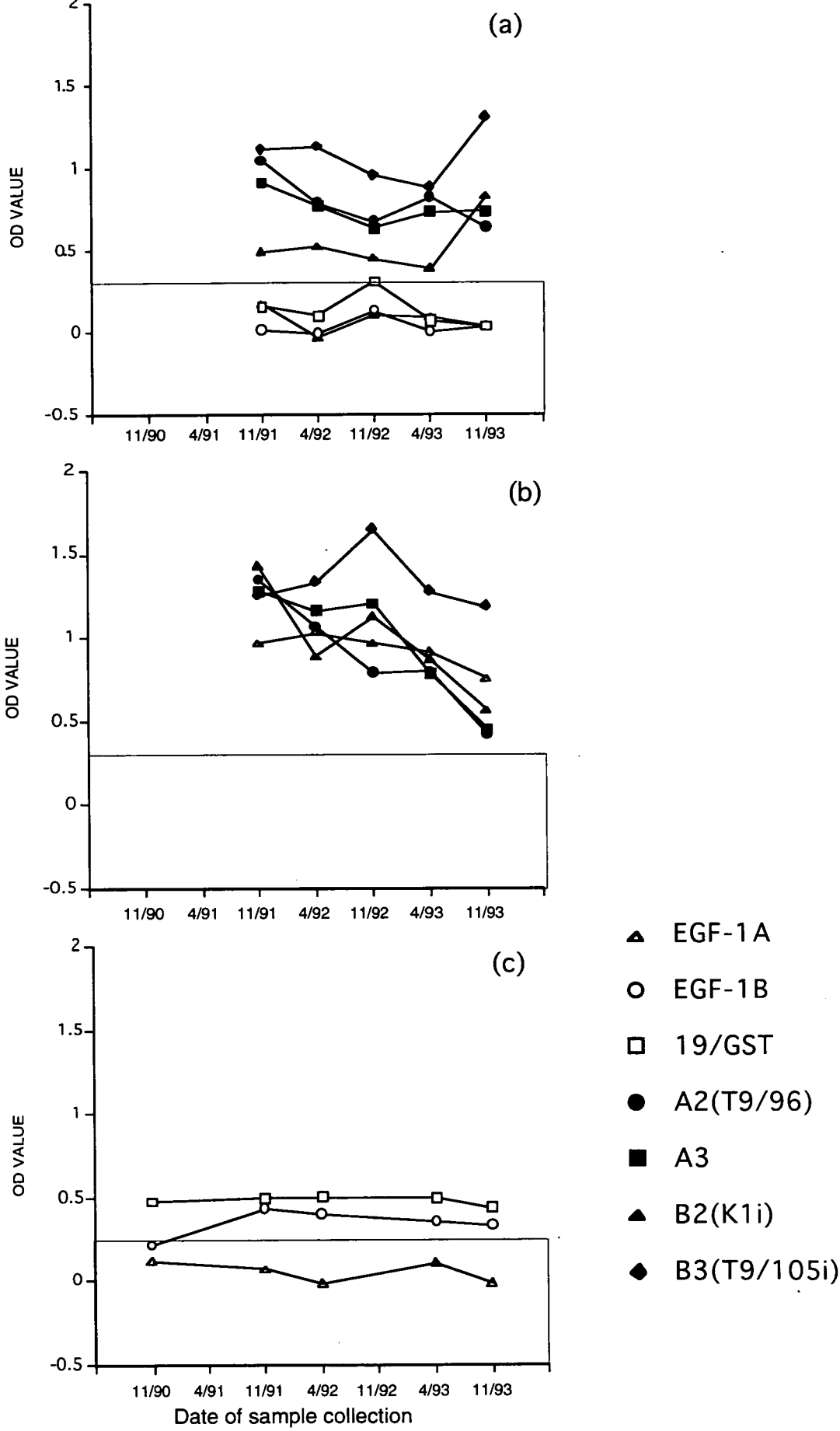


Figure 11.2: Longitudinal study of anti-malarial antibody levels in Gambian adults. Samples were collected at 6 month intervals from November 1990 to November 1993. The horizontal lines represent the upper limit of the normal range for non-immune sera.
a) Donor 45JK; b)Donor 79AJ; c)Donor110BS

11.3.2 Longitudinal recognition of *P.falciparum* merozoite surface antigens by malaria exposed children:

The recognition of merozoite surface antigens was determined for 20 children from the village of Farafenni, using sera collected over a period of 4 years during the dry seasons (June) and the wet seasons (November) (table 11.5).

As expected, the overall recognition of these proteins is much lower in children but in contrast to the adults, the recognition of these rAgs was also less stable in children (Appendix 3, table 2). Few of the donors had a stable "responder" phenotype to any of the antigens. Donor Q01008 was one of the few indivduals to show consistent positive recognition of some of the rAgs (table 11.5, figure 11.3a) but even here, boosting of antibody concentration was evident in the wet season.

The general pattern of recognition is that the antibody response fluctuates and is often boosted during the transmission season. This was apparent for donor E18003 (figure 11.3b); the antibody concentration is boosted during the transmission season in 1988, declines and is boosted again in 1991. Boosting of the antibody response can be seen clearly for donor P10016 (figure 11.3c); the antibody response to the group specific regions of MSP2 (A3 and B3) increases during the transmission season of 1989 and to a lesser extent in 1991. Donor E24009 also showed boosting of the antibody response to MSP2 in the 1990 and 1991 transmission seasons, but remains seronegative for $MSP1_{19}$.

DONOR	DATE	EGF-1A	EGF-1B	19/GST	A2 (T9/96)	A3	B2 (K1i)	B3 (T9/105i)
Q01008	6/88	0.002	0.022	0.013	0.170	1.182	0.614	1.356
	11/88	-0.117	0.051	0.199	0.226	1.342	0.593	1.689
	6/89	-0.041	0.011	-0.012	0.244	0.862	1.436	1.448
	11/89	0.121	0.435	0.513	0.799	1.358	0.873	0.839
E18003	6/88	-0.073	-0.127	-0.155	-0.222	-0.185	-0.239	-0.067
	11/88	0.957	0.906	0.764	-0.081	1.317	-0.118	0.259
	6/90	-0.238	-0.220	-0.202	-0.200	-0.209	-0.184	-0.198
	11/90	-0.197	-0.131	-0.036	-0.061	0.004	-0.068	0.112
	6/91	0.025	0.030	-0.031	0.211	-0.034	0.041	0.069
	11/91	0.672	0.701	0.446	0.074	0.214	-0.075	0.316
P10016	6/88	-0.107	-0.122	-0.119	-0.049	0.319	-0.044	0.121
	11/88	-0.051	-0.073	0.469	-0.113	1.995	-0.026	1.031
	6/89	-0.118	-0.139	-0.116	0.059	0.512	0.074	0.152
	11/89	-0.047	0.242	0.104	0.042	1.910	0.527	1.117
	6/91	-0.001	0.044	0.164	0.036	0.683	0.155	0.432
	11/91	-0.196	-0.256	0.126	0.361	1.126	-0.180	0.656
E24009	6/88	0.022	0.006	0.022	0.222	0.096	0.120	0.064
	11/88	0.021	0.006	0.026	0.005	0.115	0.156	0.107
	6/89	-0.125	-0.113	-0.135	-0.026	-0.094	0.203	-0.028
	6/90	0.004	-0.131	-0.089	0.125	0.000	0.179	-0.028
	11/90	0.002	-0.034	0.008	0.373	0.092	0.954	1.824
	6/91	-0.022	-0.105	-0.146	-0.070	-0.182	-0.140	-0.074
	11/91	0.028	0.230	0.611	0.179	1.064	-0.060	1.453

Table 11.5: Longitudinal recognition of merozoite surface proteins by children from Farafenni, The Gambia. Serum samples were collected at 6 month intervals during the dry season (June) or the wet, transmission season (November). Sera was diluted 1/1000 and IgG recognition of rAgs measured by ELISA. Positivity is determined as OD values above the mean+2SD of the European control sera; EGF-1A = 0.186, EGF-1B = 0.264, 19/GST = 0.200, A2(T9/96) = 0.138, A3 = 0.245, B2(K1i) = 0.194 and B3(T9/105i) = 0.126. Positive responses are shaded.

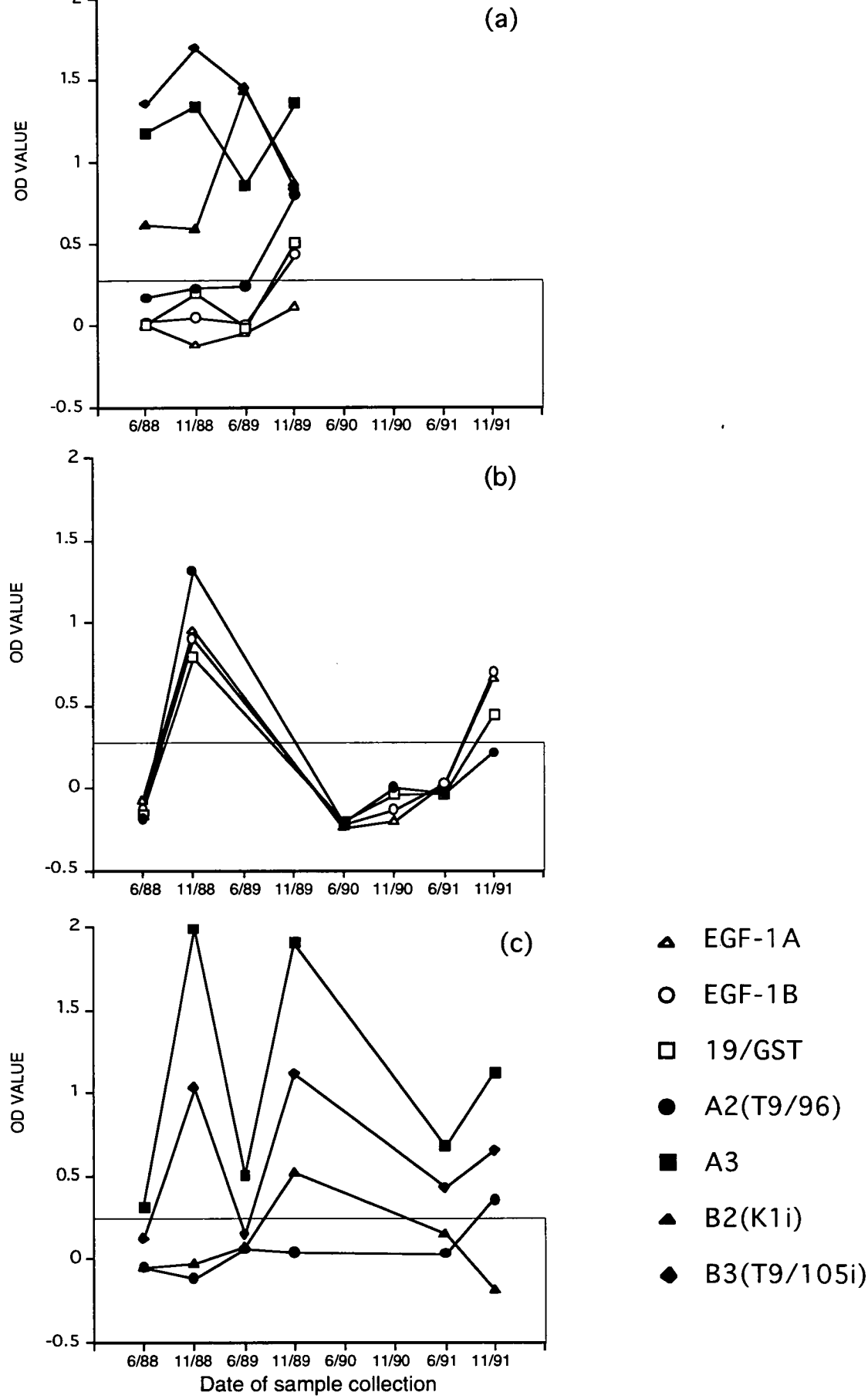


Figure 11.3: Longitudinal study of anti-malarial antibody levels in Gambian children. Samples were collected at 6 month intervals from June 1988 to November 1991. The horizontal lines represent the upper limit of the normal range for non-immune sera.
a) Donor Q01008; b)Donor E18003; c) Donor P10016

11.3.3 Is the pattern of recognition of MSP2 genetically determined?:

i) Recognition of MSP2 by twins:

The data from the adult donors suggested that some individuals were unable to make an Ab response to some of the rAgs. To investigate whether this is due to a genetically determined "non-responder" status, serum from 15 pairs of monozygous (mz) twins and 21 pairs of dizygous (dz) twins were tested for recognition of MSP2 rAgs. Typical OD values are shown in table 11.6. It is clear from this data that individual mz twin pairs can have very different antibody patterns to MSP2.

Table 11.7 gives the number of concordant pairs i.e. the number of pairs of twins that are either both positive or both negative to individual rAgs. A χ^2 test showed that there was no significant difference in the numbers of concordant (and discordant) mz or dz twins. Furthermore, if one looks at the difference in the specific OD values within individual twin pairs, there is no significant difference in the closeness of recognition of the rAgs by mz and dz twins (table 11.8). This data suggests that mz twins are no more likely to have similar anti-MSP2 Abs than are dz twins, suggesting that genetic factors are not a major determinant of recognition of MSP2. Furthermore, mz twins (with essentially identical malaria exposure, since in all cases they lived together for all their lives) can have markedly different antibody responses.

The twins study and data analysis was carried out by R.Adair, an undergraduate student, as part of his Immunology Honours project, under my supervision.

DONOR	STATUS	ODVALUE
634*	mz	-0.189
635		0.880
646	mz	1.462
647		1.480
664	mz	0.171
665		1.512
672	mz	-0.128
673		-0.094
677	mz	-0.084
678		-0.279
682	mz	1.751
683		1.383
842	mz	1.354
843		1.135
642*	dz	0.576
643		-0.109
661*	dz	1.600
671		-0.162
674	dz	0.370
679		0.508
684*	dz	0.694
685		-0.246
691	dz	0.214
692		0.374
693*	dz	-0.030
694		1.096
756	dz	0.352
758		1.712
757	dz	0.718
759		1.935

Table 11.6: Examples of specific OD values for the Ab recognition of B3(T9/105i) by monozygous (mz) and dizygous (dz) twin pairs. Positive responders are shaded.

* indicates discordant pairs. Concordancy was defined as OD values of a pair both being above or below the cut-off value (0.092), calculated as the mean +2SD of 8 European control sera.

rAg	No. of concordant pairs		χ^2 value	p
	mz (n=15)	dz (n=21)		
A2(T9/96)	7	16	3.14	>0.05
A3	12	17	0.038	>0.5
B2(K1i)	11	17	0.038	>0.5
B3(T9/105i)	11	14	0.073	>0.5

Table 11.7: Concordancy of Ab recogition of MSP2 rAgs by mz zand dz twins.
Critical χ^2 value= 3.84, p=0.05.

rAg	Rank sum		Mann-Whitney U value	p
	mz (n=15)	dz (n=21)		
A2(T9/96)	295.0	371.0	137.5	0.53
A3	250.0	416.0	140.0	0.59
B2(K1i)	301.0	365.0	134.5	0.47
B3(T9/105i)	257.5	408.5	137.0	0.57

Table 11.8: Comparison of the median values for the ranked differences in OD value within pairs of mz and dz twins to each antigen, using a non-parametric Mann-Whitney U-test.
The difference in the specific OD value between paired samples of both mz and dz twins were calculated and ranked. The sum of the ranks was calculated for mz and dz pairs separately. The Mann-Whitney test was performed to determine the difference in the sum of the ranks.

ii) Relationship between presence of anti-MSP2 antibodies and HLA classII genotype:

HLA class II typing had previously been performed (by Dr O. Olerup, Center for BioTechnology, Karolinske Institute, Sweden) for the individuals recruited for the cross sectional study (sera collected from the villages around Farafenni, October 1988) and the morbidity study (sera collected from the villages around Farafenni, May 1988) [15]. Allelic RFLP patterns at each locus (*DRB, DQA, DQB*) were designated by Roman numerals and individual *DRB -DQA -DQB* haplotypes have been ascribed Arabic numerals; the HLA class II haplotypes in the Gambian population are shown in table 11.9. For the analysis, associations between immune responses and RFLP-defined *DRB -DQA -DQB* haplotype rather than individual class II alleles or associated serological specificities were determined, and only those *DRB -DQA -DQB* haplotypes present in the sample in sufficient numbers were included (i.e. at least 20 individuals for May 1988 samples and at least 10 indiviuals for October 1988 samples).

Antibody responses to all antigens were dichotomised into responders and nonresponders; a χ^2 statistic was calculated from multiple logistic regression (table 11.9 and 11.10). The logistic regression allows for confounding effects of age, sex, and previous malaria control interventions. Ethnic group was not included as a confounder because its causal association with haplotype would have led to a masking of the association between haplotype and disease [270a].

Gambian villages are made up of compounds which accommodate large extended families. Therefore, these related individuals share HLA haplotypes, a variable number of background genes and also the same environment [270a]. The effect of shared environment and background genes were removed by stratifying on compound using conditional logistic regression, which restricts the analysis to the effect of HLA differences between individuals of the same compound (tables 11.9 and 11.10).

Since the production of anti-MSP2 antibodies appears to be a consistent phenotype within one individual the possibility that non-responsiveness to MSP2 might be due to HLA class II dependent genetic restriction of the immune response was investigated. No significant associations were observed between HLA class II haplotype and immune recognition of MSP2.

Haplotype number	RFLP pattern			Associated seologic specificity			Antibody recognition of MSP2 rAg Phenotype number (frequency)			
	DRB	DQA	DQB	DR	DQ	n	A2	A3	B2	B3
1	I	I	I	1	w5	8	3 (0.38)	2 (0.25)	1 (0.13)	4 (0.50)
2	II	II	II	w15	w6	7	1 (0.14)	1 (0.14)	1 (0.14)	1 (0.14)
7 *	VII	IV	III	w17	w2	31	13 (0.42)	9 (0.29)	6 (0.19)	17 (0.55)
8	XV	VI	IV	w18	w4	13	3 (0.23)	2 (0.15)	1 (0.08)	5 (0.38)
9	XXII	VI	IV	3	w4	5	1 (0.20)	1 (0.20)	1 (0.20)	2 (0.40)
11	XII	V	IV	4	w8	18	8 (0.44)	7 (0.39)	6 (0.33)	12 (0.67)
12 *	XII	V	VI	4	w2	26	12 (0.46)	9 (0.35)	6 (0.23)	15 (0.58)
14	XIII	V	VI	7	w2	16	3 (0.19)	3 (0.19)	3 (0.19)	10 (0.63)
15 *	XIV	V	VI	7	w2	58	21 (0.36)	21 (0.36)	13 (0.22)	31 (0.53)
17 *	V	VI	V	w8	w7	30	11 (0.37)	6 (0.20)	4 (0.13)	13 (0.43)
18	V	IV	V	w8	w7	18	6 (0.33)	8 (0.44)	5 (0.28)	12 (0.67)
21 *	XVII	V	IX	9	w2	43	18 (0.42)	11 (0.26)	9 (0.21)	21 (0.49)
22 *	III	I	I	w10	w5	60	17 (0.28)	14 (0.23)	6 (0.10)	27 (0.45)
23	VI	IV	V	w11	w7	14	6 (0.43)	2 (0.14)	4 (0.29)	10 (0.71)
26	XX	IV	V	w11	w7	9	2 (0.22)	3 (0.33)	0 (0.00)	4 (0.44)
29 *	XXI	IV	V	w11	w7	119	38 (0.32)	25 (0.21)	15 (0.13)	62 (0.52)
34	IX	III	II	w13	w6	10	1 (0.10)	0 (0.00)	0 (0.00)	1 (0.10)
35	IX	III	I	w13	w1	11	4 (0.36)	4 (0.36)	4 (0.36)	5 (0.45)
37	XI	III	II	w13	w6	9	3 (0.33)	2 (0.22)	2 (0.22)	3 (0.33)
38 *	X	II	I	w13	w6	62	26 (0.42)	9 (0.15)	9 (0.15)	35 (0.56)
39	XV	IV	V	w13	w7	13	3 (0.23)	3 (0.23)	3 (0.23)	9 (0.69)
						1)χ^2	7.29	8.66	7.08	5.31
						p	0.51	0.37	0.53	0.72
						2)χ^2	4.35	7.34	9.47	7.50
						p	0.82	0.50	0.50	0.48

Table 11.10: Association between class II haplotypes and immune responses to MSP2 rAgs for a cross-section of the population aged 1-75 years (n=119). Samples were collected at the end of the malaria transmission season (October 1988).

* denotes those haplotypes which were present in the study population in sufficient numbers (>10) to allow evaluation of their association with immune responses.

[1)] denotes standard logistic regression and [2)] denotes conditional logistic regression.

[a] indicates d.f=3 as haplotypes 14 and 21 were not included due to small group sizes.

N/A - the conditional model was not applicable to this set of data because numbers were too small.

Haplotype number	RFLP pattern			Associated seologic specificity			Antibody recognition of MSP2 rAg Phenotype number (frequency)			
	DRB	DQA	DQB	DR	DQ	n	A2	A3	B2	B3
1	I	I	I	1	w5	8	5 (0.63)	8 (1.00)	8 (1.00)	6 (0.75)
2	II	II	II	w15	w6	7	2 (0.50)	4 (1.00)	4 (1.00)	4 (1.00)
7	VII	IV	III	w17	w2	4	4 (0.57)	7 (1.00)	7 (1.00)	7 (1.00)
8	XV	VI	IV	w18	w4	1	1 (1.00)	0 (0.00)	0 (0.00)	0 (0.00)
9	XXII	VI	IV	3	w4	7	2 (0.29)	6 (0.86)	6 (0.86)	5 (0.71)
11	XII	V	IV	4	w8	5	2 (0.40)	5 (1.00)	5 (1.00)	4 (0.80)
12	XII	V	VI	4	w2	1	1 (1.00)	1 (1.00)	1 (1.00)	1 (1.00)
14 *	XIII	V	VI	7	w2	11	7 (0.64)	9 (0.82)	9 (0.82)	8 (0.73)
15	XIV	V	VI	7	w2	3	1 (0.33)	3 (1.00)	3 (1.00)	3 (1.00)
17	V	VI	V	w8	w7	5	3 (0.60)	5 (1.00)	3 (0.60)	3 (0.60)
18	V	IV	V	w8	w7	3	3 (1.00)	3 (1.00)	3 (1.00)	2 (0.67)
21 *	XVII	V	IX	9	w2	12	7 (0.58)	12 (1.00)	11 (0.92)	11 (0.92)
22 *	III	I	I	w10	w5	16	10 (0.63)	14 (0.88)	13 (0.81)	13 (0.81)
23	VI	IV	V	w11	w7	5	4 (0.80)	5 (1.00)	4 (0.80)	4 (0.80)
26	XX	IV	V	w11	w7	4	2 (0.50)	4 (1.00)	4 (1.00)	1 (0.25)
29 *	XXI	IV	V	w11	w7	60	31 (0.52)	54 (0.90)	54 (0.90)	44 (0.73)
34	IX	III	II	w13	w6	4	2 (0.50)	4 (1.00)	3 (0.75)	4 (1.00)
35	IX	III	I	w13	w1	0				
37	XI	III	II	w13	w6	3	2 (0.67)	3 (1.00)	3 (1.00)	3 (1.00)
38 *	X	II	I	w13	w6	28	16 (0.57)	25 (0.89)	26 (0.93)	25 (0.89)
39	XV	IV	V	w13	w7	9	7 (0.78)	9 (1.00)	9 (1.00)	7 (0.78)
						[1)]χ^2	2.44	4.67	3.43	5.27
						p (d.f=5)	0.79	0.46	0.63	0.38
						[2)]χ^2	5.48	0.05	N/A	2.69
						p (d.f =5)	0.36	1.00 [a]		0.75

11.4 Discussion

In this chapter I have examined the recognition of the major merozoite surface proteins of *P.falciparum*, MSP1 and MSP2, by longitudinal analysis of the seroreactivities of adults and children over a period of time.

