Molecular Diversity and Immunological Properties of the *Plasmodium falciparum* Merozoite Surface Protein 2 (MSP2)

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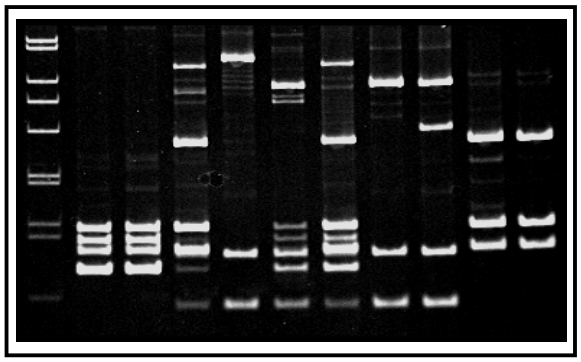

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Front page photo
Polyacrylamide gel electrophoresis
Longitudinal samples of a Tanzanian child analysed by
PCR-RFLP genotyping of the polymorphic *msp2* gene

Plasmodium falciparum parasites become distinguishable.

1. Introduction

1.1 Molecular Epidemiology

Traditionally, the interest of scientists in infectious diseases has been focusedon the role of the infectious agents as the origin of the disease in the higher organism. Their interest has been expanded to include the genetic structure, the immune response and the evolutionary consequences of public health interventions. Molecular epidemiology can be a potent tool in the understanding of infectious diseases, as e.g. *Plasmodium falciparum* malaria, and the design and evaluation of interventions for their treatment (drug trials) and prevention (exposure reducing measures and vaccines). The practical goals of molecular epidemiology are to identify the infective agents responsible for the disease (e.g. distinguish between the different human pathogenic *Plasmodium* species), determine their physical sources, their biological (phylogenetic) relationships, and their routes of transmission. Genes responsible for their virulence (e.g. var genes in *Plasmodium falciparum*), vaccine-relevant antigens (PfMSP1/PfMSP2/PfAMA1) and drug resistance (Pyrimethamine/Sulfadoxine resistance) have to be characterized. The use of molecular methods may lead to the observation that arrays of symptoms could be attributed to particular genes, as it was possible to demonstrate in other infectious diseases, e.g. *Vibrio cholerae* (Mekalanos et al. 1997) or *Escherichia coli* (Ott et al. 1991).

A detailed knowledge of the overall genetic structure of a pathogen population is not essential for epidemiological tracking. Rather, the challenge is to use typing characters that change in a range and at a rate that are informative for the particular question. The knowledge of parts of the genetic structure may be important to understand (and ideally predict) the responses of pathogen populations to selective pressures imposed by host immunity both natural and vaccine-induced, and may be important for the effective management of antiparasitic interventions. For tracking the transmission of an infectious agent in a special region, a relatively fast-changing genetic marker will be useful. On the other hand the same marker would not be valuable for looking at trends in the global population of *Plasmodium falciparum* over many decades. Polymorphic marker genes have been shown to be very useful tools to evaluate intervention strategies, to monitor prevalence of infectious agents or changes in frequencies of genotypes. But those selected markers must be distinguished from non-selected markers as microsatellites (Ferdig and Su 2000) and it depends on the scientific approach which marker system should be chosen.

A microbe proliferates in host tissue in which it is normally limited by physical and chemical barriers or by predators and competitors (phagocytic and other cells of the host immune system, and other micro-organisms). These microbial invaders often have phenotypes that enhance their survival in this habitat (virulence factors, including the ability to attach to and invade cells and to resist or evade the host's constitutive chemical and cellular defences). Evolution occurs in both the parasite population and the immune system of the host. Selection in the parasite population favours mutants (antigenic variants) that are able to evade predators or avoid predation (see below) and competition by invading different micro-habitats (cells and tissues) where the immunological predation pressure is less intense (erythrocytes do not express MHCI molecules). The key feature of science is not measurement (this is just a tool), but understanding. To understand the occurrence of malaria in human populations, the structure of the parasite population, the survival strategies of the parasite and of the host reflected by the immune response, and the implications of interventions have to be investigated.

1.2 Polymorphism and Variation - survival strategies with different goals

Polymorphism is defined as the expression of distinct alleles of a gene at a single gene locus in different clones of the parasite (Reeder and Brown 1996), whereas variation shows the ability of a clonal population to switch the antigenic phenotype with unchanged genotype. As pointed out by Brunham et al. (1993), allelic polymorphism is usually a `between-host` survival strategy, providing an individual pathogen with maximum fitness for successful infection of its host. The biological role and impact of antigenic variation is usually considered as a `within-host-mechanism` that allows parasite survival in an immunocompetent host (Brunham et al. 1993). Antigenic variation creates diverse individuals, by switching its antigenic phenotype during the course of long-term infection of a single infected host. Both strategies are realized as immune evasion mechanisms in malaria.

1.3 Immune Evasion by Multiplying Short Sequences - repeats

A further strategy for immune evasion may be based in the nature of the antigenic sequence itself. The malaria parasite may use repetitive, immunodominant epitopes as a mechanism to evade the immune response of the human host (Anders 1986) and many of malaria antigens contain tandem arrays of relatively short sequences. Maintenance of degeneracy within a repeat set, and the

existence of cross-reacting epitopes in many genes of *P. falciparum* as a consequence of short repetitive sequences and the biased amino acid composition, have been suggested to interfere with the normal maturation of high affinity antibodies. The existence of cross-reacting antibodies has often complicated the problem of identifying specific *Plasmodium* gene products and protective immune responses (Berzins and Anders 2000). In addition, repetitive epitopes may induce T cell-independent B-cell activation (Schofield 1991), suppressing antibody production to more relevant parts of the antigen. Another strategy for immune evasion of *Plasmodium falciparum* was suggested, based on the theory of 'original antigenic sin' (Good et al. 1993) as it was shown with the influenza virus. It was supposed, that the prior exposure to one strain of *Plasmodium falciparum* was able to divert the immune response to a second challenging strain by focussing the immune response on the shared (cross-reactive) epitopes.

1.4 Merozoite Surface Protein 2 (MSP2) – genetic structure

Polymorphic antigens have been described in several parasite life cycle stages but are particularly a feature of the antigens associated with the surface of the asexual blood-stage merozoites. The merozoite surface protein 2 (MSP2) is one of the well-characterized surface proteins of *P. falciparum*. MSP2 is an integral membrane (GPI anchored) protein with highly conserved carboxy- and amino-terminal regions flanking a central variable region that is composed of non-repetitive semi-conserved sequences surrounding repetitive highly variable repetitive sequences (Anders and Smythe 1989; Smythe et al. 1990). Although the protein is highly polymorphic, sequencing of MSP2-alleles and PCR-RFLP from a large number of parasite isolates from different geographical locations has shown a virtually dimorphic structure of the molecule (Thomas et al. 1990; Smythe et al. 1990; Fenton et al. 1991; Marshall et al. 1991; Smythe et al. 1991; Snewin et al. 1991; Prescott et al. 1994; Marshall et al. 1994; Dobano et al. 1997; Irion et al. 1997; Felger et al. 1999a). Thus, two allelic families exist, IC1/3D7 and the FC27 families (Smythe et al.1990), which correspond to the A and B serogroups defined by reactivity with monoclonal antibodies (Fenton et al. 1991). The finding of structural identity between *msp2* alleles isolated from human hosts geographically and chronologically separated (Dobano et al. 1997) is consistent with the idea that the evolution of *msp2* variants may be limited by functional compulsion. In addition, recently the first homologous gene for a *Plasmodium* species (*P. reichenowi*) other than *P. falciparum* was identified showing almost identical conserved regions

(Dubbeld et al.1998). However, in *Plasmodium falciparum* a high rate of amino acid changes is seen in polymorphic regions. This finding is thought to be indicative of immune selection acting to promote antigenic diversity (Hughes and Hughes 1995). As in many malarial antigens MSP2 contains extensive arrays of tandemly repeated short amino acid sequences, and much of the antibody response induced by malaria infections is directed against epitopes encoded by these repeats (Al-Yaman et al. 1994; Taylor et al. 1995). Detailed genetic structure of *msp2* is shown in figure 1.

The merozoite surface protein 2 has been verified as a suitable molecular marker when individual *Plasmodium falciparum* infections need to be distinguished currently present in a sample. Using the PCR technique and primers to conserved regions, it is feasible to characterize individual parasites obtained from a subject at one point in time and this possibility of identifying individual parasite genotypes over a time period renders detailed studies of infection dynamics. The *msp2* locus is not only a useful marker gene for molecular epidemiology purposes, but the expressed protein is also a promising vaccine candidate (Saul et al. 1999; Genton et al. 2000; Genton et al. 2001). Due to the location on the surface of the parasite and the immunological features of MSP2, the protein was suggested as part of a three component vaccine, called Combination B, recently tested in Papua New Guinea (Saul et al. 1999, Genton et al. 2000; Genton et al. 2001). In immunological studies especially concerning antigenic features of MSP2, genotyping may also offer important insides on the diversity of antigenic challenge.

1.5 The First Year of Life - the host encounters the parasite

In areas highly endemic for malaria, repeated plasmodial infections result in the development of acquired immunity which although not sterilising, protects older children and adults against severe disease (Barragan et al. 1998; Gupta et al. 1999). As a result, mortality due to malaria is mainly restricted to early childhood. Where transmission is perennial, significant clinical protection from severe morbidity was observed up to the third month of life. In the seasonal transmission area, disease rates rose after the sixth month of life (Snow et al. 1998). The period of highest susceptibility to clinical malaria among infants in such highlyendemic areas occurs between the ages of 4 and 12 months (Kitua et al. 1996), when the bulk of maternal antibodies decreases and the own immune response against the parasites is weak. Very young infants in areas holoendemic for malaria show mild symptoms when infected with *P. falciparum*, and they are comparable to

partially immune adults with respect to parasite densities and recovery rates from infection (Kitua et al. 1996). This protection is thought to be conveyed by foetal haemoglobin (Pasvol et al. 1977) or maternal antibodies, which can be detected in cord blood and which are acquired across the placenta (Desowitz et al. 1993, Rasheed et al. 1995). In a study of Tanzanian infants, the incidence rate of malaria-attributed morbidity reached a peak at six months of age (Kitua et al. 1996), and significant declines in the risk of severe malaria were demonstrated from the sixth month of age (Snow et al. 1998).

These data provide direct evidence for the very early acquisition of clinical immunity. It is hypothesised that clinical protection during the first few months of life in high transmission settings allows active immunisation to occur and contributes to a reduction in the overall risks of severe complicated malaria throughout childhood (Snow et al. 1998).

1.6 Humoral Immunity to *P. falciparum* - a general review

Acute clinical malaria, which is often life-threatening in the case of infection with *P. falciparum*, is associated with replication of the asexual blood stage parasite in circulating erythrocytes (Patino et al. 1997). The responsible parasite clone is able to reproduce to parasite numbers over the fever threshold due to the lack of appropriate defence mechanisms of the host. This parasite clone is called 'virulent'. The development of a vaccine against the erythrocytic stages has been hampered by the lack of understanding of the nature and specificity of immunity in humans, or in other words by the lack of understanding of survival strategies of the parasite. While molecular technology has permitted identification and analysis of many blood-stage proteins, their immunological significance for the host and biological function for the parasite are not well understood (Long 1993).

It is well established that humoral immunity, besides cell-mediated immunity, is important in malaria, and passive transfer of serum has been shown to have a protective or at least modifying effect on the disease (Cohen et al. 1961; Bouharoun-Tayoun et al. 1990; Druilhe and Perignon 1997; Kumaratilake et al. 1997). Experiments with antibodies purified from the sera of African adults who were clinically immune to malaria and given by passive transfer to susceptible children have established that immunoglobulin G (IgG) is at least a main component of the defence against the asexual blood stage of *P. falciparum*. Although the parasite neutralizing IgG fraction did not inhibit merozoite invasion by itself *in vitro*, an antibody-dependent cellular inhibition of parasite

growth together with monocytes was shown (Druilhe and Perignon 1994). Antibodies are directed either against a number of identified proteins on the parasite itself or against parasite-derived proteins expressed on the surface of the infected erythrocyte during intraerythrocytic development of the parasite (Giha et al. 2000). Although the potential of many antigens as targets for immune responses has been suggested, little is known about the mechanisms of protection *in vivo*. Once merozoites have been released from the schizonts, cytophilic antibodies may mediate parasite elimination by complement-dependent lysis or by cellular effector mechanisms (Druilhe and Perignon 1997). An almost exclusive restriction of the humoral immune response to MSP2 to the IgG3 subclass was observed by several investigators (Taylor et al. 1995; Ferrante and Rzepczyk 1997). Hence, these IgG3 antibodies are potentially effective mediators of protection by their cytophilic nature.

1.7 Anti-MSP2 Immune Response - the special example

Involvement of MSP2 in protective immune responses was indicated first by the merozoite invasion inhibitory effect of mAbs to the antigen (Clark et al. 1989; Epping et al. 1988; Ramasamy et al. 1990). The importance of MSP2 in the immune response is also suggested by the polymorphism of the central repeat region of the antigen (Hughes and Hughes 1995; Escalante et al. 1998). These repeat sequences have been shown to be antigenic and are recognized by antibodies induced by exposure to infection with malaria parasites (Al-Yaman et al. 1994; Taylor et al. 1995). The presence of antibodies against the conserved and semi-conserved regions was found to develop at later ages after a life long exposure to malaria and was then associated with fewer fever episodes and less anemia (Al-Yaman et al. 1994).

Many features of acquired immunity to malaria in humans indicate that the protective response may be strain-specific (Staalsoe and Hviid 1998), e.g. there is the possibility that infection induces a form of genotype-specific immune response against the MSP2 antigen that biases against reinfection by parasites bearing identical forms of MSP2 (Eisen et al. 1998). Certain *msp2* genotypes have been associated with higher parasite densities and morbidity (Engelbrecht et al. 1995) and previous studies on older children have shown that concurrent multiple infections are associated with protection against clinical malaria in endemic areas (Al-Yaman et al. 1997; Beck et al. 1997; Färnert et al. 1999).

Studies from Papua New Guinea have shown a negative correlation between antibody levels to

MSP2 and fever recall in the previous week (Al-Yaman et al. 1994). More important though is the demonstration in a prospective study of an association between antibody levels to 3D7 MSP2 at baseline and resistance to clinical malaria during the one year follow up (Al-Yaman et al. 1995). Antibody levels to 3D7 MSP2 were also found to be lower among children who died of cerebral malaria than among those who survived indicating a protective role for these antibodies against severe malaria (Al-Yaman et al. 1997a).

A prospective longitudinal study was conducted for one year in Gabon and Cameroon to examine the relationship between specific *in vitro* immune responses to two MSP2 peptides and susceptibility to clinical malaria (Migot-Nabias et al. 1999). These peptides correspond to the N-terminal conserved region and to the family specific region of alleles of the 3D7 allelic family, respectively. Clinical protection was associated with elevated antibody levels to both MSP2 peptides. In contrast to results with other antigens, there was no significant difference in the ages of individuals with a certain antibody titre to the full length recombinant or parasite-derived MSP2 molecule, but antibodies to MSP2 did correlate with parasitemia (Stowers et al. 1997).

In adults antibody levels after two infections can reach the peak levels of antibodies obtained in immune individuals (Baired 1998; Stowers et al. 1997; Gupta et al. 1999). The strength of an antibody response to antigen rMSP2 and *P. falciparum* MSP2 was shown to be very high even in youngest age groups (0-5 years) and antibody levels were significantly higher in parasitemic individuals in this age group than in nonparasitemic individuals (Stowers et al. 1997).

The location of MSP2 on the merozoite surface and the ability of human anti-MSP2 antibodies to inhibit merozoite invasion together with MSP2 specific immune protection *in vivo* makes this antigen of considerable interest as a vaccine candidate (Fenton et al. 1989; Clark et al. 1989). Hence, MSP2 is one component of a subunit vaccine against malaria which was recently assessed in the field among individuals living in an area of Papua New Guinea highly endemic for *P. falciparum* (Saul et al. 1999; Genton et al. 2000; Genton et al. 2001 Combination B).

1.8 Bed net users versus non-bed net users - an intervention strategy

In recent years, exposure reducing measures such as insecticide treated bed nets (ITNs) have been promoted as public health tools in malaria endemic areas. On the one hand several efficacy trials have given strong evidence that the use of ITNs improves survival and reduce malaria-attributable morbidity (Alonso et al. 1991; D'Allessandro et al. 1995; Binka et al. 1996; Nevill et al. 1996; Habluetzel et al. 1997; Fraser-Hurt et al. 1999; Diallo et al. 1999). On the other hand there is

some evidence for the very early acquisition of clinical immunity in children. It is hypothesized that clinical protection during the first few months of life in high transmission settings allows active immunization to occur and contributes to a reduction in the overall risks of severe complicated malaria throughout childhood (Snow et al. 1998). However, if children are protected from infections with ITNs, the development of malaria-specific immune response might be impaired. There is evidence from studies that children using a net may have significantly reduced levels of *P. falciparum*-specific IgG or IgM, when compared to fully exposed children not using a net (Genton et al. 1994; Snow et al. 1996). Blood samples of a bed net study used for genotyping parasite infections showed a significant decrease of 16.4% in microscopically determined *P. falciparum* prevalence in children in the ITN group at the end of the trial (Fraser-Hurt et al. 1999). However, no significant difference was observed in parasite density or multiplicity of infection determined by PCR-RFLP among infected children with or without ITN (Fraser-Hurt et al. 1999). It was assumed that chronic infections formed a large proportion of all infections. Hence limited reduction in exposure would not have much impact on multiplicity and premunition might be still established (Smith et al. 1999b).

1.9 Dynamics of infection - ups and downs

The number of concurrent infections with *Plasmodium falciparum* might be an important indicator of the degree of acquired immunity against malaria, since the multiplicity of infections increases with age over the first few years of life in parallel with the increase in clinical immunity (Smith et al. 1999a). In addition persisting infections have been proposed to provide protection against clinical disease when superinfected with a new parasite (Al-Yaman et al. 1997b; Beck et al. 1997; Färnert et al. 1999). The potential benefits of multiple malaria infections may have important implications for interventions aimed at reducing parasite prevalence in areas with high malaria transmission. The effect of insecticide treated bed nets on the dynamics of multiple *Plasmodium falciparum* infections was investigated by Smith et al. (1999b) using PCR-RFLP genotyping data from *msp2*. For *msp2* genotypes of the FC27 allelic family there was no difference in multiplicity of infections detectable in children aged initially 5 to 24 months with or without sleeping under a bed net. Hence multiplicity of infection seems unaffected by the infection rate. In the same study the average duration of infections of parasites with a *msp2* allele of the

FC27 family was shown to be considerably higher than in infants (Felger et al. 1999b; Smith et al. 1999b).

It was suggested that differences between individuals in parasitologic profiles may be the result of qualitative differences in protective immunity (Färnert et al. 1999). There might be a difference in the virulence of parasites expressing different MSP2 alleles to persist, reflected through the ability of the immune system to eliminate those parasites. Investigation of features of the 3D7 allelic family concerning duration of infection and recovery rates will give additional information of parasite survival strategies.

1.10 In summary,

this review of the literature has demonstrated that mechanisms of the immune response against the pathogen *Plasmodium falciparum* are not well understood. Although the potential of many antigens as targets for parasite neutralizing immune responses has been suggested, little is known about the mechanisms of protection *in vivo* against the disease and the relative importance of the different antigens as targets of this protection. The merozoite surface protein 2 of *Plasmodium falciparum* is an example for the relationship between polymorphism of an antigen and the immune response of the human host. Any attempt to elucidate the development of the immune response to this antigen may therefore yield valuable information on the evolution of the polymorphism providing the evasion from the pressure of the immune system.

Following this line of reasoning, the thesis presents MSP2 as a polymorphic marker gene which is useful to determine malariological profiles in individuals and to evaluate intervention strategies (Paper I). In addition ti being a tool, MSP2 has been identified in other studies to be a promising vaccine candidate. Therefore, the apparent different immunogenicity of the distinct regions of MSP2 (Figure 1) was investigated by vaccinating Balb/c mice and in serological assays with human sera. (Paper V). Consecutive serum samples from Tanzanian infants initially between 5 and 15 months of age from an area where transmission of malaria is intense and perennial were tested in ELISA to investigate the development of the antibody response in early childhood and to analyze the effect of the use of ITN to the immune response in the same period (Paper II). A new methodology based on a latent class model for the analysis of biomedical assays which are expected to classify samples into two groups was expounded (Paper IV). The dynamics of the antibody response to MSP2 are described in view of the dynamics of *P. falciparum* infections

measured by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the merozoite surface protein 2 (Paper III). Investigation of features of the 3D7 allelic family concerning duration of infection and recovery rates will give further information on parasite survival strategies, which may be different for parasite encoding a *msp2* allele of the FC27 family.

The methods and results of this study are finally discussed in a broad framework that includes both anti-malaria immunity and molecular epidemiology, and lastly perspectives are presented for research and analysis strategies in the future.

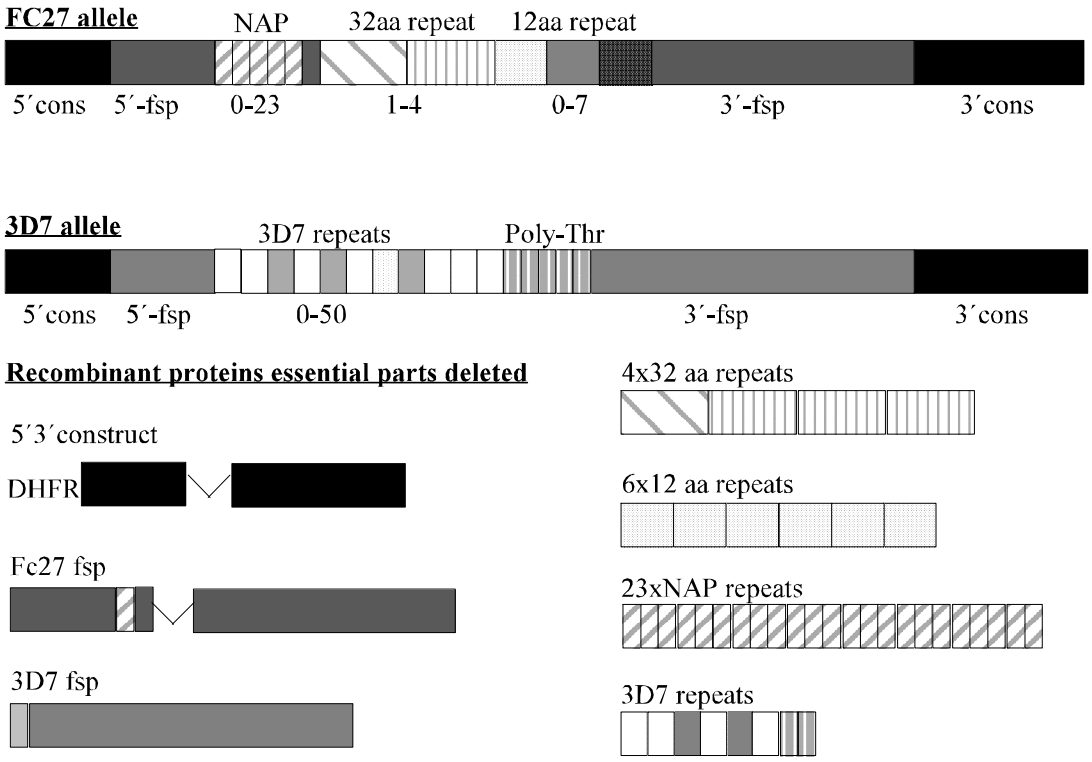

Figure 1: Schematic structure of the merozoite surface protein 2
The two allelic families and the recombinant proteins with essential parts deleted are shown. aa: amino acid, cons: conserved, fsp: family specific part, NAP: single letter code for amino acids, Thr: Threonine

2. Definitions

Some definitions of significance for this study (see Walliker 1983):

Allele: one of several alternative forms of a gene.

Allelic family: alleles of a gene are grouped with regards to similar characteristics, e.g. the allelic families of *msp2*, Fc27- and IC1/3D7-allelic families.

Clone: a set of genetically identical parasites derived from one parasite/bacterium by asexual reproduction.

Genotype: a genetic characteristic of a parasite, the type of allele found at a polymorphic locus in an individual.

Isolate: a sample of parasites collected at one time from an individual (not cultured).

Line: parasites which have been passaged *in vitro*, initially starting from one isolate. If parasites have been cloned in the laboratory, they are referred to as cloned lines.

Mother clones: commonly used in laboratory work, but may lead to confusion. Here, it is used to describe a collection of hybridoma cells in one well, that produce specific antibodies. These cell populations are not clonal.

Multiplicity of infection (MOI):
number of infecting genotypes in an isolate (Beck et al. 1997).

Strain: used only for specific bacteria applied for expression purposes.

3. Goal and Objectives

The GOAL of this study was to evaluate the molecular diversity of a molecular marker gene of *Plasmodium falciparum*, the merozoite surface protein 2 (MSP2), and its relevance to the development and the dynamic of the humoral immune response in children, initially 5-15 months old, measured by the antibody response to the antigen MSP2 in a longitudinal setting in an area highly endemic for *P. falciparum* malaria.

Objectives

1. Examine the molecular diversity of merozoite surface protein 2 (*msp2*) by PCR-RFLP.

2. Assess the immunogenicity of the rMSP2s and their similarity to parasitial PfMSP2

    A. Perform the recombinant expression and purification of different MSP2 protein constructs with defined parts deleted in a procaryotic expression system.

    B. Evaluate the immunogenicity of the produced recombinant proteins in mice.

    C. Estimate the grade of similarity of the recombinant proteins to natural PfMSP2.

    D. Achieve the production of monoclonal antibodies to the conserved parts of PfMSP2.

3. Investigate the development of the antibody responses to rMSP2 in early childhood.

    A. Establish the ELISA method for all recombinant proteins.

    B. Perform the ELISA with recombinant proteins and

        1. Sera from malaria non-exposed European individuals

        2. Sera from malaria exposed Tanzanian children initially aged 5-15 months

    C. Quantify the antibody response and assess proportion of positivity of the exposed individuals.

    D. Explore the longitudinal antibody response in correlation to genotypes of the infecting parasites.

    E. Evaluate the dynamic of *P. falciparum* infections in relationship to the dynamic of the specific antibody response.

4. Material and Methods

4.1 Material

4.1.1 Cell lines, *Plasmodium falciparum*-cloned lines and *Escherichia coli*-strains

All *P. falciparum* cloned lines were received from the laboratory of R. Brun at the Swiss Tropical Institute, Basel (Table 1.1-1). Erythrocytes for parasite cultures were obtained from members of the STI or Blutspendezentrum beider Basel, Basel, CH. EDTA-stabilized donor blood of the blood group A Rh+ was washed and used as a 50% suspension. The *E. coli* strains used are listed in table 1.1-2.

Table 1.1-1 *P. falciparum* cloned lines

Name	*msp2* genotype	Source
K1	K1-allele	MMV Laboratory of R. Brun STI, Basel, CH.
H3B	WOS3-allele	
3D7	allele of the 3D7-family (GGSA) repeat	
NF54	allele of the 3D7-family (GGSA) repeat	
ItG2.F6	allele of the 3D7-family	
FVO	allele of the 3D7-family	

Table 1.1-2 *E. coli* strains

Name	Application	Supplier
M15	protein expression	Qiagen, Basel, CH
JM109	Transformation/Sequencing	Promega, Wallisellen, CH
pMOSblue	T/A cloning/Sequencing	Amersham, Buckinghamshire, UK

Table 1.1-3 Cells for hybridoma technique

Name	Purchase	Source
PAI-myeloma cells	mouse myeloma cells	M. Brockhaus, F. Hoffmann-La Roche Ltd. Basel, CH
P388/D1 cells	mouse tumor line (macrophage)	ATCC, Rockville, ML, USA

4.1.2 Vectors

Table 1.2-1 Vectors

Name	Application	Source / Supplier
pMOSblueT-vector	T/A cloning of PCR products	Amersham, Buckinghamshire, UK
pQE30	expression of recombinant 6His tagged proteins in *E. coli*	Qiagen AG, Basel
pQE16	expression of recombinant 6His tagged DHFR-fusion proteins in *E. coli*	Qiagen AG, Basel
pUC18 (pZF 18u)	cloning of PCR products	Mead & Kemper 1988

4.1.3 Antibodies and secondary reagents

Table 1.3-1 Monoclonal antibodies

Name	Specificity	Host	Supplier
Penta-His	5xHis tag	mouse	Qiagen AG, Basel
anti-6xHistidine Clone AD 1.1.10	6xHis tag	mouse	R&D Systems, Minneapolis, MN, USA

Table 1.3-2 Conjugated secondary antibodies

Host	Specificity	Conjugate	Supplier
goat	Anti-human IgG (()	FITC	Caltag Laboratories San Francisco, CA, USA
goat	Anti-mouse IgG (()	FITC	Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA
goat	Anti-human IgG(()	HRP	Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA
goat	Anti-mouse IgG (()	AP	Sigma, Buchs, CH

4.1.4 Reagents

Table 1.4-1 List of chemicals and reagents

ABTS Peroxidase substrate system	Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD, USA
30% Acrylamide/Bis solution (37.5:1)	Gibco BRL Life Technologies, Basel, CH
Agar	DIFCO Laboratories, Detroit, MG, USA
Agarose (electrophoresis grade)	Gibco BRL Life Technologies, Basel, CH
Albumin, bovine fraction V	Sigma, Buchs, CH
Ammonium persulfate	Serva, Heidelberg, Germany
Ampicillin	Appligene Oncor, Basel, CH
8-azaguanine	Sigma, Buchs, CH
Bacto tryptone	DIFCO Laboratories, Detroit, MG, USA
Bacto yeast extract	DIFCO Laboratories, Detroit, MG, USA
$-mercaptoethanol	Fluka, Buchs, CH
BCIP	Bio-Rad Laboratories AG, Glattbrugg, CH
CAPS, 99% pure	Aldrich Chemicals, Buchs, CH
DAPI	Sigma, Buchs, CH
DMF	Sigma, Buchs, CH

DMSO	Fluka, Buchs, CH
dNTPs (100mM each)	Pharmacia, Dübendorf, CH
DTT	Calbiochem, San Diego, CA, USA
EDTA	Sigma, Buchs, CH
Ethidium bromide	Boehringer Mannheim, Mannheim, D
FCS	Gibco BRL Life Technologies, Basel, CH
GuHCL	Gibco BRL Life Technologies, Basel, CH
Giemsa solution	Fluka, Buchs, CH
HAT-medium	Boerhringer Mannheim, Mannheim, D
HEPES	BDH Laboratory Supplies, Poole, UK
IMDM	Gibco BRL Life Technologies, Basel, CH
Imidazole	Merck, Darmstadt, Germany
IPTG	Appligene Oncor, Basel, CH
L-Glutamine 200mM	Gibco BRL Life Technologies, Basel, CH
Milk powder (low fat)	Migros, Basel, CH
NBT	Bio-Rad Laboratories AG, Glattbrugg, CH
Ni-NTA	Qiagen, Basel, CH
PEG 50% w/v in 75 mM Hepes	Boehringer Mannheim, Mannheim, D
Penicilline-Streptomycin	Gibco BRL Life Technologies, Basel, CH
Phosphatase substrate pNP	Sigma, Buchs, CH
PMSF	Merck, Darmstadt, D
RPMI 1640 Medium	Gibco BRL Life Technologies, Basel, CH
Saponin (from *Glysophila*)	Sigma, Buchs, CH
SDS	Serva, Heidelberg, D
Sorbitol	Fluka, Buchs,CH
TEMED	Serva, Heidelberg, D
Tris base	Sigma, Buchs, CH
Triton X-100	Sigma, Buchs, CH

Trypanblue 0.4% solution in 0.85% saline	ICN Biochemicals Inc.,Costa Mesa, CA, USA
Urea, enzyme grade	Gibco BRL Life Technologies, Basel, CH
X-gal	Appigene Oncor, Basel, CH

4.1.5 Media, Buffers, Solutions

All solutions for cell culture work were sterilized by filtration through 0.22 :m bottle top filter (Millipore Corporation, Bedford, MA, USA). Uncompleted media was stored up to 3 months at 4/C. After every two weeks 2 mM L-glutamine was added when completed with FCS or with FCSi. FCS was heat inactivated at 56/C for 30 min.

Media for *E. coli* cultivation were autoclaved and stored at RT. Supplementation with appropriate antibiotics was done just before use.

Ten fold PBS-buffer for ELISAs was sterilized by filtration through 0.22 :m bottle top filter (Millipore, Corporation, Bedford, MA, USA) and was diluted just before use.

Table 1.5-1 List of media (per 1 litre)

Name	Composition	application
IMDM stock medium	1 box powder (17.66 g) IMDM 3.024 g $NaHCO_3$ (36 mM)	stock medium for cell culture
Cell freezing medium	stock medium 10% DMSO 30% FCS	N_2-stabilates of cells
Myeloma cell medium	stock medium for cell culture 5-10% FCS $5x10^{-2}$ mM $-mercaptoethanol	culture medium for PAI myeloma cells
Azaguanine medium	Myeloma cell medium 0.132 mM 8-azaguanine	culture medium to select for HGPRT-deficient cells
Wash medium	stock medium $5x10^{-2}$ mM $-mercaptoethanol 100 U/ml Penicillin/100 :g/ml Streptomycin	Wash medium for cell fusion

HAT-medium	wash medium 0.1 mM Hypoxanthine $0.4x10^{-3}$ mM Aminopterin 0.016 mM Thymidine 20% FCSi 5% P388/D1 conditioned medium	culture medium to select hybridoma cells after fusion procedure
Hybridoma cell medium	stock medium $5x10^{-2}$ mM $-mercaptoethanol 100 U/ml Penicillin/100 :g/ml Streptomycin 10% FCSi	culture medium for hybridoma cells
LB medium	10 g Bacto tryptone 5 g Bacto yeast extract 10 g NaCl	culture medium for *E. coli* cultures
LB agar	1 l LB medium 15 g Agar	agar plates for *E. coli* cultures
LB / 25% Glycerol	50% LB medium (with appropriate antibiotics) 50% Glycerol	freezing medium for *E. coli* cultures
Super broth medium	25 g Bacto tryptone 15 g Bacto yeast extract 5 g NaCl	culture medium for *E. coli* cultures used for protein expression
P. falciparum culture medium	11.5% RPMI 1640 0.5% Albumax 0.21% $NaHCO_3$ 50 mg hypoxanthine 5 mg neomycin sulfate (+ 2 mM L-glutamine)	culture medium for *P. falciparum* cultures
Wash medium	11.5% RPMI 1640 5 mg neomycin sulfate	washing of cultured parasites

Table 1.5-2 List of buffers

Name	Composition	Application
10x PBS	80 g NaCl 2 g KCl 11.5 g Na_2HPO_4 2 g KH_2PO_4 pH 7.2	Dialysis of recombinant proteins ELISA method
Saponin lysis buffer	0.03 g saponin 50 ml 1x SSC	saponin lysis of iRBCs
PDP lysis buffer	10 mM HEPES pH 7.9 10 mM KCL 0.1 mM EDTA 0.1 mM EGTA 1 mM DTT 0.5 mM PMSF 0.65% NP-40	preparation of parasite derived proteins
20x SSC	3 M NaCl 0.3 M Na_3-Citrat pH 7.0	various
5x TBE running buffer	5 M Tris base 4 M boric acid 10 mM EDTA, pH 8.0	gel electrophoresis
blue juice	30% glycerine 10 mM Tris-HCl, pH 8.5 10 mM EDTA, pH 8.0 pinch bromphenol blue pinch xylene cyanol	gel electrophoresis
TE	10 mM Tris-HCl, pH 8.5 1 mM EDTA	elution and storage of DNA
10x PCR buffer	0.5 M KCl 0.1 M Tris-HCl, pH 8.8 15 mM $MgCl_2$	PCR
Lysis buffer (native)	50 mM NaH_2PO_4 300 mM NaCl 10 mM imidazole pH 8.0	native protein purification

Wash buffer (native)	50 mM NaH_2PO_4 300 mM NaCl 20 mM imidazole pH 8.0	native protein purification
Elution buffer (native)	50 mM NaH_2PO_4 300 mM NaCl 250 mM imidazole pH 8.0	elution of recombinant proteins
Buffer A	6 M Gu-HCl 0.1 M NaH_2PO_4 0.01 M Tris-HCl pH 8.0	protein purification under denaturing conditions
Buffer B	8 M urea 0.1 M NaH_2PO_4 0.01 M Tris-HCl pH 8.0	protein purification under denaturing conditions
Buffer C	Buffer B pH 6.3	wash buffer for protein purification
Buffer D and E	Buffer B pH 5.9 / pH 4.5	elution of recombinant proteins
Diluting buffer	50 mM glycine-OH, pH 9.0 5 mM EDTA	renaturation of recombinant proteins
Renaturation buffer	50 mM glycine-OH, pH 10.5 5 mM EDTA	renaturation of recombinant proteins
5x SDS-PAGE sample buffer	15% $-mercaptoethanol 15% SDS 1.5% bromphenol blue 50% glycerol	protein SDS-PAGE
5x SDS-electrophoresis buffer	15 g Tris base 72 g glycine 5 g SDS	protein SDS-PAGE
4x Lower buffer	181.7 g Tris-HCl, pH 8.8 4 g SDS	SDS-PAGE
Upper buffer	60.5 g Tris-HCl, pH 6.8	SDS-PAGE
Transfer buffer	10 mM CAPS 10% MeOH pH 10.5	immunoblotting

Wash buffer IB	50 mM Tris base 5 mM EDTA 140 mM NaCl pH 7.4 0.05% NP-40 0.25% gelatine	immunoblotting
AP staining buffer	100 mM Tris-HCl, pH 9.5 0.5 mM MgCl2	immunoblotting
HRP staining buffer	23.5 ml 1x PBS 1.5 ml 0.3% chloronaphthol in MeOH 10 :l H_2O_2	immunoblotting
Blocking buffer (AP)	Wash buffer IB 1% BSA	immunoblotting with alkaline phosphatase conjugated ab
Blocking buffer (HRP)	Wash buffer IB 1% milk powder	immunoblotting with HRP conjugated ab
Blocking buffer (IFA)	1x PBS 1% BSA	IFA
Blocking buffer (ELISA)	1x PBS 5% milk powder	ELISA
Wash buffer (ELISA)	dH_2O 0.05% Tween-20	ELISA
Carbonate buffer	1.4 g Na_2CO_3 3.0 g $NaHCO_3$ 0.2 g $MgCl_2$ x $6H_20$ pH 9.6 1g pNP	AP Substrate buffer ELISA
Buffered glycerine	0.136 g KH_2PO_4 3.221 g Na_2HPO_4 x $2H_2O$ 80 ml glycerine ad 100 ml	IFA

Table 1.5-3 List of solutions

Name	Composition	Application
Comassie blue	0.25 g Serva blue 200 ml acetic acid filter before use	SDS-PAGE protein staining
Amp stock solution	100 mg / ml water	*E. coli* culture
Kan stock solution	25 mg / ml water	*E. coli* culture
Tet stock solution	15 mg / ml water	*E. coli* culture
Synchronization solution	5% D-sorbitol in dH_2O	synchronization of cultured parasites

4.1.6 Commercially available Kits

Table 1.6-1

Name	Supplier	Application
pMOSblue T-vector Kit	Amersham, Buckinghamshire, UK	Cloning
DNA Sequencing Dye Terminator Kit	Perkin Elmer, Rotkreuz, CH	Sequencing
QIAexpress Kit Type IV	Qiagen, Basel, CH	protein expression
CONCERT™ High Purity Plasmid Miniprep system	Gibco BRL, Life Technologies, Basel, CH	DNA preparation
NucleoSpin Extract 2 in 1	Macherey-Nagel Ag, Oensingen, CH	DNA elution

4.1.7 Enzymes

4.1.7.1 DNA polymerases

Table 1.7-1

Name	Supplier	Application
Ampli*Taq* DNA polymerase, FS	Perkin Elmer, Rotkreuz, CH	Sequencing
Native *Pfu*-polymerase	Stratagene	PCR
Taq DNA polymerase	Gibco BRL, Life Technologies, Basel, CH	PCR
Klenow DNA polymerase	Boehringer Mannheim, D	second strand synthesis

4.1.7.2 Restriction enzymes

Restriction enzymes and corresponding buffers were obtained from Gibco BRL Life Technologies, Basel, CH, or from New England Biolabs.

Table 1.7-2

Name	Recognition site	Application
Hinf I	5'-G | ANTC-3'	PCR-RFLP
*Bam*HI	5'-G | GATCC-3'	cloning
*Kpn*I	5'-GGTA |CC -3'	cloning
*Eco*RI	5'-G | AATTC-3'	cloning
*Bgl*II	5'-A | GATCT-3'	cloning
*Sma*I	5'-CCC | GGG-3'	cloning
*Hind*III	5'-A | AGCTT-3'	cloning

4.1.7.3 Other enzymes

Table 1.7-3

Name	Supplier	Application
Lysozyme	Appligene Oncor, Basel, CH	protein expression
T4 DNA Ligase	Amersham, Buckinghamshire, UK	ligation
RNase A	Boehringer Mannheim, D	RNA digestion
DNase	Boehringer Mannheim, D	DNA digestion

4.1.8 Primers

All primers were manufactured by Gibco BRL Life Technologies, Basel, CH.

4.1.8.1 Sequencing Primers

Table 1.8-1

Name	Sequence	Application
pQE Type III/IV	5'-CGGATAACAATTTCACACAG-3'	Sequencing pQE30 and pQE16
pQE Type II	5'-GGTCCAGGAGGAAAAAGGC-3'	
pQErev primer	5'-GTTCTGAGGTCATTACTGG-3'	
M13 fwd universal primer	5'-CCCAGTCACGACGTT-3'	Sequencing pZF 18u
M13 rev universal primer	5'-CAGGAAACAGCTATGAC-3'	
T7 promoter primer	5'-ATTAGGTGACACTATAG-3'	Sequencing pMOSblue T vector
U19mer primer	5'-GTTTTCCCAGTCACGACG-3'	

4.1.8.2 Primer pairs for recombinant protein expression

Table 1.8-2 Primer pairs for recombinant protein expression

antigen	Fwd primer (*Bam*HI)*	Rev primer (*Kpn*I)*
23xNAP repeats	5'cgggatcccgTAGTGTAGGTGCA'3	5'ggggtaccccACTAGCAATAGTA'3
3D7 fsp	5'cgggatcccgTGGTAATGGTGCT'3	5'ggggtaccccAGATTGTAATTCG'3
4x32aa repeats	5'cgggatcccgTGCTCCAAAAGCT'3	5'ggggtaccccAGGGGTATCAGCA'3
6x12aa repeats	5'cgggatcccgTACTGCTACAGAA'3	5'ggggtaccccTTGAACTTTCTGT'3
3D7 repeats	5'cgggatcccgTCCCCTACTGGTGCT'3	5'ggggtaccccGGTGGTAGCGGGAGTACT'3

FC27 fsp	5'-region	(*Bam*HI)5'cgggatcccgTAAGAGTGTAGGTGCAAATGCTCCAAAAggaattcc'3 (*Eco*RI) + GGAATTCC (*Eco*RI-Primer)	
	3'-region	5'ggaattccAGAAAGTTCAAGTT'3 (*Eco*RI)	5'ggggtaccccAGCAGGATTTTCA'3 (*Kpn*I)

3'-const. (*Bam*HI-*Kpn*I)	5'cgggatcccgTGCACCAGAGAATAAAGG'3	5'ggggtaccccTATGAATATGGCA'3
5'3'const. (*Bam*HI-*Kpn*I)	5'cgggatcccgAATGAAGGTAATT'3	5'ggggtaccccTATGAATATGGCA'3
5'3'-const w/oSP (*Bam*HI-*Kpn*I)	5'cgggatcccgCTTTAATATTAAAAATGAAAG'3	5'ggggtaccccTATGAATATGGCA'3
5'3'-const w/oSP (*Bgl*II)	5'gaagatcttcATTTAATATTAAAAATGAAAG'3	5'gaagatcttcTGAATATGGCAAA'3

DHFR-5'3'-const.	5'-region	5'gaagatcttcAATGAAGGTAATTA'3 (*Bgl*II)	5'ggaattccTAGTATTAGAACCTT'3 (*Eco*RI)
	3'-region	5'ggaattccGCACCAGAGAATAAA'3 (*Eco*RI)	5'gaagatcttcTGAATATGGCAAA'3 (*Bgl*II)

nested MSP2 (*Bam*HI-*Kpn*I)	5'cgggatcccgGAGTATAAGGAGA'3	5'ggggtaccccCTAGAACCATGCA

* Capital lettres correspond to *msp2* hybridization site, small letters build the recognition site where the restriction enzyme cuts.
aa: amino acid, fsp: family specific part, w/oSP: without sequence coding for signal peptide

4.1.8.3 Primer pairs for *msp2* genotyping PCR

Table 1.8-3

Name	Sequence	Position in MAD71 sequence
S1 (fwd)	5'-GAGTATAAGGAGAAGTATG-3'	111-129
S2 (fwd)	5'-GAAGGTAATTAAAACATTGTC-3'	3-23
S3 (rev)	5'-GAGGGATGTTGCTGCTCCACAG-3'	789-811
S4 (rev)	5'-CTAGAACCATGCATATGTCC-3'	709-728

4.1.9 Markers

Table 1.9-1

Name	Supplier	Application
1 kb ladder	Gibco BRL Life technologies	DNA gel electrophoresis
Low molecular weight marker	Pharmacia Biotech, Dübendorf, CH	protein SDS-PAGE
Rainbow marker 756	Amersham, Buckinghamshire, UK	protein SDS-PAGE immunoblotting

4.1.10 Animals

Female Balb/c mice 4-6 months old were obtained by RCC Biotechnology & Animal Breeding BRL Laboratories Ltd., Füllinsdorf, CH.

4.1.11 Other materials and equipment

Table 1.11-1

Name	Supplier
0.22 :m syringe membrane filter	Schleicher und Schuell, Dassel, D
0.22 :m bottle top filter (Steritop)	Millipore, Zürich, CH
10 well glass slides	BioMérieux, Lausanne, CH
24-, 48-, 96-well cell culture plates	Costar at Integra Biosciences, Wallisellen, CH
96-well plates Immunlon 2	Dynex Technologies, USA
ABI Prism 310 Genetic Analyzer	Perkin Elmer, Rotkreuz, CH
Dialysis tubing	Chemie Brunschwig, CH
Electroporator (EasyjecT)	Equibio, Boughton, Montchetsea Kent, UK
Electroporation cuvette	Equibio, Boughton, Montchetsea Kent, UK
Freezing vials	Nunc Inc., Naperville, IL, USA
HyBond-C extra nitrocellulose membrane	Amersham, Buckinghamshire, UK
Inverted fluorescence microscope	Zeiss, Basel, CH
Mini-PROTEAN II Electrophoresis cell	Bio-Rad Laboratories AG, Glattbrugg, CH

PAGE-chamber BRL V16	Gibco BRL Life Technologies, Basel, CH
Petri dishes (parasite culture)	Falcon Labware, Oxnard, CA, USA
Petri dishes (bacteria)	Greiner Labortechnik, Kremsmünster, Austria
policeman rubber	Costar at Integra Biosciences, Wallisellen, CH
Sonicator (Branson Sonifier)	Skan AG, Basel, CH
Titertek Multiskan MCC/340 (ELISA reader)	BioConcept, Allschwil, CH
Whatman 3MM filter paper	Whatman, Maidstone, UK
White/UV Transilluminator	UVP/Life Science, Braunschweig, D

4.2 Methods

4.2.1 PCR-RFLP-genotyping

4.2.1.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for several purposes. A negative control without DNA template was included in each set of PCR amplifications.

In general, a 100 :l reaction mix contained the following components: 1.5 U *Taq* DNA polymerase (Gibco BRL Life Technologies), 10 :l 10x PCR buffer, 0.2 mM of each dNTP, 0.5 mM of each primer, and 0.1-1 :g DNA template.

Both a primary and a nested PCR were performed to amplify the *msp2* locus from DNA extracted from blood samples. For the primary PCR, 5 :l of the extracted DNA were added to the PCR reaction mix. The primer pair corresponded to the nucleotides 3-23 and 789-811 of the MAD71 sequence of *msp2* (Foley et al. 1992). These nucleotides belong to the conserved 5' and 3' regions of *msp2*. Two :l of the primary PCR product were amplified in the nested PCR using nested primers corresponding to nucleotides 111-129 and 709-728 of the MAD71 sequence (Foley et al. 1992). The PCR conditions were the same for both PCRs, namely an initial denaturation step of 5 min at 94/C, followed by cycles of 30 sec at 94/C, 2 min at 55/C, and 2 min at 70/C. A final elongation step of 10 min at 72/C was added. The nested PCR products were separated on 10% polyacrylamide gels (BRL V16), stained with ethidium bromide and visualized by UV light.

Cycle conditions for amplification of the *msp2* fragments used in protein expression approach were different concerning the annealing temperature of the primers. A lower temperature of 42/C was chosen since the primer pairs had long non-corresponding sequences which formed the restriction site for the ligation step. Sequence of primers used are shown in table 1.8-2

4.2.1.2 Restriction digests

In general 20 :l reaction mix was used containing 10x buffer provided by the manufacturer, 2.5 U enzyme and the appropriate amount of PCR product. If the digested DNA was used for salt-sensitive applications such as ligation, the digest was precipitated prior to further use.

For *msp2* genotyping nested PCR products were digested with *Hinf* I (NEB) for 2 h at 37/C and the DNA fragments were separated on 10% polyacrylamide gels at 200 V and 50 mA. Fragment size was estimated using a DNA size marker (1kb ladder, Gibco BRL Life Technologies). Samples of one patient from longitudinal studies were run side by side on the gel in order to identify size differences of the DNA fragments.

4.2.2 Sequencing

Sequencing was done using the DNA Sequencing Dye Terminator Kit (Perkin Elmer) based on the dideoxy chain termination method (Sanger et al. 1977). In the cycle sequencing PCR reaction fluorescent dideoxy nucleotides were used as terminators. The cycle sequencing PCR reaction was conducted with a GeneAmp PCR System 2400 (Perkin Elmer). Sequence analysis was carried out with ABI Prism 310 Genetic Analyzer (Perkin Elmer) and ABI Prism software.

Two different cycle sequencing approaches were applied.

4.2.2.1 Direct sequencing

PCR amplified DNA fragments were purified by using the NucleoSpin™ Extract 2 in 1 kit according to the manufacturer's protocol. An additional purification and concentrating step was included by DNA precipitation with 2.5 vol ethanol and 0.1 vol sodium acetate. The solution was incubated for 15 min at RT. Precipitated DNA was centrifuged for 30 min at 4/C and 13'000g, washed, dried and resuspended in 25 :l dH_2O. DNA concentration was estimated by agarose gel electrophoresis. DNA sample (10-30 ng) was subjected to elongation reactions with the Ampli*Taq* DNA Polymerase, terminator nucleotides, and the appropriate sequencing primer (table 1.8-1).

4.2.2.2 Sequencing of plasmids with inserted PCR products

Plasmid DNA preparation was done as described in 2.4.4. The purification and concentration step (2.2.1 Direct sequencing) was included. Sequencing reactions were done according to the manufacturer's protocol. The amount of subjected DNA was 200-500 ng per reaction.

4.2.3 Second strand synthesis

Small DNA fragments for ligation into the expression vector were produced by second stand synthesis. The 5'end of the family specific part of FC27 alleles used for expression consists of 46 nucleotides including restriction sites at both ends (table 1.8-2). This fragment was synthesized by Gibco BRL Life Technologies (Basel, CH) plus a primer of eight nucleotides (GGAATTCC). Reaction mix containing 0.07 nmol single strand DNA fragment, 0.7 nmol primer, 0.1 mM dNTPs, 3 U Klenow DNA polymerase, 0.8 :l BSA (10 mg/ml), appropriate amount of 10x reaction buffer, was incubated for 30 min at 37/C. The Klenow DNA polymerase was inactivated 10 min at 65/C. The sample was cooled down slowly to RT to support correct re-annealing of the short double stranded DNA. The double stranded DNA product was separated by agarose gel electrophoresis from primer and single stranded DNA fragment. The desired DNA was purified by gel extraction according to manufacturer's protocol (NucleoSpin™ Extract 2 in 1, Macherey-Nagel). The following restriction digest with *Bam*HI and *Eco*RI was incubated at RT overnight to prevent denaturation of the double stranded small DNA fragment.

4.2.4 Cloning / Transformation / DNA preparation

4.2.4.1 T/A cloning

The T/A cloning method was carried out with the pMOSblue T-vector kit (Amersham) according to the manufacturer's protocol. Briefly, the method profits from the template-independent activity of *Taq* DNA polymerase adding a single adenosine nucleotide to the 3'end of double stranded DNA. Such a PCR product can be inserted into the thymidine-tailed vector pMOSblue T-vector without the requirement for restriction sites. PCR products were purified by gel extraction according to manufacturer's protocol (NucleoSpin™ Extract 2 in 1, Macherey-Nagel) and the concentration of eluted DNA was estimated by comparing the intensity of the band with the 1.6 kb band of the 1 kb ladder (100 ng / :l). The 1.6 kb band represents 10% of the whole marker applied (10 ng / :l). The molecular ratio vector : insert was adjusted to 1:10. Fifty ng of vector

were used per reaction mix. The 20 :l reaction mixture, recommended by the manufacturer's protocol, was incubated at 16/C overnight. The pMOSblue T-vector allowed for blue/white screening of recombinants, when agarose plates containing X-gal (100 :g/ml) and IPTG (100 :M) were used.

4.2.4.2 Sticky end ligation

This method was used to insert PCR products in frame into the expression vectors pQE30 and pQE16 (Qiagen) by using PCR primers with appropriate internal restriction sites (table 1.8-2). Vector and insert were digested with the restriction enzymes, purified by gel extraction (NucleoSpin™ Extract 2 in 1, Macherey-Nagel) and concentrated by ethanol precipitation. For the digestion of pQE30 the enzymes *Bam*HI and *Kpn*I were chosen. This digestion had to be performed in two consecutive reaction mixes with an ethanol precipitation in between, since the recommended reaction buffers were not compatible. The molecular ratio vector : insert was adjusted to 1:3 and to 1:6. The final volume of ligation mix was 20 :l. Two U per ligation reaction of T4 DNA Ligase and the corresponding 10x T4 DNA Ligase buffer from Amersham were used. Incubation temperature and time was chosen according to the restriction enzyme used to produce sticky ends.

Restriction enzyme	temperature	time
*Bam*HI	4/C	16 h
*Bgl*II	16/C	16 h
*Eco*RI	16/C	2 h
*Kpn*I	16/C	2 h

Ligation mix was precipitated and resuspended in 10 :l dH_2O. The DNA was stored at -20/C until use.

4.2.4.3 Transformation of *E. coli* cells by electroporation

Electrocompetent cells (M15, JM109 or pMOSblue) were prepared according to standard protocols (Seidman et al. 1997) and stored at -70/C until use. The *E. coli* cells were transformed with the Equibio electroporator using a pulse program with following specifications: voltage 2.5

kV, capacity 25 :F, shunt 201 S and pulse time 5 msec. Electrocompetent cells were thawed on ice. 40 :l of the cells were transferred to a pre-cooled electroporation cuvette and mixed with 4 :l resuspended ligation mix (incubation 1 min on ice). The cuvette was then placed into the electroporator. After the pulse the cells were transferred immediately to 1 ml LB medium and incubated for 30 min at 37/C. 200 :l were plated on an agar plate containing appropriate antibiotics. Bacteria transformed with pMOSblue T-vector or pZF 18u vector can be screened by blue/white selection of recombinants (Sambrock et al. 1989). Therefore, bacteria have to be plated on X-gal/IPTG agar plates. The plates were incubated upside down overnight at 37/C.

4.2.4.4 Analysis of transformed clones

Liquid cultures of *E. coli* clones pre-selected on agar plates were incubated overnight. The CONCERT™ High Purity Plasmid Miniprep System (Gibco BRL) was used to obtain very pure plasmid DNA for subsequent approaches (protein expression, DNA sequencing) according to the manufacturer's protocol. Plasmid DNA was digested with appropriate restriction enzymes to determine the insertion of PCR fragment. All restriction digests were performed as recommended by the manufacutrer, using the provided 10x reaction buffer. Separation of the DNA fragments was done by agarose gel electrophoresis.

4.2.5 Expression and purification of recombinant protein fragments

Expression and purification of the protein constructs were done following manufacturer's protocol (QIAexpress Kit Type IV, Qiagen). Briefly, *E. coli* cells, transformed with one of the expression vectors, were inoculated into 25 ml Super broth (25 g Trypton, 15 g yeast extract, 5 g NaCl ad 1 L) using the appropriate antibiotics, 100 mg/L ampicillin (Appligene) and 25 mg/L kanamycin (Sigma), and incubated over night. Fresh Super Broth with antibiotics was inoculated with the overnight culture (20 ml overnight culture / 1 L fresh medium) the following day. The cells were grown at 37/C to ODA600 nm = 0.5-0.8, when the protein production was induced by addition of IPTG (Appligene) to a final concentration of 1 mM. Cells were harvested by centrifugation after four hours shaking. The pelleted cells were resuspended in lysis buffer (50 mM Sodium dihydrogenphosphate pH 8.0; 300 mM Sodium chloride; 10 mM imidazole) and incubated with 1 mg/ml lysozym 30 min on ice. Bacterial cells were ultrasonically disrupted on ice. If the lysate became very viscous, Rnase A (10 :g/ml) and Dnase I (5 :g/ml) was added and

incubated for 15-30 min on ice. The protein solution was centrifuged at 10.000xg for 20 min at 4/C to pellet the cellular debris. The supernatants containing solubilized fusion proteins were mixed with appropriate amount of Ni-2+ -agarose and incubated for 1 h in a shaker. Purification was performed with imidazole (Wash buffer: lysis buffer with 20 mM imidazole, elution buffer: lysis buffer with 250 mM imidazole). The 5'3'const. recombinant protein was expressed at 25/C and denatured with 6 M guadinine hydrochloride (6 M GuHCl; 0.1 M sodium dihydrogenphosphate; 0.01 M TrisCl, pH 8.0) for 1h at 4/C with stirring . Purification was performed with 8 M Urea (decreasing pH 8.0-4.5). The recombinant protein was eluted at pH 4.5. 20 :l of each protein fraction were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to approximate the purity of the preparations. Pure protein fractions were pooled and renatured by dialysis as previously described for other MSP2 recombinant proteins (Takacs and Girard 1991). Protein preparations under native conditions were dialysed against PBS pH 7.2 to remove imidazole. The proteins were stored at −70 /C until required.

4.2.5.1 SDS-PAGE

Protein aliquots were mixed with 5x SDS-PAGE sample buffer and denatured for 7 min at 95/C. Aliquots containing imidazole were incubated for 10 min at 37/C. Proteins were separated according to their molecular mass in a Mini-Protean II Electrophoresis cell (Bio-Rad Laboratories) by running a discontinuous polyacrylamide gel (Laemmli 1970) with a acrylamide concentration of 4% and 12.5% in the stacking (upper) gel and in the separating (lower) gel, respectively. The SDS-electrophoresis buffer pH 8.3 containing 24 mM Tris base, 0.19 M glycine, and 1% SDS was used. Protein bands were visualized by staining the gel in Comassie blue solution and destaining in dH_20. The Low Molecular Weight Marker (Pharmacia Biotech) was used to determine the molecular mass of the separated proteins.