Cross-sectional analysis of the recognition of MSP2 indicated that antibody prevalence increased with age and that in each age group there were a number of non-responders. Longitudinal analysis enables us to determine if this non-responsive phenotype is stable. Serum samples were collected from adults and children from The Gambia during the dry season (minimal malaria transmission) and at the end of the wet season (maximum malaria transmission) for a period of 4 years. We found that the recognition of $MSP1_{19}$ and MSP2 in adults is, in general, a stable response. A stable antibody response is one which persists over time, even when malaria transmission is minimal. A possible explanation for this is that in these adults there is a persistence of antigen due to asymptomatic infections. Alternatively, adults may have developed an efficient memory response to malaria antigens and low level infections, when malaria transmission is minimal, are sufficient to boost the antibody levels to certain malaria antigens. The antibody levels were constant over time and many adults showed differential recognition of the antigens, and of specific epitopes within those antigens (table 11.4, figure 11.2a-c). There were certain donors who, despite being clinically immune to malaria, were consistently seronegative to certain rAgs whilst consistently recognising others. However, all the rAgs are known to be immunogenic and some individuals were consistently positive to all the rAgs tested.

In contrast to the adults, children, who were still actively acquiring immunity to malaria, had more heterogeneous antibody profiles. There was clear evidence of seasonal variation in antibody concentration, with the Ab response being boosted during the malaria transmission season (figure 11.3b-c). It is possible to make an educated guess as to the genotype of the infecting parasites, based on the increase in antibody to the MSP2 serogroup A or B proteins. In addition, it was evident from the data that $MSP1_{19}$ was less immunogenic in children than the MSP2 rAgs, despite the fact it is the least polymorphic.

Thus our data follow the patterns observed for other *P.falciparum* antigens with a proportion of adults being consistently seronegative and boosting of antibody levels being more apparent in children and non-immunes. The

phenomenon of persistent non-responsiveness to specific malaria antigens has been reported in previous studies [27, 53, 253, 267, 270]. Riley *et al* [267] found that non-responsiveness to the sexual stage antigen Pfs230 was a stable phenotype which is fixed early in life and is apparent in children as young as 3-5 years of age. Also, around 40% of malaria-immune adults tested from The Gambia were shown to be persistently seronegative to PfMSP1 with little seasonal variation despite major differences in parasite transmission rates throughout the year [270]. This is in contrast to the findings of Fruh *et al* [115] who reported that seasonal differences were evident in the antibody levels in children and adults from a rural community in Mali, West Africa. However, this data was presented as a summary of the recognition of an age group rather than the antibody profile of an individual donor and as such, persistent non-responders may have been overlooked. Thus, in general, a stable non-responder phenotype occurs in clinically immune adults. Non-immunes (i.e. children and recently exposed adults) are more likely to have fluctuating Ab levels which are boosted during the transmission season, although persistent seropositivity has been observed in children as young as 3 years [267].

In the introduction to this chapter several possible explanations were outlined for this consistent non-responsiveness in adults who have had life-long exposure to malaria.

Antigen polymorphism may play a role as the recognition of variant epitopes is lower than for some less variant epitopes, but non-recogniton of the 19kD conserved region means it is not the whole answer.

If confirmed, genetic regulation of the immune recognition of malaria antigens would have important implications for vaccine design. Comparison of the recognition of MSP2 by mz and dz twins indicates that non-responsiveness to MSP2 is not genetically determined; mz twins can clearly have discordant antibody recognition of the same rAg. Furthermore, the level of exposure to infection does not appear to be a major factor which regulates the recognition of MSP2 by these twin pairs: all twins had essentially identical malaria exposure since, in all cases, they had lived together for all their lives. In addition, the relationship between the presence of anti-MSP2 antibodies and HLA class II genotype was investigated for 444 individuals from The Gambia. A logistic regression analysis, considering the most frquent haplotypes, showed that there was no difference in the overall distribution of haplotypes between responders

and nonresponders. Furthermore, Rzepczyk *et al* [279] analysed the responsiveness of T-cells from donors in Papua New Guinea and the Solomon Islands to peptides of MSP2. They found that no peptides were preferentially recognised in association with specific HLA class II antigens.

I would like to propose an alternative explanation for stable non-responsiveness in adults.

A primary humoral immune response to most antigens results in the development of a long-lasting state of immunity. This state of immunity influences the secondary immune response to the priming antigen and may also alter subsequent immune responses to antigens that are structurally related to this priming antigen. This phenomenon has been termed "original antigenic sin" [107]. This cross-reactive property of immunity results in the previous immune experience of an individual having a direct bearing on the immune recognition of antigens that may be encountered in the future [111a].

The phenomenon of original antigenic sin, also referred to as clonal imprinting [118a], was first observed when humans vaccinated against influenza virus were shown to produce antibodies of a higher titer against variants experienced in childhood than against the immunising antigen. It appears that the primary antibody response to the initial infecting strain becomes clonally dominant and may limit, suppress or prevent the effective triggering of other B cell clones that have the potential to respond to virus variants that are presented subsequently. Thus, epitopes that are cross-reactive between the primary and boosting strain are recognised by memory B cells and antibody of the original specificity is produced. New epitopes are effectively ignored.

There are certain criteria which need to be fulfilled in order for original antigenic sin to occur; 1) the antigens must have conserved and variable regions in order for cross-reactivity to occur, 2) there should be a number of variants in the infecting population so that subsequent infections are likely to be of differing strains, and 3) antigen should be present in limiting concentrations since competition for antigen between B cells with Ag receptors of differing affinity is a crucial factor in clonal selection [107].

One can envisage how malaria infections could induce a state of clonal imprinting resulting in persistent restricted responsiveness. Firstly, many malaria Ags have conserved and polymorphic epitopes, therefore cross-reactions would be expected between variant Ags. Secondly,

polymorphisms are extensive and so it is likely that successive infections would be with parasites of differing genotypes. Thirdly, although initially there is a high antigenic load since individuals are exposed to repeated infections of high parasitaemia, as clinical immunity sets in, individuals experience infections with low, frequently subpatent parasitaemias and consequently Ag availability becomes a limiting factor, resulting in boosting of memory to cross-reactive epitopes. Thus, the observed differential recognition of epitopes in malaria-immune individuals could be the result of clonal imprinting.

This explanation is virtually impossible to confirm in the field. One would be required to follow the antibody profiles of individuals form birth and determine the genotype of the infecting malaria parasites. However, the possibility that imprinting may occur in the perinatal period, following low doses of antigen *in utero*, cannot be ignored. In terms of vaccine design, one would be required to find an antigen that was universally immunogenic and could induce an effective immune response. The phenomenon of clonal imprinting could be utilised to redirect the immune response against epitopes which could induce protective immunity. This could be achieved more easily in young vaccinees, as less imprinting would have occurred. However, in those who have had long term exposure to malaria, target antigens would need to be presented in the absence of epitopes to which the immune system has already become imprinted.

In summary, longitudinal studies have shown that patterns of malaria epitope recognition tend to become fixed in immune donors. Our data cannot easily be explained by a theory of genetically determined non-responsiveness but do fit with a model of clonal imprinting.

12. General conclusions

The purpose of this thesis has been to investigate and evaluate the immune recognition of the *P.falciparum* merozoite surface protein MSP2 by serum antibodies from a population in West Africa exposed to stable, endemic malaria. Such epidemiological studies facilitate our understanding of naturally acquired immune responses to malaria, and this knowledge is necessary for the assessment of malaria proteins for inclusion in a vaccine against malaria.

12.1 Recombinant proteins representing *P.falciparum* MSP2

Recombinant proteins representing various regions of MSP2 have been successfully expressed in *E.coli* and these have been used to investigate the immunogenicity and antigenicity of MSP2 in mice, after immunisation with rAgs, and in humans, after natural exposure to *P.falciparum* malaria. The antisera raised in mice were used to ensure that the rAgs reflected the antigenic characteristics and integrity of the native molecule, determined by IFA of mature schizont-infected erythrocytes and immunoblotting using SDS-soluble schizont extracts (chapter 4). The antibody specificities of these antisera were investigated to evaluate the serological relationship between MSP2 serogroups A and B. Serological cross-reactivity was observed in mice, mediated through epitope(s) within the conserved C terminus. However, this does not reflect the antibody specificity in humans after natural infection. Antibodies to both serogroups of MSP2 are prevalent in adults from The Gambia (chapter 5). These antibodies are serogroup-specific and do not cross-react. In this population, the conserved regions are poorly immunogenic whereas the central group-specific and repetitive sequences are immunodominant. Thus, I have shown that MSP2 rAgs are useful tools for detecting MSP2-specific antibody in individuals naturally exposed to malaria and that they permit mapping of the antibody response to defined regions of MSP2. These rAgs were used for the further investigation of qualitative and quantitative aspects of the serological immune recognition of MSP2.

12.2 Immune recognition of MSP2:

Clinical immunity to malaria has characteristic features: 1) it develops after multiple infections and 2) it requires frequent boosting; the resulting immunity is not sterile and does not completely prevent reinfection.

Many epidemiological studies have been carried out to investigate these characteristics of the development of clinical immunity to malaria. In doing so, the immune responses to particular malaria antigens are measured and their possible contribution to immunity to malaria is assessed. Individuals who have high levels of antimalarial antibodies in their serum may still be susceptible to attacks of malaria. These sera either lack antibody specificities that are critical to resistance, the antibodies are of low affinity or they have the wrong isotype to confer protection. Thus, although a large number of malaria proteins are recognised by the host's immune system during natural exposure to infection, it is likely that the immune responses to the majority of these antigens play no role in protecting the host from clinical malaria.

In The Gambia, immune responses to MSP2 are acquired in an age-dependent manner; antibody prevalence increases with age and mirror the decline in parasite density and prevalence in this population (chapter 6). In this respect, MSP2 is similar to many other *P.falciparum* antigens that have been studied [177, 263, 292].

Several hypotheses have been proposed for the slow development of immunity to malaria, these include a) poor immunogenicity, b) genetic restriction, c) induction of inappropriate immune responses, and d) antigenic diversity. I will discuss these hypotheses with regard to the immune recognition of MSP2.

Firstly, the data indicate that MSP2 is not poorly immunogenic. Individuals known to have limited experience of malaria mount high antibody responses to the molecule and young children have MSP2-specific antibodies of comparable concentration and affinity to adults (chapter 6).

Secondly, the immune recognition of MSP2 does not appear to be genetically restricted, although a proportion of individuals in all age groups remain seronegative to particular epitopes (chapter 11).

Inappropriate immune responses may also contribute to the slow development of immunity. For instance, the presence of repeat sequences in many of the immunodominant antigens may act as a decoy mechanism

which allows the parasite to escape protective immunity, inhibiting the maturation of high affinity B cells and creating a "smokescreen" effect hiding more functionally important epitopes [7, 177]. However, the repetitive domains of MSP2 are not the only immunodominant regions; there is a high prevalence of antibodies to both repetitive and group specific sequences (chapter 5). This is in contrast to RESA and the S-antigens, for example, in which the repeated sequences are immunodominant. In MSP1, the repeat region represents a small proportion of the molecule and although these sequences are immunogenic they do not appear to be immunodominant.

Therefore, I can discount several of the hypotheses mentioned above. MSP2 is immunogenic, immune responses to MSP2 are not genetically restricted and they are not necessarily inappropriate. However, the antigenic diversity of the molecule may well contribute to the gradually acquired immune recognition of MSP2.

Many *P.falciparum* proteins are antigenically diverse. It is proposed that the chronically exposed host accumulates a repertoire of memory and effector cells capable of controlling infection by any given strain or variant of the parasite, immunity thus being essentially "strain-specific". If this were true, it would be extremely difficult to estimate how many infections would be necessary to develop immunity to the repertoire of MSP2 genotypes circulating in a community, particularly considering the extent of microheterogeneity of MSP2. It appears that although immune recognition of MSP2 is essentially strain-specific in non-immunes and semi-immunes, adolescents and adults develop "serotype-transcending" immunity to MSP2 (chapter 9, 10). In adults, this presumably reflects exposure to multiple variants leading to a wide enough range of responses to cope with new infections. The rate at which this serotype-transcending immunity develops presumably depends on transmission rates and the frequency of mixed genotype infections.

Strain-transcending immunity could arise in two ways, due to: 1) antibodies which recognise cross-reacting epitopes within the repeats, such that a few antibody clones could recognise the whole repertoire, or 2) antibodies which recognise group specific regions. The development of anti-group specific antibody may arise by continual restimulation of some B cell clones. In addition, cross-reacting antibodies may arise by chance after many infections, but these would probably be of lower affinity.

An alternative view to the accepted convention of gradually acquired immunity to malaria is that a few episodes of malaria are sufficient to induce naturally acquired immunity in adults, while the immune system of a child may be constitutionally less capable of mounting a protective response against the parasite [14-16]. However, in areas such as The Gambia, where all individuals are regularly exposed to malaria infection from infancy onwards, the effects of cumulative exposure cannot be separated from any possible effects of age itself. Differences between the immature and mature immune systems which might affect the rate of development of protective immunity may include the ability to recognise complex carbohydrate antigens, the relative prevalence of Th1 versus Th2 cells and the capacity to induce switching to certain Ig isotypes.

Studies on the immune response to natural malaria infections have so far focused almost exclusively on the specificity of a response. Another important aspect is the quality of that response. Some antibody-mediated mechanisms of parasite clearance require cooperation with accessory cells and therefore it is necessary that antibodies of an appropriate subclass are produced to allow this interaction. The predominant subclass involved in the recognition of MSP2 is IgG3, an antibody subclass with cytophilic properties and high affinity for Fc receptors on monocytes and neutrophils (chapter 8). By examining a cross-section of the population, I have shown that the prevalence and mean OD of IgG3 antibodies increases with age. MSP2-specific IgG3 is the predominant subclass in adolescents and adults, but IgG1 is more common in children under 10 years old (chapter 8). A similar trend for IgG3 antibody to increase with age was observed for the response to RESA by Beck *et al* [20], but does not occur in response to $MSP1_{19}$ where the antibody response is predominantly IgG1 in all age groups [93].

The data for MSP2 supports the hypothesis that qualitative differences in the antibody responses to malaria antigens may be important in the development of protective immunity [29]. It is tempting to propose that the immune protection in children is less efficient due to the IgG subclass distribution; although IgG1 antibodies also have cytophilic properties and could also contribute to parasite clearance, it is possible that IgG3 antibodies may be more efficient at cooperating with the accessory cells [329]. Whether this reflects a constitutional difference between children and adults or is simply the result of frequent re-exposure to antigen leading to

differential class switching is not known. Further investigation is required to elucidate the cytokines which trigger the subclass switch and induce the propensity for IgG3 antibodies to MSP2.

In summary, the diversity of MSP2 appears to contribute to the gradually acquired immune response to the antigen, whereas the rate of development of serotype-transcending immunity and the rate of induction of a predominance of MSP2-specific IgG3 antibodies may well contribute to the rate at which protective immunity to malaria develops.

Another accepted characteristic of clinical immunity to malaria is that its development and maintenance requires frequent boosting. I investigated the requirement for frequent boosting of the antibody response to MSP2 (chapter 11). Malaria transmission is seasonal in many endemic areas, including The Gambia, and it is possible that antibody levels fluctuate over time, depending on the frequency of re-exposure to infection. If variation in antibody levels is considerable, then certain people may appear to be non-responders on some occasions and responders on other occasions, particularly using samples taken during cross-sectional surveys. In order to obtain a realistic estimate of antibody prevalence, serum samples need to be collected from the same individuals over a period of months or years in longitudinal studies. I found that antibody responses vary seasonally in children whilst antibody levels in clinically immune adults remain stable (chapter 11). Therefore, it appears that in semi-immune children boosting is required, whereas in adults antibody responses remain stable irrespective of recent boosting. I also found that epitope recognition is selective, with individuals consistently recognising some epitopes whilst failing to recognise adjacent epitopes from the same antigen or epitopes from distinct antigens. These patterns of antibody recognition differed from person to person but were not genetically regulated. This selective recognition of MSP2 epitopes is a stable phenotype within a single individual and is reminiscent of the clonal imprinting or original antigenic sin observed for influenza virus [107]. It seems likely that prior exposure to one form of MSP2 primes an individual for an enhanced immune response when exposed to a closely related, antigenically cross-reactive form of MSP2 in a subsequent infection. This priming phenomenon may result in a lowered response to novel, antigenically distinct epitopes encountered at

the same time since low affinity naive B cells will compete poorly with memory B cells for antigen.

The pattern in children was not unexpected; there was clear evidence of seasonal variation in antibody titres with antibody levels rising at the end of the malaria transmission season. Specific antibody boosting is apparent in the children who attended the MRC outpatients clinic with acute, uncomplicated malaria (chapter 10). The antibody profile of these children can be clearly related to the antigenic profile of the parasites with which an individual was infected. However, even in children it was evident that responses to some epitopes were being boosted more readily than for other epitopes. For example, the antigenically conserved epitope $MSP1_{19}$ would have been present during every infection but the response to it was boosted in some children but not in others.

The phenomenon of selective immune recognition has been observed for other malaria antigens: MSP1 [115, 270],RESA [26, 27, 240] and Pfs230 [267].Children lose their MSP1-specific antibodies rapidly after cessation of infection, while the adult humoral response remains high throughout the year [115]. Selective recognition of MSP1 epitopes has also been observed, with a proportion of individuals remaining persistently seronegative [270]. The level of recognition of RESA varies among the population [27, 240], and this is not readily explained by differences in exposure to malaria [253]. The authors conclude that the most probable explanation for the existence of a group of adults with low RESA response is an inherent difference in the antibody response to the antigen. The genetic regulation of the RESA antibody responses is thought to reflect the impact of factors encoded by genes outwith the HLA class II region [293, 327]. The fact that the prevalence of antibodies to RESA differs among different ethnic groups in The Gambia supports this conclusion [265]. However, there is no real evidence for genetic regulation of the response to RESA .

In Madagascar, where there was an epidemic outbreak of *P.falciparum* after 20 years without malaria transmission, clear seasonal variation in the titre of anti-RESA antibodies in children and adults was observed [53]. This supports the idea that antibody levels stabilise in malaria-immune individuals but fluctuate according to recent exposure in non-immunes or semi-immune individuals. The age at which the antibody levels stabilise would probably depend on the intensity of malaria transmission and on the

diversity of the antigen. Seropositivity to both RESA and Pf230, essentially conserved antigens, stabilise in children aged 3-5 years [26, 267], whereas the responses to MSP1 and MSP2, variable antigens, seem not to stabilise until later in life.
Hence, recognition of MSP2 does not appear to be genetically determined, but that antibody repertoires tend to become fixed in clinically immune adults as a result of clonal imprinting.

12.3 MSP2 as a vaccine candidate:

The evaluation of potential vaccine antigens requires the understanding of several factors. Information about the structural characteristics of the antigen is essential in order to select the most appropriate expression system to produce a recombinant protein which is antigenically equivalent to its native counterpart. Studies are required to elucidate the immunogenicity of the antigen during natural infection, the effect of host genetics on the ability of an individual to mount an immune response, the stability of the immune response and, importantly, the correlation between the development of the immune response and clinical markers of resistance. In addition, the effect of antigenic diversity on all of the above must be assessed.

Many of the asexual stage antigens described in section 1.4 have been structurally defined and expressed as synthetic polypeptides. The importance of conformation to protein structure, and consequently to protein antigenicity, is greatest for proteins in which secondary structure is stabilised by multiple intrachain disulphide bonds [49]. Despite the presence of multiple cysteine residues, MSP1 has been successfully expressed in eukaryotic and prokaryotic systems [38, 228, 266] and AMA-1 has been expressed using the baculovirus system [65, 323]; in both cases, immunogenicity is dependent on the correct conformation of disulphide bonds [38, 65]. The structure of MSP2 is not dependent on the formation of disulphide bonds, nevertheless, it is still necessary to ensure the structural integrity of recombinant proteins (chapter 4). Stable, soluble polypeptides of MSP2 (expressed as fusions with GST), have been produced which reflect the antigenic characteristics of native MSP2. One exception is a short rAg representing the conserved N-terminus; after immunisation, mice produced antibody of low titre against the immunogen and these antibodies

did not recognise the native protein in IFA or immunoblotting. Thus, the N-terminus appears to be poorly immunogenic. Contradictory results have been obtained in other studies. In agreement with our results, Rzepczyk *et al* [277] found that immunisation of mice with an N terminal peptide produced essentially no antibody response. In contrast, others found that peptides from the N terminus are capable of raising appropriate antibodies in mice, even in the absence of a carrier protein [167, 168, 284]. Thus, it appears that although murine B cell epitopes are present in the N terminal sequence, our construct was not recognised, even though T cell help can be provided by GST and both inbred and outbred strains of mice were used (chapter 4).

Previous studies have identified epitopes within the dimorphic and polymorphic regions of MSP2 as targets of invasion inhibitory mAb [55, 101, 109, 220, 255, 310]. These mAbs also recognised the rAgs (chapter 4). Naturally acquired antibodies to MSP2 are directed against dimorphic and polymorphic domains rather than the conserved domains. The reasons for this are not clear. Conserved epitopes of other malaria antigens are immunogenic during natural infection. For example, RESA is antigenically conserved among different strains [235] but is recognised at high frequency with repeat sequences being immunodominant [235]. Like MSP2, MSP1 contains conserved and variable regions, but antibodies are directed against epitopes mapping to polymorphic, dimorphic and conserved regions of the molecule [93, 115, 228, 266, 270, 324]. However, conserved epitopes located very close to the C terminus of MSP1 are recognised less frequently than upstream dimorphic epitopes [93, 266] suggesting that there maybe a physical or structural reason for poor immunogenicity, in that epitopes in membrane associated regions of the protein are inaccessible. Epitopes in the N terminal region of the native MSP2 molecule are perhaps also inaccessible for immune recognition and antibody binding.

Another possibility is that during the evolution of the parasite, some malaria antigens/epitopes have been selected for because they contain few T and B cell epitopes [124].

Antibody recognition of MSP2 in immune adults is stable and persists even when malaria transmission is minimal. Distinct patterns of recognition occur, but this does not reflect differences in exposure or genetic regulation (chapter 11). Stable antibody response in adults is encouraging for vaccine development as it suggests that there is good immunological memory. In

addition, the antibody response is rapidly boosted by reinfection in children, therefore vaccination in early childhood should induce a level of immunity equivalent to that occurring naturally in adults and may be enough to provide life time protection against malaria.

It is important to evaluate whether acquired immunity to a particular antigen contributes to immunity to malaria. The analysis of the association of immune recognition with resistance to clinical malaria is complex. Overinterpretation of the data must be avoided, but statistically significant associations obviously warrant further investigation. The interpretation of epidemiological studies is complicated by difficulties in defining and measuring morbidity due to malaria [266], and the assessment of the immune response is confounded by such factors as polymorphism of the host and parasite populations.
As parasite frequency and density, and severity of clinical symptoms are age-dependent, many seroepidemiological studies concentrate on the dynamics of the acquisition of antibodies with age. The acquisition of antibodies to MSP2 parallels the decline in parasitaemia and parasite density (chapter 6), suggesting a role for MSP2-specific antibodies in parasite clearance; however, the data do not indicate a direct, causative relationship. Most epidemiological studies show that antibodies to particular malaria antigens increase in childhood, but it is unlikely that all of these are involved in protective immunity. An increase in antibody with age may be associated with protection or may just be a measure of exposure. To separate these two effects, longitudinal studies of individuals, their immune responses and clinical status are required. Therefore, the immune recognition of MSP2 was correlated directly with the clinical status of a cohort of semi-immune children in a longitudinal, prospective study (chapter 7). Antibodies to epitopes in the 5´ repeat/group specific region of serogroup B (B3) appear to confer resistance to clinical malaria. However, no association was found for the other antigens tested (A2, A3 and B2). There are three possible explanations for this finding: 1) only antibodies to B3 are in fact able to mediate MSP2-associated protection; 2) antibody to the other proteins are protective, but the extensive sequence and antigenic diversity in these regions precludes the demonstration of the protective effect of any one representative sequence; 3) antibody responses to different regions of MSP2 (and possibly other merozoite surface proteins)

are closely associated, so preventing the demonstration of a protective effect of any one antigen or epitope, i.e. MSP2 may be necessary but not sufficient for a protective antibody response.
Antibodies to MSP1 [93, 115, 265], AMA-1[323] and RESA [341] are also acquired in an age-dependent manner. Antibodies to certain regions of MSP1 are associated with reduced malaria morbidity [228, 266]. Contradictory results have been reported for antibodies to RESA; increasing antibody titres are associated with decreasing parasitaemia and resistance to high parasitaemia [3, 240, 265, 341] however, antibodies to RESA epitopes have also been associated with clinical attacks of malaria [265], but this may well reflect antibody boosting by concurrent infection. In contrast, antibody responses to some antigens (such as the CS protein) have been shown repeatedly to have no association with protective immunity [197, 264]. Epidemiological studies, although not perfect, do however differentiate non protective antigens from possibly protective ones. More conclusive evidence for protection relies on clinical studies.