For immunoblotting Rainbow marker 756 (Amersham) was used and the separated proteins were transferred to a HyBond-C nitrocellulose membrane without staining the gel .

4.2.5.2 Renaturation of recombinant proteins

Renaturation of recombinant proteins was conducted as described by Takacs and Girard (1991). Briefly, solutions of recombinant 5'3'construct protein in 8 M urea were diluted with diluting buffer (50 mM glycine-OH, pH 9.0, 5 mM EDTA) to a protein concentration between 0.5-1.0

mg/ml. The pH of the solution was adjusted to 10.5 with NaOH and DTT was added to 5 mM. The solution was dialyzed against renaturation buffer (50 mM glycine-OH, pH 10.5, 5 mM EDTA) at room temperature for 3 h. Dialysis was continued against three changes of 1x PBS pH 7.2 at 4/C over two days. Protein concentrations before and after dialysis were determined photometrically by the method of Bradford at 8=595 nm (Bradford 1976). One ml reaction mix containing 800 :l Comassie brilliant blue solution (Bio-Rad) and an aliquot of the protein solutions were incubated at least 5 min at RT and the absorbance at 8=595 nm was assessed. Each protein concentration was estimated by using a protein standard curve with different BSA concentrations. Protein solutions were diluted to appropriate concentrations with 1x PBS for subsequent immunological approaches.

4.2.5.3 Preparation of dialysis tubes

The preparation of dialysis tubes was performed as previously described (Bhown et al. 1980). Briefly, tubes with appropriate specificity (excluding molecular mass 3.5 kDa and 12-14 kDa) were boiled for 1-2 min in following solutions: 1% Na_2CO_3; 0.1 M EDTA; 0.1% SDS. In between each solution change a washing step with dH_2O was included. Finally, the dialysis tubes were boiled twice in dH_2O and then stored in 0.02% sodium azide.

4.2.6 Immunological methods

4.2.6.1 ELISA method

Optimal dilutions of the immunogens and reagents were determined by checker titration with positive control serum pools and negative control sera (Quakyi 1980). Microtitre plates (Immulon 2; Dynex Technologies, USA) were coated with 50 ml of the protein preparations. Immunogen concentrations ranged from 0.1mg/ml – 10 mg/ml in PBS pH 7.2. The plates were incubated overnight at 4/C. Non-adsorbed antigen was removed and the wells were filled with 200 :l PBS pH 7.2 containing 5% non-fat milk powder to prevent non-specific protein binding to the plastic surface. After incubation for 2 h at 25/C, plates were washed four times for 20 sec with water containing 0.5% Tween 20. The excess fluid was removed. Then, 50 :l of test sera diluted 1:500 or 1:1500 with 1x PBS containing 0.5% Tween 20 and 0.5% milk powder were added to the wells in duplicates. The plates were incubated 1 h at 25/C and then washed as above. Fifty :l of the appropriate second antibody conjugated to horse radish peroxidase diluted (1:2000) in PBS-

0.5%Tween/0.5%milk powder were added to each well. Again the plates were incubated for 1 h at 25/C, then washed as above. Last, 50 :l of peroxidase substrate (ABTS peroxidase substrate, KPL) were added to each well. The absorbance at 405 nm of the content of each well was determined after incubation for 30 min in a spectrometer (Titertek Multiskan MCC/340). A positive standard was a pool of 20 sera from semi-immune adults from PNG. Titration of this standard was performed in each ELISA plate to control the quality of experiments and interexperimental variation. Sera of 36 healthy European adults, and sera from 36 healthy European children aged 5-15 months, who had not been exposed to malaria were used as negative controls to determine the specificity of the assay. All sera were stored at –20/C. Each test serum and control serum was tested against the single immunogen and, as a background control, against a protein solution consisting of DHFR-6His (5 mg/ml PBS), produced in the same way as the other seven recombinant proteins.

ELISA performed to analyze the antibody response to the recombinant proteins in immunized mice and to analyze the specificity of antibodies produced by monoclonal hybridoma cells were done in a similar way. Mouse sera were analyzed with end point titration. AP-conjugated second anti-mouse-IgG (() antibody diluted 1:1000 and the corresponding carbonate buffer containing 1mg/ml pNP were used. After incubation for 60 min in the dark absorbance at 405 nm of the content of each well was determined in a spectrometer (Titertek Multiskan MCC/340).

4.2.6.2 Immunoblotting

A semi-dry blotting protocol were used. Blotting was performed in a Trans-Blot SD Semidry Transfer Cell (Bio-Rad) for 2 h at 0.8 mA per cm^2 of gel. The separating gel was cut off the stacking gel and the separated proteins were electro-blotted in a sandwich setup onto HyBond-C extra nitrocellulose membrane. Whatman 3MM filter paper with transfer buffer (10 mM CAPS pH 10.5, 10% MeOH) pre-soaked were used above and below the pre-soaked nitrocellulose membrane. After the transfer the nitrocellulose membrane was washed two times with wash buffer IB at RT and was blocked with blocking buffer (AP) or blocking buffer (HRP) (3% BSA, or 1% MP) for 2 h, depending on the second antibody used. The appropriate dilution of the first antibody in blocking buffer was applied and incubated at least for 1 h. After an extensive washing procedure (6x 5 min) with wash buffer IB, the second conjugated antibody was applied and incubated at least 1 h. Antibody dilutions recommended for the method by the supplier were used.

Finally, the nitrocellulose membrane was washed in PBS as above. The visualization procedure depends on the conjugated second antibody used. For AP-conjugated second antibodies visualization was performed using NBT (0.3 mg/ml) and BCIP (0.15 mg/ml) in appropriate concentrations in AP staining buffer pH 9.5. Incubation in the dark was done at RT until bands were visible or maximum 30 min. For HRP-conjugated second antibodies visualization was performed in freshly made HRP staining buffer (0.018% chloronaphthol in PBS and H_2O_2) for maximum 30 min in the dark.

4.2.6.3 Immunofluorescence assay IFA

Ten-well slides were coated with *P. falciparum* infected red blood cells (cloned lines shown in table 1.1-1), dried at RT and stored at -70/C until use. Defrosted and dried slides were fixed with acetone at room temperature for five minutes. Infected erythrocytes were blocked with blocking buffer (IFA, 1% BSA) for 15 min at RT and in the next step 25 :l of diluted mouse sera or hybridoma supernatant (dilution in blocking buffer IFA according to ELISA titre) were added to each well. The slides were then incubated in a humid chamber for 1 h at 37/C. The infected erythrocytes were washed and then incubated with fluorescein isothiocyanate-labelled secondary antibody (goat anti-mouse IgG(g), KPL) for 1 h at 37/C in a humid chamber in the dark, washed, and examined with a fluorescence microscope. The multi-well slides were mounted with 50% glycerin-PBS and covered with a glass slide. As negative controls non-infected erythrocytes and pre-immunisation serum were used.

4.2.7 Vaccination

4.2.7.1 Preparation of the recombinant antigens for immunization

The purified and pooled protein solution was mixed with 5x sample buffer and incubated for 7 min at 96/C. For preparation of the immunogens 1ml of pre-heated protein fractions were loaded into a single gel-wide slot of a 8% SDS-polyacrylamide gel. A 120/120/1.5 mm gel was used and was run at low voltage (voltage 100V, current 20mA) in the cold room. A small portion of the gel was stained with Comassie blue to locate the relevant protein band. The remainder of the gel was soaked (without prior fixation) in ice-cold 250 mM KCl for 5 min at 4/C and was washed two times in ice-cold dH_2O for 15 min. At this time the protein became visible as milky band. The desired protein band was cut out with a razor blade and the material was collected into a sterile

syringe. One volume of 1x PBS was added and the semi-dry gel material was homogenized by forcing it through the syringe until the slurry was fine enough to inject into mice through a 21-gauche needle. A sample of the slurry was run side by side with the Low Molecular Marker on a gel to estimate protein concentration and purity. The slurry was stored at -20/C till the end of the experiment.

4.2.7.2 Vaccination of Balb/c mice

The immunogenicity of the recombinant proteins was investigated in female BALB/c mice (4-6 months old) by four injections (4x i.p.) of each immunogen (2.5-10 mg) without any further adjuvants. Each immunogen was tested in two mice. The immunization scale was: 0, 14, 28, 42 days. Pre-immune sera were collected from each mouse. Immune-sera were collected 14 days after the last injection. The murine sera were used in ELISA and immunoblotting for recognising the immunogen and the parasites in IFA. Sera were stored as aliquots of 20 :l at -20/C.

The immunization time scale for mice used for monoclonal antibody production were different. The mice were immunized by three injections (i.p.) of the immunogen (10 :g each) at day 0, 14, 28. Sera were collected from the tail vein. The specificity of produced antibodies was tested by ELISA, IFA and immunoblotting. Three months later, the mice received the forth injection i.v. in the tail vein of the immunogen (12 mg) suspended in PBS without any polyacrylamide. After three days the mice were dissected.

4.2.8 Monoclonal antibodies

To generate monoclonal antibodies directed against the 5'3'construct spleen cells of the immunized Balb/c mouse were fused with PAI myeloma cells (mouse myeloma). The fusion was performed according to standard procedures (Coligan et al. 1995). Instead of feeder layers, HAT medium was supplemented with 5% conditioned medium from supernatant of an LPS activated monocytic cell line (P388/D1; Koren et al. 1975; Melchers and Corbel 1983; Perstidge et al. 1984).

4.2.8.1 Production of P388/D1 conditioned medium

Monocytic P388/D1 cells were cultured to 80% confluence in Iscove's medium prepared with heat inactivated FCS (FCSi). The culture medium was removed completely and replaced by Iscove's medium supplemented with 2% FCS and 2 :g/ml LPS from *E. coli* to induce activation

of monocytes and subsequent production of B cell stimulating cytokines. After three days, the culture supernatant was harvested and filtered through 0.22 :m bottle top filter (Millipore). The P388/D1 supernatant was stored at -20/C until use.

4.2.8.2 PAI cell culture

Cells lacking HGPRT die in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium), because both the main and the salvage pathways are blocked. Selection of HGPRT deficient cells was performed according to standard protocols (Goding 1986). PAI myeloma cells used for cell fusion were cultured in myeloma medium containing the toxic base analogue 8-azaguanine, which is incorporated into DNA via HGPRT, to select for HGPRT-deficient cells. These deficient cells were harvested by scraping the cells from the bottle surface using a rubber policeman, centrifuged 5 min at 180g and resuspended with freezing medium. The cells were stored at –70/C until use. Prior to fusion, freshly thawed PAI myeloma cells were cultured in hybridoma medium to 80% confluence within one week, not longer to prevent losing the HGPRT-deficiency.

4.2.8.3 Fusion procedure

Three days after the last antigen booster injection (i.v.) the mouse was dissected under sterile conditions. The spleen was transferred into sterile wash medium. The spleen tissue was pressed through a mesh net by using a plunger of a syringe to obtain a single cell suspension. Prepared PAI myeloma cell cultures were harvested and were suspended in wash medium. 10 :l of cells were mixed with equal volume of Trypanblue solution and were transferred to a Neubauer chamber. The concentration of viable cells was calculated by counting non-stained cells in 16 fields. The number of cells per ml is calculated by cell count x2 x10'000. Both spleen and myeloma cells were washed twice by repeated centrifugation for 5 min at 180g. Before a third centrifugation step followed, the cells were pooled in equal proportions (cells per spleen $1x\ 10^8$). After complete removal of wash medium, 1 ml PEG 1500 solution (Boehringer Mannheim) was added in steps of 100 :l within one minute. During this procedure the cell pellet was slightly agitated. The tube was attached to the center of a touring wheel (45/ angle) and the two phases were mixed 10 min by slow rotation. After the addition of 35 ml wash medium the cells were centrifuged for 5 min at 180g and resuspended in 160 ml HAT medium. This cell suspension was

distributed in 200 :l portions in a 96-well culture plate and incubated at 37/C in an atmosphere containing 5% CO_2 (Carbagas, Basel, CH). Culture medium was exchanged on the third day after the fusion procedure. Growing colonies were examined for production of anti-5'-3'-const. antigen antibodies by ELISA. Mother clones producing the highest IgG titer were transferred into 48-well culture plates. The specificity of the produced antibodies was tested by IFA and immunoblotting.

4.2.8.4 Subcloning of Mother clones

The IFA positive hybridoma cells were cloned by limiting dilution. Cells of one well were resuspended by aspiration with the pipette and were diluted in HAT medium to two different final concentrations: 0.3 and 3 cells / well. Each cell suspension was distributed in one half of a 96-well culture plate. Outgrowing single colonies were screened by ELISA. Colonies with the highest titres were transferred into 24-well culture plates. At this point, culture medium was changed from HAT medium to hybridoma medium. A final transfer into 25 cm^2 tissue culture flask followed. After reaching the mid-exponential growth phase the cells were split into two 25 cm^2 flasks.

4.2.8.5 Harvesting of antibody containing supernatant

The antibody containing supernatant was removed after overgrowth of cells, centrifuged for 10 min at 180g. Aliquots were stored at −20/C until use.

4.2.8.6 Freezing of hybridoma cells

Hybridoma cells from the second flask were harvested, centrifuged for 5 min at 180g and resuspended in cold freezing medium. Aliquots were put into −70/C within tight polystyrene boxes to ensure slow freezing. For storage over longer time periods, the tubes were transferred into liquid nitrogen.

4.2.9 *In vitro* Parasite culture (Matile and Pink 1990)

EDTA-stabilized blood was centrifuged for 5 min at 180g. The supernatant and the white interphase containing the white blood cells were removed. Pelleted erythrocytes were washed twice with wash medium. The final RBC pellet was resuspended in an equal volume of culture medium to obtain a 50% suspension. Resuspended cells were stored up to one week at 4/C.

Parasites were cultured in 35x10 mm or 100x20 mm petri dishes (2.5 ml, 10 ml) with 5% human

erythrocytes (blood group A Rh+) according to the protocol of Trager & Jensen (1976). The parasite cultures were incubated at 37/C in an atmosphere consisting of 92% N2, 5% CO2, and 3% O2 (Carbagas, Basel, CH) and were split when the parasitemia reached 5-12 %.
To determine the parasitemia of cultured parasites, blood smears were made on glass slides. These were fixed in MeOH and stained in Giemsa solution for 15 min. The slides were analysedusing oil immersion microscopy.

4.2.9.1 Preparation of merozoites

Merozoites were harvested by using the supernatant of centrifuged iRBCs (10 min at 800g). The supernatant was transferred to a fresh Falcon tube and centrifuged again for 10 min at 3000g. Pelleted merozoites were resuspended and used for the preparation of parasite derived proteins or IFA slides.

4.2.9.2 Synchronization of parasite cultures

Synchronization of parasites was done in two cycles. The second synchronization was performed 8-10 h after the first according to the developmental stage of the parasites. D-sorbitol provoke osmotic lysis of all forms of the parasites except ring forms (Lambros and Vanderberg 1979). Infected RBCs (about 10% parasitemia) were centrifuged 5 min at 800g. Pelleted cells were resuspended in 5 volumes of pre-warmed 5% D-Sorbitol solution and were incubated for 5-10 min at 37/C. After centrifugation for 5 min at 800g cells were resuspended in 10 ml culture medium and 200 :l fresh RBCs.

4.2.10 Saponin lysis of *P. falciparum* infected RBCs (iRBCs)

In vitro cultured parasites were isolated by saponin lysis of iRBCs according to Goman et al. (1982). With saponin the membrane of erythrocytes is selectively lysed, whereas the parasitophorous vacuole with the parasite inside remains undamaged. Infected RBCs were centrifuged for 10 min at 800g. Pelleted cells were resuspended in 20 volumes of saponin lysis buffer and incubated 10 min at RT. Centrifugation for 10 min at 3000g was performed to pellet the parasites. The pellet was washed once in 1xSSC and stored at -70/C until use.

4.2.11 Preparation of parasite derived proteins

Preparation of parasite derived proteins was done as described previously (Lanzer et al. 1992). About $5x10^9$ parasites were suspended in 1ml of PDP lysis buffer (10 mM HEPES pH 7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.65% NP-40) and centrifuged to pellet the nuclear debris. The parasite proteins were resolved in a SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was probed with the immune sera of the immunised mice.

4.2.12 Presentation of antibody data

For all MSP2-specific ELISA assays, the positive control serum pool was included in each plate as dilution series. A regression line was fitted to the control series for each antigen, and the average of the duplicate readings of optical density at 405 nm (OD405) for each serum sample was converted to antibody units based on the standard curve. For each single plate the OD405 of the serum pool (minus corresponding background well with DHFR-6His) diluted 1:500 was defined as 1 unit. Results expressed in antibody units, calculated from the formula:

$$AU = \frac{OD405 test.serum - OD405 test.serumDHFR}{OD405 pos.serumpool - OD405 pos.serumpoolDHFR}$$

Positivity was determined from the mean reactivities plus 2 standard deviations of 36 sera of non-exposed European children. In addition the latent class model were used to determine the positivity of the test samples (Vounatsou et al. 1998, Irion et al. 2001a). For further calculations the values resulted from a modelling based on a latent class model were used.

4.2.13 Latent class model

Measurements of antibody responses in ELISA often show high levels of heterogeneity, making it impossible to clearly distinguish responders and non-responders to particular antigens. Typically a non-exposed control group is used to assign a cutoff value of positivity, calculated as the mean plus 2 or 3 standard deviations. This can give rise to extremely biased estimates of response rates when the background is variable, and especially when there is overlap between the distribution of the control levels and that of responders. We propose that such data should be analysed by modelling based on a latent class model.

Assume that the observed population consists of a mixture of non-exposed control group in proportion $(1-\theta)$, not necessarily normally distributed, together with the mixing proportion θ (the attributable fraction) of exposed (tested) individuals. The distribution of antibody units in the malaria-exposed children was resolved into two components ($g_1(.)$ and $g_2(.)$). One component corresponds to non-malaria elicited antibody levels ($g_1(.)$) and the other to malaria elicited antibody levels ($g_2(.)$). Antibody levels from control samples were assumed to be a sample from the non-malaria elicited antibodies component of the mixture. Let x_1, x_2, ... xn be the set of independent, identically distributed random variables from the two component mixture distribution

$$F(x) = (1 - \theta)\, g_1(x) + \theta\, g_2(x),$$

with $g_1(.)$ = distribution of non-malaria elicited antibody levels

$g_2(.)$ = distribution of malaria elicited antibody levels

Observations from one component distribution, $g_1(.)$ are assumed to be smaller than those from the other and there is some overlap between the two distributions.

Figure X illustrates how $F(x)$ compares with $g_1(x)$ in the case of $\theta = 0.8$ and $g_2(x)$ with the mean of 0.7 antibody units. The density ratio $D(x)=F(x)/g_1(x)$ is the ratio of the frequency of any given antibody unit value in the population, to the frequency in the control group. If there are no additional antibodies measured in the tested samples then we expect this ratio to be uniformly 1 and $\theta=0$. On the other hand, if θ is greater than 0, we expect D to be an increasing function of x. In the limiting case of no overlapping where $g_2(x)=0$ provides $D(x)=(1-\theta)$. (Another limiting case where $\theta=1$ and hence $D(x)= g_2(x)/g_1(x)$.) Children whose antibody units resulting from malaria exposure are unlikely to have very small antibody units, so that over the whole range of no

overlapping of the two subgroups D(x) may be very close to (1- 8).

A simple way to estimate 8 is to split the data into discrete categories of antibody units (x) and to plot the density ratio (D(x)) for these categories. This is calculated by taking the proportion of the tested population in that category and dividing by the proportion of the control group which falls into the same range. The average value which D(x) achieves over low antibody unit values of x can than be used to obtain a minimally biased estimate of (1- 8). Strictly, to completely eliminate bias the value of the ratio at low antibody units should be estimated by extrapolation, but in real data sets measurement errors are likely to account for a disproportionate proportion of the observed density for low values of *x*. Hence, any bias introduced by using the recommended procedure is minimal in contrast to errors depending on the laboratory method.

The average level of antibody units among the positive subset of the tested samples is equal to its mean of the antibody units divided by (1-8).

In addition to its use in estimating the prevalence of positivity, the density ratio curve can be used to estimate the probability *p* that children with a given antibody unit value have antibodies attributable to *Plasmodium falciparum* exposure. If there were no additional antibodies measured then the probability of a given antibody unit would be $g_1(x)$. However, the actual probability is F(x). The difference is attributable to the positive samples. The proportion of individuals whose antibody level can be attributed to malaria exposure, at any given value of x is equal to:

$$p(x) = \frac{l\,g2(x)}{F(x)} = \frac{F(x) - (1- l)\,g1(x)}{F(x)} = \frac{D(x) - (1- l)}{D(x)}$$

D(x) and 8 can be estimated directly from the density ratio plot (dashed line in figure 2a and 2b in paper IV) and the calculates of p(x) thus obtained can be used as an estimate of the probability that an individual shows an *Plasmodium* specific immune response. This probability depends upon both the individual's own antibody level and on the population distribution.

4.2.14 Statistical analysis

To determine if any relation between different variables and antibody unit values was significant, Spearman correlation analyses were performed on the sets of continuous data on antibody response to an antigen versus listed variables: age of the donor, net use, parasitemia, value of other antibody responses, multiplicity of infection, number of new acquired infections, number of lost infections, repeat numbers of *msp2* alleles of infecting parasites. Spearman correlation analyses were used, as the data distribution was non-normal for antibody responses and the other variables.

T-test procedure was performed to determine if there was a significant difference in antibody response between sample pairs infected and non-infected with *P. falciparum* divided by family specificity of the *msp2* allele expressed by the infecting parasites.

5. Study Populations

Malaria exposed individuals:

1. Tanzania

The study in the malaria holoendemic area was performed in the village of Kiberege, Kilombero District in the Morogoro Region of southeastern Tanzania. This region is well known as an area of high perennial malaria transmission. The village has about 6500 citizens and lies at the edge of the Kilombero river plain. The epidemiology of malaria has been investigated in this area since decades (Tanner et al. 1991). The annual entomological inoculation rate (EIR) is around 300 (Smith et al. 1993) and more than 60% of the children of five months of age are infected with *Plasmodium falciparum* (Kitua et al. 1996). The study design has been described in detail by Fraser-Hurt et al. (1999). The trial has been lasted from May 1996 to November 1996. In total, 122 children initially aged 5-24 months were split randomly in two groups: the ITN using group and the control group. For our immunological study, we selected 30 children from each group, initially aged 5-15 months.

2. Papua New Guinea

Twenty sera from semi-immune adults inhabiting the Wosera region, a highly endemic area for *P. falciparum* malaria in Papua New Guinea, were used as positive controls in various immunological assays. These sera originate from one of seven community surveys for immunological and parasitological baseline monitoring of a potential study population (Genton et al. 1995).

Malaria non-exposed individuals

Sera from non-exposed adults originated from the serum bank of the Swiss Tropical Institute. The donors have never been travelled to a tropical country.

Sera from non-exposed European children aged 5-15 months were obtained as a generous gift from the Universitäts-Kinderspital beider Basel, Department of Virology and Serology, Basel, Switzerland. The healthy children were part of a study concerning allergic disorders.

Sera storage condition

All sera were stored as aliquots at -20/C until use.

Genotypes of Merozoite Surface Protein 2 in Tanzania

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Abstract

The merozoite surface protein 2 (MSP2) of *Plasmodium falciparum* is extremely polymorphic: 82 different *msp2* alleles were found in four studies of molecular epidemiology conducted in Tanzania. This diversity renders *msp2* suitable as a marker gene for genotyping of parasite infections. Amplification of *msp2* by PCR, and subsequent restriction digests of the PCR product (PCR-RFLP genotyping), have proved to be an informative tool for enumerating multiple concurrent infections in a blood sample, and distinguishing individual alleles.

Depending on the specific questions asked in a genotyping study, analytical techniques of different degrees of complexity are employed. The restriction fragments resulting from a single *Hin*f I digest generally allow the enumeration of multiple concurrent infections and the determination of their allelic family. When a restriction pattern is too complex to be resolved, owing to the high number of concurrent infections, or due to the appearance of previously undescribed alleles, one or more additional digests (*Dde* I, *Rsa* I, *Scr*f I) may be necessary. To unequivocally determine individual alleles, in particular in longitudinal studies, where several consecutive samples need to be compared with each other, a more detailed analysis involving all three additional digests is applied. The methodological experience and results gained in four epidemiological field studies involving *msp2* genotyping are summarized. We also provide the *Hin*f I restriction patterns and some nucleotide sequences of the alleles found so far in our studies in Tanzania.

Keywords: *Plasmodium falciparum*, merozoite surface protein 2, genotyping method, restriction patterns, sequences, new alleles.

Introduction

Alleles of the merozoite surface protein 2 (MSP2) are grouped into two allelic families according to the dimorphic structure of the variable non-repetitive region (Smythe et al. 1990). The extensive diversity is due to an allele-specific central region which comprises tandem repeats of varying size. Different copy numbers of these repeats result in length polymorphism. These characteristics of *msp2* have been exploited by several genotyping methods. Common to all methods of *msp2* genotyping is a PCR amplification of the central part of the *msp2* gene, which comprises the allele-specific repetitive region and conserved flanking sequences. The major methods used for *msp2* genotyping are (1) analysis of size polymorphism with subsequent hybridisation with family specific probes (Foley et al. 1992; Babiker at al. 1994; Ntoumi et al. 1995; Viriyakosol et al. 1995), or (2) restriction fragment length polymorphism of the amplified product (Felger et al. 1993). *msp2* has been used extensively as polymorphic marker gene in different field studies in different geographic locations either within a multilocus-analysis or in a single-locus analysis. All studies showed extensive polymorphism at this locus, even in areas of low endemicity.

The use of *msp2* as marker has proven to be of great use when individual *Plasmodium falciparum* infections need to be identified. PCR-RFLP genotyping of parasites for *msp2* makes it possible to distinguish the individual parasite infections concurrently present in a blood sample. The possibility of tracing individual parasite clones over time allows detailed studies of infection dynamics. In immunological studies genotyping can also provide important information on the diversity of antigenic challenge. Most importantly, genotyping makes it possible to determine the multiplicity of infection, which can be used as an outcome measurement of interventions, such as drug trials, vaccine trials, or trials to determine the effect of exposure-reducing interventions.

The *msp2* gene is not only an extremely polymorphic marker gene, but its product is also a vaccine candidate. MSP2 as part of a subunit vaccine is currently under field trial in Papua New Guinea (PNG) (Genton et al. 1996). Two genotyping studies from PNG have already shown that the two allelic families of *msp2* are differently associated with morbidity (Engelbrecht et al. 1995; Al-Yaman et al. 1997). Thus, using *msp2* for genotyping offers both genetic diversity for high resolution typing, and additional information on the biological role and significance of MSP2 as an immunological target. In this paper we describe in detail the PCR-RFLP genotyping technique and its possible applications, as well as the diversity and RFLP patterns of the *msp2* alleles detected so far in blood samples from the Kilombero District, Tanzania.

Methods

Extraction of deoxyribonucleic acid

Infected erythrocytes (RBC) from samples of packed cells (stored at -20° C) were used for isolation of parasite deoxyribonucleic acid (DNA). After thawing the pellets, 5 :l of packed cells (equivalent to about 12.5 :l of whole blood) were added to 50 :l 4 M guanidine-isothiocyanate containing 25 mM sodium-citrate (pH 7.0) and 0.5% sodium-sarcosylsulfate; 5 :l of 0.2 M sodium-acetate (pH 4.4) were then added. The mixture was left on ice for 10 minutes and then extracted once with 100 :l phenol (pH8.0):chloroform (1:1), precipitated with isopropanol and resuspended in 50 :l of distilled water.

PCR amplification

Both a primary and a nested PCR were performed for each sample. For primary PCR, 5 :l of the extracted DNA (corresponding to 0.5 :l RBC pellet) were added to 95 :l of reaction mix including 1.5 units *Taq* polymerase (GIBCO BRL/Life Technologies), 50 mM potassium chloride, 10mM Tris-HCl (pH 8.8), 1.5 mM magnesium-chloride, 0.2 mM of each deoxynucleotide (dNTP), and 0.5 mM of each primer. The primer pair for primary PCR corresponded to nucleotides 3-23 and 789-811 from the 5' and 3 ' conserved region of the MAD71 sequence of *msp2* (Foley et al. 1992). The PCR conditions were: 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 2 min at 55°C, 2 min at 70°C. Two :l of the primary PCR product were reamplified in a nested reaction (total volume 100 :l) with nested primers (Foley et al. 1992) corresonding to nucleotides 111-129 and 709-728 of the same sequence. The cycle conditions for nested PCR were the same as for the primary reaction. In earlier *msp2* genotyping studies in PNG, PCR conditions with a lower annealing temperature, according to Foley et al. (1992), were used. However, due to the complexity of Tanzanian *P. falciparum* infections, a higher annealing temperature was chosen to increase specificity, in spite of reduced yields of PCR products. The nested PCR products were separated on a 10% polyacrylamide (PAA) gel. Samples from which no detectable PCR product was obtained were re-examined at least once, starting from the DNA preparation. For quality control, DNA extraction, both rounds of PCR, and restriction digests were repeated for 10% of all samples.

Restriction digests

Nested PCR products were routinely digested with *Hin*f I for 2 hours and then run on 10% PAA

gels (BRL V16) using 1 mm spacers. The separation was improved if the gels were prepared at least 1 day before use. Gels were stained with ethidium bromide and photographed or electronically recorded. Fragment sizes were estimated by comparison with a DNA size marker (1 kb ladder, Gibco BRL Life Technologies). If samples from longitudinal studies were to be compared with each other, they were run side by side on the gel. When a higher discriminatory power of the RFLP pattern was desired, usually in the case of new alleles or ambiguities, additional *Dde* I, *Rsa* I and *Scr*f I restriction digests were performed. When direct comparison of RFLP patterns with those of already defined alleles was needed, restriction digests of both the known and the unknown PCR product were repeated, and run side by side on a PAA gel. Similarly, when consecutive blood samples from a longitudinal study had to be compared with each other, the digested PCR products of these samples were run on the same gel side by side, in order to identify size differences of a few nucleotides.

Results

Diversity of Tanzanian msp2 *alleles*

We performed several genotyping studies with blood samples from Ifakara or neighboring villages in Tanzania. In all studies the same PCR-RFLP typing method was used. Taking the four studies listed in Table 1 together, 3506 individual infections with *P. falciparum* were genotyped. In these samples 82 different *msp2* alleles were detected, of which 41 belonged to the FC27 family, and 41 to the 3D7 family.

Principles of PCR-RFLP genotyping

To determine the number of concurrent infections per blood sample (multiplicity of infection), or to discriminate whether an allele belongs to the 3D7 or FC27 allelic family, only a single restriction digest (*Hin*f I) is performed on the nested PCR product of the *msp2* gene. The dimorphic region of *msp2* shows family-specific *Hin*f I restriction sites which are located in the variable non-repetitive region and which are shared by most alleles of the same family. Only rare point mutations within a *Hin*f I site, or the appearance of a new repeat type in the FC27 family, were found to cause deviations from the ubiquitous family-specific restriction fragments. In all other cases, size deviations were caused by crossing over between alleles of different families. The family-specific restriction fragments resulting from *Hin*f I digests of FC27-type alleles are two fragments with lengths of 137 bp and 115 bp, and of 3D7-type alleles, two fragments of 70 bp and

108 bp. Fragments of these sizes can easily be discriminated on a 10% PAA gel. Thus, either one pair of fragments or the other indicates the presence of at least one allele of the respective allelic family. If both fragment pairs appear in one sample, at least one allele of each family is present. The total number of different alleles found in a given blood sample is determined by the remaining *Hin*f I restriction fragments which form an allele-specific pattern. These restriction patterns of individual alleles will be presented below.

FC27 family of msp2 *alleles*

Table 2 lists all 41 FC27-type alleles found in these studies, their restriction fragments obtained after *Hin*f I digestion of the nested PCR product, and the Genbank™ accession numbers of those alleles already sequenced. Some alleles contained more than one 96 bp *Hin*f I fragment, as indicated in Table 2. A 96 bp *Hin*f I fragment is generated if an allele contains at least two copies of a 96 bp repeat unit in tandem array. Therefore, the actual number of repeats equals the number of 96 bp fragments +1.

*Hin*f I restriction digestion of a nested PCR product of FC27-type alleles normally produces two conserved fragments of 137 bp and 115 bp, which represent the 5' and 3' ends of the amplification product, respectively. A selection of *Hin*f I digested FC27-type alleles from Tanzania is shown in Fig.1. The family-specific fragments (115 bp and 137 bp) are present in all allelesin lanes Wos6 to K1. Two repeat units (96 bp and 36 bp) are characteristic of all FC27-type alleles. At least one copy of each repeat is normally present in an allele of the FC27 family, while different alleles vary in the number of copies of 96 bp and 36 bp repeats present in the central region of the PCR product. Thus, this central region gives rise to allele-specific restriction fragments. Due to variation in the copy number of a 36 bp repeat between one and 7, the largest *Hin*f I fragment in FC27-type repeats varies from 162 bp in Ifa46 to 378 bp in Ifa4. Lanes Ifa46 to K1 in Fig. 1 represent a selection of alleles, which differ only in the number of the 36 bp repeats present. The smallest allele, Ifa46, has one copy of the repeat, the longest allele, K1, has 5 copies. A 96 bp *Hin*f I fragment (as seen in Fig.1, lanes Wos6 and D10) represents two or more copies of the 96 bp repeat unit.

In many FC27-type *msp2* alleles from Tanzania, a third repeat type was discovered, which increased the size of the family-specific 137 bp *Hin*f I fragment at the 3' end of the amplification product (Irion et al. 1998a). Substituting for the 137 bp fragment, new fragment sizes were generated by different copy numbers of this 9 bp repeat encoding the amino acids asparagine (N), alanine (A) and proline (P). As a result, the size of this normally conserved fragment can range

from 146 bp in Ifa1, 182 bp in Ifa13, and 200 bp in Ifa11 to up to 335 bp in Ifa23 (see Fig.1), which contains 23 copies of the NAP repeat.

3D7 family of msp2 *alleles*

*Hin*f I restriction digestion of a 3D7-type allele produces two conserved restriction fragments of 70 bp and 108 bp, which both derive from the 3' end of the *msp2* nested PCR product. One further large *Hin*f I fragment is obtained, which varies between alleles. Individual alleles were named according to the size of this fragment, e.g. $3D7_{370}$. Other, unusual restriction patterns can also, though rarely, be found in this allelic family, mostly due to point mutations, creating new *Hin*f I restriction sites. 3D7-type alleles with deviations from the conserved *Hin*f I pattern are presented in Table 3.

Recombinant forms between the two allelic families

Alleles which represent recombinations between the FC27 and the 3D7 family have also been found in Tanzanian blood samples. These recombinations are revealed by *Hin*f I restriction fragment analysis by their unusual fragment lengths. The *Hin*f I restriction digests of two recombinant alleles (Ifa18, Ifa42) are shown in Fig. 1. Nucleotide sequencing revealed that in all examples analysed so far, the site of recombination was located at the 5' end of the 96 bp repeat unit (Irion et al. 1998a). In all recombinations for which sequence data are accessible, the 5' end was derived from a 3D7-type allele, and the 3' end was of the FC27 type. PCR products of *msp2,* representing recombination between the 3D7 and the FC27 allelic families, are grouped with FC27-type alleles and listed in Table 2, because they contain mainly FC27-type sequences, in particular the FC27-family specific 36bp and 96bp repeats.

Multiple infections in Tanzanian blood samples

To illustrate the use of our RFLP genotyping method for *P. falciparum* infection in Tanzanian blood samples, an example of a multiple infection (multiplicity of 4) is shown in Fig. 2. The photograph shows *Hin*f I restriction digests separated on a 10% PAA gel. The fifth lane represents a naturally-occurring mixed infection in a Tanzanian blood sample. The restriction patterns of all four individual alleles contained in this mixed infection are shown separately in lanes Wos7, Wos12, Wos10 and $3D7_{400}$.

When analysing multiple infections, the intensity of ethidium bromide staining of individual bands in the PAA gel has to be considered. All *Hin*f I fragments of an allele show the same intensity, and can therefore be grouped together. Fragments which are shared by several alleles, also appear as dominant bands, as can be seen in the mixed sample of Fig. 2. Here the 115 bp and 137 bp *Hin*f I fragments stood out as the strongest bands, because these two fragments were present in 3 of the 4 co-infecting alleles. Wos 7 (Fig. 2) contained three copies of the 96bp repeat, resulting in two 96 bp *Hin*f I fragments, indicated by the increased intensity of staining of this fragment compared to the other *Hin*f I fragments of this allele.

Additional restriction digests increase the resolution of the genotyping scheme

In some studies, not only multiplicity and allelic family were to be determined, but it was also necessary to trace individual alleles in consecutive blood samples. For this purpose, a more complex genotyping technique was applied, involving four different restriction digests and side-by-side runs of the samples to be compared on the gel. Because the variable, allele-specific *Hin*f I fragment of 3D7-type alleles is too large (250 to 550 bp) to identify an allele unequivocally by gel electrophoresis, further restriction digests (*Dde* I, *Rsa* I, *Scr*f I) are necessary which yield smaller and allele-specific fragment sizes. This allows further differentiation between 3D7-type alleles showing a Hinf I fragment of similar size (differences < 10 bp). The conserved and variable restriction fragments resulting from *Rsa* I, *Dde* I and *Scr*f I digests of 3D7-type alleles have been described earlier (Felger et al. 1994). These additional restriction digests are generally done, if infections from different blood samples need to be compared to each other. Further restriction digests in addition to Hinf I are also useful for the determination of recombinant, new, or 3D7-type alleles. These extra digests increase the discriminatory power of this genotyping scheme considerably and allow the identification of individual alleles.

Potential use of msp2 *genotyping by PCR-RFLP*

The potential of PCR-RFLP typing for direct comparison of blood samples is shown in Fig. 3. This example derives from a drug test where recrudescent and new infections were discriminated (Irion et al. 1998b). Each lane represents a consecutive blood sample from the same donor. In this example, baseline samples A and B (day 0 and day 3 were defined as baseline) clearly showed RFLP patterns different from samples C and D. Sample A contains 4 different infections, two of which belonged to the FC27 family (Ifa1, Ifa4), and two were of 3D7 type ($3D7_{320}$, $3D7_{430}$). The second baseline sample B showed the same alleles with the exception of Ifa1, which was missing. All baseline genotypes had disappeared from subsequent samples (C and D), which both contained two new and distinctively different infections (Ifa40 , $3D7_{340}$). By conventional microscopy, these samples would have been identified as recrudescences and treatment failures.

Comparison of PCR-RFLP with two-loci-genotyping

In a previous study (Beck et al. 1997) genotyping results were available for both loci, *msp2* genotyped by PCR-RFLP (Beck et al. 1997), and *msp1* (W. Huber, personal communication), which was genotyped for allelic family by family-specific nested PCR amplification as described by Huber et al. (1998). A comparison of multiplicity obtained from each locus made it possible to assess the gain in resolution by genotyping an additional locus. The analysis of *msp1* was based on the determination of allelic families only, size variations of the *msp1* PCR products not being considered. Only in 7 of 269 infections did the additional *msp1* genotyping improve the resolution obtained by the *msp2* PCR-RFLP method (Table 4). This increased resolution was due to infections defined as single clone infections by *msp2* PCR-RFLP typing. 7 of which were defined as mixed infections when defined by *msp1* typing. In these cases, two different parasite clones shared the same *msp2* allele, or at least, produced the same RFLP pattern. However, in all cases of multiple simultaneous infections, additional *msp1* typing did not increase the multiplicity of infection determined by *msp2* PCR-RFLP genotyping.

Discussion

Genotyping *P. falciparum* field isolates is useful for a wide variety of applications. While we were mainly interested in detecting multiplicity of mixed clone infections and in studying infection dynamics, other research applications could include finding markers for virulence or drug resistence. Since only limited amounts of blood are available in epidemiological studies, amplification of a marker sequence by PCR is the method of choice. Potential genotyping markers are sequences showing polymorphism within a parasite population. Polymorphisms in both repetitive sequences and unique sequences have been used for genotyping (Robert et al. 1996). When polymorphic repetitive sequences are amplified by PCR, a clone-specific pattern of multiple bands is seen after separating the PCR products on a gel. However, for the multiple concurrent infections, normally found in areas of high malaria transmission, these banding patterns superimpose each other and become too complex to analyse. A selection of unique DNA sequences, suitable as polymorphic markers, is available in *P. falciparum* because of its highly diverse surface antigens. The most promising marker for genotyping is the one showing the most extensive polymorphism. We chose *msp2* as a marker gene for two reasons: (1) with 82 different alleles detected so far in Tanzania, *msp2* offers the genetic diversity necessary to allow single locus genotyping. The polymorphism is of such an extent that superinfection with the same genotype as the previous one is highly unlikely (Smith et al. 1999b). This fact allowed the use of *msp2* as a marker to discriminate between new *P. falciparum* infections and recrudescenes in a drug trial (Irion et al. 1998). (2) Besides information on multiplicity of infection, further information about the biological role of MSP2 can be gained from epidemiological studies which involve *msp2* as a marker gene, i.e., morbidity associations of certain *msp2* alleles or an allelic family.

In analyzing genotyping data it has to be remembered that there are intrinsic limitations of the PCR technique. It has to be noted that the number of concurrent infections detected by PCR is always a minimum estimate of the number of different parasite clones which are present in a carrier. Some clones might be missed owing to the detection limit of the PCR technique, which might miss infections of very low density, or to sequestration of parasites at sampling as shown by Farnert et al. (1997). Furthermore, Contamin et al. (1995) showed that in multiple infections, the most abundant allele in a blood sample can suppress the amplification of an allele which is less abundant in the sample. However, statistical analyses of longitudinal data can be used to estimate the extent of such imperfect sensititvity (Smith et al. 1999b). Our knowledge of heterogeneities in densities of individual genotypes in multiple infections remains limited, and further development of

quantitative genotyping techniques is needed to fully understand the interdependence in multiple clone infections.

We are also aware that alleles resulting from recombination between the allelic families remain undetected if no obvious alteration in the restriction patterns is caused. PCR amplification with mixed family-specific primers provided evidence for a high frequency of recombination (Ntoumi et al. 1997). This was not observed in our studies. A further limitation of genotyping by RFLP consists in sequence diversity located outside the restriction sites. In order to test this, we have previously conducted single-strand conformation polymorphism (SSCP) experiments, using the FC27 allele (defined by PCR-RFLP) from different isolates. We were not able to detect intraallelic variation within 36 samples (Felger et al. 1997). In order to establish the full extent of sequence diversity in a marker gene, direct sequencing is necessary. However, this is not feasible in epidemiological studies, and direct sequencing is not possible if multiple infections are simultanously present. A more detailed critical evaluation of PCR-RFLP genotyping can be found in Snounou & Beck (1998).

In spite of the high resolution of PCR-RFLP genotyping of *msp2*, there is an obvious inherent limitation in using only one locus, since two infections sharing the same *msp2* allele will be determined as one. This problem can be reduced by genotyping a second gene in addition to *msp2*. Other studies have used multiple locus genotyping, with low resolution for each individual locus. This introduces the problem of estimating the total number of concurrent infections. The probability of recombination increases the possible maximum number of concurrent infections to the number of multiple genotypes found at one locus times the number of multiple genotypes found at a second locus. This rarely reflects the real situation in the individual host. However, advanced statistical analysis allows to estimate the mean multiplicity for the host population (Hill & Babiker, 1995). Using this method, an estimate of the mean multiplicity of 3.3 was obtained from 53 sampes from the same area in Tanzania (Babiker et al. 1997). This estimation was based on genotyping data generated by hybridisation with oligonucleotide probes specific for allelic families to blotted PCR products from the two loci *msp1* and *msp2*. This result is closely comparable to our own findings (Fraser-Hurt et al. 1999). However, this method does not provide usable estimates of multiplicity for individual carriers, and the result depend on assumptions about the population structure of the parasite.

With any genotyping method available so far, only a lower limit to the number of concurrent infections can be determined. By adding more polymorphic markers, such as *msp1*, the apparent

degree of diversity can be further increased, at least in single infections, and thus can contribute to a more exact determination of multiplicity. However, for studies of infection dynamics or comparative studies, where individual parasites need to be traced in consecutive blood samples, the amplification of several polymorphic genetic loci does not improve the information already gained by the most diverse marker gene. This is due to the fact, that all marker genes used to date are unlinked, and therefore a multi-locus genotype of an individual parasite clone cannot be identified in multiple infections.

Furthermore, a comparison of results obtained by PCR-RFLP genotyping of *msp2* with results from genotyping for *msp1* allelic families revealed that the additional locus did not contribute much to the determination of multiplicity. *msp1* typing improved the resolution of PCR based genotyping only in 2.6 % of the samples, all of which appeared to be single infections with respect to *msp2*, but were shown to be double infections with respect to *msp1*. We therefore concluded that in Tanzanian blood samples, with an average of 5 detected infections per child, the single locus PCR-RFLP typing using *msp2* has adequate discriminatory power. There are some purposes for which multilocus typing is necessary, for instance studies of linkage disequilibrium, searching for markers of virulence of pathogenicity, or checking whether factors modifying parasite multiplicity or diversity selectively affect particular loci (e.g., locus-specific efects of a vaccine). Hill & Babiker (1995) also described how the mean multiplicity in a population can be estimated from such multilocus typing data. Using this method, Babiker et al. (1997) estimated the mean multiplicity in 53 samples from Michenga village inTanzania to be 3.3, based on data generated by hybridization of oligonucleotide probes specific for allelic families to blotted PCR products of the 2 loci *msp1* and *msp2*. While this result was very similar to direct estimates of multiplicity for this area from PCR-RFLP genotyping of *msp2* alone (Smith et al. 1999a), the multilocus approach has several disadvantages when the objective is to relate the number of infections in a host to individual characteristics such as age, morbidity risks, or risk factors for infection. It is much more straightforward to analyse relationships of other variables with multiplicity when it is measured by a single number, and the Hill & Babiker (1995) approach does not lend itself to providing estimates of multiplicity for individual carriers. Moreover, the estimates of multiplicity depend on assumptions about the population structure of the parasite.

The analysis of studies involving longitudinal tracking of individual infections is also much more straightforward if high resolution single-locus typing is used. Such applications include comparisons of baseline and recrudescent genotypes in vaccine and drug trials in order to

distinguish treatment failure from reinfection (Irion et al. 1998; Snounou & Beck 1998), and studies of infection dynamics (Smith et al 1999b). High resolution typing of a single locus gives rise directly to patterns of appearance and disappearance of individual infections. In contrast, multi-locus genotypes of individual parasite clones cannot be identified in multiple infections, since the marker genes used to date are unlinked. Hence longitudinal studies using multilocus genotyping in areas of high parasite multiplicity can give rise to patterns which are difficult to interpret.

The extreme genetic polymorphism detected in particular in *msp2*, but also in other surface antigens of *Plasmodium*, raises further questions. For example, do individual alleles persist in time and space, or are new forms generated constantly de novo? A third possibility consists of a combination of both alternatives: a panel of defined conserved alleles plus newly generated ephemeral genotypes. Questions about the molecular evolution of *msp2* alleles have been addressed (Conway 1997; Felger et al. 1997), but not yet fully been answered. Nucleotide sequence comparisons between alleles of distant geographical origin indicated that alleles persist (Dobano et al. 1997). A large body of information on *msp2* genotypes is now available from the four studies summarized here, which we have conducted in Ifakara and Kilombero valley, Tanzania, in different years, using the same PCR-RFLP method for genotyping. The diversity of *msp2* was similar in all four studies. This suggests that though the genetic diversity is extensive, it is nevertheless limited by structural constraints or immune selection. This hypothesis has been previously proposed for the FC27-type alleles (Felger et al. 1997). The results of our molecular epidemiological studies (listed in table 1) clearly showed that most of the alleles, in particular the most frequent ones, were found in all studies at comparable prevalence (data not shown). The fact that some rare alleles were not found in all studies might be due to sampling variation, which might miss alleles of low frequency. Alternatively it could indicate that these rare alleles existed only for a short period of time. Even in studies comparing genotypes found in PNG and Tanzania, no major differences were observed, except for the group of new FC27-type alleles containing different numbers of a new 9 bp repeat unit, which was not detected in PNG. This extensive but restricted diversity might be due to functional constraints of MSP2 and might therefore help to elucidate the biological role of this molecule.

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Table 1: Comparison of numbers of different *msp2* alleles of Plasmodium falciparum found in four studies of children from the Kilombero valley, Tanzania.

Reference	total no. of individual *P. falciparum* infections	no. of different alleles	no. of FC27-type alleles	no. of 3D7-type alleles
Irion et al. 1997	1034	58	26	32
Beck et al. 1997	292	51	18	33
Felger et al. 1999	365	50	24	26
Fraser-Hurt et al. 1999	1815	68	34	34
total	3506	82	41	41

Table 2: Details of 41 *msp2* genotypes of the *Plasmodium falciparum* FC27 allelic family from Tanzania

msp2 genotypes	Genbank™ accession No.	Hinf I restriction fragments (bp)			
D10	JO3828	1x96	115	137	162
Ifa1	AF010462		115	146	234
Ifa2			115	308	162
Ifa4			115	137	378
Ifa10			115	164	162
Ifa11	AF010454		115	200	198
Ifa13	AF010455		115	182	162
Ifa14[1]	AF010456	2x96	115	131	162
Ifa15			115	218	162
Ifa16			115	146	270
Ifa17		1x96	115	146	126[2]
Ifa18[1]	AF010457		115	239	162
Ifa23	AF010463		115	335	162
Ifa27		1x96	115	107	126[2]
Ifa28			115	137	342
Ifa30		1x96	115	191	162
Ifa31	AF010458		115	191	198
Ifa32		1x96	115	137	198
Ifa33		1x96	115	137	150[2]
Ifa34			115	191	234
Ifa36			115	218	198
Ifa38			115	137	126[2]
Ifa40			115	254	162
Ifa41	AF010459		115	272	198
Ifa42[1]	AF010460		115	31+82	162

Ifa43			115	-	371
Ifa44			115	146	306
Ifa45		3x96	115	137	162
Ifa46	AF010461		115	137	162
Ifa47[1]		1x96	115	-90	126[2]
Ifa49			115	254	162
Ifa50			115	173	198
Ifa51		1x96	115	137	234
Ifa52			115	281	162
Ifa54		2x96	115	137	126[2]
K1	M59766		115	137	306
Wos10	U07010		115	137	198
Wos12	U16696		115	137	270
Wos3	U07003		115	137	234
Wos6	U07006	3x96	115	137	126[2]
Wos7	U07007	2x96	115	137	162

[1]) cross over

[2]) deletion

Table 3: Details of 9 msp2 genotypes of the Plasmodium falciparum 3D7 allelic family from Tanzania[a]

msp2 genotypes	Genbank accession no.	Hinf I restriction fragments (bp)
Ifa5		70, 108, -550
Ifa6		51, 70, 108, -310
Ifa7	AF033860	51, 70, 108, 386
Ifa8		51, 70, 108, -400
Ifa20		70, 23+85, -370
Ifa24	AF033859	70, 108, 266
Ifa39		51, 70, 108, -290
Ifa48		51, 70, 108, -330
Ifa53		70, 108, -250

[a]Only genotypes with unusual restriction fragments are listed.

Table 4: Numbers of concurrent infections detected by genotyping *msp1* and *msp2* of *Plasmodium falciparum* in multiple infections in 269 blood samples from Tanzania

	msp2								
	1	2	3	4	5	6	7	8	
msp1									total
1	38	24	15	14	13	4	3	1	112
2	7*	15	20	17	32	13	16	23	143
3	0	0	0	1	3	4	3	3	14
									269

*Samples in which genotyping for two loci resulted in a gain in the resolution of multiple infections.

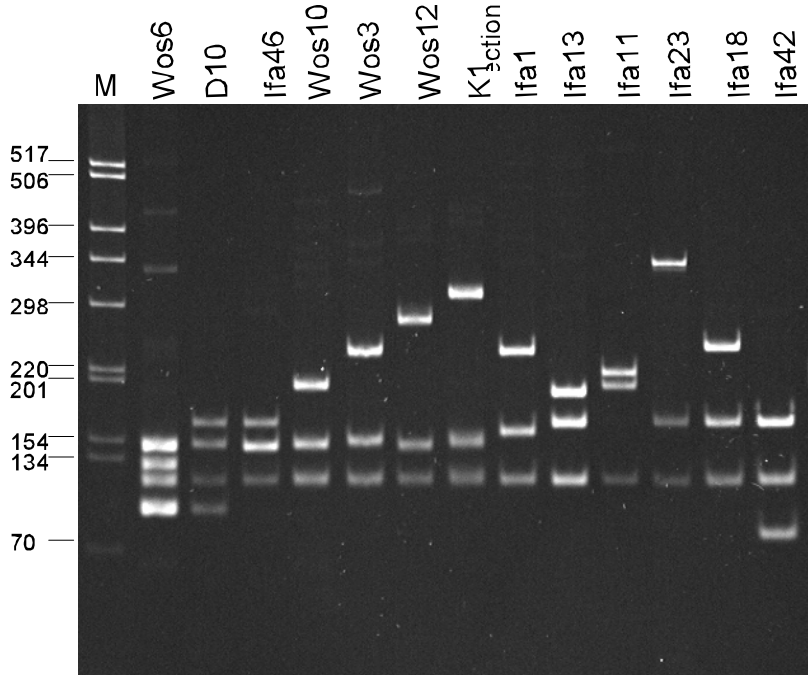
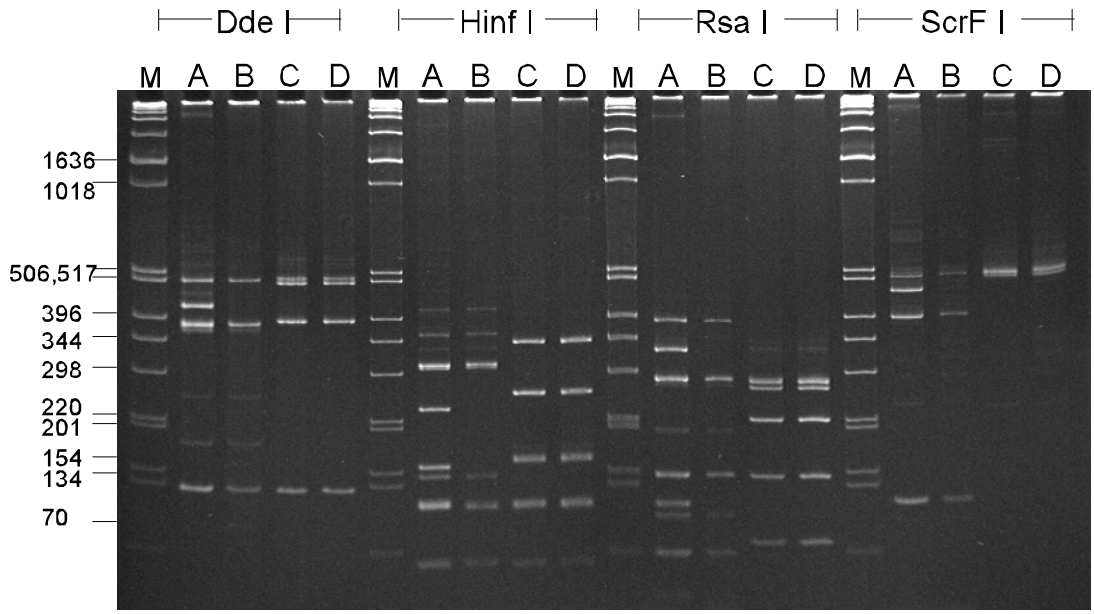


Figure 1: *Hin*f I restriction patterns of selected FC27-type *msp2* alleles of *Plasmodium falciparum* from Tanzania. Restriction fragments were separated on a 10 % PAA gel. The fragment sizes and Genbank™ accession numbers of these alleles are listed in Table 2. Lane M: DNA size marker (1 :g of 1 kb ladder, Gibco BRL Life Technologies); lanes Wos6 and D10: alleles with 4 (Wos6) or 2 copies (D10) of the 96 bp repeat; lanes Ifa46-K1: increasing copy numbers of the 36 bp repeat ranging from Ifa46 (1 copy) to K1(5 copies); lanes Ifa1-Ifa23: increasing numbers of the 9 bp NAP repeat with 2, 6, 8, 23 copies, respectively; lanes Ifa18 and Ifa42: recombined alleles.

Figure 2: *Hin*f I digest of *msp2* alleles of a *Plasmodium falciparum* multiple infection from a Tanzanian blood sample, which shows a multiplicity of four concurrent infections. In order to



demonstrate how a complex restriction pattern is analysed, digests of corresponding alleles (Wos7-3D7$_{400}$) were loaded beside the RFLP pattern of the multiple infection. Lane M1: 1 :g of 1 kb ladder (Gibco BRL Life Technologies); lane M2: 1 :g of 100 bp DNA Marker (Gibco BRL Life Technologies).

Figure 3: Comparison of four subsequent blood samples (days A, B, C, and D) from a patient

infected with Plasmodium falciparum and participating in a longitudinal drug efficacy study. A and B show genotypes of parasites at baseline, C and D represent parasites appearing after treatment. PCR products of *msp2* were digested with the restriction enzymes *Dde* I, *Hin*f I, *Rsa* I, and *Scr*F I. The resulting fragments were separated side by side on a 10 % PAA gel. On days C and D, all baseline genotypes had disappeared, while two new genotypes were found. Lane M: 1 :g of 1 kb ladder DNA marker (Gibco BRL Life Technologies).

Longitudinal study of antibody responses to distinct regions of the polymorphic Merozoite Surface Protein 2 of *Plasmodium falciparum* in young children from malaria-endemic area

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Abstract

In areas highly endemic for malaria, repeated infections with *Plasmodium falciparum* result in the development of acquired immunity which, although not sterilising, protects older children and adults against severe disease. The immunological basis of this immunity is still unknown, but it has been speculated that the accumulated immune responses against antigenically diverse parasite proteins would be an important factor. In addition, the establishment of a persistent protection may depend on the presence of concomitant infections. Here we report a study examining antibody responses to different domains of different merozoite surface protein 2 (MSP2) in consecutive serum samples from 60 Tanzanian children initially aged 5-15 months from an area with intense and perennial malaria transmission. The MSP2 alleles of the corresponding were also analysed PCR-RFLP genotyping.

The pattern of response in these children was extremely complex with antibodies produced to different parts of the merozoite surface protein 2 by different children. The frequency of responders was high, although the magnitude of the antibody responses was generally low. Levels of antibody responses were quite variable to any one part of the protein.

In addition, we demonstrated that the use of insecticide treated bed nets does influence the development of antibody responses to MSP2. Hence, transmission blocking interventions in infants and children should be monitored concerning the development of immune response to *P. falciparum.*

Key words: malaria, *Plasmodium falciparum*, genotypes, merozoite surface protein 2, insecticide-treated bed nets, IgG antibody response, ELISA, children, Tanzania.