It can be argued that if antigenic polymorphism is indeed an important immune evasion mechanism for the parasite, it should correlate with the degree of immune pressure imposed on the parasite antigens by naturally acquired anti-parasite immunity [215]. This is true, to the extent that the highest degree of antigenic polymorphism has been shown in stages of the parasite, including the asexual blood stages, against which potent immunity is eventually mounted, particularly antigens on the surface membrane of extracellular forms of the parasite exposed to the vertebrate immune system. However, there are also some relatively invariant antigens/epitopes that are thought to be the targets of anti-parasite immunity. Many of the parasite molecules that are critical for biological function are probably conserved. Any change in the antigenicity of critical molecules to avoid immune attack would need to preserve this function. Ironically, the deployment of antimalarial vaccines itself may provide the most direct evidence as to whether polymorphism is an important immune evasion mechanism in malaria [215].
Polymorphism of malaria antigens among natural parasite populations is extensive and has been described for both B and T cell epitopes of many antigens of all stages of the parasite. The extent to which this diversity could affect the efficiency of a vaccine would depend on the degree of

variability (in natural isolates) of the targets of immunity and the extent to which mutations in the target genes are compatible with parasite viability. Malaria antigens can be divided into three groups with regard to their antigenic polymorphism:

1. those that are essentially conserved, perhaps exhibiting minor polymorphisms involving point mutations;
2. those that exhibit allelic dimorphism, with regions (which are usually repetitive) of truly polymorphic sequence;
3. those where the polymorphism is extensive, and where only limited C and N terminal sequence conservation indicates that these genes are in fact alleles at a single locus, e.g. S antigens and probably also PfEMP1.

Any of these antigens may also contain regions of tandemly repeated sequences.

Each group has advantages and disadvantages for vaccine development. Those antigens which are essentially conserved make attractive targets because immune responses induced against such antigens would be effective against all isolates. However, it may be that minor amino acid changes could significantly alter the antigenicity whilst maintaining the biological function of the molecule. Therefore, the trick is to identify functionally important molecules.

Antigens that exhibit allelic dimorphism have the advantage that the dimorphic sequences are conserved within each allelic family. The fact that these genes can be grouped into allelic families indicates that some selective constraints apply [10]. The different allelic forms of these antigens would not become fixed in the parasite population and be widely dispersed unless they provide a biologic advantage for the parasite. However, these dimorphic sequences often flank repetitive sequences which could divert the immune response from critical epitopes through immunodominance or induce T-independent responses by cross-linking surface Ig on B cells. It may be that immunisation with epitopes from the dimorphic domains, delivered in the absence of repetitive sequences, could enable the rapid development of protective immunity [179].

Antigens that are extremely polymorphic would be less useful as targets of vaccine induced immunity, as the immune responses would only be effective against a limited repertoire of parasites. On the other hand, a vaccine may be able to selectively exploit any sequence conservation at the N and C termini.

Among current contenders, EBA-175, the rhoptry protein complex antigen RAP-1 and RESA are non-polymorphic [263, 292]. AMA-1 exhibits limited polymorphisms (diversity is generated by non-conservative point mutations) [8] and MSP1 consists of 17 blocks of conserved, dimorphic or polymorphic sequences [315] with additional polymorphism is created by intragenic recombination. However, it appears that the conserved 19kDa fragment may be an important target of MSP1-specific antibodies [28, 38, 93]. Of these, several clinical and preclinical trials with RESA have been disappointing and this antigen is now generally considered as unsuitable for a malaria vaccine. Evaluation of the other antigens is continuing, with particularly promising recent results for AMA-1 and $PfMSP1_{19}$.

The extent of diversity in MSP2 is considerable; although the molecule can be classified into two basic prototypes, the tandemly repeated sequences can vary extensively in number and sequence. Like MSP1, within an allelic family point mutations, insertions, deletions and intragenic recombination increase variability. The effect of antigenic diversity of MSP2 on immune recognition remains to be fully elucidated, but the data presented in this thesis indicates that polymorphism plays a major role in the immune recognition of the molecule. Preliminary studies indicate that the antibody profile of semi-immune children is clearly related to the specific serotype of the parasite with which that child was infected (chapter 10). Nonetheless, antibodies to epitopes in the dimorphic regions are prevalent and, if these were associated with protection (as may be the case for serogroup B), it may only be necessary to include sequences from the two major serogroups in a vaccine. Success may come from artificially raising antibodies to the conserved or semi-conserved sequences to which the natural immune response is rare or suppressed, thus circumventing the problem of antigenic diversity. Studies in mice have shown the conserved C-terminus of MSP2 to be immunogenic (chapter 4) and to induce protective antibodies [284]. The fact that the natural immune response to these regions is poor and the sequence is highly conserved, indicates that these domains may be essential for parasite survival.

12.4 Future directions:

To conclude, I think that MSP2 is an antigen worthy of further investigation to fully clarify its potential as a vaccine candidate.

I have proposed that antibodies to MSP2 may be important in parasite clearance and the subclass of these antibodies may be critical in conferring protection. Protective antibodies may have a limited direct effect upon parasite growth and invasion, but rather act in cooperation with accessory cells. Therefore, it would be of interest to investigate the ability of MSP2-specific antibodies to exert an antibody-dependent inhibitory effect on parasite growth in cooperation with normal blood monocytes.

In addition I have shown that antibody levels in children are boosted by current infection and the specificity of these antibodies can be clearly related to the antigenic profile of the infecting parasites. The effect of these antibodies on subsequent infections needs to be assessed in longitudinal epidemiological studies. The hypothesis would be that pre-existing antibody to a particular MSP2 serotype would prevent further infection with that serotype, but would not protect against infection with an antigenically distinct serotype.

Direct immunisation studies are also required in animal models and humans. In practical terms it is necessary to determine the factors which influence the switch to production of IgG3 antibodies and the immunisation methods required to mimic this. Of course vaccination against MSP2 may encourage the emergence of mutant forms of the parasite. We have already seen that the parasite is viable without the R2 repeat region of serogroup B (chapter 10). Thus, identification of regions of the molecule that are functionally important and on which parasite survival is dependent , is a strategy by which we may prevent the evolution of such variants.

To vaccinate against malaria is to interfere in a host-parasite system of evolutionary success. Measures that sustain a selection pressure on the parasite may be countered by the parasite through the evolution of new variants. The use of a multi-stage vaccine may prevent this, or at least slow it down, and inclusion of a transmission-blocking component would stop the transmission and spread of these mutants.

Whatever the vaccine of choice, the interest of science and society should not take precedence over the consideration of the individual's well-being. It is possible that while a malaria vaccine trial may reduce transmission and benefit the community as a whole, the vaccinated individual may, as artificial immunity wanes, become more susceptible to severe malaria than he was before being vaccinated. Furthermore, short-lived immunity could

result in a shift of the age related pattern of malaria-related disease and mortality. An unsuccessful vaccination campaign could convert an area of stable endemic malaria to one which is subject to unstable epidemic malaria. Thus, vaccine trials have to be considered in an environmental and sociological setting, where controlling malaria may eliminate some problems and introduce new ones. For example, significant reduction in childhood mortality may worsen the effects of overpopulation in some communities.

Whilst awaiting the results of trials of the synthetic malaria vaccine SPf66 in The Gambia and Thailand, the results from Tanzania are encouraging, showing at the very least that vaccination against malaria is a reality [5]. Of course modifications and improvements will be needed. By normal standards, a vaccine that reduces cases by only one third (as obtained for SPf66) would not be acceptable. Vaccines against polio or measles, for example, would be expected to reduce cases by 90%. But malaria is perhaps a special case because even natural immunity is only partial, years of research have failed to produce a vaccine and the world situation worsens. We need to ask: is the need to tackle the disease so great that a suboptimal vaccine (such as SPf66) should be given to all those that want it in the hope that millions of cases of malaria might be averted? Or should scientists regard SPf66 as the first step, the proof of a principle that should now be refined and therefore redouble their efforts to find a more effective vaccine? [36]. There are ethical arguments for and against the use of a suboptimal vaccine. Arguments for are that infections (and therefore presumably deaths) would be prevented. Arguments against are the false feeling of security engendered by vaccination and the possible decrease in alternate malaria prevention methods, the use of limited Third World resources for an ineffective vaccine and the difficulty of subsequently introducing a (possibly) more effective alternative.
SPf66 does have its limitations. It currently cannot be recommended for use before a child's first birthday, since there is no evidence that it is safe or efficacious in this age group [316]. An age restriction would seriously limit vaccine effectiveness in areas of extremely high transmission where a large proportion of the malaria morbidity is in infants. Furthermore, it is not known how SPf66 mediates protection, and there is no evidence that the vaccine protects against death due to malaria. The duration of SPf66-

mediated protection under different levels of exposure is not known, nor is it known to what extent natural boosting occurs [316]. There is a danger that public opinion, in assuming that there is now an effective vaccine against malaria, will turn against investment of scientific resources in further malaria vaccine research. It is vital therefore that researchers in the field stress that SPf66 - whilst a vindication of previous attempts to make a vaccine - is only the beginning of the process.

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Appendix 1

1A: Schematic representation of MSP2 recombinant proteins - see inside back cover.

1B: Schematic representation of putative epitopes of MSP2-specific monoclonal antibodies - see inside back cover.

Appendix 2

Antibody*	Working dilution	Isotype	Epitope and location
Anti-MSP1			
9.8-4-4-1	1000	IgG1	conserved
12.2-1-1	200	IgG1	block 2 (3D7-type)
123D3.10	1000	IgG2b	block 2 (CAMP-type)
CE2.1§	50	IgG1	block 2 (CAMP-type)
31.1-8†	neat	IgG1	block 2 (RO33-type)
13.2-3	2000	IgG1	block 3 (K1-type)
9.5-1-5-1	500	IgG2b	block 3 (MAD-20-type)
10-2B	1000	IgG2a	block 4 (K1-type)
12.1-5-4	2000	IgG1	block 4 (MAD-20-type)
127B11.1[a]	300	IgG1	block 6-16 (MAD-20-type)
9.2-6-2[a]	1000	IgG1	
9.7-1[a]	500	IgG1	
10.3-2[a]	500	IgG1	
127F1.1[a]	100	IgG1	
1-1C[b]	500	IgG1	block 6-16 (K1-type)
7.3-7[b]	500	IgG2a	
7.6-2[b]	1000	IgG1	
6.1-1-3[b]	1000	IgG1	
13.1-2[b]	10^4	IgG1	
17.1-3[b]	1000	IgG1	
111.4	1000	IgG1	block 16-17 (K1-type)
Anti-MSP2			
13.4-2-1	500	IgG1	T9/96 specific -R1 repeat GSAG
4-4F[d]	200	IgM	FVO specific - R1 repeat
8-5D[d]	200	IgM	
12.3-1-2-4[e]	500	IgG1	Serogroup A specific - dimorphic
12.5-1-2[e]	500	IgG1	
12.7-1-2-4	500	IgG1	
8G10/48	1000	IgG2b	Serogroup B - STNS
8F6/49	50	IgG3	Serogroup B - DTPTATE
anti-7G8	300		Serogroup A -R1 repeats
anti-ThaiTn	300	mouse sera	Serogroup A -R1 repeats
anti-T9/96	400		Serogroup A -R1 repeats
Anti-Exp1			
5.1-4	500	IgG1	

Characteristics of monoclonal antibodies used for parasite typing.
mAb epitopes marked by idenctical symbols (a,b,c,d,e) exhibit identical allelic distributions.
Mouse sera were pooled from bleeds 2 & 3 after the second immunisation.
*Details of mAbs are given in Conway *et al* [66] and references therein.
§ mAb produced by Locher, Hawaii; † mAb produced by McBride, Edinburgh.
Access to mAb and serum stocks was kindly provided by Dr J McBride.

Appendix 3

Donor	Sample date	MSP1 rAgs EGF-1A	EGF-1B	19/GST	A2	MSP2 rAgs A3	B1	B3
Mean + 2SD		0.249	0.229	0.196	0.290	0.139	0.111	0.146
12 TS	11/90	0.090	-0.054	-0.031	0.020	0.063	-0.056	-0.058
	11/91	-0.350	-0.352	-0.250	0.305	0.538	1.113	0.573
	4/92	-0.187	-0.199	-0.213	0.171	0.520	0.238	0.188
	11/92	-0.242	-0.173	-0.279	-0.060	0.526	0.435	0.689
	4/93	-0.168	-0.027	-0.121	0.061	0.487	1.048	0.842
	11/93	-0.088	-0.113	0.125	0.125	0.399	0.996	0.952
13 AJ	11/90							
	11/91	0.264	0.042	0.786	-0.004	0.582	1.609	1.020
	4/92	0.067	0.125	0.761	-0.022	0.624	1.573	0.866
	11/92	-0.003	0.197	0.861	0.033	0.460	1.156	0.470
	4/93	0.051	0.244	0.824	0.051	0.421	1.309	0.639
	11/93	-0.110	-0.113	0.701	-0.084	0.326	1.073	0.706
127 JK	11/90							
	11/91	0.866	0.999	0.378	0.425	0.675	1.133	0.221
	4/92	0.940	1.474	0.701	0.189	0.459	0.267	0.021
	11/92	0.819	0.834	0.498	0.061	0.475	0.332	-0.035
	4/93							
	11/93	0.816	0.836	0.417	0.304	0.430	0.498	0.034
109 FWS	11/90	0.321	0.145	0.179	0.222	0.081	0.051	0.129
	11/91	0.036	0.189	0.469	0.114	0.507	0.094	0.057
	4/92	0.120	0.011	0.095	0.024	0.021	-0.004	0.064
	11/92	0.047	0.057	0.442	0.122	0.329	0.090	0.076
	4/93							
	11/93	0.153	0.033	0.456	0.109	0.209	0.062	0.095
31 S	11/90	0.316	0.101	0.264	0.233	0.385	0.312	0.046
	11/91	0.093	0.077	0.411	0.168	0.552	0.580	0.038
	4/92	-0.088	-0.112	0.112	-0.045	0.346	0.406	-0.025
	11/92	-0.062	-0.040	0.218	0.033	0.363	0.706	0.011
	4/93	0.224	0.485	0.613	0.372	0.825	0.598	0.000
	11/93	0.350	0.319	0.596	1.155	0.621	0.552	-0.044
104 FK	11/90	0.153	0.143	0.199	0.984	0.148	0.379	0.240
	11/91	0.110	-0.034	0.008	0.250	0.054	0.370	-0.067
	4/92	0.152	0.132	0.090	0.209	0.214	0.933	0.105
	11/92	0.676	0.727	0.476	0.872	0.457	0.614	0.095
	4/93							
	11/93	0.453	0.285	0.322	0.036	0.772	0.498	0.210
53 MC	11/90	0.020	-0.034	-0.026	0.594	0.433	0.001	0.107

Appendix 3

Donor	Sample date	MSP1 rAgs EGF-1A	EGF-1B	19/GST	MSP2 rAgs A2	A3	B1	B3
Mean + 2SD		0.249	0.229	0.196	0.290	0.139	0.111	0.146
12 TS	11/90	0.090	-0.054	-0.031	0.020	0.063	-0.056	-0.058
	11/91	-0.350	-0.352	-0.250	0.305	0.538	1.113	0.573
	4/92	-0.187	-0.199	-0.213	0.171	0.520	0.238	0.188
	11/92	-0.242	-0.173	-0.279	-0.060	0.526	0.435	0.689
	4/93	-0.168	-0.027	-0.121	0.061	0.487	1.048	0.842
	11/93	-0.088	-0.113	0.125	0.125	0.399	0.996	0.952
13 AJ	11/90							
	11/91	0.264	0.042	0.786	-0.004	0.582	1.609	1.020
	4/92	0.067	0.125	0.761	-0.022	0.624	1.573	0.866
	11/92	-0.003	0.197	0.861	0.033	0.460	1.156	0.470
	4/93	0.051	0.244	0.824	0.051	0.421	1.309	0.639
	11/93	-0.110	-0.113	0.701	-0.084	0.326	1.073	0.706
127 JK	11/90							
	11/91	0.866	0.999	0.378	0.425	0.675	1.133	0.221
	4/92	0.940	1.474	0.701	0.189	0.459	0.267	0.021
	11/92	0.819	0.834	0.498	0.061	0.475	0.332	-0.035
	4/93							
	11/93	0.816	0.836	0.417	0.304	0.430	0.498	0.034
109 FWS	11/90	0.321	0.145	0.179	0.222	0.081	0.051	0.129
	11/91	0.036	0.189	0.469	0.114	0.507	0.094	0.057
	4/92	0.120	0.011	0.095	0.024	0.021	-0.004	0.064
	11/92	0.047	0.057	0.442	0.122	0.329	0.090	0.076
	4/93							
	11/93	0.153	0.033	0.456	0.109	0.209	0.062	0.095
31 S	11/90	0.316	0.101	0.264	0.233	0.385	0.312	0.046
	11/91	0.093	0.077	0.411	0.168	0.552	0.580	0.038
	4/92	-0.088	-0.112	0.112	-0.045	0.346	0.406	-0.025
	11/92	-0.062	-0.040	0.218	0.033	0.363	0.706	0.011
	4/93	0.224	0.485	0.613	0.372	0.825	0.598	0.000
	11/93	0.350	0.319	0.596	1.155	0.621	0.552	-0.044
104 FK	11/90	0.153	0.143	0.199	0.984	0.148	0.379	0.240
	11/91	0.110	-0.034	0.008	0.250	0.054	0.370	-0.067
	4/92	0.152	0.132	0.090	0.209	0.214	0.933	0.105
	11/92	0.676	0.727	0.476	0.872	0.457	0.614	0.095
	4/93							
	11/93	0.453	0.285	0.322	0.036	0.772	0.498	0.210
53 MC	11/90	0.020	-0.034	-0.026	0.594	0.433	0.001	0.107

	11/91							
	4/92	0.039	-0.030	0.000	0.493	0.434	0.027	-0.007
	11/92	-0.020	-0.076	-0.100	0.245	0.210	-0.093	-0.040
	4/93	0.004	-0.065	0.080	0.217	0.120	-0.090	-0.012
	11/93	0.143	-0.015	0.039	0.908	0.431	-0.012	0.005
15 TC	11/90							
	11/91							
	4/92	0.139	0.229	0.426	0.449	0.121	0.073	0.487
	11/92	0.175	0.199	0.760	0.240	0.218	0.144	0.818
	4/93	0.057	0.136	0.479	0.188	0.169	0.048	0.515
	11/93	0.429	0.425	0.777	0.486	0.146	0.130	0.550
7 SC	11/90	-0.062	-0.083	0.004	0.177	0.245	-0.034	-0.021
	11/91	0.091	0.077	0.010	0.152	0.734	0.158	0.129
	4/92	0.051	0.045	0.129	0.185	0.546	0.168	0.077
	11/92	0.035	0.118	-0.013	0.130	0.541	-0.014	0.078
	4/93	-0.181	-0.181	-0.223	-0.080	0.244	-0.119	-0.032
	11/93	0.107	0.003	0.101	0.045	0.535	0.089	0.112
79 AJ	11/90							
	11/91	0.740	0.625	0.971	1.355	1.286	1.437	1.259
	4/92	0.810	0.806	1.035	1.071	1.170	0.890	1.340
	11/92	0.909	0.886	0.971	0.800	1.212	1.129	1.654
	4/93	0.589	0.536	0.919	0.803	0.792	0.873	1.281
	11/93	0.253	0.296	0.757	0.418	0.453	0.565	1.194
45 JK	11/90							
	11/91	0.171	0.017	0.159	1.042	0.912	0.490	1.114
	4/92	-0.032	-0.008	0.100	0.791	0.775	0.521	1.127
	11/92	0.103	0.132	0.311	0.677	0.635	0.451	0.955
	4/93	0.089	0.009	0.072	0.821	0.734	0.387	0.880
	11/93	0.040	0.036	0.037	0.640	0.737	0.826	1.300
167 WK	11/90							
	11/91	0.070	-0.025	-0.047	0.252	0.021	1.170	0.016
	4/92	-0.203	-0.187	-0.198	-0.157	-0.148	0.033	0.015
	11/92	0.013	-0.034	-0.009	0.131	-0.011	0.235	-0.020
	4/93	0.021	0.062	0.047	0.195	-0.004	0.545	0.021
	11/93	-0.217	-0.199	-0.198	0.470	-0.104	0.789	-0.096
35 BS	11/90	0.658	0.769	0.452	0.545	0.278	0.501	-0.026
	11/91	0.369	0.254	0.279	0.674	0.258	0.427	0.016
	4/92	0.125	0.142	0.328	0.391	0.056	0.215	0.008
	11/92	-0.157	-0.081	-0.135	0.121	-0.167	0.196	-0.232
	4/93	-0.017	0.057	0.033	0.297	0.120	1.057	-0.134
	11/93	0.354	0.460	0.409	0.473	0.202	0.366	-0.024
114 MSS	11/90							
	11/91	0.722	0.743	0.407	1.072	0.660	0.087	0.435

	11/92	0.044	0.004	0.059	-0.038	0.429	0.912	0.674
	4/93	-0.108	-0.123	0.277	-0.104	0.269	1.375	0.541
	11/93	-0.125	-0.148	0.300	0.394	0.893	1.143	0.053
34 BS	11/90							
	11/91	-0.064	-0.150	0.109	0.858	0.805	1.235	0.264
	4/92	-0.135	-0.048	0.023	0.756	0.409	0.990	0.082
	11/92	-0.136	-0.148	0.026	0.910	0.344	1.201	0.254
	4/93							
	11/93							
4 FB	11/90	0.331	0.353	0.268	0.079	0.005	0.240	0.202
	11/91	0.700	0.633	0.692	0.032	-0.009	0.973	0.178
	4/92	0.391	0.425	0.482	0.064	-0.036	0.339	0.064
	11/92	0.370	0.584	0.447	0.134	0.301	0.549	0.122
	4/93	0.189	0.261	0.304	0.027	-0.045	0.238	0.214
	11/93	0.363	0.373	0.388	0.049	0.169	0.263	0.453
117 KB	11/90	0.047	0.057	-0.009	0.066	0.500	0.022	0.180
	11/91	-0.065	-0.037	-0.056	-0.040	0.953	0.489	0.646
	4/92	-0.061	-0.004	0.000	0.266	0.656	0.253	0.123
	11/92	0.103	0.298	0.216	0.374	1.167	0.924	0.668
	4/93	-0.052	0.020	0.042	1.075	0.797	0.141	0.375
	11/93	0.049	0.138	0.106	1.030	1.097	1.521	0.198
1 MS	11/90							
v. high	11/91	-0.529	-0.723	-0.659	-0.246	-0.185	-0.200	-0.325
GST								
	4/92	-0.881	-0.875	-0.778	-0.202	-0.403	-0.408	-0.616
	11/92	-0.850	-0.853	-0.783	-0.154	0.080	-0.610	-0.732
	4/93	-0.829	-0.901	-0.812	-0.509	-0.279	-0.733	-0.747
	11/93	-0.743	-0.684	-0.623	-0.170	0.437	-0.192	0.288
110 BS	11/90	0.121	0.218	0.482	-0.140	0.990	0.786	0.321
	11/91	0.072	0.438	0.501	-0.159	1.025	1.261	0.286
	4/92	-0.012	0.406	0.504	0.105	0.793	0.665	0.195
	11/92							
	4/93	0.111	0.360	0.500	-0.007	0.677	0.701	1.043
	11/93	-0.017	0.338	0.442	0.180	0.824	0.848	0.630
105 NK	11/90	0.234	0.440	0.240	0.487	0.191	1.267	0.825
	11/91	-0.017	0.089	0.313	1.553	1.031	1.131	0.005
	4/92	-0.058	-0.044	-0.039	0.144	0.023	0.841	0.073
	11/92							
	4/93	-0.104	-0.096	-0.139	0.158	-0.045	0.896	0.223
	11/93	-0.468	-0.410	-0.409	0.145	0.001	0.549	0.440
50 0S	11/90	0.140	-0.053	-0.067	1.122	1.170	0.590	0.139
	11/91	-0.268	-0.277	-0.294	0.660	0.447	0.391	0.121
	4/92	-0.154	-0.157	-0.110	1.004	0.957	0.508	0.351
	11/92							

4/93	-0.208	-0.196	-0.210	1.184	1.173	0.549	-0.017
11/93	-0.261	-0.267	-0.256	0.936	1.082	0.463	0.101

Table 1: Longitudinal recognition of merozoite surface proteins by malaria exposed adults from The Gambia. Samples were collected at 6 month intervals from November 1990 to November 1991.

Donor	Sample date	MSP1 rAgs			MSP2 rAgs			
		EGF-1A	EGF-1B	19/GST	A2	A3	B2	B3
mean + 2SD		0.186	0.264	0.200	0.138	0.245	0.194	0.126
E07008	6/88	0.198	0.114	0.076	0.162	0.167	-0.027	0.172
	11/88							
	6/89	0.187	0.013	0.117	0.110	-0.069	-0.039	-0.079
	11/89	0.856	1.045	0.657	-0.051	1.739	0.168	0.175
	6/90	-0.089	0.162	-0.104	-0.167	0.789	-0.200	-0.003
	11/90	0.023	-0.023	-0.054	-0.195	0.106	-0.105	-0.130
	6/91	-0.235	-0.181	-0.182	-0.172	-0.013	-0.121	-0.153
	11/91							
E08010	6/88	0.359	0.603	0.173	-0.322	0.199	-0.473	0.671
	11/88	-0.152	-0.133	0.619	0.513	1.664	1.567	0.517
	6/89	1.389	1.352	0.937	-0.027	0.101	-0.055	1.197
	11/89							
	6/90	0.163	0.336	0.174	-0.176	-0.011	-0.154	0.779
	11/90	0.085	0.210	0.230	-0.229	-0.098	-0.177	0.488
	6/91	0.004	0.078	0.001	-0.061	-0.066	-0.202	0.097
	11/91	0.232	0.242	0.104	-0.043	0.000	-0.025	0.250
E09009	6/88	-0.229	-0.171	-0.108	-0.165	-0.236	-0.218	-0.105
	11/88							
	6/89	-0.059	-0.141	-0.055	-0.035	-0.015	0.051	0.129
	11/89							
	6/90							
	11/90	-0.081	-0.143	-0.168	-0.160	-0.170	-0.177	-0.126
	6/91	-0.282	-0.267	-0.250	-0.261	-0.289	-0.215	-0.185
	11/91	-0.117	-0.125	-0.086	-0.059	-0.107	-0.075	-0.101
E17004	6/88	0.020	-0.139	-0.159	-0.175	-0.158	-0.148	-0.177
	11/88							
	6/89	-0.283	-0.241	-0.261	-0.123	0.014	-0.023	-0.092
	11/89	-0.188	-0.254	-0.244	-0.025	0.192	-0.040	-0.016
	6/90	-0.147	-0.166	-0.150	-0.023	0.145	-0.119	0.064
	11/90	-0.170	-0.143	-0.130	-0.075	-0.103	-0.161	0.089
	6/91	-0.104	-0.035	-0.111	-0.066	-0.060	-0.064	-0.062
	11/91	-0.132	-0.288	-0.271	-0.185	-0.255	-0.301	-0.181
E18003	6/88	-0.073	-0.127	-0.155	-0.222	-0.185	-0.239	-0.067
	11/88	0.957	0.906	0.794	-0.081	1.317	-0.118	0.259
	6/89							
	11/89							
	6/90	-0.238	-0.220	-0.202	-0.200	-0.209	-0.184	-0.198
	11/90	-0.197	-0.131	-0.036	-0.061	0.004	-0.068	0.112
	6/91	0.025	0.030	-0.031	0.211	-0.034	0.041	0.069
	11/91	0.672	0.701	0.446	0.074	0.214	-0.075	0.316
E20015	6/88	0.007	0.233	0.201	-0.011	-0.041	0.111	0.176

	11/88							
	6/89	-0.061	-0.106	0.016	-0.062	-0.077	0.186	0.101
	11/89	0.196	0.229	0.349	0.163	0.340	1.633	1.705
	6/90							
	11/90	-0.077	-0.028	-0.099	0.963	0.160	1.181	1.154
	6/91							
	11/91	-0.085	-0.061	-0.179	1.151	0.029	0.110	1.686
E24008	6/88	-0.035	-0.205	-0.239	0.111	-0.080	-0.191	-0.233
	11/88	0.112	0.062	0.190	0.477	0.428	0.262	0.562
	6/89	0.087	0.293	0.214	0.280	0.208	-0.013	0.910
	11/89							
	6/90	-0.176	-0.173	-0.204	-0.079	-0.058	-0.089	-0.054
	11/90	0.000	-0.073	-0.042	0.055	0.062	0.092	0.260
	6/91	-0.174	-0.239	-0.150	-0.109	-0.125	-0.091	-0.113
	11/91	-0.196	-0.181	-0.327	-0.246	-0.198	0.027	-0.189
E24009	6/88	0.022	0.006	0.022	0.222	0.096	0.120	0.064
	11/88	0.021	0.006	0.026	0.005	0.115	0.156	0.107
	6/89	-0.125	-0.113	-0.135	-0.026	-0.094	0.203	-0.028
	11/89							
	6/90	0.004	-0.131	-0.089	0.125	0.000	0.179	-0.028
	11/90	0.002	-0.034	0.008	0.373	0.092	0.954	1.824
	6/91	-0.022	-0.105	-0.146	-0.070	-0.182	-0.140	-0.074
	11/91	0.028	0.230	0.611	0.179	1.064	-0.060	1.453
E25007	6/88	-0.102	-0.079	0.058	-0.038	-0.021	-0.086	-0.046
	11/88							
	6/89	-0.050	-0.131	-0.131	0.095	-0.104	0.015	0.007
	11/89	-0.451	-0.431	-0.450	-0.447	-0.215	-0.402	-0.389
	6/90	0.008	-0.024	-0.033	0.075	-0.012	-0.004	0.094
	11/90							
	6/91	-0.082	-0.149	-0.159	0.007	-0.145	-0.168	-0.149
	11/91	-0.016	-0.042	-0.063	-0.124	-0.056	0.019	0.175
E25009	6/88	-0.068	-0.037	-0.049	-0.056	0.094	0.030	-0.014
	11/88	-0.065	0.029	0.231	0.018	0.012	-0.059	0.189
	6/89	-0.135	-0.138	-0.088	0.087	0.326	-0.050	0.208
	11/89							
	6/90	-0.191	-0.283	-0.218	-0.179	0.082	-0.166	0.047
	11/90	-0.359	-0.304	-0.336	-0.295	0.027	-0.154	0.072
	6/91	-0.042	0.017	0.040	0.010	0.116	-0.015	0.038
	11/91	-0.196	-0.119	-0.211	-0.156	-0.190	-0.227	-0.259
P06008	6/88	0.086	0.216	0.071	0.104	0.133	0.059	0.056
	11/88	-0.026	0.080	0.176	0.388	0.205	0.056	0.395
	6/89	0.025	-0.020	0.024	0.139	0.150	0.121	0.055
	11/89							
	6/90							
	11/90							
	6/91	-0.037	-0.049	-0.048	0.232	0.146	0.002	0.126

	11/91	0.058	0.032	0.112	0.000	1.362	0.193	0.617
P06009	6/88	-0.117	-0.046	0.318	-0.056	-0.061	-0.034	-0.008
	11/88	-0.135	-0.030	-0.044	-0.059	1.185	-0.106	0.631
	6/89	-0.320	-0.200	-0.267	-0.262	-0.203	0.000	-0.003
	11/89	0.028	-0.017	-0.030	0.038	-0.043	0.062	-0.031
	6/90							
	11/90							
	6/91	0.211	-0.051	-0.022	0.045	-0.097	-0.021	0.099
	11/91	-0.182	-0.190	-0.104	-0.027	-0.167	-0.168	0.446
P06010	6/88	-0.098	-0.092	-0.073	0.156	0.108	0.002	-0.032
	11/88	-0.337	-0.242	-0.216	-0.248	0.389	-0.174	0.451
	6/89							
	11/89	-0.076	-0.032	-0.074	-0.010	0.036	-0.018	0.135
	6/90							
	11/90							
	6/91	0.021	0.077	0.216	-0.018	-0.045	0.008	0.130
	11/91	0.262	0.169	0.222	0.626	0.666	0.001	0.924
P10016	6/88	-0.107	-0.122	-0.119	-0.049	0.319	-0.044	0.121
	11/88	-0.051	-0.073	0.469	-0.113	1.995	-0.026	1.031
	6/89	-0.118	-0.139	-0.116	0.059	0.512	0.074	0.152
	11/89	-0.047	0.242	0.104	0.042	1.910	0.527	1.117
	6/90							
	11/90							
	6/91	-0.001	0.044	0.164	0.036	0.683	0.155	0.432
	11/91	-0.196	-0.256	0.126	0.361	1.128	-0.180	0.656
P10017	6/88	-0.003	-0.072	-0.066	-0.067	0.015	0.019	0.162
	11/88	0.002	-0.018	0.041	-0.165	0.416	-0.106	0.398
	6/89	-0.226	-0.283	-0.272	-0.269	-0.221	-0.172	-0.154
	11/89							
	6/90							
	11/90							
	6/91	0.021	-0.029	-0.016	0.101	0.034	-0.009	0.275
	11/91	0.396	0.379	0.594	0.055	2.322	0.654	1.095
P11005	6/88	-0.047	-0.020	-0.052	-0.005	-0.005	-0.046	0.030
	11/88	0.019	-0.002	0.002	0.158	0.109	0.067	0.071
	6/89	-0.091	-0.032	-0.062	0.035	0.045	0.078	-0.038
	11/89	0.023	-0.020	-0.036	0.021	0.152	-0.066	0.106
	6/90							
	11/90							
	6/91							
	11/91							
P12009	6/88	-0.217	-0.196	-0.112	-0.044	-0.023	-0.140	0.523
	11/88	0.548	0.669	0.556	0.240	0.593	-0.008	1.567
	6/89	0.106	0.117	0.122	0.230	0.219	0.521	0.367
	11/89							

	6/90							
	11/90							
	6/91	0.176	0.128	0.159	0.471	0.811	0.015	0.721
	11/91	-0.093	-0.050	0.138	0.977	0.660	0.665	0.408
Q01008	6/88	0.002	0.022	0.013	0.170	1.182	0.614	1.356
	11/88	-0.117	0.051	0.199	0.226	1.342	0.593	1.698
	6/89	-0.041	0.011	-0.012	0.244	0.862	1.436	1.448
	11/89	0.121	0.435	0.513	0.799	1.358	0.873	0.839
	6/90							
	11/90							
	6/91							
	11/91							
Q03005	6/88	-0.132	-0.024	-0.029	0.006	0.206	-0.042	0.284
	11/88							
	6/89	-0.217	-0.107	-0.146	-0.189	-0.082	-0.139	0.024
	11/89	0.269	0.476	0.247	0.134	0.911	-0.023	0.305
	6/90							
	11/90							
	6/91	-0.159	-0.222	-0.078	-0.142	-0.166	-0.120	-0.079
	11/91	1.065	1.092	0.970	0.213	1.157	-0.157	1.086
N05034	6/88	-0.079	-0.106	-0.078	-0.108	-0.038	-0.091	-0.012
	11/88	-0.164	-0.060	-0.102	0.697	0.749	-0.036	0.396
	6/89	-0.113	-0.136	-0.139	0.106	0.002	0.101	0.382
	11/89							
	6/90							
	11/90							
	6/91	-0.119	-0.088	-0.059	0.280	0.112	-0.095	-0.012
	11/91							

Table 2: Longitudinal recognition of merozoite surface proteins by malaria exposed children from The Gambia. Samples were collected at six month intervals from June 1988 to November 1991.

Appendix 4

Papers submitted for publication

Human antibody response to *Plasmodium falciparum* merozoite surface protein 2 is serogroup specific and predominantly of the IgG3 subclass[1]

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ABSTRACT

MSP2 is a merozoite surface protein of *Plasmodium falciparum* and, as such, is a potential component of a malaria vaccine. In this study we have used a panel of recombinant MSP2 antigens in ELISA assays to investigate the recognition of MSP2 by antibodies from malaria-immune human serum. These recombinant antigens include full length proteins of serogroups A and B, and fragments representing the conserved, group-specific or repeat regions of each serogroup. Ninety five percent of the sera tested contained MSP2-specific antibodies; 81% of sera tested responded to serogroup A and 86% responded to serogroup B.

The antibody response is directed almost exclusively towards dimorphic and polymorphic regions of MSP2; the conserved regions are rarely recognised and antibodies to serogroups A and B do not cross-react. Interestingly, the antibody response is predominately of the cytophilic and complement fixing subclass IgG3.

INTRODUCTION

Antigens on the surface of malaria merozoites are of interest as potential targets for vaccine induced immune responses. Since these antigens may be involved in merozoite adherence to, and invasion of, red blood cells [1], antibodies specific for merozoite surface antigens could prevent invasion of erythrocytes and thereby interrupt the asexual cycle of parasite proliferation.

One such antigen, of the human malaria parasite *Plasmodium falciparum*, is the merozoite surface protein 2 (MSP2 , also called MSA2, gp35-56 or G3). Several pieces of evidence suggest that antibodies to MSP2 may be involved in protective immunity to malaria. Monoclonal antibodies (mAb) to MSP2 have been shown to inhibit parasite growth *in vitro* [2, 3], MSP2 is among the antigens recognised by antibodies that inhibit merozoite dispersal [4] and mice immunised with peptides corresponding to the conserved regions of MSP2 of *P.falciparum* are protected against challenge with the rodent parasite *P.chabaudi* [5].

Sequencing data and serological characterisation of malaria parasites with MSP2-specific mAbs [4, 6-9] show that MSP2 is a polymorphic protein which can be classified into two allelic families corresponding to two antigenically distinct serogroups. Serogroup A includes the isolates T9/96, Camp, 3D7 and IC1, and serogroup B includes the isolates FCQ-27, K1 and Dd2. The protein has conserved sequences, at the amino- and carboxy-termini, which are common to both serogroups. These conserved regions flank dimorphic, group-specific sequences containing two central regions of polymorphic, tandemly arranged repetitive sequences. Fenton *et al* [9] designated the two regions of tandem repeats as R1 and R2. The R1 region of serogroup A is glycine, serine and alanine rich and varies considerably from isolate to isolate; the R2 region is a relatively conserved repeat, rich in the amino acid threonine. The repeat sequences of serogroup B are unrelated to those of serogroup A and consist of either 32 amino acids (R1) or 12 amino acids (R2). Although the sequences of group B repeats are relatively conserved between isolates, they vary in number.

The extensive sequence polymorphism of MSP2 raises the important question of whether immune reponses to the protein might be sequence specific. If this is the case, then responses to MSP2 may be implicated in the notion that the slow development of protective immunity to malaria, in people living in malaria endemic areas, is due to gradual acquisition of specific immunity to all the variant genotypes of the parasite circulating in the community [10]. In order to better understand the nature of protective immunity to malaria it is neccessary to determine whether naturally acquired antibody responses to MSP2 are cross-reactive within and between serogroups. Such information would also be useful for determining which, if any, regions of MSP2 might usefully be included in a malaria vaccine. To date, there is very little published data on naturally acquired immunity to MSP2 and no information on the relative immunogenicity of conserved, group specific (dimorphic) and repetitive (polymorphic) regions of the molecule. A study of adult, malaria exposed, Melanesians showed that 82% had antibodies against MSP2 serogroup B [11], but the specificity of these antibodies was not fully characterised. A recent study in a highly endemic area of Papua New Guinea found high

antibody prevalence (≥ 90%) to recombinant antigens representing the full length proteins of both MSP2 serogroup A (3D7) and serogroup B (FC27)[12]. Using a serogroup A construct lacking the central repeats, this group also showed that a proportion of immune individuals responded only to epitopes within the repeat region. Experimental immunisation of *Aotus* monkeys with *P.falciparum* results in an anti-MSP2 antibody response directed primarily to repeat and group-specific regions of MSP2 [4]. Antibodies purified from immune clusters of merozoites also recognise group specific sequences, suggesting that this region is accessible at the surface of the intact parasite and may therefore be a target for parasite-inhibitory immune responses [4]. However, immunoblotting studies using affinity purified human serum suggest that naturally immunodominant epitopes are encoded within the repetitive sequences of the molecule [7]. None of these studies has addressed the crucial question of whether sequence polymorphism gives rise to antigenic diversity and whether epitopes in one allelic form of the protein cross-react with similar epitopes from other parasites.

Here we report a comprehensive study of the recognition of MSP2 by sera from malaria-immune adults from The Gambia, West Africa, and the identification of regions of the molecule that are immunogenic during malaria infections. We find that MSP2 is naturally immunogenic in man, inducing IgG antibodies which predominantly recognise epitopes located in the dimorphic and polymorphic regions of the molecule. Importantly, antibodies to the two main serogroups of MSP2 (A and B) do not appear to cross-react. Anti-MSP2 antibodies were found to belong mainly to IgG subclasses that have opsonising and complement fixing properties, suggesting that they might play a role in regulating the proliferation of intraerythrocytic parasites.

MATERIALS AND METHODS

Sera

Serum samples were obtained from 70 adults (aged 15-65 years) living in rural and peri-urban areas of The Gambia, West Africa, where malaria transmission is intense during the short wet season (from July to November) and minimal at other times of the year [13]. Control serum samples were obtained from 15 European adults who had not been exposed to malaria.

Antigens

Recombinant MSP2 proteins

pGEX expression vectors were used to direct the synthesis of MSP2 polypeptides in *E.coli* as fusions with the C-terminus of glutathione S-transferase (GST) of *Schistosoma japonicum* [14, Smith *et al,* in preparation]. This permits the purification, on a glutathione column, of recombinant antigens as stable, soluble fusion polypeptides. Expression of the protein was initiated by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG) to cultures of transformed *E.coli* . After 3-5 hours, cells were pelleted, resuspended in PBS and lysed on ice by mild sonication in the presence of 10% (v/v) Triton X-100 (BDH Chemicals, Poole, UK.). The fusion proteins were purified from the supernatant by absorption onto glutathione agarose beads (Sigma, Poole, UK.). The proteins were eluted by competition with free glutathione and their concentrations estimated by the intensity of coomassie blue staining on acrylamide gels.

As a control, the fusion protein partner, GST, was purified from pGEX plasmids lacking an MSP2 insert.

The immunogenicity and antigenic integrity of the fusion proteins were assessed by mouse immunisation and testing of the mouse sera in ELISA, Western blotting and immunofluoresence assays (using acetone-fixed, mature *P. falciparum* schizonts) [Smith *et al,* in preparation]. All the recombinant proteins used in this study were shown to reflect the antigenic character of the native protein and are represented in figure 1. It has not yet been possible to produce a recombinant protein which accurately reflects the antigenic structure of the N-terminal conserved sequence of the protein, hence no analysis of responses to this region is reported.

MSP1 - p190.1

As a positive control for prior exposure to *Plasmodium falciparum,* sera were also tested for reactivity with a conserved sequence from another merozoite surface protein, MSP1. p190.1 represents a non-variable region of MSP1 (gp190) which has previously been shown to be recognised by more than 90% of malaria exposed individuals [15] and was produced in *E. coli* as a free polypeptide [16]. p190.1 was a kind gift of Dr J.R.L Pink, F. Hoffman La Roche, Basel, Switzerland.

Enzyme-linked Immunosorbent Assay (ELISA)

Microtitre plates (Immulon-4 (Dynatech)) were coated overnight at 4ºC with 100µl/well of antigen at 0.5µg/ml in 0.1M carbonate ($Na_2CO_3/NaHCO_3$) buffer (pH9.6), and blocked for 5 hours at room temperature with 200µl/well

of blocking buffer (1% (w/v) milk powder in PBS/0.05% Tween 20). At the same time, sera were diluted in blocking buffer and incubated at room temperature for 5 hours. Plates were washed three times with PBS/Tween and 100µl/well of diluted serum was added to duplicate wells and incubated overnight at 4°C. Plates were washed and incubated with horse-radish peroxidase-conjugated rabbit anti-human IgG antibody (Dako Ltd., High Wycombe, UK) for 3 hours at room temperature.
For the detection of specific IgG subclasses, plates were incubated for 3 hours with murine monoclonal antibodies to specific human IgG subclasses, followed by HRP-conjugated rabbit anti-mouse Ig antibody for 3 hours. The subclass specificity of these mAbs [IgG1 (NL16, Boehringer Mannheim, Germany), IgG2 (ROM1, Boehringer), IgG3 (HP6050, Serotec, Oxford, England), IgG4 (RJ4, Boehringer)] has been widely reported [17-20] and was reconfirmed before their use in this study. Each mAb was titrated against varying concentrations of purified IgG of the appropriate subclass; all mAbs gave parallel titration curves. For each mAb, a working concentration was selected such that the relationship between OD and IgG concentration was approximately the same for each subclass. This allows the amounts of antibody in each subclass to be assessed, on a roughly quantitative basis, by comparing the OD values.
All plates were developed with H_2O_2 as substrate and o-phenylenediamine (OPD) as chromagen at 4°C and the reaction was stopped after 10 minutes with 20µl/well of 2M H_2SO_4. The optical density (OD) was measured at a wavelength of 492 nm.
Optimal concentrations of antigen and antibody were determined by chequer board titrations.

Statistical analysis

The reactivity of the sera with various MSP2 fusion proteins in ELISA was calculated by subtracting the OD value for the GST control from the value obtained for the MSP2 fusion protein, to obtain specific OD values. Positive samples were defined as those giving a specific OD above the normal range for control European sera. The normal range was taken as the mean ± 2 standard deviations of 15 control sera.
The reactivity of individual sera with different antigens was compared by means of Spearman's rank correlation test [21].

RESULTS

1. Reactivity of human serum antibodies with MSP2 fusion proteins.

Serum IgG antibodies from Gambian and European adults were tested for recognition of recombinant MSP2 proteins in ELISA. MSP2 proteins were specifically recognised by sera from individuals who have been exposed to malaria (Fig 2). The cut-off level for positive sera for each antigen ranged from 0.117 to 0.384 OD units (Fig 2). The proportion of sera recognising each of the proteins is shown in table I.

These results show that MSP2 is well recognised by sera from individuals who have been exposed to malaria. Serogroup A and serogroup B are recognised by 81% and 86% of sera respectively, indicating that MSP2 is highly antigenic during natural infections. MSP2-specific antibodies predominantly recognise polymorphic and dimorphic regions of the protein; the conserved C terminus is recognised by only 36% of sera and the OD values obtained with this protein were significantly lower than for the full length and group specific proteins (only 3 sera recognised this protein with an OD value greater than 0.300).