Introduction

In areas highly endemic for malaria, repeated infections with *Plasmodium falciparum* result in the development of acquired immunity which, although not sterilising, protects older children and adults against severe disease (Barragan et al. 1998). In infants in the highly endemic area of the Kilombero valley of Tanzania, the period of highest susceptibility to clinical malaria among infants occurs between the ages of 4 and 12 months, and the incidence of malaria-attributable morbidity reaches a peak at six months of age (Kitua et al. 1996). The underlying mechanisms of protection in older children are poorly understood. Passive transfer experiments with purified antibodies from hyperimmune sera to susceptible children provided evidence for the protective or at least modifying effect of the IgG fraction in combination with monocytes on the disease (Bouharoun-Tayoun et al. 1990; Kumaratilake et al. 1997). Whilst many blood-stage proteins have been identified, their significance as immunological targets is unknown (Long 1993). It is believed that the total responses against polymorphic antigens and against parasite-derived variable antigens on the surface of the infected erythrocyte contribute to the development of immunity (Giha et al. 2000). Recently, we proposed that the concomitant infections may also be important for the development of protective immunity (Smith et al. 1999c). This hypothesis implies that limited cross-protection between given polymorphic antigens exist. Therefore, we examined humoral immune responses to the merozoite surface protein 2 (MSP2), a promising vaccine candidate (Genton et al. 2001) and one of the most diverse antigens of *P. falciparum* which may be involved in protection. MSP2 is an integral GPI-anchored membrane protein with conserved carboxy- and amino-terminal regions flanking a central variable region. The variable region comprises non-repetitive, semi-conserved sequences and highly variable repetitive sequences (Anders and Smythe 1989; Smythe et al. 1990). The basic structure can be classified as dimorphic, falling into two allelic families: IC1/3D7 and FC27 (Thomas et al. 1990; Smythe et al. 1990). The role of this dimorphism is unknown, but FC27-type *msp2* genotypes have been associated with higher parasite densities and morbidity (Engelbrecht et al. 1995) and Felger et al. (1997) suggested that these allelic families are subject to different selection mechanisms. In a prospective study in Papua New Guinea, Al-Yaman et al. (1995) demonstrated a positive association between antibody levels to 3D7-type MSP2 at baseline and reduced clinical malaria during one year follow-up. Antibody levels to 3D7-type MSP2 were also found to be lower

among children who died from cerebral malaria than among those who survived, indicating a protective role for these antibodies against severe malaria (Al-Yaman et al. 1997a).

To investigate the role of dimorphism and of the distinct domains of MSP2 in immunological recognition and to assess any correlation with observed parasitemia, we expressed different MSP2 constructs in *E. coli*. These constructs were tested in serological assays with multiple serum samples from children living in a highly endemic area in order to identify the domains recognized and to investigate the development of antibody responses in early childhood. All infecting parasites were genotyped for *msp2* by PCR-RFLP. Because this study was performed within the context of an insecticide treated bed net (ITN) study we were able to test the effect on antibody dynamics of long term use of ITNs.

Study design and Methods

Serum samples: serum samples were collected in the village of Kiberege, Kilombero District, southern Tanzania. The area is holoendemic for malaria with approximately 300 infectious bites per year with perennial transmission and little seasonal changes of parasite prevalence and density (Smith et al. 1993). The study was previously described in detail by Fraser-Hurt et al. (1999). Seven consecutive finger-prick blood samples collected at one month intervals were selected from 30 children initially aged 5 to 15 months who used insecticide-treated nets, and from 30 children of the same age group not using ITNs. All blood samples were genotyped by PCR-RFLP on *msp2* as described and reported elsewhere (Felger et al. 1999, Smith et al. 1999b). Serum samples from semi-immune adults from PNG were used as positive controls. Sera from 36 healthy European adults and from 36 European children aged 5-15 months, all never exposed to malaria, were used as negative controls.

Production of recombinant msp2-clones: seven different *msp2* constructs as shown in figure 1 were expressed in the procaryotic pQE expression system (Qiagen) in *E. coli* M15. Corresponding primer pairs with restriction sites (5' *Bam*HI, 3' *Kpn*I) were used to amplify distinct regions in the *msp2* gene (Table 1) derived from different *msp2* alleles. Subsequently, PCR products were cloned in the pQE30 expression vector as a fusion protein with six histidine residues at the 5' end. For an improved yield, the 5'3'-constant recombinant protein was fused to

the mouse DHFR sequence (pQE16) using the *Bgl*II restriction site. All expressed constructs were confirmed by DNA sequencing on an ABI-Sequencer (Perkin Elmer). The recombinant constructs 23xNAP repeats, 3D7 family specific part, 4x32 aa repeats, 6x12 aa repeats, and 3D7 repeats were identical to the original protein regions and contained amino acids resulting from the restriction sites at the ends of the sequences. A single nucleotide change from guanosine to adenosine occurred at position 68 in the 5'3'construct, resulting in an amino acid change from glycine to glutamic acid also found in naturally occurring *msp2* alleles. The sequence coding for the family specific part of the FC27 allele showed a duplication of the last 14 amino acids.

Expression and purification of rMSP2: all constructs were produced according to the manufacturer's protocol (Qiagen). Briefly, after transformation by electroporation Super broth (25 g Trypton, 15 g yeast extract, 5 g NaCl ad 1 L) with 100 mg/l ampicillin (Appligene) and 25 mg/l kanamycin (Sigma), was inoculated with an over night culture and incubated in a shaker at 37°C to an $OD_{600\,nm}$ = 0.5-0.8. Protein expression was induced by addition of IPTG (Appligene) to a final concentration of 1 mM. Cells were harvested by centrifugation after four hours. Cells were resuspended in lysis buffer (50 mM Sodium dihydrogenphosphate pH 8.0; 300 mM Sodium chloride; 10 mM imidazole), incubated with 1 mg/ml lysozym for 30 min on ice, and broken up on ice by ultrasound. The solution was spun at 10.000xg for 20 min at 4 °C, the supernatant containing the fusion protein was mixed with Ni^{2+}-agarose and incubated for 1 h in a shaker. The protein-Ni^{2+}-mixture was washed with 20 mM imidazole, and the protein was eluted with 250 mM imidazole. The 5'3'constant recombinant protein was expressed at 25°C, denatured with 6 M Guadininehydrochloride (6 M GuHCl; 0.1 M sodium dihydrogenphosphate; 0.01 M TrisCl, pH 8.0), and purified with 8 M Urea with decreasing pH. The recombinant protein was eluted at pH 4.5. Twenty :l of each protein fraction were analysed by SDS-PAGE electrophoresis to approximate the purity of the preparations. Pure protein fractions were pooled and renatured by dialysis as previously described for other MSP2 recombinant proteins (Takacs and Girard 1991). Protein preparations under native conditions were dialysed against PBS pH 7.2 to remove imidazole. The protein content was estimated by the method of Bradford at 595 nm (Bradford 1976). Expression levels were found to vary, ranging from approx. 10 mg/l to 0.1 mg/l. Proteins were stored at -70°C until used.

Immunological assays: all recombinant protein constructs were recognized by

anti-6His-antibodies (Qiagen, R&D). Two point five to ten :g of each recombinant protein was used to immunize female balb/c mice by four injections (i.p.). Sera were collected 14 days after the last injection. All sera recognized the respective protein specifically in ELISAs and on immunoblots using protein preparations from strain K1 and NF54. High ELISA titres (1:12 800 - 1:25 600) were obtained with the 5'3'construct, the 6x12 aa repeats, the 4x32 aa repeats, and the FC27-family specific construct. A median titre (1:1600) was obtained with the 3D7-repeats, and low titres (1:100) with the 23xNAP repeats and 3D7-family specific construct. Except for the latter two constructs, all sera recognized mature parasites in a grape-like structure in IFAs. Specificity was high since sera recognised only parasites with the corresponding MSP2 allele, except sera against the 3D7-repeat construct, which showed cross-reactivity with FC27 alleles. All experiments were controlled with non-immune sera and with protein fractions from non-infected erythrocytes.

ELISA with sera from infants: Optimal dilutions of the immunogens and reagents were determined by checker titration with positive control serum pools (20 semi-immune adults from PNG) and negative control sera (36 European non-exposed children aged 5-15 months and 36 European non-exposed adults) (Quakyi 1980). Microtitre plates (Immulon 2; Dynex Technologies, USA) were coated with 50 :l of protein preparations. Protein concentrations ranged from 0.1 :g/ml to 10 :g/ml in PBS pH 7.2, but these concentrations were not associated with the absorption levels of antibodies. Plates were incubated overnight at 4°C, and non-adsorbed antigen was removed. Plates were blocked with 200 :l PBS pH 7.2, 5% nonfat milk powder and incubated for 2 h at 25°C. Fifty :l of test sera in 1:500 dilutions (1:1500 dilutions for the 5'3'construct and 6x12 aa repeats construct) in PBS, containing 0.5% Tween 20 and 0.5% milk powder, was added in duplicates and incubated for 1 h at 25°C. After washing, 50 :l horse radish peroxidase-conjugated second antibody (dilution1:2000 in PBS-0.5% Tween20/0.5% milk powder) was added and plates were incubated for 1 h at 25°C. After washing, 50 :l peroxidase substrate (ABTS peroxidase substrate, KPL) was added to each well, and the absorbance was measured at 405 nm after 30 min in a spectrophotometer (Titertek Multiskan MCC/340). Twenty sera from semi-immune adults from PNG were used as standard, and the European sera were used as negative controls. Each sera was also tested against a DHFR-6His preparation (5 :g/ml in PBS) produced similarly as the recombinant proteins.

Data presentation: a positive control serum pool was included on each plate in serial dilutions in all MSP2-specific ELISA assays. A regression line was fitted to the control series for each antigen, and the average of the duplicate readings of optical density at 405 nm (OD_{405}) for each serum sample was converted to antibody units based on the standard curve. For each single plate the OD_{405} of the serum pool (minus corresponding background from the DHFR-6His-control) diluted 1:500 was defined as 1 unit. Results were expressed in antibody units and positivity was initially calculated as mean values of sera from 36 non-exposed children + 2 standard deviations. For improved accuracy, the latent class model was used (Vounatsou et al. 1998). Adjustment of the model to the ELISA data and the comparison to standard calculation of cutoff values (mean+2sd) is reported elsewhere (Irion et al. 2001a).

Statistical analysis: to determine if any relation between different variables and antibody unit values was significant, Spearman's correlation analyses were performed on sets of continuous data on antibody response to a particular antigen versus listed variables: age of the donor, net use, parasitemia, value of other antibody responses, multiplicity of infection, and repeat numbers of *msp2* alleles of infecting parasites. Spearman's correlation analyses were used, as the data distribution was non-normal for antibody responses and the other variables.

Two sets of analysis were done: In the first analysis, the samples were considered as independent samples with their observed antibody value. In the second analysis, subsequent sample pairs of individuals were formed and the changes of antibody titres from the previous to the current sample of an individual were assessed. The change of antibody titres in sample pairs was correlated with several parameters, including the number of infections and the genotype determined in the current sample. The correlation of infection dynamics such as acquisition and loss of infections measured by PCR-RFLP analysis of the *msp2* locus and anti-MSP2 immune responses is described elsewhere (Irion et al 2001b).

Results

Controls: titration of the positive standard serum pool in each assay was highly reproducible and allowed inter-experimental comparison. Antibody values of 36 sera from non-exposed children were used to calculate the proportion of antibody-positive Tanzanian children. Two antigens showed high and highly variable measurements with the negative control sera: the FC27 family

specific region and the 5'3'construct (Table 2). Thus, determination cut-off values as the mean plus 2 standard deviations using non-immune sera was not appropriate and ELISA data were analysed with the methodology based on a latent class model (Irion et al 2001a).

Frequency and magnitude of responses: the frequency of responders in the Tanzanian children samples was high, although the magnitude of the responses expressed in antibody units was generally low (Table 2). For the various antigen constructs positivity determined by the latent class model ranged from 53% (4x32 aa repeats) to 92% (6x12 aa repeats). Low antibody values were obtained with the 3D7 family specific part, the 23xNAP repeats, and the 4x32 aa repeats. Medium antibody unit values were found with the 6x12 aa repeats, 3D7 repeats, and FC27 family specific regions. High antibody unit values were observed with the conserved regions of MSP2.

Antibody correlation with malariometric indices: the antibody response against all antigen constructs was positively correlated with age (Spearman's correlation $p<0.001$ for all antigens), except for 6x12 aa repeats ($p=0.108$), and for the 5'3'construct ($p=0.063$). The age coefficient for the 5'3'construct was rs= ! 0.19 and suggests that antibody responses against the conserved regions of MSP2 decrease with age.

We also tested the correlation between the change of antibody values from the previous to the current sample of an individual. There was a negative correlation with the 3D7 family specific part, the 3D7 repeats, and the FC27 family specific part ($p=0.048$, $p=0.003$, and $p<0.001$, respectively) indicating no further boosting within the sampling interval. The other antigen constructs showed no significant correlation between two consecutive samples.

The antibody values were higher in parasitemic individuals than in individuals without current parasite presence. All associations were significantly higher ($p<0.001$), except for the 5'3'construct and 6x12 aa repeats. Antibody values were also significantly correlated with parasite density (Spearman's correlation: $p<0.001$ for all except the 5'3'construct with $p=0.01$). Only the 6x12 aa repeats was not significantly correlated with parasite density. Spearman's correlation revealed no significant correlation of the antibody dynamic between the previous and the current sample and parasitemia and parasite density, respectively, suggesting that the change of antibody levels is independent from newly acquired infections or changes are not that fast.

Antibody responses against one antigen were correlated with antibody responses against any other

antigen in individual samples (Spearman's correlation $p<0.05$) with the exception of the antibody response against the 5'3' construct, which was only correlated with responses against the constructs representing the 4x32 aa repeats, and the 23xNAP repeats.

Antibody titres increased with the number of infections detected by *msp2* PCR-RFLP-genotyping, called the multiplicity of infections. Antibody concentrations against all antigen constructs were positively correlated with overall multiplicity, but also multiplicity within both allelic families, except for the 6x12 aa repeats and 5'3'construct and the number of infections expressing 3D7 alleles (Table3a).

A relatively weak but still significant correlation was observed between the change of antibody response of two consecutive samples and the number of *msp2* alleles in the corresponding blood sample (Table 3b). The change of antibody levels to 3D7 repeats correlated positively with the number of *msp2* alleles belonging to the 3D7 allelic family ($p<0.05$). A positive correlation was also found for antibody responses against the 3D7 family specific part, 3D7 repeats, 4x32 aa repeats and the number of *msp2* alleles belonging to the FC27 allelic family.

Correlation of antibody concentrations and FC27 alleles containing the highest number of repeats: the association between antibody responses to any antigen and the presence of an FC27 allele with the highest number of both the 32 aa repeats and the 12 aa-repeats was also analysed by Spearman's correlation analysis. Antibody responses to all tested antigens were significantly higher in those samples containing infections with parasites having the FC27 allele with the highest number of the different repeat types (all $p<0.03$).

When the Spearman's correlation analyses of antibody change were conducted with paired consecutive samples, antibody response to 4x32 aa repeats showed to correlate with all FC27 alleles possessing the highest number of repeats ($p<0.05$). The change of antibody response to the 5'3'construct antigen was significantly higher in samples with alleles containing the highest number of 12 aa repeats, and the change of antibody response to 3D7 repeats was significantly higher in samples with the highest number of 32 aa repeats (both $p=0.033$). A borderline significance was calculated for the correlation of the change of antibody levels to 3D7 family specific part between the previous and the current sample and the presence of infections expressing alleles with the highest number of 32 aa repeats ($p=0.05$).

Antibody correlation with net use: children sleeping under an ITN showed significantly lower antibody levels to the 3D7 family specific part, the 3D7 repeats, the 4x32 aa repeats, and the 23xNAP repeats (all $p<0.001$). There was a non-significant reduction in antibody levels against the 5'3'construct in ITN users, and a non-significant positive association with antibody responses against the 6x12 aa repeats and to FC27 family specific part. There was no correlation observed in the change of antibody levels of two consecutive samples and net use.

Discussion

The extensive antigenic diversity of *P. falciparum* and the repetitive structure of many malaria antigens imposes a major challenge to identify protein regions which might elicit protective antibodies. The merozoite surface protein 2 of *P. falciparum* contains distinct regions which are both highly conserved and highly polymorphic, as well as domains which show distinct dimorphism. We attempted to determine the immunological recognition of these individual domains in naturally occurring infections in young children of a highly malaria endemic area. We produced six recombinant proteins in *E. coli* which elicited antibodies in mice recognizing mature parasites or parasite proteins in Western blots. Expression in *E. coli* seems to be acceptable for malaria proteins since *P. falciparum* probably does not modify MSP2 and other proteins by post-translationally N- and O-glycosylation (Dieckman-Schuppert et al. 1992; Berhe et al. 2000).

We found that all domains of MSP2 were readily recognized by sera from young children living in a highly endemic area. However, antibody titres were quite low, the response was variable against the distinct protein domains, and the antibody dynamics was extremely complex as has been recently shown in a small study from Irian Jaya (Weisman et al 2001). The most frequently recognized antigen was the 5'3' construct. Antibody titres against this antigen were high, but it was also frequently recognized by sera from non-exposed individuals, indicating cross-reactivity. In fact, this serum also recognized parasites in IFAs. Similar high background levels in ELISAs and IFAs with plasmodial antigens were also found by others (Biggar et al. 1985; Howard et al. 1993; Saul et al. 1999). Whether this is a natural phenomena or it is due to the fusion of the 5'region with the 3'region and the DHFR molecule is unclear. But *P. falciparum* cross-reacting antibodies have also been described with many human pathogenic viruses, such as Rift valley

fever, HTLV, HIV, Polio, Influenza (McLaughlin et al. 1987) and related parasites like *Babesia microti* (Chisholm et al. 1978). Such shared sequences could possibly function in mimicry, decoy, or immunosuppression.

The apparent contradictory results obtained with antibodies measurements directed against the MSP2 conserved region might be due to different recombinant antigen products and production protocols, or to different study populations. Stowers et al. (1997) reported a strong correlation of antibody levels with both age and the recognition of other antigens, whereas others found no antibodies against the conserved regions of MSP2 (Thomas et al. 1990; Taylor et al. 1995; Nagendran and Ramasamy 1996; Lougovskoi et al. 1999). Migot-Nabias et al. (1999) reported a frequency of responders against a N-terminal peptide in children aged 5-17 years similar to ours. In our study, antibody titres against this construct showed no age dependance, nor were they associated with parasite presence. This suggest that the responses are either not specifically boosted or the antibody titres are already at an equilibrium. On the other hand, a weak but significant correlation of the antibody response and parasite density or number of FC27 like infections suggests a boosting effect by the total parasite load. Since our recombinant 5'3'construct elicited antibodies in mice which recognised mature parasites in IFAs, we believe that the measured response reflects at least partial recognition of malaria relevant epitopes.

The overall frequency of responders to most parts of the protein was high but the magnitude of responses was low in most individuals. Frequencies were generally higher than reported in other studies (Stowers et al. 1997; Migot-Nabias et al. 1999). This might be the result of our more precise method of calculating positivity using the latent class model (Vounatsou et al. 1998, Irion et al. 2001a). The increased length of the peptides used which consequently might represent more linear and non-contiguous epitopes might also influence antibody binding.

Immunity against severe malaria is acquired early in life in highly endemic areas (Kitua et al. 1996; Snow et al. 1998), and the observed significant correlation of all responses except against the 5'3'construct and the 6x12 aa repeats with age and parasite presence might reflect the developing immunity. Because of limited morbidity records and a generally low morbidity in these children we were not able to determine whether any of the observed anti-MSP2 responses were associated with protection against clinical disease. On the contrary, the correlation with parasite density

would suggest that measured antibodies rather reflect exposure than protection. It is difficult to reconcile since repeated and continuous exposure to *P. falciparum* may be required to reach and keep high IgG reactivities.

In this study, parasite genotyping allowed to assess the effect of parasite genotypes on the response to the various MSP2 domains. We were interested to see whether the infection dynamics affects immune responses, and to what degree these responses were specific. We previously speculated that established infections offer cross-protection against newly invading clones in older children (Smith et al. 1999c), and we and others showed that multiple infections seem to protect against clinical disease (Beck et al. 1997; Al-Yaman et al. 1997b; Färnert et al. 1999). It also has been shown that both prevalence and concentration of antibodies to the non-repetitive part of the 3D7 antigen correlated with reduced morbidity (Al-Yaman et al. 1994, 1995, 1997a; Taylor et al. 1998). In our study, the antibody titres to the 3D7 family specific part were marginal compared to the titres in adults, and might reflect the susceptibility of these children, due to a lack of antibodies against the 3D7 family specific part. In this respect, it is interesting to note that a recent subunit vaccine trial in Papua New Guinea including the 3D7 form of MSP2 showed a significant reduction of 3D7-like infections in the follow up period (Genton et al. 2001).

The antibody titres against the repeats except the FC27 4x32 aa repeat were all comparatively high. This would support the speculation that presence of these repeats might divert the immune response from critical epitopes within these or other parasite molecules (Anders 1986; Kemp et al. 1987; Bickle et al. 1993). We observed various pattern in responses against these repeats, in particular the lack of age dependence of antibody titres to the FC27 6x12 aa repeats. High levels and a high rate of responders to these repeats are in accordance with findings from other groups (Ranford-Cartwright et al. 1996). This group also showed that antigens with increasing numbers of repeats yielded higher antibody titres. We immunized mice with the 6x12 aa repeats and this sera highly specific recognized MSP2 alleles with 5x12 aa repeats but hardly MSP2 alleles with only 3x12 aa repeats (data not shown). FC27-like parasites observed in our study mostly had *msp2* alleles contained more than three 12 aa repeat units. However, nearly 70 % of detected FC27-like parasites contained only one 32 aa repeat, whereas our test antigen contained 4x32 aa repeats. The immune response against this antigen showed a highly significant correlation with the presence of *msp2* allele containing four 32 aa repeats. This might explain the overall low titre

against this antigen and suggests a similar importance of the repeat number. It is further supported by the fact that mouse sera against the 4x32 aa repeat poorly recognized parasites containing one 32 aa repeat only, whereas parasites with 4 repeats were strongly recognized. We must assume that most antibodies produced in mice upon immunization react with conformational epitopes created by more than one 32 aa repeat or with epitopes located at the junction of two repeat units. A similarly low response was observed with the 23xNAP repeats, and again MSP2 antigens with more than two NAP repeats were relatively rare.

The 3D7 repeats were recognized by 90 % of sera, and the change of antibody titres against these 3D7 repeats between two consecutive samples was significantly correlated with the number of present FC27 alleles. This clearly implies that parasites expressing FC27-like MSP2 provoke production of antibodies cross-reacting with the 3D7 repeats. Such cross reactivity of affinity purified human antibodies to the 3D7 repeat with FC27 alleles has been shown previously (Smythe et al. 1990). The 32 aa repeat of FC27 alleles contains the sequence Ala-Ser-Gly-Ser which is also present in the 3D7 sequence and might be responsible for the cross-reaction. This sequence has also been identified as a hot spot of recombination (Irion et al. 1997). The FC27 12 aa repeat in turn seems to stimulate responses to the FC27 4x32 aa repeats and the constant region. It is conceivable that such broad cross-stimulation might be an evasion strategy to divert the immune responses, but also will influence parasite dynamics and co-infections to a large degree. Whether these antibodies have a protective value remains to be elucidated.

Within this study, we were also able to test whether transmission blocking interventions might affect the development of potentially protective immune responses. There is much debate over the long-term effects of control measures such as ITNs, which reduce malaria transmission and possibly interfere with the development of immunity to malaria (Snow and Marsh 1995; Trape and Rogier 1996). During six months use of ITNs we observed no reduction in multiplicity despite a significant reduction in the parasite density and patent prevalence of *P. falciparum* infections (Fraser-Hurt et al. 1999; Smith et al. 1999b). This led to the speculation that prevention of only a small proportion of infections may not hinder the establishment of premunition or concomitant immunity (Smith et al. 1999c). But by analysing immune responses against MSP2 we demonstrate that longer term ITN use does indeed influence the development of these responses to some extent. Antibody titres to the 3D7 family specific part, the 3D7 repeats, the 4x32 aa repeats, and

the 23xNAP repeats were significant lower in children sleeping under a bed net. A similar reduction in antibody titres was shown for other antigens in a previous study in Papua New Guinea (Genton et al. 1994). The antibody responses to the 5'3'construct, the FC27 family specific part, and the 6x12 aa repeats were not affected. This demonstrates that investigating the antibody response to one or two antigens is not sufficient for the assessment of the effect of an intervention (Kitua et al. 1999), since immunogenicity varies between different antigens and even between different parts within one antigen.

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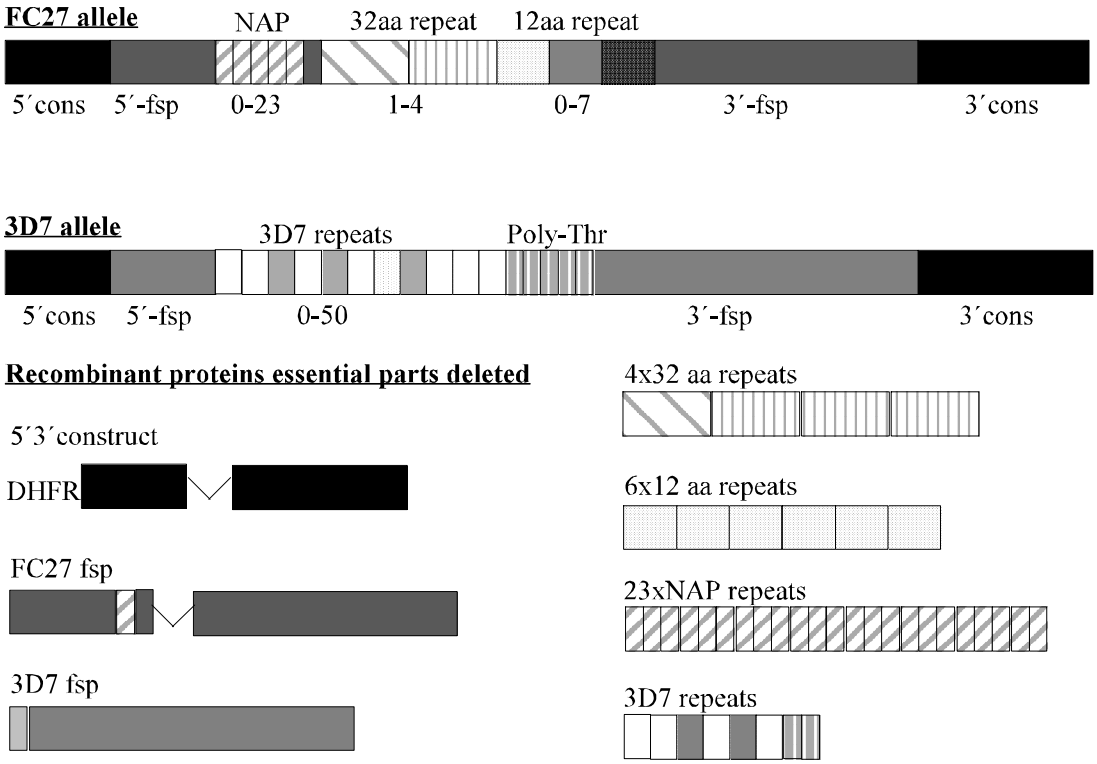

Figure 1: recombinant antigens representing distinct parts of the merozoite surface protein 2 (MSP2) of *P. falciparum*.

aa: amino acid, cons: conserved regions, DHFR: Dihydrofolate reductase, fsp: family specific part, NAP: single letter code of amino acids, Thr: Threonine.

Table 1. PCR primer pairs used to amplify distinct parts of *msp2*

antigen	Fwd primer (*Bam*HI)*	Rev primer (*Kpn*I)*	Allele	aa sequence
23xNAP repeats	5'cgggatcccgTAGTGTAGGTGCA'3	5'ggggtaccccACTAGCAATAGTA'3	Ifa23	SVGA-$(NAP)_{23}$ - NADTIASG
3D7 fsp	5'cgggatcccgTGGTAATGGTGCT'3	5'ggggtaccccAGATTGTAATTCG'3	FCR3	aa109-214
4x32aa repeats	5'cgggatcccgTGCTCCAAAAGCT'3	5'ggggtaccccAGGGGTATCAGCA'3	Ifa45	APK-(32aa repeat)$_4$-ADTP
6x12aa repeats	5'cgggatcccgTACTGCTACAGAA'3	5'ggggtaccccTTGAACTTTCTGT'3	K1+1	TAT-(ESNSRS PPITTT)$_6$-ESS
3D7 repeats	5'cgggatcccgTCCCCTACTGGTGCT'3	5'ggggtaccccGGTGGTAGCGGGAGTACT'3		PPT- 49aa (GAS rich)-GGSSTPATT

FC27 fsp	5'-region	(*Bam*HI)5'cgggatcccgTAAGAGTGTAGGTGCAAATGCTCCAAAAggaattcc'3 (*Eco*RI) + GGAATTCC (*Eco*RI-Primer)		K1	KSVGANAPK-GIP-(FC27fsp)-APQEPQTAE NENPA
	3'-region	5'ggaattccAGAAAGTTCAAGTT'3 (*Eco*RI)	5'ggggtaccccAGCAGGATTTTCA'3 (*Kpn*I)		

DHFR-5'3'-construct	5'-region	5'gaagatcttcAATGAAGGTAATTA'3 (*Bgl*II)	5'ggaattccTAGTATTAGAACCTT'3 (*Eco*RI)	K1	(5'-region K1 aa 1-50)-RNS -(3'-region K1 207-280aa) aa 23 Glu
	3'-region	5'ggaattccGCACCAGAGAATAAA'3 (*Eco*RI)	5'gaagatcttcTGAATATGGCAAA'3 (*Bgl*II)		

* Capital letters correspond to *msp2* hybridization site, small letters build the recognition site where the restriction enzyme cuts.