2. Lack of antigenic cross-reactivity between serogroups A and B:

Seventy-nine percent of sera from adult Gambians recognised both the A and B serogroups (proteins A1 and B1, which represent the full length molecules). To determine whether this 'dual' recognition was due to a single population of antibodies which react with epitopes common to both proteins or whether the serum contained two separate, non-cross-reacting, populations of antibody, we compared the reactivity of individual sera with the full length proteins from both serogroups (A1 and B1) (Fig 3). It is clear that although some sera recognise the two proteins apparently equally, other sera clearly recognise one protein but not the other.

Using Spearman's rank correlation test, a positive correlation was obtained for responses to A1 and B1 ($r=0.555$; $r_S=0.307$, $p=0.005$). However, we consider that the high level of statistical significance is the result of the large sample size and the considerable number of double negative sera; the association is in fact quite weak and may simply reflect independent exposure to both serogroups of *P.falciparum*. As an estimate of association due to exposure, we compared the recognition of MSP2 proteins with the recognition of a recombinant protein representing a conserved region of an unrelated merozoite surface protein (MSP1, p190.1). Correlation coefficients of up to 0.492 were obtained. Thus the correlation coefficient obtained for A1 versus B1 was only slightly higher than that obtained for MSP2 versus MSP1, suggesting that the correlation is indeed due to exposure rather than to cross-reacting antibodies.

To confirm that antibodies to the two serogroups are not cross-reactive, individual sera were tested in competition ELISAs. Sera which were known to contain antibodies to both A1 and B1 were selected; these sera were preincubated with either A1 or B1 and tested in ELISA for recognition of the other protein. The example shown in Figure 4 was typical of the sera tested: whilst preincubation with increasing concentrations of A1 prevents subsequent binding of antibodies to A1-coated plates, it has no effect on

binding of antibody to B1-coated plates, and vice versa. Thus, in double positive sera, there appear to be two distinct populations of antibodies, one specific for serogroup A and the other specific for serogroup B.

3. Subclass of anti-MSP2 IgG antibodies:

The IgG subclass of anti-MSP2 antibodies was determined for sera that had been shown to contain MSP2-specific IgG. Figure 5 shows the IgG subclasses of antibodies to proteins A1, A2 and A3. The distribution was similar for the corresponding serogroup B proteins (data not shown).

Although the IgG subclass pattern differs slightly for antibodies to the different proteins (Fig. 5) the predominant MSP2-specific antibody subclass is IgG3. 77% and 80%, respectively, of A1 and A3 positive sera contained only IgG3. The remainder of the A1 and A3-positive sera contained only IgG1. In comparison, 44% of A2-positive sera contained A2-specific IgG4. The pattern of recognition is similar for the serogroup B proteins, in that IgG3 is the predominant subclass. However, approximately 57% of serogroup B positive sera were IgG1 positive; in the majority of cases, IgG1 was coexpressed with IgG3.

This predilection for induction of IgG3 antibodies is unusual and suggests that there may be something about the antigenic structure of the MSP2 molecule which preferentially triggers isotype switching to IgG3 in MSP2-specific plasma cells.

DISCUSSION

The aim of this study was to characterize the reactivity of serum antibodies from malaria-exposed adult individuals with the merozoite surface protein, MSP2. Evaluation of MSP2 as a potential component of a subunit malaria vaccine requires an understanding of the naturally occuring immune response to MSP2 and, most importantly, the immunological significance of amino acid sequence polymorphisms. In particular, it is important to determine whether Abs against one MSP2 serogroup will cross react with the other serogroup, or whether the Ab response is group-specific or allele-specific. Such information may also help us to understand the relative importance of allele-specific ("strain-specific") immune responses in the acquisition of clinically protective immunity to malaria by people living in malaria endemic areas.

There are two main theories to explain the slow development of protective immunity to malaria which is typically seen in individuals living in endemic areas: (i) polymorphism of antigens which are the targets of protective immune responses and, (ii) intrinsically poor immunogenicity of the target molecules. The data presented here indicate that the latter explanation is not true, at least with respect to MSP2. Sixty seven of the 70 individuals tested had clearly detectable anti-MSP2 antibodies with end point titrations of > 1/9,000 for most sera (data not shown). It is possible that the three seronegative individuals had not been exposed to parasites carrying the MSP2 variants tested here. In addition, we have screened a small number of sera collected 2 to 4 weeks after a known primary malaria infection and all contained detectable levels of antibody to MSP2 (R. Taylor and J. Carlsson, unpublished data).

Antibodies tended to recognise epitopes within the dimorphic and polymorphic regions of MSP2; the conserved C-terminus seems to be poorly antigenic. This is in agreement with Thomas *et al* [4] who reported that although intact MSP2 is recognised by monkey and human antibodies eluted from intact merozoites, such antibodies did not recognise peptides representing the N- and C-terminal regions of the molecule. Similarly, Saul *et al* [5] were unable to detect Abs against synthetic peptides representing the conserved regions of MSP2 in sera from 18 people with high titres of anti-parasite antibodies (as determined by immunofluorescence assays).

Importantly, the sequences which are conserved within each allelic family (detected using A3 and B3 proteins) are highly immunogenic. Ninety percent of the sera tested contained Abs which recognise either A3 or B3 (or both); thus a vaccine based on MSP2 may need to contain only two different antigens - representing the group-specific sequences of each serogroup. Interestingly the group-specific A3 protein is recognised by significantly more individuals than the B3 protein. This may reflect more frequent or more recent exposure of the donors to parasites of serogroup A since approximately 60% of parasites isolated in The Gambia belong to the A serogroup [22; R.Taylor, unpublished]. Antibodies eluted from immune clusters of merozoites recognise dimorphic MSP2 sequences [4], indicating that epitopes associated with these sequences are accessible at the surface of intact merozoites, and may therefore be a target for inhibitory antibodies.

Proteins which represent the polymorphic R1 repeat regions of both serogroups (i.e. A2 and B2) were recognised by a substantial proportion of the sera, a greater proportion in fact than recognised the dimorphic regions. This finding was somewhat unexpected since the sera were tested against only one variant for each serogroup and, at least for serogroup A, the amino acid sequence of the repeats from different isolates varies extensively [23, 24]. One likely explanation of this finding is that the antibodies detected were in fact recognising epitopes within a short N-terminal segment of group specific sequence contained in the protein A2 (see figure 1). Alternative explanations include extensive immunological cross-reactivity between different repeat sequences or a very high frequency of parasites in The Gambia expressing the tested R1 repeat sequence. This latter explanation is unlikely since considerable microheterogeneity within repetitive sequences has been reported for parasite populations and isolates from all over the world [7-9, 23-25; R. Taylor, unpublished]. Immunological cross-reactivity may well occur among the R1 repeats of serogroup B, where amino acid sequences are relatively conserved, but is less likely for serogroup A. Further studies are underway to study the effect, on antibody recognition, of variation in the sequence and number of repeats.

Quantification of absolute amounts of Ab by ELISA is difficult since the OD value obtained is dependent on both the concentration of Ab and its' affinity. It is not possible to compare the amounts of Ab in sera which react with different recombinant antigens since the number of epitopes in each assay (and thus the avidity of the reaction) is unknown [26]. Therefore, in order to make comparisons between the Ab responses to different proteins, we used a nonparametric rank correlation test to compare specific OD values for individual sera, tested at a single dilution, against the full length proteins of serogroup A and serogroup B. Such comparisons show that the correlation between responses to the two serogroups is weak and is probably due to exposure to parasites of both serogroups rather than to cross-reactive antibodies. Competition experiments clearly demonstrate that antibodies to serogroup A and B are not cross-reactive, since protein A1 cannot compete for binding of B1-specific antibodies, and vice versa.

In theory, antibodies specific for MSP2 could inhibit merozoite invasion of erythrocytes by a number of mechanisms including merozoite agglutination, complement-mediated lysis, opsonisation or blocking of receptors involved in the adherence of merozoites to erythrocytes. These various mechanisms would be mediated by antibodies of different IgG subclasses - only IgG1 and IgG3 are opsonising and complement-fixing but all four subclasses could mediate agglutination or receptor blocking. Since functional differences may exist among Abs of the same specificity, we have determined the subclass of MSP2-specific IgG in sera from individuals immunised by natural exposure to malaria. Our work indicates that antibodies which recognise MSP2 are predominantly of the IgG3 subclass. It has been suggested that the ratio of IgG1 and IgG3 to IgG2 and IgG4 may be important in immunity to asexual blood stages of *P. falciparum* [27, 28] since, whilst IgG1 and IgG3 can mediate opsonisation and phagocytosis of parasitised erythrocytes or free merozoites, IgG2 and IgG4 antibodies (of the same epitope specificity) may block the binding of the protective subclasses. IgG3 is considered to be the most effective subclass for

activating the complement pathway [29], and it is known to mediate cell lysis by monocytes or Fc receptor bearing lymphocytes [30]. Thus, the predominance of the IgG3 response to MSP2 in adults with a high degree of protective immunity to malaria, suggests that this molecule may be a target of protective antibody reponses.

The predominance of the IgG3 response is unusual and is noticeably different from the response to MSP1 [31]. The only other examples of which we are aware, of an antibody response which is significantly skewed towards IgG3, are the response to the outer membrane protein of *Branhamella cattarhalis* where IgG3 antibodies represent approximately 70% of the total response in individuals over 4 years of age [32] and responses to the streptolysin M protein where IgG3 predominated in the response of more than half of the individuals tested [33]. Interestingly, in the case of *Branhamella*, the switch to IgG3 production seemed to be age-related, specific IgG3 being essentially undetectable in children under the age of 4 years [32]. IgG1and IgG3 are typically produced in response to protein antigens [34] with IgG1 present in significantly greater amounts than IgG3 but, as yet, little is known about factors which may preferentially induce the production of IgG3. Although specific switch factors have been described for different IgG subclasses in the mouse, much less is known about this system in humans. Isotype switching to both IgG1 and IgG3 appears to be controlled by similar processes which may be regulated by the T cell-derived cytokine IL-10 [35]. Goldblatt *et al* [32] suggest that the membrane-bound nature of the *Branhamella* proteins, and their mitogenic activity for B cells, may be partly responsible for the IgG3 antibody response. The propensity for MSP2 to induce IgG3 antibodies suggests that this antigen may be a useful tool for investigating subclass specific switch mechanisms.

The most important question regarding the potential of MSP2 as a vaccine antigen is whether or not MSP2-specific immune responses are involved in protective immunity to malaria. We have shown here that MSP2 is naturally antigenic, that the immune response is directed to dimorphic as well as polymorphic regions of the molecule and that these antibodies are of appropriate subclasses. Longitudinal epidemiological studies are now underway to determine whether these antibodies are able to mediate immunity to clinical malaria and to investigate the hypothesis that polymorphism within the repeat sequences of MSP2 is a significant factor in the slow development of protective immunity to *P. falciparum.*

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LEGENDS

Figure 1: Schematic representation of the MSP2 fusion proteins. Isolates from which the recombinant proteins were derived are indicated in bold. A1-A3 and B1-B3 are the codes for the rAgs.

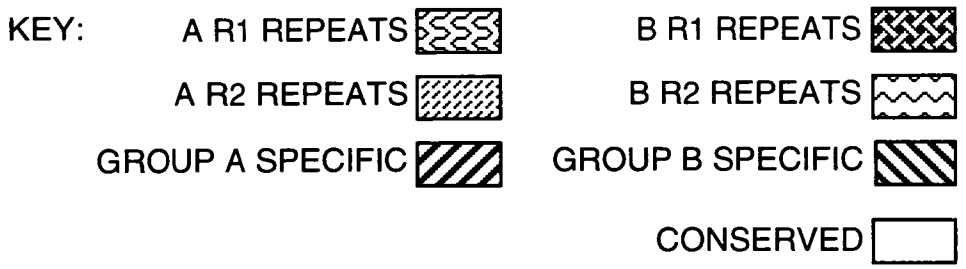


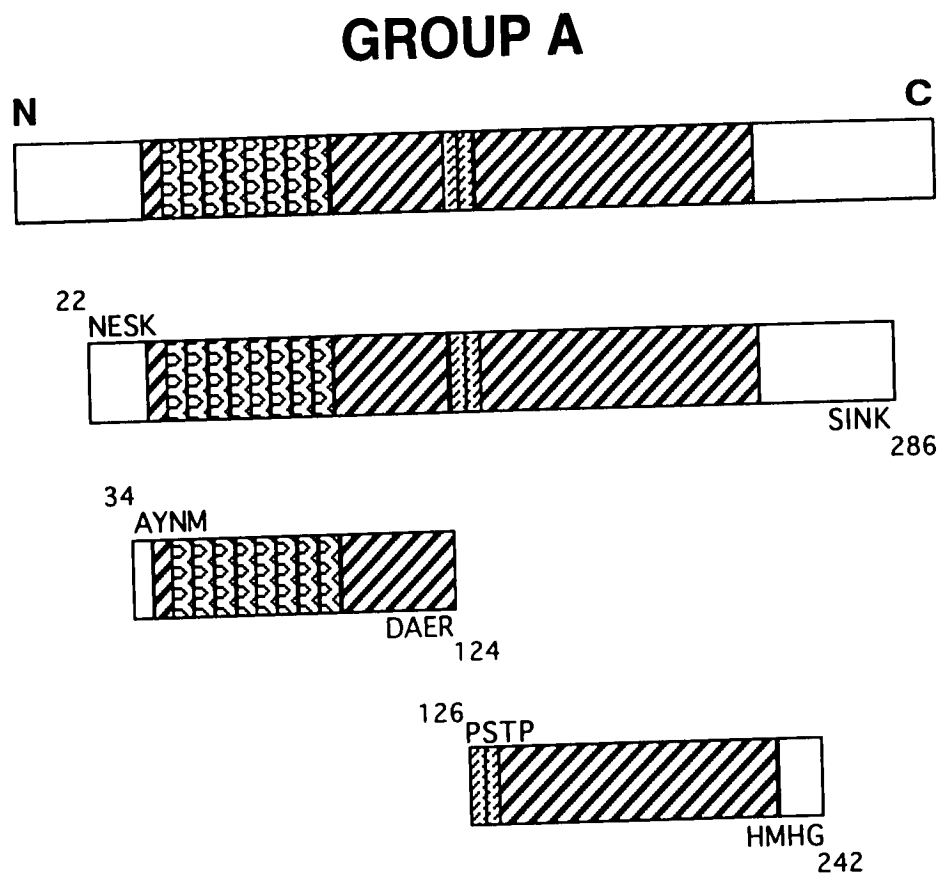
Figure 2: Dot plots showing specific IgG responses (OD $_{492}$) to MSP2 fusion proteins of serogroup A (a) and B (b) in 70 Gambian sera.
The cut off level for positive sera is indicated by a horizontal line.
Sera were tested at a dilution of 1/1000.

Figure 3: Comparison of OD values for A1 versus B1 for sera from 70 Gambian adults. Solid lines indicate the cut-off level as determined from the mean+ 2SD of 15 European sera. Arrow indicates the serum shown in the competition ELISA (Figure 4).
Each circle represents a single serum tested at a dilution of 1/1000.

Figure 4: Competition assay to determine the extent of cross-reactivity between antibodies recognising A1 and B1. The results for a single, typical, serum are shown. Sera were preincubated with antigen at concentrations from 0µg/ml - 5 µg/ml. Sera were tested at a dilution of 1/1000.

Key: ● A1(on plate) vs A1 (in serum), ○ A1 vs B1, ■ B1 vs B1, □ B1 vs A1, Δ GST vs GST.

Figure 5: Dot plots of IgG subclass of antibodies to serogroup A proteins :
a) A1 (n=30) b) A2 (n=25) c) A3 (n=30)
Horizontal lines represent the mean + 2SD of 15 control sera.



rAg	ISOLATE
A1	T9/96
A2	T9/96
A3	RO33

Figure 1a)

R R Taylor, D B Smith, V J Robinson, J S McBride & E M Riley

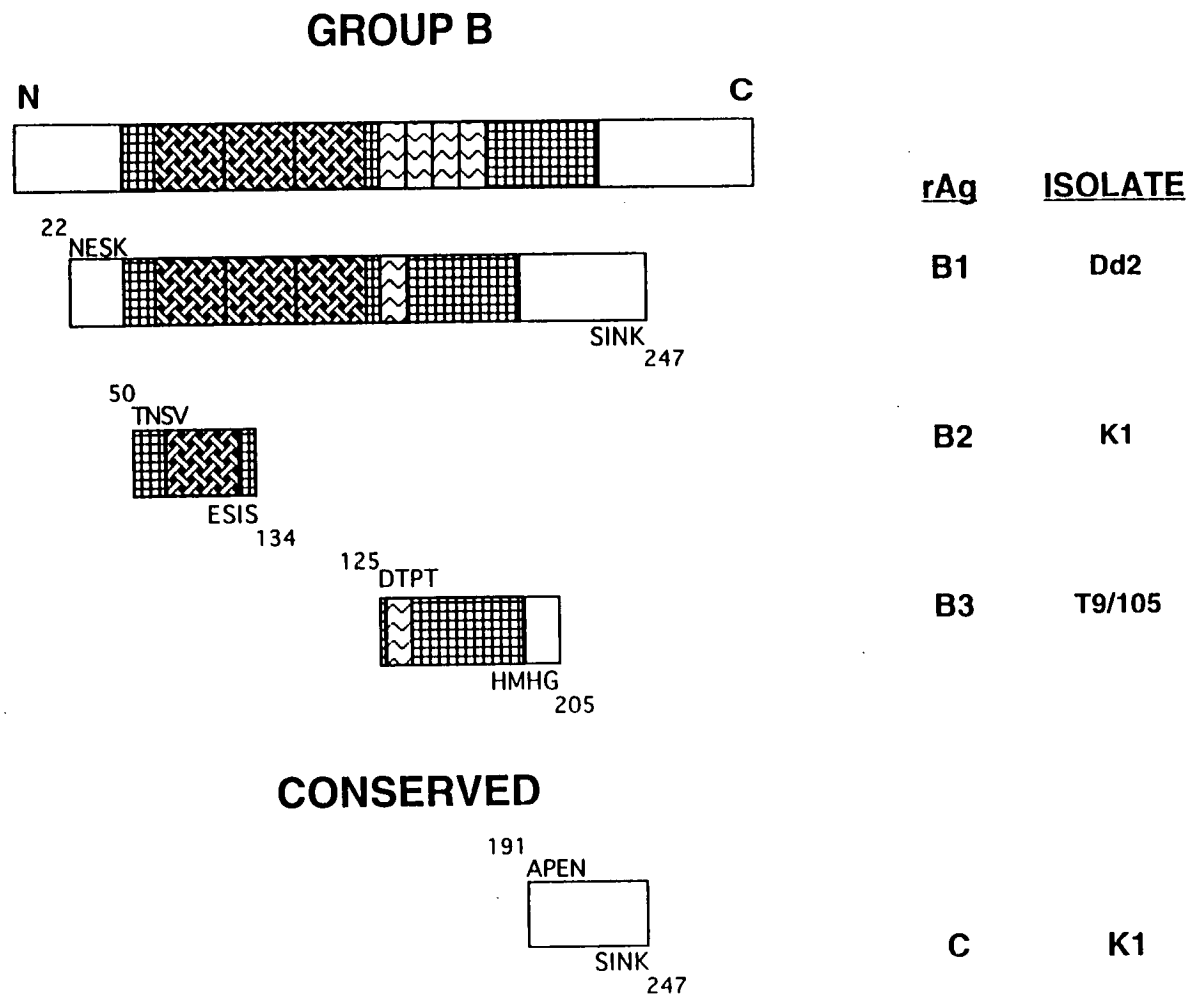


Figure 1b)