Table 2. Mean titres and estimates of positivity

	Mean titre in negative controls (s.d.)	**Estimated Proportion of test sera positive[1]**	**Mean titre in positive test sera[1] (units)**	**Mean titre in negative test sera[1] (units)**	**Overall mean titre in test sera (units)**
6x12 aa repeats	0.032 (0.022)	0.923	0.481	0.049	0.447
3D7 fsp	0.003 (0.003)	0.754	0.067	0.004	0.052
3D7 repeats	0.009 (0.005)	0.895	0.210	0.012	0.190
5'3' construct	0.281 (0.280)	0.856	1.866	0.344	1.647
4x32 aa repeats	0.004 (0.003)	0.526	0.071	0.004	0.039
FC27 fsp	0.090 (0.094)	0.786	0.356	0.106	0.302
23xNAP repeats	0.007 (0.005)	0.660	0.162	0.010	0.111

[1]As estimated from latent class models;

Table 3a: Spearman correlation analyses of achieved antibody titres with multiplicity of infection (MOI)

antigen	**N_3D7**		**N_FC27**	
	rs	**(p=)**	**rs**	**(p=)**
6x12 aa repeats	0.04	0.469	**0.14**	**0.007**
3D7 fsp	**0.49**	**<0.001**	**0.40**	**<0.001**
3D7 repeats	**0.33**	**<0.001**	**0.34**	**<0.001**
5'3'-construct	0.05	0.392	**0.17**	**0.001**
4x32 aa repeats	**0.29**	**<0.001**	**0.39**	**<0.001**
FC27 fsp	**0.24**	**<0.001**	**0.24**	**<0.001**
23xNAP repeats	**0.31**	**<0.001**	**0.36**	**<0.001**

N: number of infections expressing one *msp2* of the allelic families, p: P value, rs: correlation coefficient

Table 3b: Spearman correlation analyses of antibody unit change (difference between current and previous sample) with multiplicity of infection (MOI)

antigen	**N_3D7**		**N_FC27**	
	rs	**(p=)**	**rs**	**(p=)**
6x12 aa repeats	0.01	0.9	0.03	0.5
3D7 fsp	0.10	0.08	**0.12**	**0.030**
3D7 repeats	**0.11**	**0.04**	**0.12**	**0.029**
5'3'-construct	-0.02	0.7	0.11	0.07
4x32 aa repeats	0.08	0.14	**0.14**	**0.012**
FC27 fsp	0.03	0.5	0.08	0.13
23xNAP repeats	-0.03	0.5	0.00	0.9

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Dynamics of *Plasmodium falciparum* infections and anti-MSP2 immune response in young children

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Abstract

In order to investigate the correlation between *Plasmodium falciparum* infection dynamic and serological stimulation, we conducted a longitudinal study with children aged initially between 5-15 months in a highly malarious endemic area of Tanzania. All infections were genotyped by *msp2*-PCR-RFLP and sera of the identical children were tested in IgG-ELISA against various recombinant constructs of MSP2. It seems that *msp2* alleles of the FC27 allelic family stimulate the antibody response to distinct parts of both allelic families, including the conserved regions and the 3D7 family specific part. In contrast, *P. falciparum* infections expressing 3D7 alleles stimulated only the antibody response to the antigen which represented the 3D7 repeats. The dynamic of infections itself showed significant differences in their duration between infections expressing MSP2 alleles of the different allelic families.

Key words: duration of infection, recovery rate, dynamic of antibody response, merozoite surface protein 2, *Plasmodium falciparum*, malaria.

Introduction

In areas highly endemic for *Plasmodium falciparum*, age variation in the outcome of the malaria disease and in parasitological profiles have been reported (Baired et al. 1993; Trape and Rogier 1996; Konaté et al. 1999; Smith et al. 1999a). *P. falciparum* infected infants in highly endemic areas have higher parasite densities than older children (Molineaux & Gramiccia 1980; Kitua et al. 1996; Smith et al. 1998), but they show lower multiplicity of infection and microscopically sub-patent infections are not present (Felger et al. 1999b). As children grow older, parasite densities decrease and the prevalence of microscopically sub-patent parasitemia increases (Felger et al. 1995). The period of highest susceptibility to clinical malaria among infants in highly endemic areas occurs between the ages of four and 12 months, when the mean parasite density and the risk of fever reach their maximum (Kitua et al. 1996). During this period the bulk of maternal antibodies decreases but the specific immune response of the child is still weak. It was hypothesized that clinical immunity in highly endemic areas depends on cross protection by concomitant infections (Al-Yaman et al. 1997a; Smith et al. 1999c; Färnert et al. 1999). Hence, the higher susceptibility of infants to clinical malaria in comparison with older children may be largely a consequence of the short duration of infections which precludes the establishment of concomitant immunity and the lower multiplicity of infection reported in children might be rather the consequence of the rapid clearance than the slow accumulation of infections in infants (Smith et al. 1999c).

Most data on the immune response to *Plasmodium falciparum* antigens during the first two years of age are described indirectly by the prevalence of asymptomatic children, infection multiplicity or parasite densities. Actively acquired immunity to malaria in humans are believed to be strain-specific, partly mediated by recognizing polymorphic epitopes in merozoite proteins (Berzins and Anders 1999). The merozoite surface protein 2 (MSP2) is one of the well-characterized surface proteins of *P. falciparum*. MSP2 contains a single, centrally located block of tandem repeats (Smythe et al. 1988; Fenton et al. 1989). And despite of being highly polymorphic the molecule has essentially a dimorphic structure (Thomas et al. 1990; Marshall et al. 1994), which defines the two allelic families, FC27 and IC1/3D7 (Smythe et al. 1990; Fenton et al. 1991). *msp2* genotypes of the FC27 allelic family have been associated with higher parasite densities and morbidity (Engelbrecht et al. 1995), which might be prevented by simultaneous infections with *P. falciparum* expressing MSP2 alleles of the 3D7 allelic family.

Involvement of MSP2 in protective immune responses was indicated by the merozoite invasion inhibitory effect of monoclonal antibodies to the antigen (Clark et al. 1989; Epping et al. 1988). The antibody response to MSP2 in humans is mainly directed against the central polymorphic region (Al-Yaman et al. 1994; Taylor et al. 1995), although, in some populations, antibodies to the conserved and semi-conserved regions develop at later ages after a life long exposure to malaria and were associated with fewer fever episodes and less anaemia (Al-Yaman et al. 1994). Associations between antibody level to 3D7 alleles and resistance to clinical malaria were also demonstrated indicating a protective role for these antibodies (Al-Yaman et al. 1994, 1997b).

However, little is known about the dynamic of specific immune responses in natural settings with frequent new infections and loss of existing infections. Similarly, it has never been studied in detail, which domains of the molecule elicit an antibody response with subsequent analysis of the specificity and cross-reactivity. We report here the detailed PCR analysis of longitudinal blood samples from young children with a detailed analysis of elicited antibody responses. With this study we provide a first insight into the development of a specific immune response in early childhood using recombinant proteins in serological assays with children sera. The presented data were part of a longitudinal study of immune response to distinct parts of the merozoite surface protein 2 (MSP2) reported elsewhere (Irion et al. 2001a).

Study Design and Methods

Subjects: the samples for this study originated from a insecticide treated bed nets (ITN) trial which was carried out in Kiberege Village in the Kilombero valley in southeastern Tanzania, an area highly endemic for *P. falciparum* malaria (Tanner et al. 1991). The design of the study has been described in detail by Fraser-Hurt et al.(1999). Seven consecutive finger-prick blood samples collected at one month intervals were selected from 60 children initially aged five to 24 months who used insecticide-treated nets, and from 60 children of the same age group not using ITNs. All blood samples were genotyped by PCR-RFLP on *msp2* as described and reported elsewhere (Felger et al. 1999a, Smith et al. 1999c). Twenty serum samples from semi-immune adults from PNG were used as positive controls. Sera from 36 healthy European adults and from 36 European children aged 5-15 months, all never exposed to malaria, were used as negative controls.

Estimation of the probability of recovery, M, and the sensitivity of detection, S

M and S were estimated by maximum likelihood as described previously (Smith et al. 1999c)

Protein expression and ELISA: expression in the procaryotic pQE expression system (Qiagen) in *E. coli* and purification of seven different recombinant *msp2*-clones were described previously (Irion et al. 2001a). Briefly, PCR products corresponding to distinct parts of *msp2* (table 1), were cloned in the pQE30 expression vector as a fusion protein with six histidine residues at the 5' end. Protein expression and purification were done according to the manufacturer's protocol (Qiagen). Pure protein fractions were pooled and renatured by dialysis as previously described for other MSP2 recombinant proteins (Takacs and Girard 1991). Protein preparations under native conditions were dialysed against PBS pH 7.2 to remove imidazole. Protein content was estimated by the method of Bradford at 595 nm (Bradford 1976).

All immunological assays were carried out as described previously (Irion et al. 2001a). For ELISA optimal dilutions of the immunogens and reagents were determined by checker titration with positive and negative control sera (Quakyi 1980). After coating with antigens and blocking with 5% milk powder in PBS, 50 :l of test sera in 1:500 dilutions (1:1500 dilutions for the 5'3'-construct and the 6x12 aa repeats) in PBS, containing 0.5% Tween 20 and 0.5% milk powder, were added in duplicates. IgG antibody binding was revealed by addition of 50 :l horse radish peroxidase-conjugated second antibody (dilution1:2000 in PBS-0.5% Tween20/0.5% milk powder). Subsequently, 50 :l peroxidase substrate (ABTS peroxidase substrate, KPL) were added to each well, and the absorbance was measured at 405 nm in a spectrophotometer (Titertek Multiskan MCC/340). Each sera was also tested against a similarly produced DHFR-6His preparation. Presentation of antibody units was carried out as described previously (Irion et al. 2001a). A latent class model based method was used to determine positivity of test samples (Vounatsou et al. 1998, Irion et al. 2001b).

Statistical analysis: to determine if any relation between different variables and antibody unit values was significant Spearman's correlation analyses were performed on the sets of continuous data on antibody response to an antigen versus listed variables: number of new acquired infections and number of lost infections separately for the allelic families. Spearman's correlation analyses were used because the data distribution was non-normal for antibody responses and the other variables.

Subsequent sample pairs of an individual were formed and divided in three groups: (A) sample

pairs with an increasing number of infections with *P. falciparum* expressing *msp2* alleles of the FC27 allelic family or of the 3D7 allelic family, (B) sample pairs with a decreasing number of infections expressing FC27 alleles or 3D7 alleles, and (C) sample pairs with a constant number of infections. First, the antibody titres of group (A) or (B) were compared with the antibody titres of the current samples of group (C) (table 3). Second, the changes of antibody titres from the previous to the current sample of an individual of the sample pairs with non-constant number of infections (group (A) or (B)) were compared with the changes of antibody titres in samples from group (C) (table 4).

Results

*Duration of infection*s: The distribution of duration estimates was significantly different between age groups 6-18 months and 18-37 months ($p<0.05$) when *P. falciparum* infections were separated according to the expressed MSP2 allelic family (table 2). Estimates of the duration of infections expressing different allelic families reverses when the children getting older. Infections expressing MSP2 alleles of the 3D7 allelic family show a decreased duration of 80 days in children older than 18 months whereas infections expressing FC27 alleles persist longer in this age group (120 days). In children under 18 months of age, the situation is different.

Dynamics of immune response: The results of Spearman correlation analyses are summarized in table 3 and 4. Overall, both the number and acquisition of infections with an *msp2* allele of the FC27 allelic family seem to be correlated with an increase of titres against all constructs. An acquisition of an FC27 expressing infection however seems to stimulate only antibodies against the 5'3'construct, the 4x32 aa repeats and surprisingly against the 3D7 family specific and 3D7 repeats domain. Hence, MSP2 of the FC27 allelic family seems to boost the IgG immune response to both allelic families.

In contrast, the acquisition of 3D7 allele expressing infections stimulates only the antibody response to the 3D7 repeats. This is also reflected by the correlation of the change of antibody response between the previous and the current sample and the number of infections expressing *msp2* alleles of the 3D7 allelic family. The numbers of infections expressing a 3D7 allele is positively correlated with the change of antibody level only against the 3D7 repeats representing antigen ($p=0.04$). Whereas the number of infections expressing a FC27 allele is correlated with

the antibody level change against the antigens representing 4x32 aa repeats, 3D7 repeats and the 3D7 family specific part (p=0.012, p=0.029, p=0.030, respectively).

During the monthly intervals in which loss of infections were determined by PCR, no decrease of antibodies was observed. There was a borderline positive correlation between loss of 3D7 allele expressing infections and titres against the 5'3'construct and a negative correlation between loss of FC27 allele expressing infections and antibodies against the 4x32 aa repeats.

Discussion

The pronounced dichotomy between the two allelic families of MSP2 led us to analyse their biological significance by estimating the duration of infections with the different alleles. Since the immune response of the host seems to be one of the limiting factors for the surviving of the parasites, we analysed, additionally, the dynamics of the MSP2 specific antibody response in respect to the present and recent *P. falciparum* infections with genotyped *msp2* alleles.

The duration of *P. falciparum* infections expressing MSP2 alleles of the two families seems to be considerably different and reverses when the children grow older. We showed that infections expressing MSP2 alleles of the 3D7 allelic family present a decreased duration in children older than 18 months in contrast to the situation in younger children whereas infections expressing FC27 alleles persist longer in the older age group. If this is a continuing process, our finding explains an earlier observation of Felger et al. (1994; 1999b), when the distribution of the two allelic families of MSP2 differed significantly between semi-immune adults and children. FC27 alleles were predominantly found in adults, whereas 3D7 alleles were found more frequently in children.

In addition the sensitivity of detection increases for the 3D7 alleles and decreases for FC27 alleles with age. This might indicate that infections expressing a 3D7 allele do not persist as low density infections, whereas the FC27 allele infections are able to persist with densities below the detection limit.

In infants members of both allelic families are affected in the same extent by innate immune mechanisms, hence the duration of infections are of equal length (Smith et al. 1999b). Then the duration of infections with both allelic families increases significantly when the innate immune

mechanisms are losing their importance and the acquired immunity is only little developed. The immune response of the host seems early to be able to limit *P. falciparum* infections expressing 3D7 alleles, beginning gradually in the first and a half year of life, when the duration of infections with 3D7 alleles decreases. At this time point the duration of infections with FC27 alleles increases indicating that these infections profit from the limitation of 3D7 infections or FC27 infections evade the immune response by mechanisms such as original antigenic sin (Good et al. 1993) or altered ligand peptides (Plebanski et al. 1997). The ability of limiting infections expressing FC27 alleles needs probably another process in which other antigens might also be involved. The negative correlation of the change of antibody titres to 32 aa repeats from the previous to the current sample of one individual with the loss of infections expressing MSP2 of the FC27 allelic family might indicate a short duration of this antibody response, which might need reinfection with *P. falciparum* expressing a FC27 allele for boosting. Additionally, the lowest antibody level measured in the study was that against 32 aa repeats (Irion et al.2001a).

It was suggested that current infections protect, enhanced by the multiplicity of infection, against superinfection and prevent clinical malaria (Ntoumi et al. 1995; Al-Yaman et al. 1997a; Beck et al. 1997; Färnert et al. 1999). Observed differences in parasitologic profiles between single children might be rather qualitative differences in protective immunity than differences in the genotypes of infecting parasites (Färnert et al. 1999). This might be related to continuous presence of diverse parasite clones, which in turn may depend on immunogenetic properties. In older children low density chronic infections resulting in high multiplicity of infection have a protective effect against clinical malaria, but in infants acute episodes of clinical malaria are positively associated with multiplicity of infection (Felger et al. 1999b) and the protective effect of concomitant infections is not observed. The cross-protecting potential might be developed at later ages, since adults show considerable higher antibody levels to all recombinant antigens tested than the children enrolled in the study (Irion et al. 2001a).

Recently, the cross-protection to FC27 alleles mediated by 3D7 alleles (Engelbrecht et al. 1995) could not be induced by vaccination with a recombinant 3D7 protein (Genton et al. 2001).This and the difference in duration of infections might indicate the lack of a protective role for antibodies to the conserved regions of the molecule. It might be speculated that the observed protective effect of 3D7 alleles bases only on the absorption of FC27 specific antibodies by the repeat structure of present *P. falciparum* infections expressing a 3D7 allele and not on the

induction of protective antibodies.

Additionally, we showed that *msp2* alleles of the FC27 allelic family seemed to stimulate the antibody response to parts of its own molecule and in addition a stimulation of the antibody response to parts of the 3D7 alleles was observed in children under 21 months. Due to the observed difference in the duration of infections, further investigations comparing antibody response of older children are needed. In contrast, *P. falciparum* infections expressing 3D7 alleles stimulate only the antibody response to the antigen which represents the 3D7 repeats. This might reflect the ability of FC27 alleles to confuse the immune system of the host by broadening the immune response, which was thought to be responsible for the lack of sterile immunity to malaria (Anders 1986). *P. falciparum* infections expressing FC27 alleles might displace infections expressing the 3D7 alleles by influencing the immune response to the 3D7 family specific part. Hence, a protective mechanism of 3D7 alleles observed in children with less morbidity might be prevented (Engelbrecht et al. 1995). There is an indirect evidence that the loss of infections with 3D7 alleles allows infections with FC27 alleles to reproduce. The induction of antibodies to the antigen representing the conserved regions by FC27 alleles, especially by a high number of 12 aa repeats (Irion et al. 2001a), whereas a positive correlation of these antibodies to the loss of infections expressing 3D7 alleles was shown. Shifting the immune response towards the 3D7 allelic family could be a strategy of the parasite to limit competing infections. Furthermore a correlation between the number of 32 aa repeats of the infecting parasite and the stimulation of the specific antibody response to 3D7 repeats was shown (Irion et al. 2001a). Polyclonal antibodies induced by vaccinating mice with the recombinant antigen representing the 3D7 repeats were interacting with cloned lines of *P. falciparum* expressing FC27 alleles (Irion et al. 2001a). These findings indicate a shared epitope that might be located at the 5'end of the 32 aa repeat (Irion et al. 1997).

Our study clearly shows that there are differences in the duration of infection and in the induction of immune response between the two allelic families of MSP2. This is of additional evidence that the two allelic families represent different evolutionary strategies (Felger et al. 1997). The 3D7 alleles have very divers repeat regions but alleles are of low allelic frequencies. In contrast, the alleles of the FC27 allelic family are of high frequencies and of a relative conserved repeat structure. Infections expressing 3D7 alleles might respond to the immune pressure of the host by diversification of their repeat sequence, repeat length, and number of repeats, whereas infections

with FC27 alleles partly count on shifting the immune response towards 3D7 alleles or evading the FC27 specific antibody response.

Table 1: recombinant antigens corresponding to distinct parts of MSP2

antigen	amino acid sequence (allele)
23xNAP repeats	MRGS-HHHHHH-GSR-SVGA-$(NAP)_{23}$-NADTIASG-YPGSTCSQA (Ifa23)
3D7 fsp	MRGS-HHHHHH-GSR-(3D7allele FCR3 aa109-214)-GYPGSTCSQA (FCR3)
4x32 aa repeats	MRGS-HHHHHH-GSR-APK-$(32aa\ repeat)_4$-ADTP-GYPGSTCSQA (Ifa45)
6x12 aa repeats	MRGS-HHHHHH-GSR-TAT-$(ESNSRSPPITTT)_6$-ESS-GYPGSTCSQA (K1+1)
FC27 fsp	MRGS-HHHHHH-GSR-KSVGANAPK-GIP-(FC27fsp)-APQEPQTAENENPA-GYPGSTCSQA (K1)
5'3'construct	MRGS-DHFR-(5'-region K1 aa 1-50)-RNS-(3'-region K1 aa)-RRS-HHHHHH
3D7 repeats	MRGS-HHHHHH-GSR-PPT-GAGASGRAGA-GA-GAGASGRAGA-GAGASGRAGS-GSGDGAVASA-GSGADAEGGSSTPATT-GYPGSTCSQA (84aa)

Table 2: Duration of infections expressing MSP2 alleles of both allelic families

		Sensitivity of detection S	**recovery probability M**	**recovery rate :**	**duration [days]**
3D7	**<18 months**	0.49	0.23	0.01	100
3D7	**>18 months**	0.6	0.29	0.0126	79.4
FC27	**<18 months**	0.73	0.27	0.0117	85.5
FC27	**>18 months**	0.64	0.19	0.0083	120.5
3D7	**no net**	0.53	0.24	0.0104	96
3D7	**net**	0.55	0.28	0.0122	82
FC27	**no net**	0.6	0.2	0.0087	115
FC27	**net**	0.78	0.25	0.0109	92

Table 3: Correlation of achieved antibody titres with acquisition and lost of *P. falciparum* infections expressing MSP2 of different allelic families (Spearman correlation analysis)

		group (A)		**group (B)**	
antigen		**FC27 aq**	**3D7 aq**	**FC27 lost**	**3D7 lost**
6x12 aa repeats	**r**	0.08	-0.01	0.05	0
	P	0.14	0.848	0.395	0.955
3D7 fsp	**r**	**0.32**	**0.41**	**0.22**	**0.39**
	P	**0**	**0**	**0**	**0**
3D7 repeats	**r**	**0.32**	**0.3**	**0.2**	**0.25**
	P	**0**	**0**	**0**	**0**
5'3'construct	**r**	**0.22**	0.02	**0.18**	0.01
	P	**0**	0.781	**0.002**	0.926
4x32 aa repeats	**r**	**0.27**	**0.25**	**0.24**	**0.24**
	P	**0**	**0**	**0**	**0**
FC27 fsp	**r**	**0.16**	**0.18**	0.09	**0.16**
	P	**0.003**	**0.001**	0.098	**0.003**
23xNAP repeats	**r**	**0.25**	**0.26**	**0.25**	**0.24**
	P	**0**	**0**	**0**	**0**
n		**335**	**335**	**335**	**335**

Statistically significant correlations are stressed by bold letters ($p<0.05$). *: borderline significance, aa: amino acid, aq: acquisition, fsp: family specific part, n: number of analysed samples, NAP: single letter code for amino acids, p: p value, r: correlation coefficient.

Table 4: Correlation of the change of antibody titres between current and previous sample with acquisition and loss of *P. falciparum* infections expressing MSP2 of different allelic families (Spearman correlation analysis) sample1 ← antibody sample 2.

		group (A)		group (B)	
antigen		**FC27 aq**	**3D7 aq**	**FC27 lost**	**3D7 lost**
6x12 aa repeats	**r**	0.04	-0.005	-0.018	0.044
	P	0.4	0.9	0.8	0.4
3D7 fsp	**r**	**0.12**	0.09	-0.04	-0.04
	P	**0.029**	0.1	0.5	0.5
3D7 repeats	**r**	**0.14**	**0.12**	0.02	-0.02
	P	**0.009**	**0.022**	0.7	0.7
5'3'construct	**r**	**0.13**	-0.04	0.1	**0.11**
	P	**0.031**	0.5	0.09	**0.053***
4x32 aa repeats	**r**	**0.17**	0.09	**-0.1**	0.01
	P	**0.002**	0.096	**0.060***	0.8
FC27 fsp	**r**	0.08	0.04	0.07	0.05
	P	0.16	0.5	0.2	0.4
23xNAP repeats	**r**	0	-0.04	0.01	-0.01
	P	0.9	0.4	0.9	0.8

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Assessment of positivity in immuno-assays with variability in background measurements: a new approach applied to the antibody response to *Plasmodium falciparum* MSP2

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Abstract

Measurements of immune responses often show high levels of heterogeneity, making it impossible to clearly distinguish responders and non-responders to particular antigens. Typically, in, e.g. ELISA assays, a non-exposed control group is used to assign a cutoff value of positivity, calculated as the mean plus either 2 or 3 standard deviations. This can give rise to extremely biased estimates of response rates when the background is variable, and especially when there is overlap between the distribution of the control levels and that of responders. This problem is compounded when results of assays with different background levels are compared. We illustrate this with hypothetical data sets reflecting frequent patterns seen in laboratory and epidemiological studies.

We propose that such data should be analysed by statistical modelling of the ratio of numbers of test samples:control samples as a function of the readout from the assay. Rather than classifying samples dichotomously as negative or positive, this provides estimates of the prevalence of positivity θ, and the probability, for each sample, that the measured activity is above background. Several statistical methods can provide such estimates. Analyses of simulated data sets using our preferred estimation method (a latent class model), demonstrate that this gives more reliable results than the traditional assignment using cutoff values. We have applied this approach to the analysis of ELISA assessments of antibodies against distinct regions of the *Plasmodium falciparum* merozoite surface protein 2 (MSP2) in human sera from Tanzania.

Key words: ELISA, malaria, latent class model, cutoff, prevalence of positivity, probability of positivity.

1. Introduction

Many laboratory methods, for instance enzyme-linked immunosorbent assays (ELISA) can be used as diagnostic tools to assess the prevalence of a disease and/or the extent of the immune responses in populations exposed to the infective agents. Measurements made on samples from an exposed group are compared with those from a non-exposed control or standard group, in order to determine which samples are positive.

In ELISA antibody assays a common practice is to report an individual sample as positive when its titre or optical density is higher than the mean of the corresponding measurements for a negative control group plus either 2 or 3 standard deviations (the 'classical' approach). When there is a good separation between negative and positive samples, and when the background levels measured in the controls are not very variable, this gives clear and unbiased results. However, these conditions are not always met. Figure 1 illustrates several scenarios that can arise in epidemiology and in the application of diagnostic assays when an exposed group is compared to a non-exposed control group. All the samples of the non-exposed control group are by definition negative, whereas the individuals of the exposed group comprise two subgroups: the negative and the positive ones (in a ratio of (1- 8) : 8).

Immune responses encountered in human populations are extremely heterogeneous, as is the performance of immunoassays. This can lead to three kinds of problems with the 'classical' approach. (i) The average difference in measurement between negative and positive samples may be so small that there is insufficient separation between controls and positive samples (Figure 1 a,b,c). (ii) The difference in measurements may be variable so that not all positive samples can be clearly distinguished (Figure 1 d,e,f). (iii) The measures for control samples (background) may be highly variable (i.e. with a high standard deviation) (Figure 1 b,e,h) so that even if the separation is good, many positive samples have measurements below the cutoff (Figure 1h). Only if none of these problems arise (Figure 1 g,i), does the classical approach correctly identify the group of positive samples. In all other cases, a substantial proportion of the test samples cannot be assigned with confidence.

If the assay is intended for diagnostic purposes and cannot be optimized, the appropriate response to such problems may be to abandon the assay. However in epidemiological surveys the objective is usually to estimate the number of positive samples, rather than to assign each individually. Even

if it is not obvious exactly which samples are positive, it may be possible to estimate the probability, θ_i, for each sample *i*, that the measured activity is above background. The average of the values of θ_i provides an estimate of the prevalence of positivity, θ.

As an example, we consider data from a study of antibody levels to distinct parts of the *Plasmodium falciparum* merozoite surface protein 2 (MSP2) among children in Tanzania. The data were described previously by Irion et al. (2001). Antibody levels in both the malaria exposed Tanzanian and non-exposed European children of the same age were measured. The objective of the analysis is to determine the proportion of the Tanzanian children with higher levels of specific antibodies than those in the European controls.

Optimally, an estimate of θ should be consistent (converge on the correct value when the sample size is large), and unbiased (should not show any systematic tendency to over- or under-estimate when the sample size is small). There are several ways of making such estimates. Our preferred approach is a latent class model for non-parametric decomposition of two-component mixture distributions (Vounatsou et al. 1998). We demonstrate the use of this to estimate the proportion of children with antibodies attributable to distinct regions of MSP2. The principles illustrated with this example can also be applied to a wide range of other assays.

2. Theory

Assume that an observed population consists of a mixture of samples from non-responders and from responders to some stimulus, in proportions 1-θ and θ respectively, where θ is the mixing proportion or equivalently, the prevalence of positivity or the attributable fraction. The distribution of immunological measurements, F(.), thus comprises two components (g_1(.) and g_2(.)), neither of them necessarily normally distributed. g_1(.) corresponds to the distribution of measurements in control individuals who have not been exposed to the stimulus, and g_2(.) to the levels in responders, and hence:

$$F(x) = (1 - \theta)\, g_1(x) + \theta\, g_2(x),$$

where x denotes the value of the chosen measure of immune response.

For the assay to be meaningful, observations from g_1(.) must be smaller on average than those from g_2(.) but there may be overlap between the two distributions.

The density ratio $D(x)=F(x)/g_1(x)$ is the ratio of the frequency of any given antibody unit value in the population, to the frequency in the control group. If there are no additional antibodies measured in the tested samples then we expect this ratio to be uniformly 1 and θ=0. On the other hand, if θ is greater than 0, we expect D to be an increasing function of x. The limiting case of no overlapping, where $g_2(x)=0$, provides $D(x)=(1-\theta)$ for all x. Figure 2a illustrates $D(x)$ for the example of Figure 1e.

A simple way to estimate θ for real data is to group the measurement (x) into distinct categories and to plot $D(x)$ for these categories. This is calculated by taking the proportion of the tested population in that category and dividing by the proportion of the control group which falls into the same range (Figure 2b). The average value that $D(x)$ achieves over low values of x can than be used to obtain a minimally biased estimate of $(1-\theta)$ and hence of θ.

Besides its use in estimating the overall prevalence of positivity, the density ratio curve can be used to estimate the probability that samples with a given measurement value have an immune response to the stimulus. If there were no effect of the stimulus then the probability of obtaining measurement value x would be $g_1(x)$. However, the actual probability is $F(x)$. The difference is attributable to the positive samples. The proportion of those samples with measurement x that are positive is therefore equal to:

$$p(x) = \frac{\lambda g_2(x)}{F(x)} = \frac{F(x) - (1-\lambda) g_1(x)}{F(x)} = \frac{D(x) - (1-\lambda)}{D(x)}$$

It follows, that if D(x) and 8 can be estimated, that p(x) can also be obtained, and used as an estimate of the probability that a test sample is positive. This probability depends not only on the sample's value, but also on both the distribution of measurements made on control samples and that of the other test samples.

Unfortunately, when there is only a limited amount of data then estimates of 8 made graphically, as described above, will be very imprecise. In such situations it is necessary to use a statistical model to fit a curve to D(x) or p(x). Several different statistical methods are available. If the distributions $g_1(x)$ and $g_2(x)$ are known to belong to particular families (e.g. Normal distributions) then their parameters, together with 8, can be estimated by maximum likelihood. However, more usually there is no reason to suppose that either $g_1(x)$ or $g_2(x)$ belong to any particular type of distribution, and the strongest assumption which we would like to make is that as x increases, so do D(x) and p(x). We have already investigated various statistical approaches to this problem, which incorporate this assumption but do not assume particular distributional forms for $g_1(x)$ and $g_2(x)$ (Smith et al. 1994; Vounatsou et al. 1998; Smith and Vounatsou 1997). Our preferred approach (Vounatsou et al. 1998) fits a latent class model (LCM) to the data, and can be implemented in the freely available software package Winbugs (Spiegelhalter et al. 2000).

3. Examples

3.1. Simulated example

To illustrate the performance of our proposed method, we simulated a data set corresponding to that of Figure 1e. We drew 500 samples from the distribution F(x), and 100 samples from $g_1(x)$. The columns in Figure 2a illustrate the resulting distributions, binned into intervals of length 2. The distributions obtained differ a little from those shown in Figure 1e because of chance sampling variations.

In this example the density ratio in the $0<x<2$ category is by chance exactly equal to 0.2, corresponding to the correct value of (1- 8). The density then increases with x. Corresponding to this density ratio curve, the model estimates the function p(x) (the probability that a sample is

positive), as a smooth increasing function of *x*. The method of (Vounatsou et al. 1998) also provides an overall estimate of θ, which for this example was 0.717. This estimate is much closer to the true value of θ=0.8, than is the value obtained from the classical cutoff approach (Figure 1e; estimate 0.395) but is not exactly equal to the correct proportion positive. In general, this LCM approach will give estimates of θ which are much closer to the true value than those obtained using the cutoff approach, but the sampling variation means that these will not be exactly equal to the true value especially when the sample size is small.

3.2 Levels of antibodies against MSP2

As part of a study of the antibody response to malaria antigens in Tanzania, levels of total IgG were measured by ELISA against a series of seven recombinant proteins comprising parts of the merozoite surface protein 2 (MSP2) of *Plasmodium falciparum*. The study involved more than four hundred sera from children aged 5-21 months. The procedures for these ELISA assays have been described in detail elsewhere (Irion et al. 2001). Briefly, microtitre plates (Immulon 2; Dynex Technologies, USA) were coated with 50 :l of one of the set of protein preparations. Immunogen concentrations ranged from 0.1 :g/mL – 10 :g/mL in PBS at pH 7.2. Titrations of a positive standard of 20 pooled sera from semi-immune adults from Papua New Guinea were performed on each ELISA plate to standardise for differences between plates. Sera of 36 healthy European children aged 5-15 months, who had not been exposed to malaria were used as negative controls. Each test serum and control serum was tested against the single immunogen and, as a background control, against a protein solution consisting of DHFR-6His (5 mg/ml PBS), produced like the other recombinant proteins.

Estimates of positivity of test sera against the antigens calculated by the proposed approach in comparison to the classical approach are shown in table 1.

Using estimates of θ it is also possible to assess the levels of response in the positive samples, without being biased by the effects of non-responders. The mean antibody levels of test sera in our study are shown in table 1. The positive test sera have particularly high antibody units to the antigens 6x12 aa repeats and 5'3'construct. Moderate antibody units were seen with the antigens FC27fsp, 3D7repeats and 23xNAP repeats. Low unit levels were found with the antigens 3D7fsp and the 4x32 aa repeats.

4. Discussion

The performance of immunological tests is commonly assessed by examining the ability of the test to correctly classify individuals into two subgroups, for example, a subgroup of individuals affected by some disease and a second subgroup of unaffected individuals. If there is no overlap in test results from these two subgroups, then the test can identify all individuals correctly, that is, distinguish the two subgroups perfectly, otherwise false positive and false negative results must be considered.

Some data sets exhibit a clear distinction between responders and non-responders, and then there is a strong argument for using a threshold value to define positivity, on the grounds of clarity of presentation, or where an unambiguous diagnosis is needed for clinical purposes. However for many others there is no evidence of dichotomy. Defining a cut-off value when there may be no obvious division of individuals into two groups, is potentially misleading and a rather arbitrary process, which can have profound effects on the results.

Different attempts have been made to manage this problem. Where a gold standard exists, predictive values can be calculated, describing the likely correctness of positive (or negative) test results. The efficiency, i.e. the percentage of all test results which will be correct for a particular population, can also be calculated. Receiver Operating Characteristic (ROC) curves can also be used to assess the sensitivity and specificity of the assay in a particular population (Zweig and Robertson 1987; Xu et al. 1997).

Where there is no gold standard, the assessment of diagnostic accuracy represents a more challenging statistical problem. Latent class models have been applied in a number of such situations, for instance assessment of true disease status (Boelaert et al. 1999), and comparison of several diagnostic tests or of several readers (Yang and Becker 1997, Qu et al. 1996, Walter et al. 1999). The objective here is to assess the extent of misclassification, or to optimise the assignment to classes, whilst accepting that there will be a certain level of misdiagnosis.

Where the objective is to estimate the proportion of positive samples, however, it is not necessary to assign each sample using a dichotomous grouping. This is very often the case in epidemiological studies. We propose the use of statistical models to assess the relationship between positivity and the measured response in the analysis of immunoepidemiological data, and illustrated this with ELISA data from a study of malaria. This approach assigns a probability of positivity and hence

those samples that cannot be assigned with certainty need not be allocated to one or other category. We believe such methods could be useful in the characterization of antibody responses measured by many different immunological methods with quantitative readouts.

One advantage of this approach is that it automatically introduces consistency into the definition of a positive responder, making it easier to compare results between studies, and between tests, and reduces the sensitivity of the results to outliers in the distributions of control values. Moreover, when fixed cutoffs are used to analyze ELISA tests, rates of responders are often seriously under-estimated, depending on the absorbance cut-off method applied for negative controls. This is especially a problem when the immune responses are weak and the differences between responders and non-responders small, as with our malaria data set.

Statistically, it is more efficient to compare average levels of response rather than proportions of "responders" when comparing groups where all individuals are at least partially immune or "exposed" (Bennett and Riley 1992). However, such analyses presupposes that the level of response is important and that we are not interested in knowing whether this response is accounted for by contributions from the whole population, or from only a few individuals. Mechanisms such as genetic restriction, altered peptide ligands antagonism (Kersh and Allen 1996; Plebanski et al. 1997) or original antigenic sin (Good et al. 1993; McMichael 1998) predict that responses (against e.g. malaria antigens) should be limited to some individuals, and it can therefore be relevant to know the proportion of responders. Estimation of θ for different specific antibody responses in different populations may thus represent a valuable step towards understanding immune mechanisms.

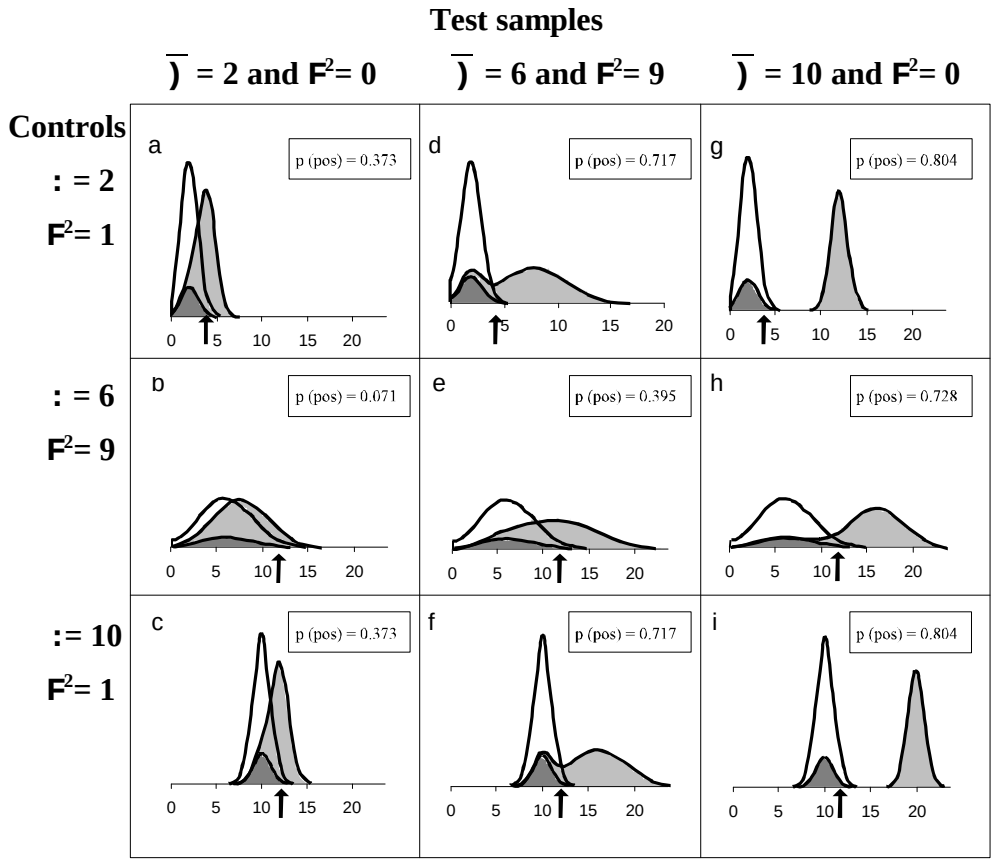

Figure 1: Comparisons of hypothetical data sets

Each sub-figure illustrates the hypothetical distributions of measurements observed using an assay where the test samples comprise a mixture of 80% positive samples and 20% negative samples (true value of 8=0.8). The unshaded peak is the distribution of the controls, the dark grey area corresponds to the true negative samples, and the light grey area to the true positives. The arrow indicates the location of the mean + 2 s.d. cutoff, and P is the percentage of test samples with measurements greater than the cutoff.

$\bar{)}$: mean increase in) above background for the test samples, :: mean of the control samples, F²: variance of) .

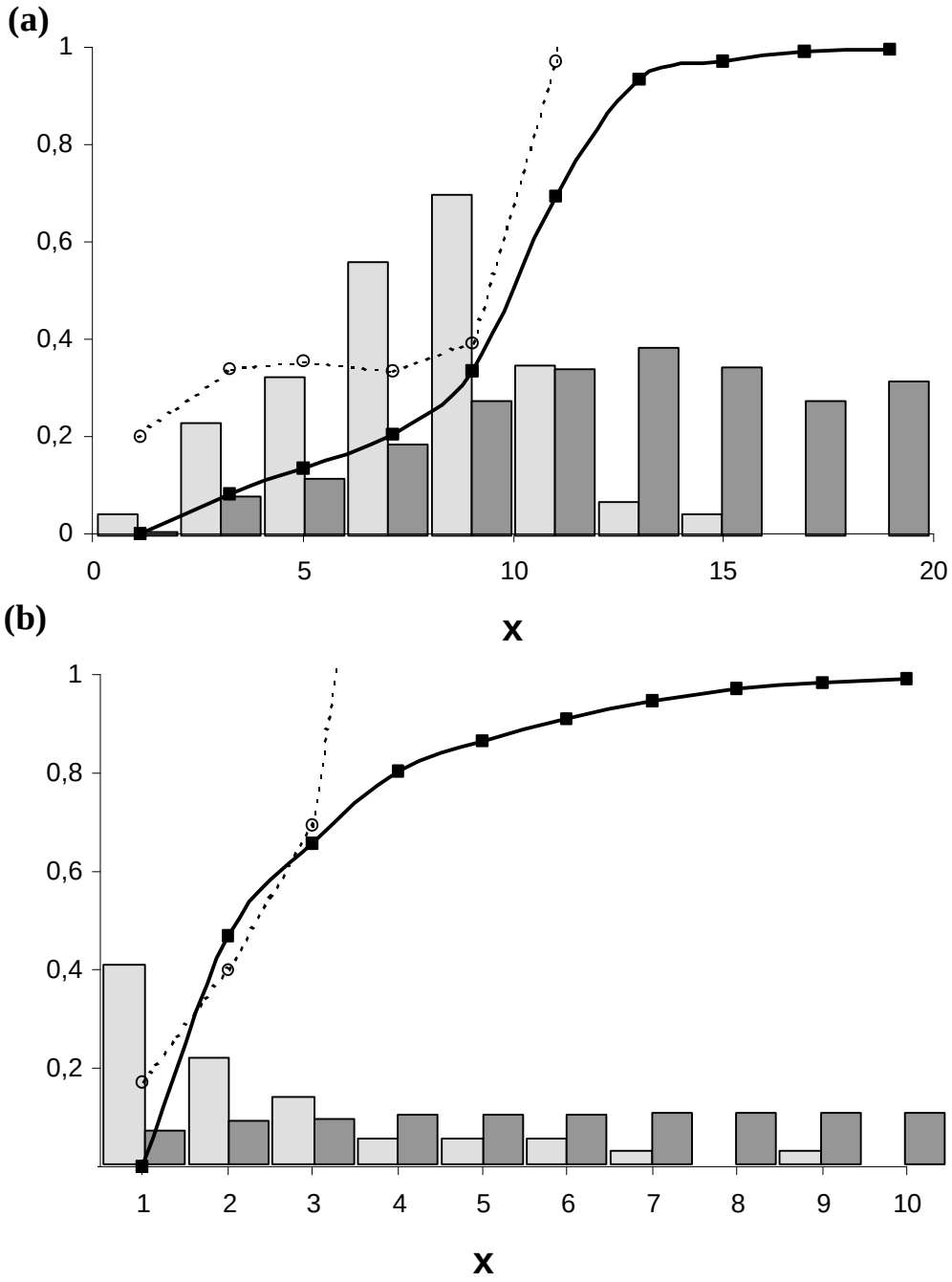

Figure 2: A simple way to estimate 8 for real data is to group the measurement (x) into distinct categories and to plot D(x) for these categories. This is calculated by taking the proportion of the tested population in that category and dividing by the proportion of the control group which falls into the same range. The average value that D(x) achieves over low values of x can than be used to obtain a minimally biased estimate of (1- 8) and hence of 8.
(a) illustrates the density ratio D(x) for the example of Figure 1e.
(b) illustrates the density ratio D(x) for the data set of antibody response to the antigen representing the FC27 family specific part.
Dashed line: density ratio D(x)
Thick line: 8 as estimated from the latent class model

Table 1: Mean titres, estimates of positivity and cutoffs.

antigens representing defined regions of the MSP2 molecule	Mean titre in negative controls (s.d.)	Classical aproach used to define positivity		Estimations from latent class model			Overall mean titre in test sera (units)
		Cutoffs x+2s.d.	Proportion of test sera positive	Estimated Proportion of test sera positive	Mean titre in positive test sera (units)	Mean titre in negative test sera (units)	
6x12 aa repeats	0.032 (0.022)	0.077	0.928	0.923	0.481	0.049	0.447
3D7 fsp	0.003 (0.003)	0.010	0.579	0.754	0.067	0.004	0.052
3D7 repeats	0.009 (0.005)	0.020	0.864	0.895	0.210	0.012	0.190
5'3' constant	0.281 (0.280)	0.840	0.540	0.856	1.866	0.344	1.647
4x32 aa repeats	0.004 (0.003)	0.010	0.396	0.526	0.071	0.004	0.039
FC27 fsp	0.090 (0.094)	0.278	0.441	0.786	0.356	0.106	0.302
23x NAP repeats	0.007 (0.005)	0.018	0.134	0.660	0.162	0.010	0.111

The antigens used in ELISA are described and defined in Irion et al. 2001.

x+2s.d.: mean of the negative controls + 2 standard deviations; aa: amino acid; fsp: family specific part; NAP: single letter code for amino acids.

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Immunogenicity of recombinant protein domains derived from merozoite surface protein 2 (MSP2) and production of monoclonal antibodies to the conserved protein regions

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Introduction

Merozoite surface protein 2 (MSP2) of *Plasmodium falciparum* is a potential component of a malaria vaccine (Genton et al. 2000, 2001). The MSP2 is a highly polymorphic 45-52 kDa integral membrane protein located on the surface of merozoites. The molecule has highly conserved N- and C-terminal regions with a central block composed of variable repeat domains flanked by dimorphic, non-repetitive, family-specificsequences. Even though MSP2 is naturally antigenic in man and antibody responses to conserved regions do occur during malaria infection, these responses predominantly recognize the dimorphic and polymorphic regions of the molecule (Taylor et al. 1995; Al-Yaman et al. 1995). Several studies were performed to assess the antibody response to corresponding peptides (Ramasamy et al. 1998; Lougovskoi et al. 1999) or recombinant MSP2 protein (Al-Yaman et al. 1994, 1995, 1997a, 1997b; Rzepczyk et al. 1997) representing all parts of the molecule. Several B cell epitopes have been identified in different parts of the molecule by peptide mapping. In order to improve our understanding of MSP2 as an antigen and vaccine candidate, which elicit antibodies with different protecting qualities in natural infections (Al-Yaman et al. 1994, 1995), the distinct domains of the molecule must be

Key words: merozoite surface protein 2, immunogenicity, monoclonal antibodies, conserved regions, malaria, *Plasmodium falciparum*.

investigated separately. Especially those regions of the protein, which are conserved even in different species of plasmodia, could be responsible for functions important for the survival of the parasite and might be a promising base for a malaria vaccine. Therefore, we have expressed several recombinant MSP2 proteins in *Escherichia coli* representing the entire sequence of the mature protein and assessed the immunogenicity of seven of these proteins by immunisation of mice. In addition the protein construct with the dimorphic and variable central parts deleted was used to generate monoclonal antibodies. To investigate the importance of the conserved regions, these monoclonal antibodies could be further used in inhibition and competition assays.

Methods

Production and purification of recombinant proteins

Several different MSP2 protein constructs with essential parts deleted (Fig. 1) were expressed in a procaryotic expression system (Qiagen) in *E.coli* M15 strain, initially transformed with pREP4 repressor plasmid. Corresponding primer pairs with specific restriction sites (5' *Bam* HI, 3' *Kpn* I) were used to amplify distinct regions in the *msp2* gene (Table 1) derived from different *msp2* alleles. The PCR products were cloned in the pQE30 expression vector as fusion proteins with six histidine residues at the 5'end. For better expression yield the 5'3'construct recombinant protein was fused to the gene coding for mouse DHFR protein (pQE16 expression vector). For cloning into this vector the *Bgl* II restriction site was used. The expression of the fusion proteins was under control of the lac promoter and hence inducible by addition of IPTG. The constructs are shown in Fig.1. Their positions relative to the *msp2* gene are presented. It should be noted that the restriction sites (*Bam* HI, *Kpn* I, *Eco* RI, *Bgl* II), by which the fragments were ligated into the pQE vector, are not represented in the *msp2* gene itself, but come from the primer used for amplification of the DNA fragments.

Sequences of rMSP2 proteins

All constructs were confirmed by DNA sequencing with ABI-Sequencer (Perkin Elmer) and the deduced amino acid sequences of the recombinant proteins were compared to that of MSP2 of the origin allele (Table 1). Recombinant proteins 23xNAP repeats, 3D7 family specific part, 4x32

aa repeats, 6x12 aa repeats, and 3D7 repeats have amino acid sequences identical to the origin protein regions plus the amino acids resulting from the restriction sites at the ends of the sequences. Sequence encoding protein 5'3'construct contains a single non-synonymous nucleotide change leading to an amino acid change in the protein, which is found in other *msp2* alleles, also. The antigen representing the family specific part of FC27 alleles showed an additional doubling of the last 14 amino acids.

Expression and purification of rMSP2

Expression and purification of the protein constructs were done according to the manufacturer's protocol (Qiagen) and were described in detail elsewhere (Irion et al. 2001). Briefly, *E. coli* cells, transformed with one of the expression vectors, were grown in Super broth (25 g Trypton, 15 g yeast extract, 5 g NaCl ad 1 L) using the appropriate antibiotics, 100 mg/L ampicilline (Appligene) and 25 mg/L kanamycin (Sigma) at 37/C to $OD_{600\ nm}$ = 0.5-0.8, when the protein production was induced by addition of IPTG (Appligene) to a final concentration of 1 mM. The harvested cells were resuspended in lysis buffer (50 mM Sodium dihydrogenphosphate pH 8.0; 300 mM Sodium chloride; 10 mM imidazole). Bacterial cells were broken by lysozym digestion and ultrasound on ice. The fusion proteins were purified by binding to Ni-2+ -agarose and elution was performed with imidazole (250 mM imidazole). The 5'3'construct recombinant protein was expressed at 25/C to prevent inclusion body formation and denatured with 6 M Guadininehydrochloride (6 M GuHCl; 0.1 M sodium dihydrogenphosphate; 0.01 M TrisCl, pH 8.0). Purification was performed with 8 M Urea (decreasing pH 8.0-4.5). The recombinant protein was eluted at pH 4.5. Pure protein fractions were pooled and renatured by dialysis as previously described for other MSP2 recombinant proteins (Takacs and Girard 1991). Protein preparations under native conditions were dialyzed against PBS pH 7.2 to remove imidazole. Immunoblots and ELISAs showed a good recognition of the recombinant proteins by serum from semi-immune adults from PNG and Tanzania. Sera from healthy Europeans (36 adults and 36 children aged 5-15 months) non-exposed to malaria were used as negative controls. The proteins were stored at –70/C until required.

Immunogenicity in BALB/c mice

The immunogenicity of the recombinant proteins was investigated in female BALB/c mice (4-6 months old) by four injections (4x i.p.) of each immunogen (2.5-10 mg) without adjuvants except polyacrylamide. Each immunogen was tested in two mice. The immunisation scale was: 0, 14, 28, 42 days. Pre-immune serum was collected from each mouse. Immune sera were collected 14 days after the last injection. The murine sera were used in ELISA and immunoblotting to test for recognition of the immunogens and parasites in IFAs. Sera were stored as aliquots at -20/C.

The immunisation time scale for mice used for monoclonal antibody production was different. Four mice were immunized by three injections (i.p.) of the immunogen (10 :g each) at day 0, 14, 28. Sera were collected from the tail vein. The specificity of produced polyclonal antibodies was tested by ELISA, IFA and immunoblotting. Three months later, the mice received the forth injection i.v. in the tail vein of the immunogen (12 mg) suspended in PBS without any polyacrylamide. After three days the mice were dissected. The murine sera were also tested in ELISA and immunoblotting to assess the recognition of the immunogen and parasites in IFAs. Some sera were tested against longer recombinant proteins, since no parasite derived antigen with the corresponding *msp2* gene was available.

Enzyme-linked immunosorbant assays (ELISAs)

ELISAs were performed as described by Irion et al. (2001). Microtitre plates (Immulon 2; Dynex Technologies, USA) were coated with 50 :l of antigen. Protein concentrations ranged from 0.1 :g/ml to 10 :g/ml in PBS pH 7.2. Sera from mice were used in serial dilution starting from 1:50 in two-fold steps. The second antibody was alkaline phosphatase conjugated, and 1mg/ml p-nitrophenyl in a carbonate buffer (Na_2CO_3, $NaHCO_3$, $MgCl_2$ pH 9.6) was used for colour development. The absorbance was measured at 405 nm in a spectrophotometer (Titertek Multiskan MCC/340). Each antiserum was tested against the single immunogen and, as a background control on the same plate, against a protein solution consisting of DHFR-6His (5 :g/ml PBS) produced in the same way like the other recombinant proteins. Pre-immune control sera were included in each ELISA assay. The antibody levels were reported as the reciprocal antibody titre which gave an OD_{405} of 0.1 over background.

Immunoblot analysis of recombinant immunogens and of parasite derived MSP2

Recombinant protein constructs were tested by immunoblotting for reactivity with specific anti-6His-antibody (Qiagen, R&D) and with anitsera of immunized mice. Purified protein was subjected to SDS-PAGE under reducing and non-reducing conditions. Proteins were transferred to a nitrocellulose membrane, which was then blocked overnight in blocking buffer (3% BSA in PBS pH 7.2). Diluted anti-6His-mouse-antibody (1:1000) was incubated with the membrane for one hour, washed, and subsequently incubated with alkaline phosphatase-conjugated secondary antibody for an additional hour and washed. Alkaline phosphatase substrate (NBT, BCIP, Biorad) was added in development buffer (100mM TrisCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) and incubated until bands became visible.

In vitro cultured parasites of the cloned lines K1 and NF54 were isolated by saponin lysis (Goman et al. 1982). Preparation of parasite derived proteins was done as described previously (Lanzer et al. 1992). About $5x10^9$ parasites were suspended in 1ml of lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.65% NP-40) and centrifuged to pellet the nuclear debris. Parasite proteins were resolved in an SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was probed with immune sera of the immunized mice as above.

Immunofluorescence assay (IFA)

Each murine serum sample was incubated at the appropriate dilution (according ELISA titres) with acetone-fixed cultured parasites expressing different *msp2* alleles (K1= K1-allele, HB3 = wos3-allele, NF54 = 3D7-allele, ITG2F6= 3D7-allele) for one hour at 37/C. IFA slides were washed and then incubated with fluorescein isothiocyanate-labelled secondary antibody (goat anti-mouse IgG(g), KPL) for one hour at 37/C in a humid chamber, washed, and examined with a fluorescence microscope. As negative controls non-infected erythrocytes and pre-immunization serum were used.

Monoclonal antibodies

To generate monoclonal antibodies directed against the 5'3'construct protein spleen cells of the immunized BALB/c mouse were fused with PAI myeloma cells (mouse myeloma HGPRT-deficient cells). The fusion was performed according to standard procedures (Coligan et al. 1995). Instead of feeder layers, HAT medium was supplemented with 5% conditioned medium from supernatant of an LPS activated monocytic cell line (P388/D1; Koren et al. 1975; Melchers and Corbel 1983; Perstidge et al. 1984). Three days after the last antigen booster injection (i.v.) the mouse was dissected under sterile conditions and the spleen tissue was pressed through a mesh net by using a plunger of a syringe to obtain a single cell suspension. Both spleen and prepared PAI myeloma cells were washed twice in wash medium by repeated centrifugation for 5 min at 180g. Prior to a third centrifugation step cells were pooled in equal proportions (cells per spleen 1x 10^8). After complete removal of wash medium, 1 ml PEG 1500 solution (Boehringer Mannheim) was added in steps of 100 :l within one minute. The resulting two phases were mixed by slow rotation during 10 min. After addition of 35 ml wash medium the cells were centrifuged for 5 min at 180g and resuspended in 160 ml HAT medium (IMDM, 36 mM $NaHCO_3$, $5x10^{-2}$ mM $-mercaptoethanol, 100 U/ml Penicillin/100 :g/ml Streptomycin, 0.1 mM Hypoxanthine, $0.4x10^{-3}$ mM Aminopterin, 0.016 mM Thymidine, 20% FCSi, 5% P388/D1 conditioned medium). This cell suspension was distributed in 200 :l portions in a 96-well culture plate and incubated at 37/C in an atmosphere containing 5% CO_2 (Carbagas, Basel, CH). Growing colonies were examined for production of anti-5'3'construct antigen antibodies by ELISA. Mother clones producing the highest IgG titer were transferred into 48-well culture plates. The specificity of the produced antibodies was tested by IFA and immunoblotting. The IFA positive hybridoma cells were cloned by limiting dilution in HAT medium to two different final concentrations: 0.3 and 3 cells / well. Growing single colonies were screened by ELISA. Cell lines with the highest titres were transferred into 24-well culture plates. At this point, culture medium was changed from HAT medium to hybridoma medium. A final transfer in 25 cm^2 tissue culture flasks followed. The antibody containing supernatant was removed after overgrowth of cells, centrifuged for 10 min at 180g, and aliquots were stored at –70/C until use.

Results

Immunogenicity of recombinant MSP2 constructs

Mice were immunised with the purified recombinant MSP2 antigens with essential parts deleted (except recombinant 3'end protein) to show that all seven protein constructs were immunogenic. This also established the protein similarity to parasite derived MSP2 (PfMSP2) as seen on immunoblotting using parasite derived proteins and to the native MSP2 on the parasite itself by IFA. Antibody titres against the seven recombinant MSP2 protein constructs were measured by ELISA, immunoblot, and IFA after four injections in BALB/c mice. High antibody titres were observed in ELISA and immunoblot with rMSP2 constructs against the immunogens 5'3'construct, 6x12 aa repeats, 4x32 aa repeats, FC27 family specific part (table 2), medium antibody titres were reached against 3D7 repeats and only low antibody titres were achieved against the immunogens 23xNAP repeats and 3D7 family specific part even after the fourth injection. IFA was used to assess stage specificity and subcellular distribution of positive reactions. All IFA positive sera resulted in reactions typical of PfMSP2 (Smythe et al 1988), a grape-like pattern on segmented schizonts and extracellular merozoites. Antibodies against the 5'3'construct showed a distinct pattern with stained schizonts, extracellular merozoites, but also all other parasite stages. The strength of response induced by different rMSP2 constructs varied. Both mice immunized with 5'3'construct, 6x12 aa repeats, 4x32 aa repeats, FC27 family specific, 3D7 repeats showed positive reaction with parasites. Antibodies to the 4x32 aa repeats recognised also parasite derived protein containing 4x32 aa repeats (Wos6). All sera from mice immunized with 23xNAP repeats and 3D7 family specific failed to react with parasites. Antisera against 23xNAP repeats were tested to a longer recombinant MSP2 antigen with 23xNAP repeats (nested Ifa23) and showed recognition in ELISA and immunoblot. The specificity of all antisera was good since polyclonal antisera recognised only parasites with corresponding MSP2 alleles except antisera against 3D7 repeats where a cross-reaction with FC27 alleles was observed. In addition, for the assessment of natural immune recognition eighty sera from patients attending the Policlinic of the Swiss Tropical Institute (traveller and migrants from endemic areas) with a diagnosed *P. falciparum* malaria, were used in immunoblot analysis against the 5'3'construct and a recombinant protein representing the entire conserved 3'-end. Eight individuals (10%) showed an antibody response to the 5'3'construct antigen, and two individuals (2.5%) recognized the 3'end.