R. Taylor, D.B. Smith, V.J. Robinson, J.S. McBride & E.M. Riley

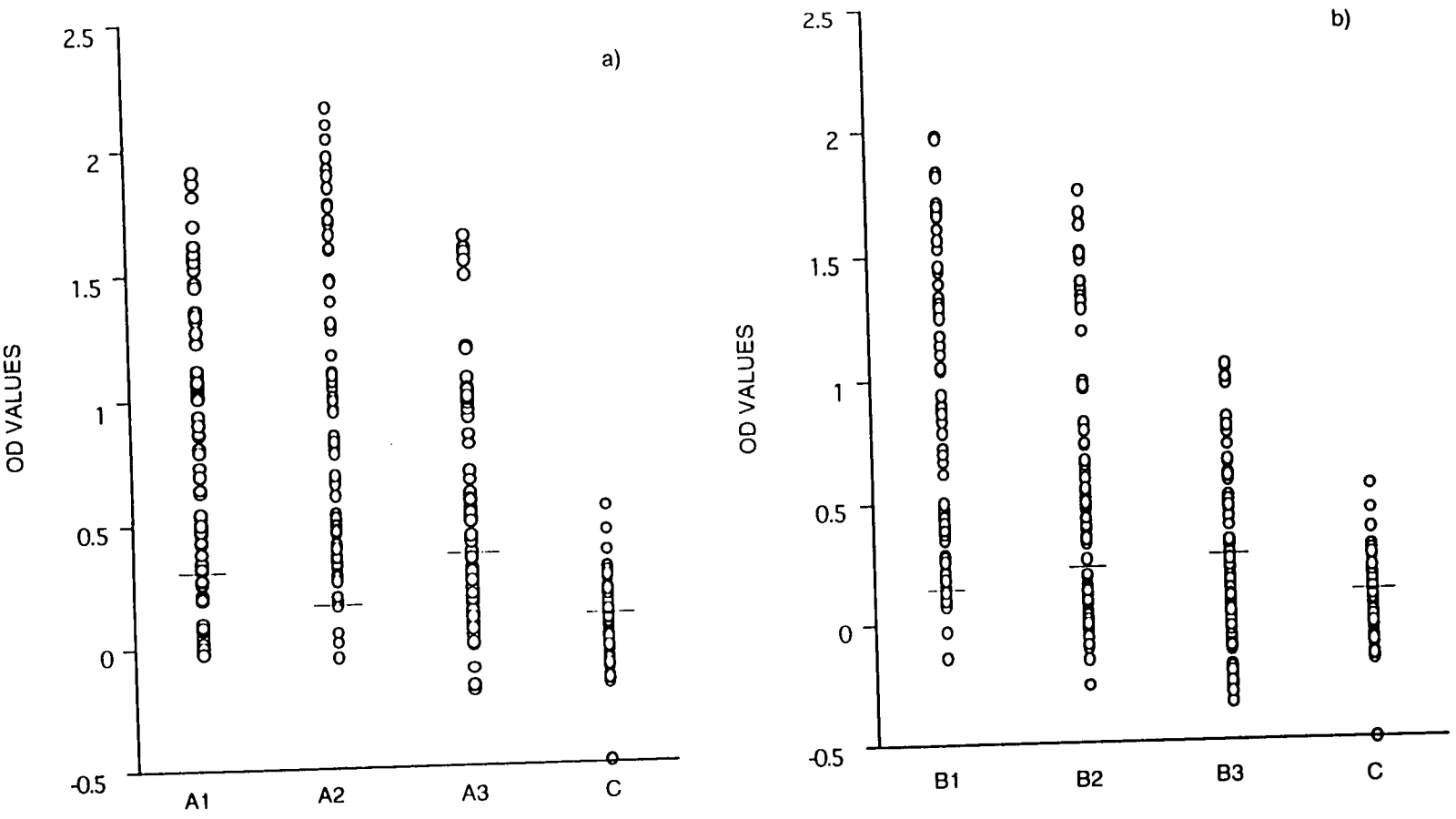


Figure 2

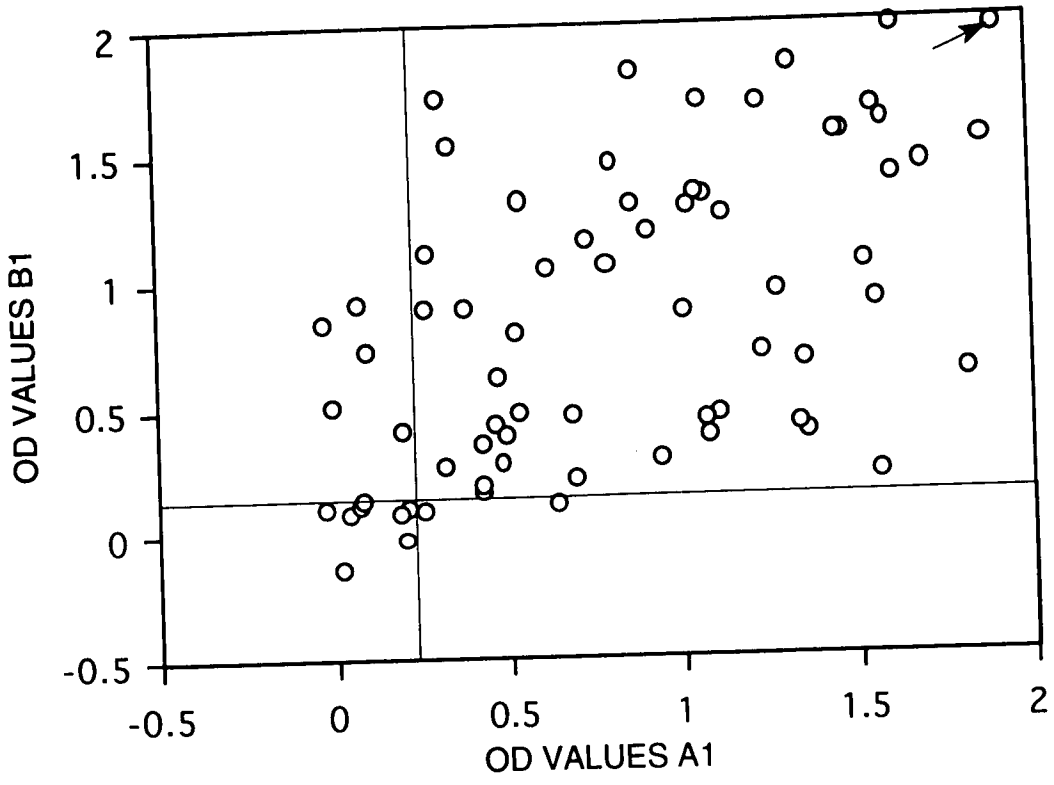


Figure 3
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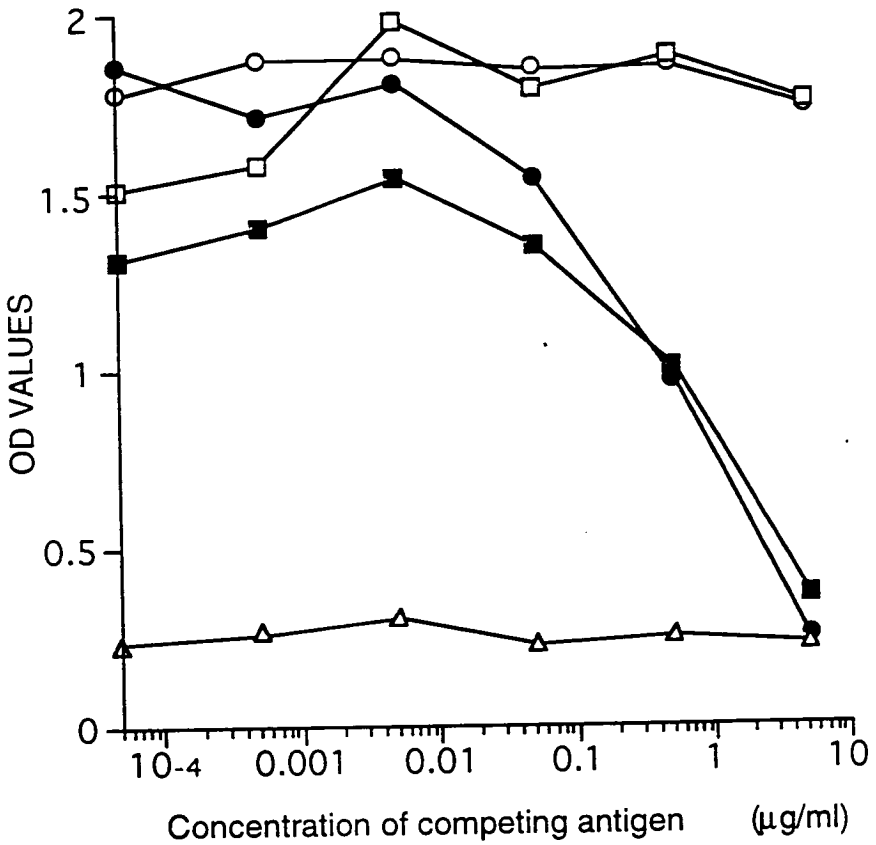
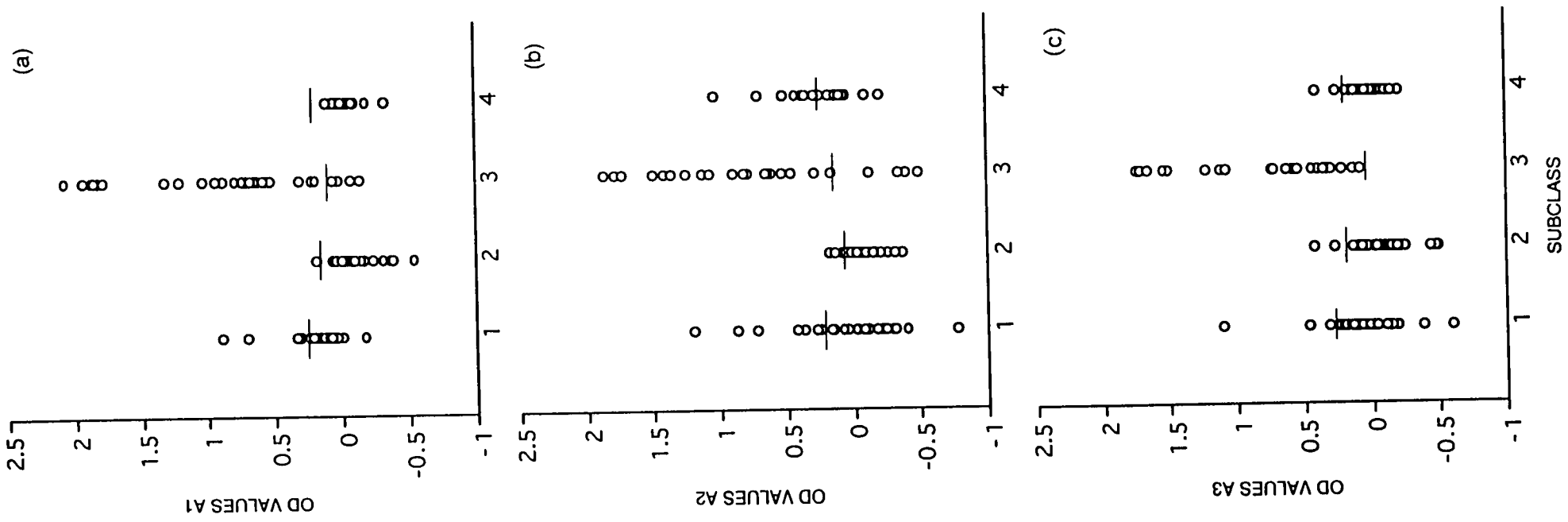


Figure 4
R R Taylor, D B Smith, V J Robinson, J S McBride & E M Riley


(a)
OD VALUES A1
2.5
2
1.5
1
0.5
0
-0.5
-1
1
2
3
4
(b)
OD VALUES A2
(c)
OD VALUES A3
SUBCLASS

		SEROGROUP A			SEROGROUP B			CONSERVED
PROTEIN		A1	A2	A3	B1	B2	B3	C
% RESPONDERS		81	81	73	86	64	43	36
RANGE	-min.	-0.026	-0.053	-0.194	-0.140	-0.256	-0.339	-0.493
	-max.	1.911	2.157	1.632	1.981	1.755	1.044	0.542

TABLE 1: The percentage of malaria immune sera recognising recombinant MSP2 proteins.

70 Gambian sera were tested in ELISA for recognition of MSP2 proteins. The percentage of responders was calculated as those sera giving OD values greater than the mean+2SD of the OD values obtained for 15 European (malaria non-exposed) sera (see figure 2). The range of OD values (minimum and maximum) obtained for each protein are shown. (Negative values occur when the OD value of the GST control protein exceeds the OD value of the fusion protein).

Selective recognition of malaria antigens by human serum antibodies.

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SUMMARY

Malaria infection induces the production of serum antibodies to a variety of malaria antigens but the prevalence of antibodies to any particular antigen is typically much less than 100%. It has been assumed that non-responsiveness to defined antigens in malaria immune subjects is due to HLA-mediated restriction of the immune response, but numerous studies of the relationship between antibody responses and HLA genotype have failed to show any significant associations. In this study we show that whilst antibody levels vary in accordance with seasonal variations in malaria transmission in semi-immune children, antibody levels remain stable in clinically immune adults. Antigen recognition is selective with individual donors showing consistent high titre responses to some antigens/epitopes whilst consistently failing to recognise adjacent regions/epitopes from the same protein. We have investigated the role of HLA and non-HLA genes in the antibody response to two merozoite surface antigens (MSP1 and MSP2) and a sexual stage antigen (Pfs260/230) of *Plasmodium falciparum* and conclude that host genotype is not a major determinant of responsiveness. Thus, we propose that clonal imprinting (original antigenic sin) is an alternative explanation for selective antibody responses to malaria antigens in immune individuals.

INTRODUCTION

Protective immunity to blood stage malaria parasites is at least partly antibody dependent (1). Inhibition of merozoite invasion into erythrocytes, opsonisation and/or antibody-dependent cellular cytotoxicity are believed to be the principle mechanisms of antibody-mediated immunity (2-4) and antibody responses to a number of defined erythrocyte or merozoite surface antigens correlate with acquisition of clinical immunity to malaria (5-8).

Malaria infection induces antibodies directed against both conserved and variable epitopes of merozoite antigens. However, the prevalence of antibodies to any particular antigen or epitope is typically much less than 100%, even where the epitope is highly conserved between parasite isolates and where malaria transmission rates are high enough to ensure that the study population is frequently re-exposed to infection (9-15). Since immunization of MHC (H-2) congenic mice with short polypeptides derived from malaria antigens has shown that non-responsiveness can, in certain circumstances, be linked to H-2 genotype (16,17) it has been assumed that non-responsiveness to malaria antigens in humans is also the result of HLA-dependent genetic restriction of the immune response (10,18,19). However, when immune responses of humans immunised by natural exposure to malaria are compared with their HLA class II genotype very few associations are found between responsiveness (or non-responsiveness) and HLA gene expression (14,20-24). Since widespread non-responsiveness to defined malaria antigens could pose difficulties for the implementation of subunit vaccines, it is important that alternative explanations for individual non-responsiveness should be explored.

Most antibody prevalence data has been collected by cross-sectional analysis of a population at a single point in time. Malaria transmission is seasonal in many endemic areas and it is possible that antibody levels fluctuate over time such that people appear to be non-responders on some occasions and responders on other occasions. To obtain reliable estimates of antibody prevalence it is neccessary to conduct longitudinal studies, collecting serum from the same individuals over a period of months or years. Few such studies have been reported.

It is clear that some regions of any given protein are more potent activators of B cells than are other regions, giving rise to immunodominant epitopes. For example, the repetitive amino acid sequences which are common in malaria proteins tend to be immunodominant (25). However, even epitopes which are immunodominant at a population level are not universally recognised (11,26,27), so immunodominance (or lack thereof) is not, in itself, a sufficient explanation for selectivity of antibody responses. In this study we have investigated the effect of three potential causes of apparent non-responsiveness to malaria antigens, (i) poor immunogenicity of malaria antigens (ii) temporal variations in antibody levels and (iii) host genotype, on the antibody response

to conserved or semi-conserved sequences of two *P. falciparum* merozoite surface proteins (PfMSP1 and PfMSP2) and a sexual stage specific antigen (Pfs260/230). The inherent immunogenicity of the antigens has been assessed by looking for specific antibody in the serum of individuals recovering from a primary malaria infection. In longitudinal serological studies, we have found that whilst antibody responses vary seasonally in children antibody levels remain stable in clinically immune adults. Antigen recognition is selective, with some individuals consistently recognising specific regions of some antigens whilst failing to recognise adjacent epitopes from the same protein. To determine whether selective recognition of malaria antigens is primarily due to host genetic factors, or to external "environmental" factors, we have looked for associations between HLA class II genotype and antibody responses in malaria immune donors and have compared antibody responses in identical and non-identical twins. Antigen recognition does not appear to be genetically regulated. We therefore propose an alternative explanation for selective antibody responses in malaria immune individuals - clonal imprinting, or original antigenic sin.

MATERIALS AND METHODS.

1. Subjects

i. Naive donors

Serum samples were obtained from six patients convalescing from primary malaria infections at the Department of Infectious Diseases and Tropical Medicine, Northwick Park Hospital. Samples were obtained 14-21 days after successful chemotherapy and were stored at -20°C.

ii. Malaria immune/semi-immune donors

All subjects lived in rural areas of The Gambia, West Africa, where malaria transmission is seasonally endemic with the majority of new infections occurring during and after the annual rainy season (July to October) (28). The level of malaria transmission is such that individuals are exposed to between one and five infective mosquito bites per year (28).

a). HLA class II study: Blood samples were obtained from a group of 355 semi-immune children (group A) and a separate group of 283 children and adults (Group B) (for details see (22)). HLA typing for *DRB*, *DQA* and *DQB* was performed by Southern blot analysis of *Taq 1*-cleaved DNA from peripheral blood leucocytes as described previously (22). Carriage of the sickle cell trait, which is a potential confounder of the immune response to malaria, was determined by haemoglobin electrophoresis.

b). Twin study: Serum samples were obtained from 36 pairs of same-sex adult twins. In each case, the twins had been raised together and were still living in the same, or adjacent, house(s); thus it is likely that their past history of malaria infection is very similar.

Fifteen pairs were shown to be monozygous by DNA probing with five separate minisatellite probes (29). Sera were collected during the dry season, when malaria transmission was minimal and stored at -20°C.

c). Longitudinal studies

The first longitudinal study involved 20 children, all of whom were under the age of 10 years at the end of the study. The second study was of 22 adults (all over the age of 16 years at the beginning of the study). Serum samples were collected twice yearly - at the end of the dry season (when malaria transmission is minimal) and at the end of the wet season (when transmission is maximal).

iv. Controls

Control sera were collected from 12 European (malaria unexposed) adults and 12 European children (aged 3-8 years).

2. Antigens

All the antigens (Figure 1) were recombinant proteins, fused to glutathione S transferase (GST) or maltose binding protein (MBP), expressed in *E. coli*, transfected with either pGex or PIH-902 plasmids(30,31). Previous studies have confirmed that the recombinant proteins express essentially the same B cell epitopes as the native proteins (15,31) (R.

Taylor *et al* submitted for publication).

i. PfMSP1 is the major merozoite surface protein of *P. falciparum* and, except for a short N terminal polymorphic region, is dimorphic. The two allelic types are defined as MAD20-type and Wellcome-type. The C-terminal 42kDa region of PfMSP1 is processed into a dimorphic 33kDa fragment and a 19kDa fragment which is composed of two epidermal growth factor-like motifs and which is essentially antigenically conserved (15,32). 19-GST represents the Wellcome sequence of the 19kDa fragment (33) and was a gift of Dr P. Berghaus, NIMR, London. EGF-1 and EGF-2 represent the MAD20 sequences of the first and second EGf-like motifs respectively and were a gift from Dr J. Chappel, NIMR, London (34). Finally, MAD33 represents the MAD20 sequence of the C-terminal, 33kDa dimorphic region (Egan *et al*, in preparation). All the PfMSP1 antigens were GST fusion proteins.

ii. PfMSP2

Four polypeptides, derived from a second dimorphic merozoite surface protein PfMSP2 (35) and fused to GST, were a kind gift of Dr Jana McBride, University of Edinburgh. A2 represents the amino-terminal group-specific sequence of the A (3D7-like) serogroup of PfMSP2 together with the highly polymorphic tetrapeptide R1 repeat region from the Thai isolate T9/96. A3 represents the carboxy-terminal group-specific and R2 repeat region of serogroup A. B2 and B3 represent the equivalent regions of MSP2 serogroup B (FCQ27-like). All four MSP2 antigens thus contain both semi-conserved (group-specific) sequences and repetitive sequences which show varying degrees of polymorphism.

iii. Pfs260/230

The r260 construct (31) represents the glutamic acid-rich tetrapeptide repeat region of the gametocyte/gamete surface antigen Pfs260/230 (36) fused to MBP and was kindly provided by Dr Kim Williamson, Loyola University, Chicago. The repeats ($[EEVG]_n$) share sequence homology with other glutamic acid-rich malaria proteins and appear to be immunodominant in natural human infections (31).

iv. Fusion protein controls.

For all immunoassays, a control GST or MBP peptide, purified from *E. coli* transformed with vectors lacking an insert, was used to determine the response to the fusion protein alone.

3. Enzyme linked immunosorbent assays (Elisa)

Microtitre plates (Immulon-4, Dynatech) were coated at 4°C with 100µl/well of antigen at 0.5µg/ml in 0.1M carbonate ($Na_2CO_3/NaHCO_3$) buffer (pH9.6), blocked at room temperature with 200µl/well of blocking buffer (1% w/v milk powder in PBS/0.05% Tween 20) and washed three times in PBS/Tween20. Sera were diluted 1:1,000 in blocking buffer. One hundred µl of diluted serum was added to duplicate wells and incubated at 4°C overnight. Plates were washed and incubated with an optimal concentration of horse-radish peroxidase-conjugated rabbit anti-human IgG antibody (Dako Ltd, High Wycombe, UK) for 3 hours at RT. Plates were developed with H_2O_2 as substrate and o-phenylenediamine (OPD, Sigma, UK) as chromagen and the reaction was stopped after 10 minutes with 20µl/well 2M H_2SO_4. The optical density (OD) was measured at a wavelength of 492 nm.

To minimise interassay variation, sera from each pair of twins were tested in parallel on the same microtitre plate. For the longitudinal studies, consecutive samples from the same donor were all tested on a single plate.

4. Data analysis

The reactivity of the sera with malaria antigens was calculated by subtracting the OD value for the GST or MBP control from the value obtained for the fusion proteins, to obtain specific OD values. Positive sera were defined as those giving a specific OD above the normal range (mean plus 2 SD) for 24 control European sera.

The association between HLA class II genotype and antibody response was tested as

described previously (22). Briefly, HLA genotype was defined by the *DRB-DQA-DQB* haplotype and only those haplotypes which were present in at least 20/355 children in group A or 10/283 people in group B were included in the analysis. 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 the potential confounding effects of age, sex and, in group A, sickle cell status. To allow for linkage of HLA genotype and malaria exposure within members of a single family group, the data were stratified by household and analysed by conditional logistic regression.

Differences between monozygous (mz) and dizygous (dz) twins were assessed in two ways. Firstly, the proportion of discordant pairs (i.e. where one twin was seronegative and the other seropositive for any particular antigen) in each group (mz vs dz) was compared by Fisher's exact test. Secondly, the absolute difference in OD values for each twin pair (for each antigen) was calculated and the significance of the median difference between mz and dz pairs was assessed using a non-parametric Mann-Whitney test.

RESULTS

1. How immunogenic are merozoite and gametocyte surface proteins?

In determining the significance of apparent non-recognition of malaria antigens by serum antibodies from malaria exposed individuals it is important to assess the relative immunogenicity of the antigens. It is not necessarily appropriate to assess the immunogenicity of a particular antigen on the basis of the proportion of responders in an immune population since even poorly immunogenic antigens may be recognised after long term exposure. Alternatively, down regulation of responses following frequent re-infection may underestimate the initial immunogenicity of the antigen. An alternative method for assessing the natural immunogenicity of these antigens is to examine convalescent serum samples from people who are known to have had only one or two malaria infections. We have examined convalescent sera from six such individuals (Table 1).

MSP2 is highly immunogenic, with all six convalescent sera recognising either the serogroup A or serogroup B proteins. Five sera recognised the highly conserved double EGF motif construct (19-GST) whilst the individual EGF motifs and the dimorphic MAD33 protein were each recognised by 3 sera. None of the sera from non-immune malaria patients recognised the Pfs260/230 construct, suggesting either that the sequence is poorly immunogenic or that their infections had been cleared (by drug therapy) before significant differentiation of the asexual parasites into sexual stages.

Thus, with the possible exception of Pfs260/230, poor immunogenicity seems an unlikely explanation for non-responsiveness to these particular proteins in malaria-immune individuals.

2. Are antibody levels, within one individual, stable over time?

In order to confirm that non-responsiveness to malaria antigens is a real phenomenon, it is important to determine whether responder or non-responder status is a consistent phenotype within an individual. To do this we collected multiple serum samples from children and adults, at intervals of approximately six months, i.e. both high and low malaria transmission seasons.

In children there was clear evidence of seasonal variation in antibody responses with oscillation between seropositivity and seronegativity (Table 2) and boosting of the antibody response during the malaria transmission season (Figure 2). It is possible to make a shrewd guess as to the MSP2 genotype of the infecting parasites, based on the rise or fall of antibodies to MSP2 serogroup A or B proteins. For example, donor E07008 shows boosting of antibodies to serogroup A whilst E24009 shows boosting of the response to serogroup B in 1990 and to A and B in 1991. It is also clear that some antigens are more frequently recognised than others. Surprisingly, the dimorphic antigens (MAD33, MSP2-A3 and MSP2-B3) appear to be more frequently recognised than the conserved antigens ($MSP1_{19}$, r260, EGF-1 and EGF-2) but this may simply reflect the

fact that they are somewhat larger and presumably express a more diverse array of epitopes.
In contrast to the variable responses seen in children, adult responses were much less variable over time (Figure 3; Table 3). Individuals tend to be either consistently seropositive or consistently seronegative for specific antigens. What is evident in the adults however, is some kind of epitope selection. Donor 13 (Fig 3a) is consistently antibody positive for MSP2-A3, B2 and B3 but negative for MSP2-A2. Donor 45 (Table 3, Fig 3b) recognises MAD33 and MSP2 but fails to recognise the $MSP1_{19}$ proteins. Some donors (e.g. 79) make strong antibody responses to all the antigens tested and all donors (with the exception of donor 109) make consistent responses to at least one of the antigens tested.

3. Is the pattern of antibody recognition of MSP1 and MSP2 genetically determined?
To determine whether persistent non-responder status in adults is genetically determined, the effects of HLA Class II genes and non-HLA genes on antibody responses were examined. Table 4 summarises the results of the statistical analysis of the relationship between HLA class II haplotype and antibody responses to MSP1 and MSP2. No significant associations were seen between any of the antigens tested and any HLA class II antigen. One marginally significant association was observed, for MAD33, in Group A but this was not apparent in Group B and is therefore unlikely to be a true association. r260 was analysed for Group B only but few individuals were tested for each haplotype (data not shown) and although no association was found ($\chi^2 = 3.04$, $p = 0.55$), the group sizes were too small for any definitive conclusions to be drawn.
Sera from 15 pairs of adult monozygous (mz) twins and 21 pairs of adult dizygous (dz) twins were tested for recognition of $MSP1_{19}$ and MSP2 (Table 5). Overall, the level of concordance is high (36 pairs tested against 5 antigens = 180 comparisons, of which 142 were concordant, = 79%), this presumably reflects the similarity of past malaria exposure within the pairs. However, some pairs are clearly discordant with one twin showing a strong antibody response to a particular antigen and the other twin being seronegative. If these differences were the result mainly of genetic differences between the twins, one would expect that more dz than mz pairs would be discordant, but this is not the case. There was no significant difference between mz and dz twins in either the number of discordant pairs (Fisher's exact test, $p > 0.05$ for all antigens) or the median difference in OD values (Mann-Whitney; $p > 0.07$ for all antigens). In other words, mz pairs are no more alike than are dz twins and genetically identical mz twins can have very different antibody responses.
Sufficient serum was available from eight pairs of twins (3 mz, 5 dz) for testing for responses to r260. All eight pairs gave concordant responses (i.e. both negative or both positive). However, in a larger number of twins, we have demonstrated discordant responses to native Pfs260/230 within monozygous pairs (14).

DISCUSSION

Limited antibody recognition of malaria merozoite antigens is a potential problem for vaccine development, particularly if non-responsiveness is genetically determined, since a proportion of any given population may be unable to respond to vaccination. Although non-responsiveness to malaria antigens has been widely reported (9,10,14,18,20) there has been little attempt to determine the cause. In this study we have investigated the role of three factors - immunogenicity of the antigens, temporal fluctuations in antibody titre and host genotype - in the response to defined epitopes of two merozoite surface antigens, MSP1 and MSP2 and a gametocyte surface antigen Pfs260/230. In order to overcome the confounding effects of antigenic polymorphism and heterogeneity of malaria exposure, we have deliberately selected antigens representing semi-conserved or conserved protein sequences which are commonly expressed by parasites circulating in the study area (37). We also selected antigens which we believed would be highly immunogenic in that more than 50% of immune adults have antibodies to them (15,31)

(R. Taylor *et al*, submitted for publication, A. Egan *et al*, unpublished). The immunogenicity of the antigens used is confirmed by our finding that high titre antibody responses can be detected during the convalescent phase of primary malaria infections. Previous studies in human populations have not shown any significant association between naturally acquired antibody responses to defined malaria antigens and HLA class II genotype (14,20-24). Similarly, in this study, we have looked for relationships between HLA class II and responses to MSP1, MSP2 and r260 antigens in some 500 malaria-immune individuals and found no significant associations between the two variables. However, demonstration of HLA associated non-responsiveness in outbred human populations is hindered by the extreme genetic diversity of even quite small communities (38-40) and by the heterogeneity of antigenic challenge, particularly for polymorphic micro-organisms such as malaria parasites (37). In this study we have overcome this problem by comparing immune responses of twins which have a shared genetic background and similar malaria exposure histories. Although the number of twin pairs tested was rather small, comparison of antibody responses in identical and non-identical twins indicates that genetic background is not a major factor in determining responsiveness to MSP1, MSP2 and r260. This is in agreement with data from a much larger twin study (see below) and with our previous observations for antibody responses of twins to native Pfs260/230 (14). We have shown that, despite life long exposure to malaria, monozygous twin pairs can have clearly discordant antibody responses to the same antigen. (Recent boosting of the antibody response in one twin but not the other is unlikely since samples were collected several months after the end of the transmission season.)

In this, rather small, twin study it is not possible to dissociate the MHC (HLA class II) effects from non-MHC effects. However, in a related, but much larger, study of antimalarial immune responses in Gambian twins (41), it was found that although T cell responses to a wide variety of malaria antigens are influenced by genetic factors lying outside the HLA class II region, neither HLA or non-HLA genes contributed significantly to antibody responses. This is probably because environmental factors outweigh genetic factors in determining the level of the antibody response at any particular point in time. Thus, we conclude that genetic factors do not play a major role in determining serological responsiveness or non-responsiveness to these particular malaria antigens. We therefore turned to an examination of temporal (environmental) factors which may influence the response.

We compared patterns of antibody responsiveness over time in a group of 20 partially-immune children who were still susceptible to clinical malaria infection (and who experience high levels of parasitaemia when infected) with the pattern of responses in clinically immune adults who experienced only asymptomatic, low level, infections. The pattern of responses in children was not unexpected - there was clear evidence of seasonal variation in antibody levels with titres rising at the end of the malaria transmission season. However, even in children it was evident that responses to some antigens were being boosted more readily than others. For example, epitopes within $MSP1_{19}$ would have been present during every infection but the response to this protein was boosted in some children but not in others. Antibody levels were remarkably stable in adults with individuals remaining consistently seropositive or consistently seronegative with respect to individual antigens. Individual donors were clearly able to recognise different antigens in a selective manner, recognising some regions of an antigen but failing to recognise adjacent regions of the same protein.

Few immuno-epidemiological studies have examined antibody responses to malaria antigens over a period of months or years in the same person, but in a two year study in Liberia Björkman *et al* (42) found that antibodies to the erythrocyte membrane antigen Pf155/RESA were consistent on consecutive surveys and they were able to divide their cohort into responders or non-responders. These workers ascribed the stability of responses to genetic restriction but provided no supporting evidence. Interestingly, in a longitudinal study conducted in Madagascar, where an epidemic outbreak of *Plasmodium falciparum* occurred after 20 years without malaria transmission, clear seasonal variation

in the titre of anti-Pf155/RESA antibodies was seen in both children and adults (43). This supports our conclusion that antibody levels stabilise in malaria-immune individuals but fluctuate according to recent malaria exposure in non-immunes or semi-immunes. The age at which antibody levels begin to stabilise may depend on the intensity of malaria transmission and on the antigen in question. In an area of perennial, holoendemic malaria transmission, Björkman *et al* (44) found that seropositivity to Pf155/RESA was consistent from season to season in children aged 3 to 5 years. We have previously found stable antibody responses to Pfs260/230 in children as young as 3 to 5 years old (14), and in this study responses of some children to MSP2-B3 were very consistent, but on the whole responses to merozoite surface antigens, and to the repeat region of Pfs260/230, do not seem to stabilise until later in life.

Since recognition of malaria epitopes does not appear to be genetically determined, and since antibody repertoires appear to become fixed in clinically-immune adults, we have considered the possibility that the selective recognition of these antigens may be the result of clonal imprinting (original antigenic sin).

The term "original antigenic sin" was first used to explain the observation that adult humans vaccinated against influenza produced antibodies with higher affinity for strains to which they had been exposed in childhood than for the vaccine strain (45,46). The molecular basis of original antigenic sin was subsequently shown to be the presence of cross-reactive epitopes within the polymorphic viral haemagglutinin (47,48). On primary infection, specific clones of naive T and B cells proliferate and transform into memory cells. Memory B cells have higher avidity for antigen than naive B cells since they express more surface Ig (sIg) and somatic mutation of the Ig genes results in expression of sIg of higher affinity (49). On secondary infection, memory B cells successfully compete with naive B cells for antigens which cross-react with the original infection and antibody of the original specificity is produced. Epitopes which were not present in the original infection, or which induced memory cells of lower affinity, are effectively ignored. Since relative affinity of sIg for antigen is crucial to this process, it follows that the effect can be overcome by high doses of antigen (48).

There are many features of malaria infection which make clonal imprinting a likely explanation for the selective antibody responses seen in malaria-immune individuals. Firstly, malaria antigens are composed of both conserved (or semi-conserved) and polymorphic epitopes (50), so cross-reactive immune responses would be expected. Secondly, people are infected many times by malaria and polymorphism within malaria parasite populations is extensive enough (51) that it is highly likely that successive infections would be of differing genotypes. Finally, in clinically immune individuals blood stage infections are of very low density, such that competition for antigen may occur between B cells of differing affinity. In this case, high affinity responses would be boosted and low affinity responses would eventually be lost. This may explain why the prevalence of antibodies to, for example, $MSP1_{19}$, appears to be higher in individuals recovering from a primary malaria infection (5/6 in this study) than in immune or semi-immune individuals.

As yet, there is no direct evidence that clonal imprinting occurs to malaria antigens, but the data presented here are consistent with such an explanation. Confirmation requires longitudinal studies where the development of antibody responses can be monitored in individuals whose infection history is carefully documented in terms of parasite genotype. We have recently initiated such a study in West Africa to confirm or refute the clonal imprinting hypothesis.

If the low prevalence of antibodies to certain antigens is due to clonal imprinting, injudicious vaccine design may cause problems. However, antibody responses to a synthetic vaccine antigen may be more widespread than responses to the same antigen following natural infection since presentation of an antigen in the absence of the epitope to which the immune system has become imprinted, would allow imprinting to be circumvented.

Acknowledgements.
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Figure legends.

Figure 1: Schematic representation of the MSP1 and MSP2 antigens used in this study.

Key to MSP2 proteins:

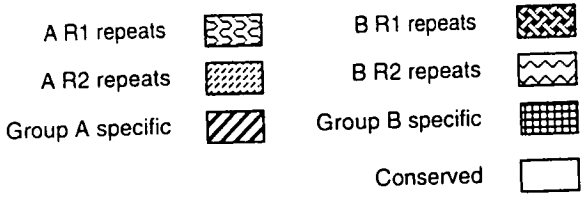


Figure 2: Longitudinal study of anti-malarial antibody levels in Gambian children. Samples were collected at 6 month intervals from June 1988 to November 1991. Values are OD units for serum tested in ELISA at a dilution of 1/1000. The horizontal lines represent the upper limit of the normal range for non-immune sera.

a. E07008, child aged 5 years in 1988

b. E24009, child aged 4 years in 1988

19-GST ● A3 ■ B3 ▲

Figure 3:

Longitudinal study of anti-malarial antibody levels in Gambian adults (aged more than 20 years). Samples were collected at 6 month intervals from November 1991 to November 1993. Values are OD units for serum tested in ELISA at a dilution of 1/1000. The horizontal lines represent the upper limit of the normal range for non-immune sera.

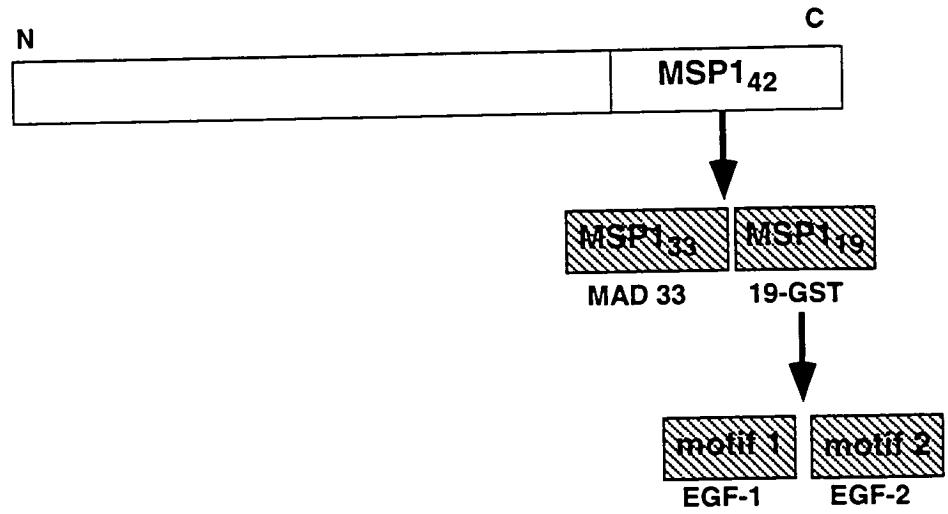
a. Donor 13AJ

b. Donor 45JK

A2 □ A3 ■ B2 △ B3 ▲ 19-GST ● MAD33 ○

Figure 1

a) MSP1



b) MSP2

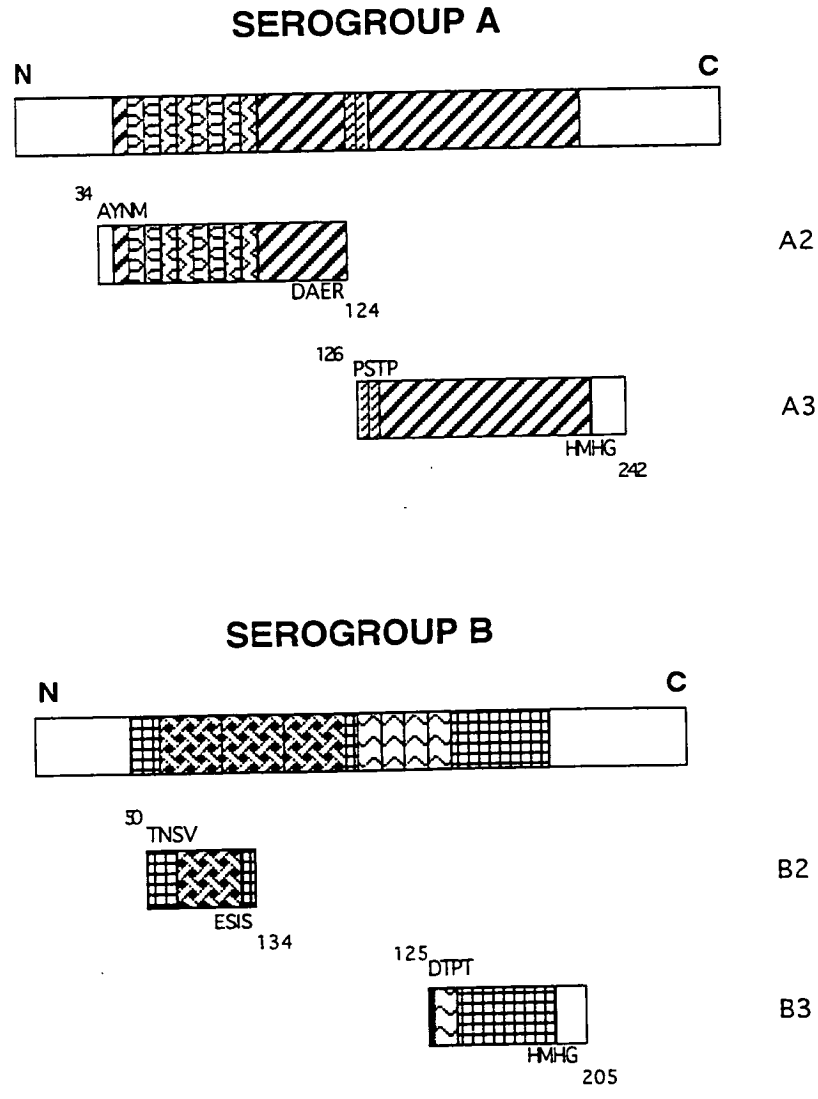
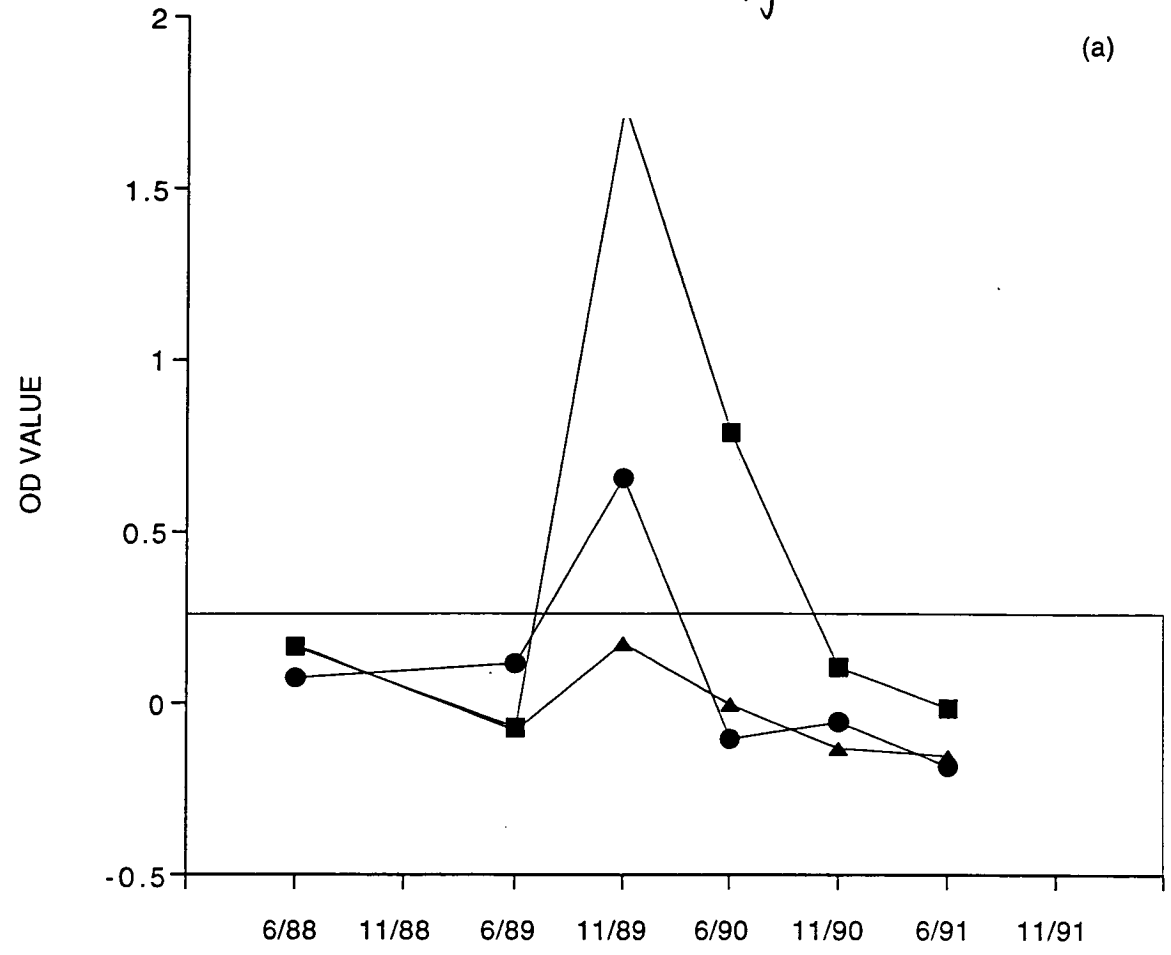
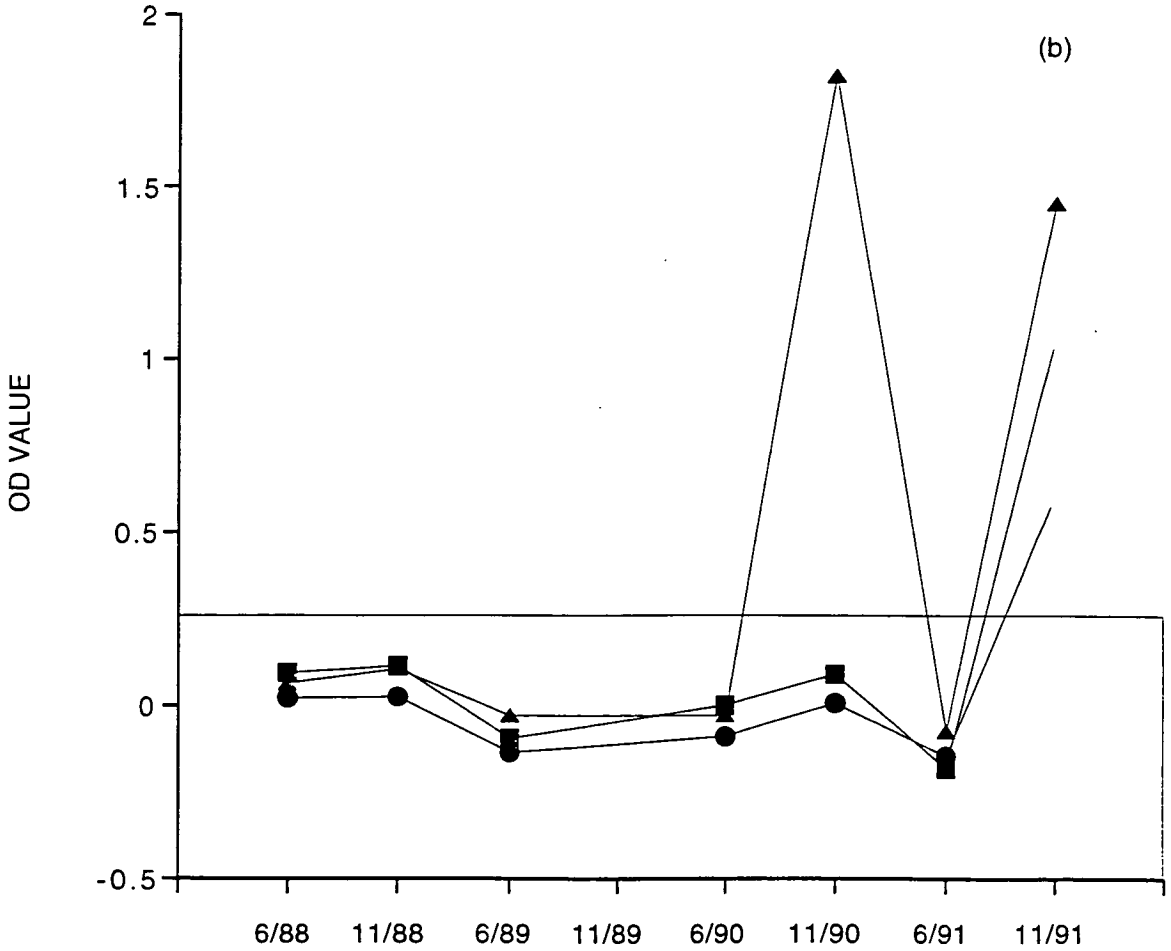


Figure 2


(a)
OD VALUE
2
1.5
1
0.5
0
-0.5
6/88
11/88
6/89
11/89
6/90
11/90
6/91
11/91


(b)
OD VALUE
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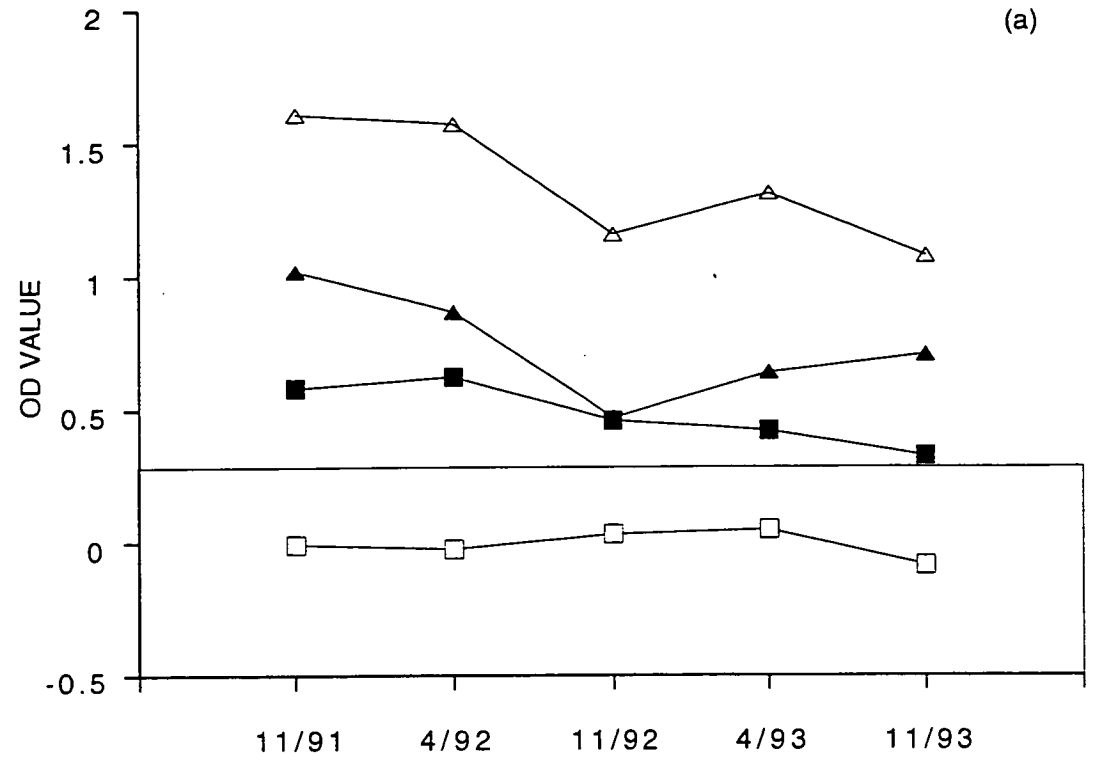
1.5
1
0.5
0
-0.5
6/88
11/88
6/89
11/89
6/90
11/90
6/91
11/91

Figure 3


(a)
2
1.5
1
0.5
0
-0.5
OD VALUE
11/91
4/92
11/92
4/93
11/93

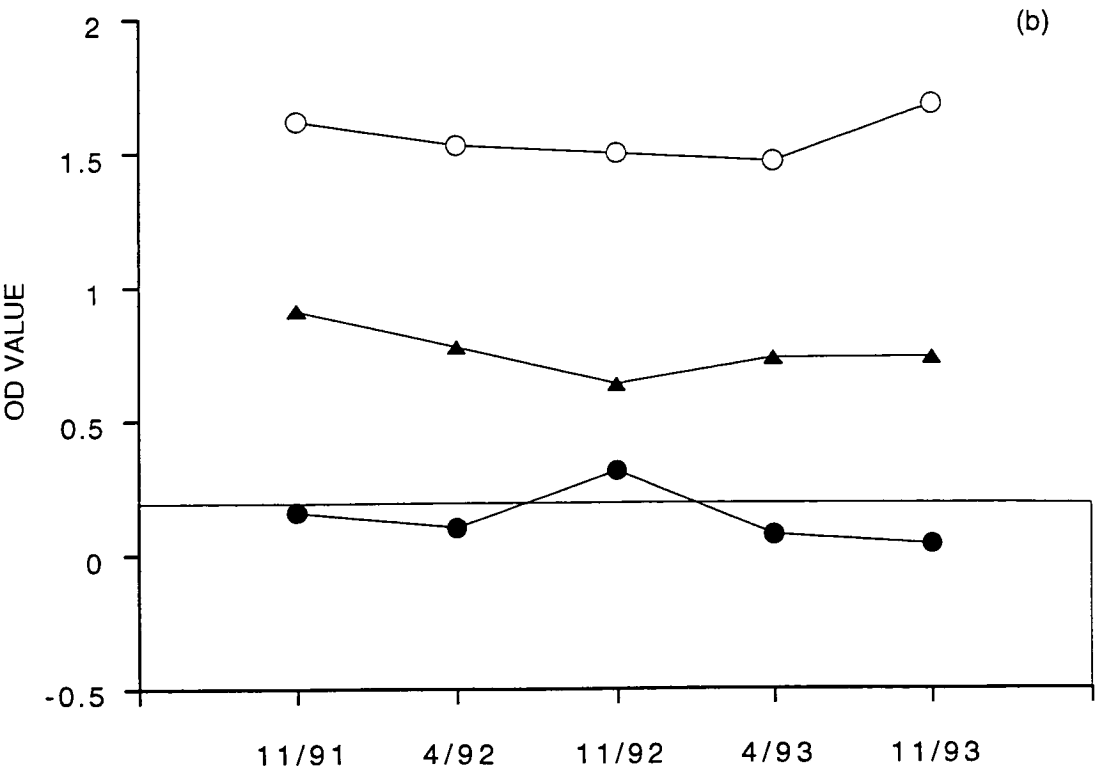

(b)
2
1.5
1
0.5
0
-0.5
OD VALUE
11/91
4/92
11/92
4/93
11/93

Table 1: Antibody recognition of recombinant MSP1 and MSP2 antigens by acute and convalescent sera from individuals experiencing their first or second attack of *P. falciparum* malaria. Sera were tested at a dilution of 1:1000. Values given are specific OD values. Values shown in bold are positive (i.e. greater than the mean plus 2SD of the naive European control sera).

	MSP1				**MSP2**				**Pfs260/230**
Serum	EGF-1	EGF-2	19-GST	MAD33	A2	A3	B2	B3	r260
92/21	**0.343**	-0.005	**0.865**	**0.701**	0.002	**0.722**	0.278	-0.125	-0.104
93/28	0.047	-0.084	**0.285**	0.403	0.425	**0.615**	**0.374**	-0.300	0.160
92/10	0.049	0.166	0.028	0.149	0.312	-0.042	**0.807**	**0.396**	-0.254
92/38	**2.119**	**0.501**	**2.113**	**1.523**	**1.288**	**1.079**	0.271	**0.639**	0.126
92/12	**0.423**	**0.322**	**0.723**	**1.123**	**0.909**	**1.108**	-0.144	-0.402	-0.005
92/33	0.071	**0.439**	**0.285**	0.086	**0.536**	0.082	0.113	-0.195	-0.301
Cut off for +ve	0.150	0.300	0.100	0.614	0.509	0.330	0.311	0.167	0.186

Table 2: Longitudinal survey of antibody responses to MSP1 and MSP2 antigens in Gambian children. Each symbol represents a separate serum sample. Samples were collected at six month intervals. - = antibody negative, + = antibody positive, + = strongly antibody positive.

		MSP1			MSP2				Pfs260/230
Donor	Age	EGF-1	$MSP1_{19}$	MAD33	A2	A3	B2	B3	r260
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	--+-+	--+-+	--+-+	-----	--+-+	-----	+-+-+	-+-++

Table 3: Longitudinal study of antibody responses to MSP1 and MSP2 antigens in Gambian adults. Each symbol represents a separate serum sample. Samples were collected at six month intervals.
- = antibody negative, + = antibody positive, + = strongly antibody positive.

		MSP1			MSP2				Pfs260/230
Donor	Age	EGF-1	$MSP1_{19}$	MAD33	A2	A3	B2	B3	r260
12	60	-----	-----	+++++	-----	+++++	+++++	+++++	----+
13	55	+++++	nd	nd	-----	+++++	+++++	+++++	-----
45	45	-----	--+--	+++++	+++++	+++++	+++++	+++++	nd
50	38	-----	-----	+++++	+++++	+++++	+++++	--+--	-----
79	50	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
110	17	+++++	+++++	+++++	-----	+++++	+++++	+++++	-----
167	16	-----	-----	+++++	----+	-----	+-+++	-----	---
109	16	----	-+-+	----	----	-+-+	----	----	-----
31	44	---++	+-+++	+++++	---++	+++++	+++++	-----	-----
104	16	---++	---++	+++++	---+-	+-+++	+++++	-----	nd
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: Association between HLA class II haplotype and antibody responses to MSP1, MSP2 and r260 proteins. Number (proportion) of individuals expressing each haplotype who were seropsitive for each antigen. χ^2 is conditional logistic regression statistic for overall association between HLA type and immune response.

a) Group A.

				MSP1				MSP2			
HLA II	Serolog specif. DR	DQ	n	EGF-1	EGF-2	$MSP1_{19}$	MAD33	A2	A3	B2	B3
1	1	w5	8	4(0.50)	5(0.63)	2(0.25)	4(0.50)	3(0.38)	2(0.25)	1(0.13)	4(0.50)
2	w15	w6	7	2(0.29)	1(0.14)	0(0.00)	1(0.14)	1(0.14)	1(0.14)	1(0.14)	1(0.14)
7	w17	w2	31	16(0.52)	16(0.52)	16(0.52)	14(0.45)	13(0.42)	9(0.29)	6(0.19)	17(0.55)
8	w18	w4	13	7(0.54)	7(0.54)	4(0.31)	4(0.31)	3(0.23)	2(0.15)	1(0.08)	5(0.38)
9	3	w44	5	0(0.00)	2(0.40)	2(0.40)	2(0.40)	1(0.20)	1(0.20)	1(0.20)	2(0.40)
11	w8		18	11(0.61)	10(0.56)	9(0.50)	11(0.61)	8(0.44)	7(0.39)	6(0.33)	12(0.67)
12	4	w2	26	16(0.62)	11(0.42)	14(0.54)	17(0.65)	12(0.46)	9(0.35)	6(0.23)	15(0.58)
14	7	w2	16	7(0.44)	8(0.50)	4(0.25)	11(0.69)	3(0.19)	3(0.19)	3(0.19)	10(0.63)
15	7	w2	58	28(0.48)	26(0.45)	27(0.47)	33(0.60)[a]	21(0.36)	21(0.36)	13(0.22)	31(0.53)
17	w8	w7	30	13(0.43)	14(0.47)	12(0.40)	15(0.50)	11(0.37)	6(0.20)	4(0.13)	13(0.43)
18	w8	w7	18	14(0.78)	11(0.61)	8(0.44)	9(0.50)	6(0.33)	8(0.44)	5(0.28)	12(0.67)
21	9	w2	43	27(0.63)	25(0.58)	21(0.49)	19(0.44)	18(0.42)	11(0.26)	9(0.21)	21(0.49)
22	w10	w5	60	36(0.60)	29(0.48)	33(0.55)	25(0.42)[b]	17(0.28)	14(0.23)	6(0.10)	27(0.45)
23	w11	w7	14	11(0.79)	7(0.50)	5(0.36)	8(0.57)	6(0.43)	2(0.14)	4(0.29)	10(0.71)
26	w11	w7	9	5(0.56)	2(0.22)	3(0.33)	4(0.44)	2(0.22)	3(0.33)	0(0.00)	4(0.44)
29	w11	w7	119	53(0.45)	54(0.45)	50(0.42)	53(0.45)[c]	38(0.32)	25(0.21)	15(0.13)	62(0.52)
34	w13	w6	10	4(0.40)	4(0.40)	2(0.20)	3(0.30)	1(0.10)	0(0.00)	0(0.00)	1(0.10)
35	w13	w1	11	4(0.36)	4(0.36)	3(0.27)	4(0.44)[d]	4(0.36)	7(0.64)	4(0.36)	5(0.45)
37	w13	w6	9	3(0.33)	4(0.44)	1(0.11)	4(0.44)	3(0.33)	0(0.00)	2(0.22)	3(0.33)
38	w13	w6	62	31(0.50)	29(0.47)	24(0.39)	27(0.44)[e]	26(0.42)	18(0.29)	9(0.15)	35(0.56)
39	w13	w7	13	10(0.77)	9(0.69)	5(0.38)	9(0.69)	3(0.23)	2(0.15)	3(0.23)	9(0.69)
χ^2				11.99	10.14	5.00	13.23	4.35	7.34	9.47	7.50
df				8	8	8	8	8	8	8	8
p				0.15	0.26	0.76	0.10	0.82	0.50	0.30	0.48

[a] $n_p = 55$; [b] $n_p = 50$; [c] $n_p = 118$; [d] $n_p = 9$; [e] $n_p = 61$

Table 4: b) Group B.

HLA II	Serolog. specif. DR	DQ	n	MSP1 EGF-1	MSP1 EGF-2	MSP1 $MSP1_{19}$	MSP1 MAD33	MSP2 A2	MSP2 A3	MSP2 B2	MSP2 B3
1	1	w5	8[a]	1(0.14)	6(0.86)	5(0.71)	5(0.71)	5(0.63)	8(1.00)	8(1.00)	6(0.75)
2	w15	w6	4	2(0.50)	3(0.75)	4(1.00)	4(1.00)	2(0.50)	4(1.00)	4(1.00)	4(1.00)
7	w17	w2	7	4(0.57)	5(0.71)	7(1.00)	7(1.00)	4(0.57)	7(1.00)	7(1.00)	7(1.00)
8	w18	w4	1	1(1.00)	1(1.00)	1(1.00)	1(1.00)	1(1.00)	0(0.00)	0(0.00)	0(0.00)
9	3	w4	7[a]	1(0.17)	4(0.67)	2(0.33)	6(1.00)	2(0.29)	6(0.86)	6(0.86)	5(0.71)
11	4	w8	5[a]	2(0.50)	3(0.75)	4(1.00)	2(0.50)	2(0.40)	5(1.00)	4(0.80)	4(0.80)
12	4	w2	1	1(1.00)	1(1.00)	1(1.00)	1(1.00)	1(1.00)	1(1.00)	1(1.00)	1(1.00)
14	7	w2	11[a]	3(0.30)	10(1.00)	8(0.80)	10(1.00)	7(0.64)	9(0.82)	9(0.82)	8(0.73)
15	7	w2	3[a]	1(0.50)	2(1.00)	2(1.00)	2(1.00)	1(0.33)	3(1.00)	3(1.00)	3(1.00)
17	w8	w7	5[a]	2(0.50)	3(0.75)	4(1.00)	4(1.00)	3(0.60)	5(1.00)	3(0.60)	3(0.60)
18	w8	w7	3	2(0.67)	3(1.00)	3(1.00)	3(1.00)	3(1.00)	3(1.00)	3(1.00)	2(0.67)
21	9	w2	12[b]	3(0.30)	9(0.90)	9(0.90)	9(0.90)	7(0.58)	12(1.00)	11(0.92)	11(0.92)
22	w10	w5	16	5(0.31)	14(0.88)	13(0.81)	15(0.94)	10(0.63)	14(0.88)	13(0.81)	13(0.81)
23	w11	w7	5[b]	1(0.33)	3(1.00)	3(1.00)	3(1.00)	4(0.80)	5(1.00)	4(0.80)	4(0.80)
26	w11	w7	4	2(0.50)	2(0.50)	3(0.75)	3(0.75)	2(0.50)	4(1.00)	4(1.00)	1(0.25)
29	w11	w7	60[c]	22(0.41)	46(0.85)	42(0.78)	53(0.98)	31(0.52)	54(0.90)	54(0.90)	44(0.73)
34	w13	w6	4	0(0.00)	3(0.75)	2(0.50)	4(1.00)	2(0.50)	4(1.00)	3(0.75)	4(1.00)
37	w13	w6	3	0(0.00)	3(1.00)	3(1.00)	3(1.00)	2(0.67)	3(1.00)	3(1.00)	3(1.00)
38	w13	w6	28[a]	10(0.37)	23(0.85)	24(0.89)	27(1.00)	16(0.57)	25(0.89)	26(0.93)	25(0.89)
39	w13	w7	9	3(0.33)	7(0.78)	6(0.67)	9(1.00)	7(0.78)	9(1.00)	9(1.00)	7(0.78)
χ^2				2.82	6.13	2.79	4.44	5.48	0.05	3.43*	2.69
df				5	5	5	5	5	3	5	5
p				0.73	0.29	0.73	0.49	0.36	1.00	0.63	0.75

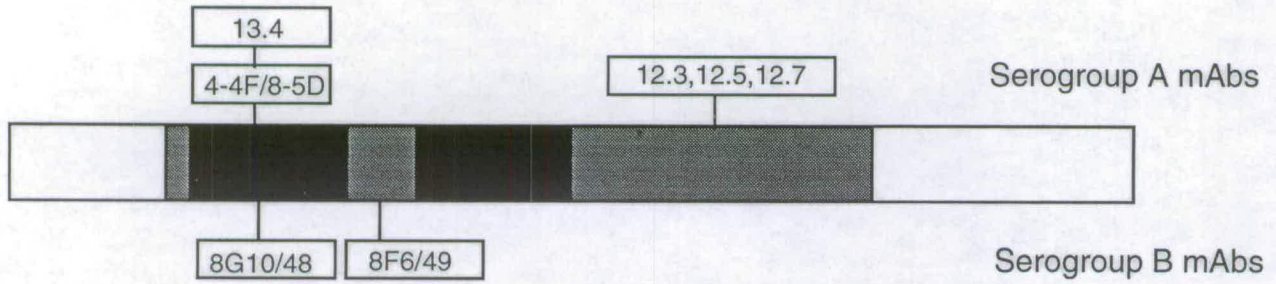
a n = 1 less for MSP1 proteins; b n = 2 less for MSP1 proteins; c n = 3 less for MSP1 proteins.
* results for unconditional (standard) logistic regression since conditional model did not converge for any combination of haplotypes.

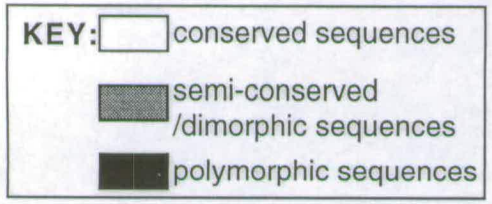
Table 5: Comparison of antibody responses to MSP1 and MSP2 antigens in adult, monozygous and dizygous Gambian twins. The first figure in each column represents the OD value for one twin, the second figure is the OD value for the other twin in the pair. Discordant results (one above and one below cut off) are underlined. * Cut off values for positive responses (mean + 2SD of control sera).

	$MSP1_{19}$		MSP2-A2		MSP2-A3		MSP2-B2		MSP2-B3	
MZ1	0.123	0.138	0.010	0.569	0.068	0.086	0.010	0.637	0.189	0.880
MZ2	0.769	0.822	1.992	1.854	1.088	1.184	2.481	2.228	1.462	1.480
MZ3	0.114	0.163	0.509	0.849	0.223	0.269	0.680	0.850	0.171	1.512
MZ4	0.128	0.106	0.526	0.311	0.172	0.002	0.007	0.069	0.128	0.094
MZ5	0.289	0.138	0.461	1.216	0.120	0.792	0.121	1.747	0.084	0.279
MZ6	1.220	0.482	0.700	0.601	0.254	0.072	0.418	2.313	1.751	1.383
MZ7	0.047	0.681	0.718	0.986	1.472	0.970	1.760	2.219	0.718	1.935
MZ8	1.358	1.738	1.986	0.254	2.261	0.150	1.932	1.359	1.354	1.135
MZ9	0.079	0.341	0.881	1.050	1.571	1.241	0.361	2.482	0.967	1.864
MZ10	0.075	0.085	0.053	0.513	0.059	0.213	0.049	0.300	0.061	0.359
MZ11	0.315	0.129	0.792	0.957	0.560	1.173	1.758	0.339	2.339	0.012
MZ12	0.457	0.843	0.181	0.788	2.185	2.039	1.267	0.434	2.083	1.673
MZ13	0.153	0.205	0.100	0.841	0.689	0.929	0.082	0.636	1.029	2.055
MZ14	0.230	0.571	0.148	0.480	1.425	2.165	1.675	0.544	2.188	1.159
MZ15	0.225	0.103	0.387	0.129	0.858	0.404	1.357	0.673	0.694	0.455
DZ1	0.171	0.139	0.486	0.374	0.265	0.052	0.328	0.613	0.576	0.109
DZ2	0.339	0.147	0.541	0.266	0.237	0.248	2.140	0.329	1.600	0.162
DZ3	0.146	0.157	0.301	1.837	0.107	0.305	0.576	2.273	0.370	0.508
DZ4	0.099	0.106	0.203	0.163	0.537	0.092	0.021	0.084	0.694	0.246
DZ5	0.105	0.256	0.185	0.077	0.010	0.054	0.393	0.120	0.214	0.374
DZ6	0.140	0.123	1.176	0.596	0.506	0.486	0.411	0.704	0.030	1.096
DZ7	0.369	0.806	0.873	1.066	0.555	0.285	0.148	0.744	0.352	1.712
DZ8	0.096	0.082	1.391	0.936	1.874	1.250	0.089	0.819	0.041	0.327
DZ9	0.222	0.390	0.858	2.118	1.291	2.166	0.751	2.290	0.292	1.919
DZ10	0.097	0.076	1.411	0.417	1.703	2.267	1.215	1.514	2.193	0.826
DZ11	1.414	0.197	0.557	0.486	1.136	0.958	1.935	0.754	1.842	1.607
DZ12	0.348	0.145	0.120	0.027	0.808	0.121	0.301	2.121	1.957	2.076
DZ13	0.579	0.607	1.164	1.989	1.500	1.449	0.958	1.379	0.828	1.341
DZ14	0.181	0.332	1.558	0.674	1.077	1.990	1.374	1.031	1.407	2.023
DZ15	0.073	0.168	0.119	1.146	0.321	2.069	1.976	0.649	1.194	2.264
DZ16	0.123	0.115	0.072	0.109	2.035	0.542	2.078	0.051	2.101	0.128
DZ17	0.067	0.077	0.029	0.032	0.495	0.716	0.030	0.561	0.574	0.997
DZ18	0.086	0.080	0.607	0.125	0.831	0.723	0.194	0.045	0.478	0.227
DZ19	0.332	0.167	0.087	0.351	0.739	0.256	0.322	0.433	0.763	1.214
DZ20	0.086	0.163	0.574	0.657	1.497	0.722	0.603	0.622	1.694	0.452
DZ21	0.061	0.061	0.030	0.270	0.117	0.369	0.148	0.148	0.124	0.104
* >	0.098		0.296		0.185		0.096		0.130	
χ^2	0.129		3.14		0.038		0.038		0.073	
p	> 0.5		>0.05		>0.5		>0.5		>0.5	
U	101.5		137.5		140.0		134.5		137.0	
p	0.07		0.53		0.59		0.47		0.5	

Appendix 1B

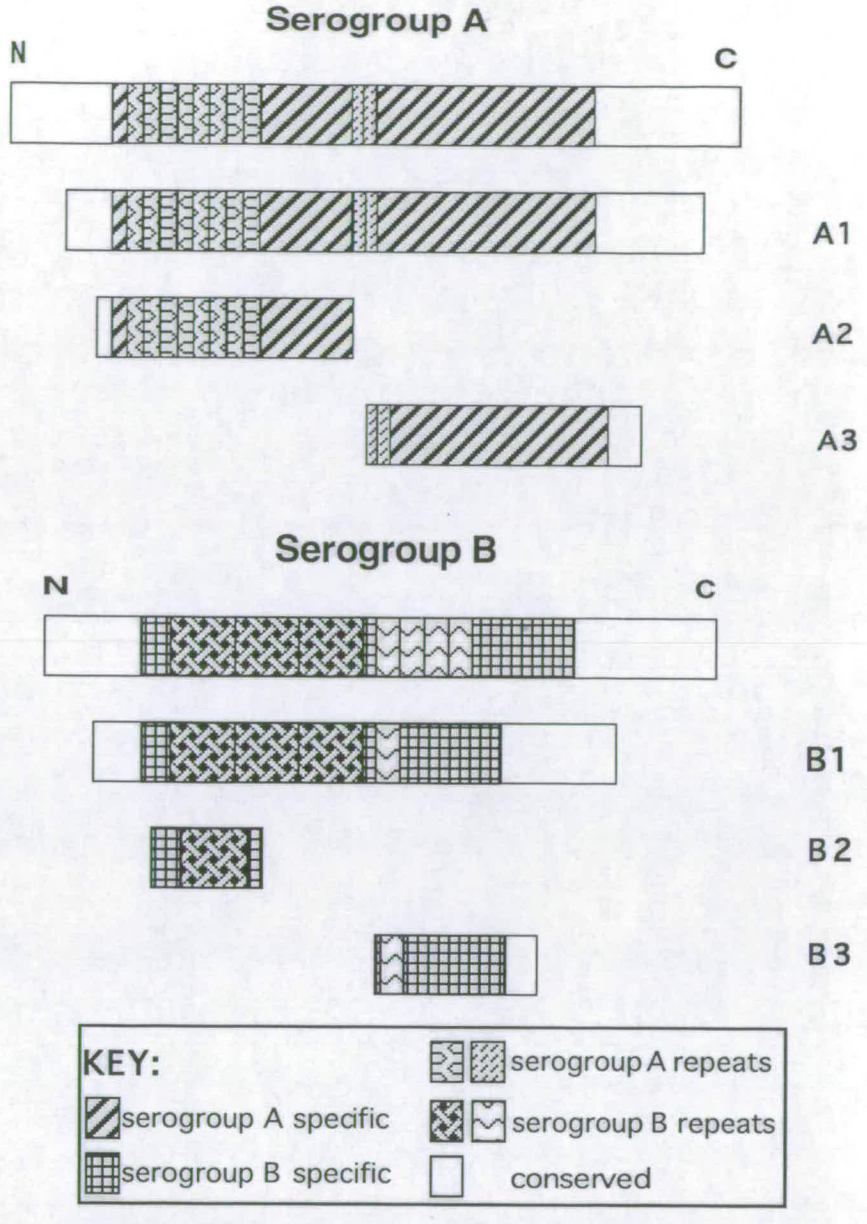
Epitope specificities of MSP2 monoclonal antibodies for serogroup A and B dimorphic and polymorphic domains.





Appendix 1A

Schematic representation of MSP2 recombinant proteins



R.Taylor, PhD Thesis 1995 "Immune responses to the *Plasmodium falciparum* antigen MSP2"