Monoclonal antibodies

Polyclonal antisera raised in 4/4 BALB/c mice recognized the 5'3'construct in immunoblot and ELISA. Additionally, these antisera recognized several proteins with the maximum apparent mass (Mr) of ca. 45 kDa by immunoblotting of *P. falciparum* K1 antigen extracts. The levels of antibody response raised against the immunogen were estimated by ELISA, and the results are presented as reciprocal titre in table 3. For the antisera, a grape like pattern of fluorescence was observed in IFA with *P. falciparum* cloned lines K1 (FC27 allele) and NF54 (3D7 allele), suggesting recognition of protein(s) on the surface of merozoites within schizont stage parasites. Parasites in other stages were also recognized by staining the cytoplasma of the cells.

Five hybridoma cell lines produced antibodies which specifically recognized the immunogen in ELISA (mab A1.108, A1.3, A1.27, A1.36, A1.81, see table 3). Three antibodies were also tested as positive against the 3'protein in ELISA (mab A1.108, A1.3, A1.36). There are two groups of monoclonal antibodies. One group of antibodies recognized the recombinant immunogen and the recombinant 3'end protein in immunoblots, but did not recognize parasites in IFA. The other group of monoclonal antibodies recognized all stage parasites in IFA, but did not recognize neither the recombinant immunogen nor the recombinant 3'end protein in immunoblots. In immunoblots with parasite derived proteins these antibodies recognized several bands including the MSP2 corresponding band (Figure 2).

Discussion

The aim of this study was to characterize the antibody response to distinct parts of the merozoite surface protein 2 in mice to assess the immunogenicity of the recombinant proteins. In addition the production of monoclonal antibodies to the chimeric protein representing the conserved regions of the molecule was performed to estimate the significance of the conserved regions for the function of the MSP2 molecule by *in vitro* inhibition assays. The antibody responses obtained in mice immunized with recombinant proteins were analysed by IFA, immunoblot and ELISA.

Sequencing studies have shown that many malarial antigens contain extensive arrays of tandemly repeated short amino acid sequences, and much of the antibody response induced by malaria infections is directed against epitopes encoded by these repeats (Cowman et al. 1984; Anders et al. 1985). It is known that short amino acid repeats elicit a rather ineffective T-cell-independent

antibody response, whereas a T-cell-dependent response against non-repeat regions would be more effective (Vergara et al. 1985; Bachmann et al. 1995). Thus, it had been argued that the primary function of the repeats is the induction of a strong T-cell-independent response to the repeat region in order to prevent development of a T-cell-dependent response (Enea & Arnot 1988), thereby favouring the parasite's evasion. It is reported that the fine specificities of the humoral immune response in *P. falciparum* infected individuals are dominated by the repeat peptide structures and the conserved regions seem to be cryptic B epitopes, at least during the course of natural infections (Ballou et al. 1985; Bharadwaj et al. 1998).

In our hands, the repetitive sequences of MSP2 showed high immunogenicity in mice and elicited high level of antibody titres. In addition the protein which represents the family specific region of FC27 alleles was highly immunogenic in mice, with high antibody level and high specificity. The only exceptions were 23xNAP repeats and the family specific part of 3D7 alleles, where only marginal antibody titres were reached and no recognition of parasites could be demonstrated. The negative IFA with polyclonal antisera against the 3D7-family specific part could be due to the low antibody titres. Since the serum samples of semi-immune adults showed a very good reaction with the recombinant antigen representing the 3D7 family specific part in contrary to the sera of non-exposed children and adults (together with a very low background), we rated this antigen as at least partly similar to the PfMSP2 and therefore useable for ELISA (Irion et al. 2001). But this could indicate, that the 3D7 family specific part is little immunogenic and requires frequent natural infection or boosting to reach high antibody titres. Unfortunately a parasite culture expressing MSP2 with more than one NAP-repeat was not available. However, a longer recombinant protein with 23xNAP repeats was recognised in immunoblot. In summary, immunisation of mice showed that all seven recombinant proteins were immunogenic, and antibodies to five proteins recognised PfMSP2, indicating that at least these proteins had epitopes of the native protein. Once the antigenic similarity of the rMSP2 proteins to PfMSP2 was estimated, we were confident in using these proteins for serological assays. Hence, the proteins could be used in an ELISA assay to detect MSP2 specific antibodies in consecutive sera of children initially aged5-15 months from Tanzania. This study is described elsewhere (Irion et al. 2001).

IFA positive polyclonal antisera (32 aa repeats, 12 aa repeats, FC27 fsp, 3D7 repeats) showed a typical MSP2 staining pattern with grape like schizonts and merozoite surface staining. However, antisera to 5'3'construct reacted with all parasite stages indicating the recognition of a shared epitope of several proteins. Further investigations with monoclonal antibodies originated from mice immunized with 5'3'construct elucidated these findings.

Antibodies that recognized *P. falciparum* parasites by IFA were produced by all of the four BALB/c mice immunized with the 5'3'construct. Produced monoclonal antibodies could be divided in two groups. On the one hand monoclonal antibodies recognizing the recombinant immunogen in immunoblots and ELISA did not show any recognition of parasites in IFA. The recognition of the recombinant 3'end protein showed that the C-terminal region and not the N-terminal region is the immune target for these antibodies. Previously it was reported that antisera from mice immunized with an antigen similar to the 5'3'construct responded to an amino acid sequence at the C-teminus which is cleaved from the mature MSP2 protein (Lawrence et al. 2000). This might explain the lack of recognition of the parasites in IFA and of the parasite derived protein in immunoblot although the monoclonal antibodies recognized the recombinant immunogen in ELISA and immunoblotting. Another explanation could be neoantigens created by fusing the different parts of the molecule to DHFR, but the 3'-end recombinant protein was expressed without fusion to the DHFR-protein. Hence, we favour the first explanation, and this has to be proven using short peptides representing the whole 3'end of the MSP2 molecule.

On the other hand, those monoclonal antibodies recognizing all stage parasites in IFA did not recognize the immunogen in immunoblots, but in ELISA. In addition, the 3'protein was also recognized in ELISA, indicating the recognition of a conformational epitope, which is located within the 3'region of the protein. In addition, this second group of antibodies showed a reaction with several bands of the parasite derived protein preparation, including the MSP2 band. This indicates the recognition of an epitope which is shared by several plasmodial proteins of all parasite stages. Therefore these antibodies are not expected to be protective or the target epitope is not immunogenic like a cryptic epitope in natural infections. Hence, these antibodies might not be present in malaria patients. The nature of this shared epitope has to be investigated. Whether these antibodies show an inhibitory effect on *P. falciparum* culture, has to be further examined in inhibition and competition assays.

Only, ten percent and two and a half percent of sera from travellers and migrants with a diagnosed *P. falciparum* malaria reacted with recombinant 5'3'construct and 3'protein in immunoblots, respectively. These results are in agreement with previous findings from Lawrence et al. (2000). Whereas sera from children, non-exposed and exposed to malaria, showed a moderate antibody level against the 5'3'construct in ELISA, even the non-exposed children (Irion et al. 2001) indicating a conformational epitope as immune target. Whether this target is equivalent to the conformational epitope of the monoclonal antibodies has to be investigated. Affinity purification of these human antibodies and subsequent IFAs with parasitized erythrocytes will elucidate this finding.

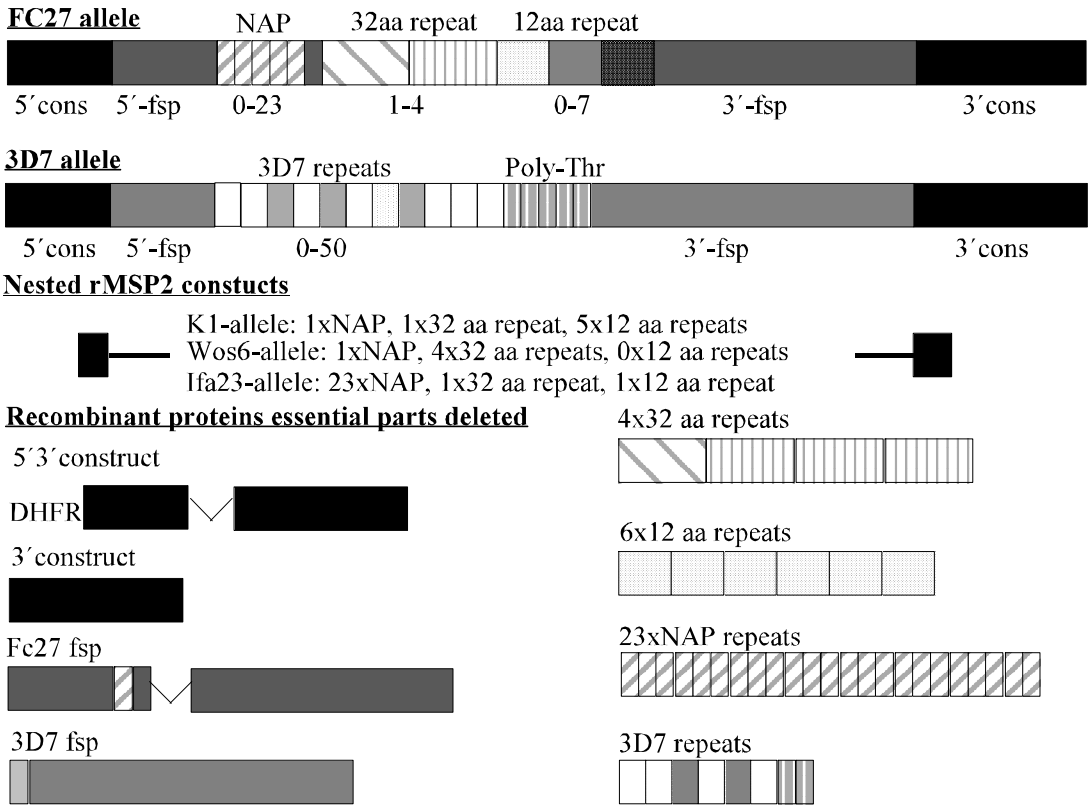

Figure 1: Schematic structure of the merozoite surface protein 2
The two allelic families and the recombinant proteins with essential parts deleted are shown. aa: amino acid, cons: conserved, fsp: family specific part, NAP: single letter code for amino acids, Thr: Threonine

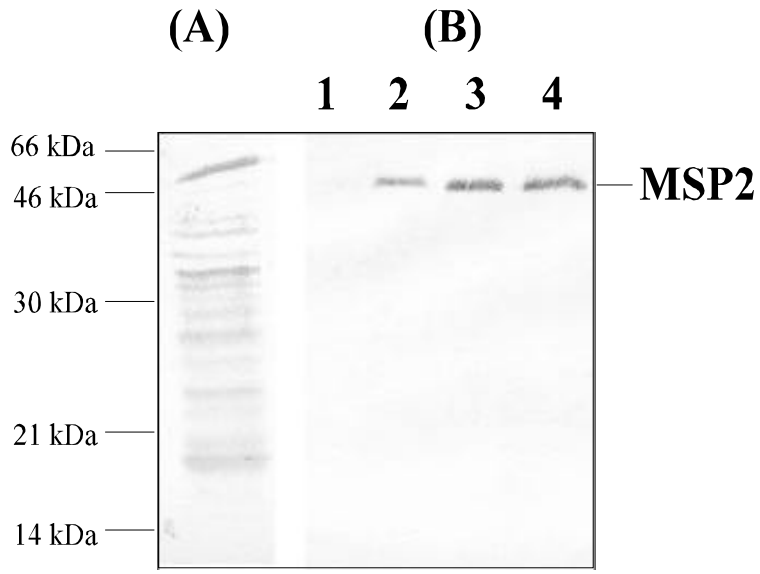

Figure 2: Immunoblot of parasite derived protein preparation MSP2 of the cloned line K1
(A) monoclonal antibody A1.3 on protein preparation of a non-synchronized cloned line K1;
(B) anti-6x12 aa repeats antiserum:
Lane 1: Ring stages and trophozoites
Lane 2: Trophozoites with segmented nuclei
Lane 3: Schizonts and merozoites
Lane 4: Schizonts and merozoites

Table 1: Deduced amino acid sequences of tested antigens

antigen	deduced amino acid sequence (allele)
23xNAP repeats	MRGS-HHHHHH-GSR-SVGA-$(NAP)_{23}$-NADTIASG-YPGSTCSQA (Ifa23)
3D7 fsp	MRGS-HHHHHH-GSR-(3D7allele FCR3 aa109-214)-GYPGSTCSQA (FCR3)
4x32 aa repeats	MRGS-HHHHHH-GSR-APK-(32aa repeat)$_4$-ADTP-GYPGSTCSQA (Ifa45)
6x12 aa repeats	MRGS-HHHHHH-GSR-TAT-(ESNSRSPPITTT) $_6$-ESS-GYPGSTCSQA (K1+1x12 aa repeat)
FC27 fsp	MRGS-HHHHHH-GSR-KSVGANAPK-GIP-(FC27fsp)-APQEPQTAENENPA-GYPGSTCSQA (K1)
5'3'construct	MRGS-HHHHHH-GSR-(5'-region K1 aa 1-50)-RNS-(3'-region K1 aa 207-280)-GYPGSTCSQA aa 23 E (like FC27-genotype)
DHFR-5'3'construct	MRGS-(mouseDHFR)-GSRSS-(5'-region K1 aa 1-50)-RNS-(3'-region K1 aa 207-280)-RRS-HHHHHH aa 23 E (like FC27-genotype)
5'3'const w/oSP	MRGS-HHHHHH-GSR-(5'-region K1 aa 18-50)-RNS-(3'-region K1 aa 207-280)-GYPGSTCSQA aa 23 E (like FC27-genotype)
3'end	MRGS-HHHHHH-GSR-(3'-region K1 aa 207-280)-GYPGSTCSQA
3D7 repeats	MRGS-HHHHHH-GSR-PPT-GAGASGRAGA-GA-GAGASGRAGA-GAGASGRAGS-GSGDGAVASA-GSGADAEGGSSTPATT-GYPGSTCSQA (84aa)
nested rMSP2	MRGS-HHHHHH-GSR- (SIR-- //--MHGS) -RGYPGSTCSQA
DHFR control	MRGS-(mouseDHFR)-RRS-HHHHHH

aa: amino acid, const.: construct, fsp: family specific part, IFA: immuno fluorescence assay, NAP: single letter code for amino acids, nd: not done, PfMSP2: parasite derived protein MSP2, w/oSP: without signal peptide

Table 2: Reciprocal antibody titres and reaction of polyclonal antisera from immunized mice in ELISA, immunoblot and IFA.

immunogen	ELISA	immunoblot				IFA	
		immunogen	PfMSP2		nested rMSP2	K1/ HB3	3D7/ NF54
			K1	NF54			
5'3'construct	12 800	pos (500)	several bands	several bands	K1 pos.	++/++	++/++
FC27 fsp	12 800	pos (500)	pos	neg	K1 pos	++/++	neg
3D7 fsp	100	neg (50)	nd	nd	nd	neg	neg
23xNAP repeats	100	pos (50)	nd	nd	Ifa23 pos	nd	nd
4x32 aa repeats	12 800	pos (500)	nd	nd	Wos6 pos	(+)	neg
6x12 aa repeats	25 600	pos (500)	pos	neg	K1 pos	++/+	neg
3D7 repeats	1 600	pos (500)	pos	pos	nd	+/+	++/++

Table 3: Monoclonal antibodies produced against the 5'3'construct

clone	ELISA					immunoblot				IFA	
	DHFR 5'3' const	5'3' const	5'3' const w/oSP	3'end	DHFR	DHFR 5'3' const	3'end	DHFR	Pf MSP 2	K1	NF54
A1.108	pos	pos	pos	pos	neg	pos	pos	neg	neg	neg	neg
A1.3	pos	pos	pos	pos	neg	neg	neg	neg	pos	pos	pos
A1.27	pos	pos	pos	nd	neg	neg	nd	neg	neg	neg	neg
A1.36	pos	pos	pos	pos	neg	neg	neg	neg	pos	pos	pos
A1.81	pos	pos	pos	nd	neg	pos	nd	neg	neg	neg	neg

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7. General Discussion

The key feature of science is not measurement (this is just a tool), but understanding. To understand the occurrence of disease in human populations we need to know something about biology, but we also often need to know something about populations, including the population of the host organism and the population of the infecting parasite. During the last two decades large research efforts have been made to identify malaria antigens involved in protection and to define mechanisms by which the immune system may neutralize the parasite, however the success was only small. The extreme antigenic diversity within *P. falciparum* imposes major challenges to identify which of its numerous proteins are important antigens eliciting protective antibodies. This diversity (and variation) of antigens might be one of the cornerstones for understanding of how semi-immunity against malaria develops. Polymorphic antigens have been described in several parasite life cycle stages but are particularly a feature of the antigens associated with the surface of the asexual blood-stage merozoites.

In this thesis, the merozoite surface protein 2 (MSP2), one of the well-characterized surface proteins and a vaccine candidate of *P. falciparum*, is illustrated from two viewpoints:

(i) the epidemiological viewpoint, which uses *msp2* as a marker gene for PCR-RFLP genotyping as an outcome measurement of interventions or of the *status quo* concerning malariometric indices and (ii) additionally, the immunological viewpoint, which provides further information on the biological role and significance of MSP2 as an immunological target. Both can contribute to the understanding of evolution and ecology of *P. falciparum*, especially of survival strategies of the parasite in the host organism and hence of the development of semi-immunity of the host.

7.1 Methodological Aspects

7.1.1 The appropriate analytic tool

Using objective methods for analysing biomedical research data would increase the power of research studies and by that improve the outcome measurements of interventions. Therefore we used a latent class model based methodology which promises almost unbiased analysis of biomedical research data. This methodology can be applied to outputs of biomedical assays, like the ELISA method, which are expected to classify samples into two groups which overlap each

other to some extent (Vounatsou et al. 1998). Using real data sets we have considered the application of a latent class model as a better method to calculate the probability of a sample to be positive in an immunological assay. Although some data sets might give a clear distribution in responder and non-responder, for many others no evidence of dichotomy occurs. Hence, defining a cut-off value when there may be no obvious division of individuals into two groups, is potentially unjustified and a rather arbitrary process. Since the latent class model does not classify the sample dichotomously, it may be applied to any data set, regardless of the level of dichotomy. We showed the usefulness of this methodology particularly for the analysis of variable background measurements (Paper IV), which arise frequently in immunology, since immune responses encountered in human populations display a high level of heterogeneity.

This does not mean that the cut-off method should not be used as a measure of the rate of positivity in immunological methods. There may still be some argument for using a threshold value, for reasons of clarity of presentation, or in a situation where some real evidence of a dichotomy exists. This approach may be appropriate when the assay is used for diagnostic purposes, but its suitability for epidemiological studies is less well-founded.

Since there is no agreement on how to define a "positive responder", results from different studies may be not comparable. Besides being valuable in evaluating a single test, a latent class model is extremely useful in comparing tests with another one without having to choose any specific decision level. Especially in small sample sets of the control group, calculating the cut-off value as mean + 2sd is difficult, since out layers increase the cutoff value.

We demonstrated that the rates of responders could be seriously underestimated from ELISA tests, depending on the absorbance cut-off method applied for negative controls. Especially in children where the immune response (to malaria antigens) is expected to be weak and the differences between "responders" and "non-responders" may be small, it is important to choose the appropriate analysis method. In a study such as ours where the development of an immune response is the main issue, one cannot select a cutoff value simply on the basis of examining the data.

The observed differences between the data sets are mainly based on the nature of the antigens themselves and not on the performance of the assay. Comparing the latent class model to the cut-off value method, the estimated proportions of test sera positive to the antigens 6x12 aa repeats

and 3D7 repeats are very similar (Paper IV). In contrast there is a big difference in the proportion of test sera positive to the other five antigens. The proportion of test sera positive was about 0.13 (4x32 aa repeats) to 0.53 (23xNAP repeats) higher when the latent class model was used. Misclassifying 53% of samples by the cutoff method is considerably high.

Especially in surveys evaluating intervention strategies, determining probability of positivity 8in the considered population is important. Interventions that reduce the force of infection may affect the disease rate adversely by reducing boosting that may be required for the development and maintenance of immunity to severe malaria. We found a highly significant decrease in antibody response to the antigens 3D7 fsp, 3D7 repeats, 4x32 aa repeats, and 23xNAP repeats in the bed net user group (Paper II). This difference might not be so clear when the cut-off value method is used for estimating the rate of positivity.

In addition when two groups of individuals were investigated, which are both at least partially immune or "exposed", it would be much more efficient to compare the mean level of response rather than the proportion of "responders" (Bennett and Riley 1992). The mean antibody levels of test sera positive and negative are easily calculated by the latent class model (Paper II and IV). Overall, estimation of probability of positivity 8or the mean level of response for different specific antibody responses in different populations would represent a valuable step towards understanding immune mechanisms acting against *Plasmodium falciparum.*

7.1.2 The polymorphic gene - The appropriate marker gene

The development, accuracy and effective use of PCR-based diagnostic markers in the study of malaria epidemiology are dependent upon an understanding of the assumptions and limitations of the techniques used to generate the markers and the use of appropriate controls to test them.

One of the major advantages of a PCR-based approach is the possibility of investigating from circulating blood stages a large number of genetic characters, whatever the stage of the life cycle at which these loci are expressed. In addition, PCR generates the material to be further analysed during the analysis procedure instead of consuming it, as in all other methodologies (e.g., isoenzyme typing or monoclonal typing). Single PCR reactions amplifying regions from single copy genes routinely detect below the microscopic threshold and allow a semi-quantitative amplification as in such reactions the intensity of the ethidium bromide staining roughly reflects

the quantity of genomic template DNA added (Contamin et al. 1995).

In practice, one of the major limitations of PCR is that the detection of a band surely denotes the presence of parasites carrying this allele, but the failure to detect any specific band is more difficult to interpret. This may be due to either the absence of this parasite type, to the presence of other more abundant alleles or to an impossibility to amplify this allele because of a mutation in the sequence where the primer should hybridize. This might be circumvented by using more than one typing reaction. In addition, one should keep in mind, that due to very low density or sequestration of parasites at the time of sampling one sample may only partly reflect the whole parasite population in an infected individual (Färnert et al. 1997).

Genotyping *P. falciparum* infections in field isolates is suitable for various applications (Paper I). Assessing the multiplicity of infections and, hence in longitudinal surveys, the infection dynamics is only one example (Smth et al. 1999b; Paper III). For typing purposes, loci presenting a large number of alleles with distinct size and sequence polymorphism, such as *msp1* and *msp2* are valuable markers. Hence, the high number of different alleles of *msp2* detected so far in Tanzania offers the genetic diversity necessary for single locus genotyping and the problem of superinfection with the same genotype as the previous is prevented since the polymorphism is prominent and distinct (Smith et al. 1999b). Epidemiological studies which involve *msp2* genotyping give additional insights about the biological role of the expressed protein, e.g., morbidity association or protective potential of an allelic family, since the parasite's genotype affects its phenotype, growth, population size and toxin production. But, host and parasite genetic factors are both of importance, since the virulence of a particular parasite strain will depend in part on the genetic makeup of the host.

Finally, there is the immediate issue of how candidate vaccines are to be evaluated in the field and genotyping the *msp2* locus provides one big advantage: the marker gene is a vaccine candidate itself (Genton et al. 2000).

7.2 The Polymorphic Gene - The Target of the Immune System

Besides using *msp2* for genotyping as an epidemiologic tool, this method offers additional information of the biological role and significance of MSP2 as an immunological target. Since MSP2 is a prominent antigen on the surface of the merozoites, it is exposed to the selective pressure of the immune system, resulting in a polymorphic structure of the antigen (Hughes and Hughes 1995). In addition, the observed dimorphism of the msp2 locus might be the effect of the competition between parasite genotypes mediated by the immune system of the semi-immune host. Hence, understanding of immune response to MSP2 will provide valuable insights in the surviving strategies of *Plasmodium falciparum*, e.g. evasion from the immune system of the host and in the evolution of the polymorphic sequence.

Immunisation of mice showed that all recombinant proteins representing various parts of the MSP2 sequence are immunogenic (paper V). Elicited antibodies recognized parasite derived MSP2 and IFA positive polyclonal antisera showed a typical MSP2 staining pattern. Antisera and monoclonal antibodies to the 5'3'construct showed a recognition of all parasite stages indicating the recognition of a shared epitope. Cross reactions between malaria antigens have been seen with a variety of monoclonal antibodies (Hope et al. 1984; Epping et al. 1988). Further investigations with monoclonal antibodies from mice immunised with 5'3'construct would elucidate the nature of this shared epitope(s). Whether these antibodies show an inhibitory effect on *P. falciparum* culture, has to be examined. In addition the protein which represents the family specific region of FC27 alleles was highly immunogenic in mice, with high antibody level and high specificity. The only exceptions were the recombinant antigens representing 23xNAP repeats and the family specific region of 3D7 alleles, where only marginal antibody titres were reached and no recognition of parasites could be demonstrated. This observed lack of antibody response might be due to restriction in Balb/c mice, which was also reported by others (Lawrence et al. 2000) or a repeated exposure to these antigens might be necessary to reach higher antibody levels. Since the serum samples of semi-immune adults showed a very good reaction with these recombinant antigens in contrary to the sera of non-exposed children and adults, we rated these antigens as partly similar to the PfMSP2 and therefore useable for ELISA.

The high values of probability of positivity 8obtained for the antibody response in children aged 5-21 months to each of the antigens encourages the direct evidence for the early acquisition of an antibody response to the tested malaria antigens (paper II) in contrast to the antibody response

to RESA (Al-Yaman et al. 1995b). It was hypothesized that clinical protection during the first few months of life in high transmission settings allows active immunization to occur and contributes to a reduction in the overall risks of severe complicated malaria throughout childhood (Roberts et al. 1994; Snow et al. 1998; Gupta et al. 1999). The fact that protection from life-threatening disease is apparently acquired rapidly and early in life indicates that the reduction of severe malaria through vaccination may be an achievable goal. Most of the tested children, initially aged 5-15 months, have antibodies to distinct parts of the merozoite surface protein 2, but the extent of the antibody response was quite different compared to the antibody response of semi-immune adults. In other words, the frequency of responders was high, although the magnitude of the responses was generally low, but increases with age. The high proportion sera positive to 6x12aa repeats provides some evidence for an age-independent marker of those antibodies for exposure, which is in agreement with previous findings (Ranford-Cartwright et al. 1996). These antibody levels may reflect an early history of exposure in combination with high immunogenicity of the antigen plus the predominance of FC27 alleles with more than three 12 aa repeats. A different situation was seen with the 4x32 aa repeats antigen. Although, this antigen is highly immunogenic the antibody levels in sera of Tanzanian children were weak. This finding may be due to the predominance of FC27 alleles with only a single 32 aa repeat unit and specific antibodies might have a short half life or might be elicited only after several infections. In contrast, high antibody levels to the recombinant construct representing the conserved parts of MSP2 might be due to cross-reactive antibodies elicited by a ubiquitous antigen, since the background measurements with the European control group were quite high. Cross-reactivity might be part of an explanation for the age dependence of semi-immunity to malaria. People lacking exposure to malaria may recognise malaria antigens, perhaps because of antigenic cross-reactivity between epitopes in malarial parasites and other microorganism (Good et al. 1987; Jones et al. 1990; Good 1991, 1995; Beverley 1994; Elm et al. 1998; Good and Doolan 1999). Exposure to a myriad of immunogenic microorganisms throughout life is unavoidable. Accordingly, the accumulation of memory to these antigens and the likelihood of ”incidental” cross-reactivity with antigens from malarial parasites probably increases with age (Baired et al. 1993; Baired 1998).

In several studies, both the prevalence and concentration of antibodies to the non-repetitive part of the 3D7 antigen appeared to correlate with reduced morbidity due to malaria (Al-Yaman et al. 1994; Al-Yaman et al. 1995a; Al-Yaman et al. 1997; Taylor et al. 1998). In our study the antibody response to the recombinant 3D7 family specific part was marginal in children aged 5-15 months initially in comparison to the strong IgG antibody response in adults. The mean antibody level to 3D7 family specific part was much lower than those to FC27 family specific part and antibody levels correlated with the presence and acquisition of infections expressing FC27 alleles. The antibody responses to both family specific parts differ in background level, probability of positivity, and ability to be induced by *P. falciparum* infections. These differences might reflect those observed in the morbidity association of the different genotypes. Since morbidity data from the tested children in our study are missing an association of a specific antibody response and protection cannot be drawn. Therefore, a longitudinal prospective study with children aged 0.5-5 years is needed to assess the impact of the humoral immune response on protection against malaria disease.

It might be postulated that *Plasmodium falciparum* infections expressing *msp2* of the FC27 allelic family distract the antibody response to the MSP2 molecule towards antibodies against parts of the 3D7 allelic family, which might support protection to infections expressing 3D7 alleles. The part of the MSP2 molecule responsible for the induction of 3D7 specific antibodies might be the 32 aa repeat, where some similarities to the 3D7 repeat sequence are observed. 3D7 alleles with this homologous sequence are more frequently observed in PNG than 3D7 alleles with other repeat sequences (Felger pers. com.). It has to be investigated whether 3D7 alleles with repeats containing the homologous sequence also predominate in Tanzania and whether these genotypes have any advantages related to this homology.

Cross reactivity of affinity purified human antibodies to the 3D7 repeat Gly Gly Ser Ala and other 3D7 repeat sequences and to FC27 alleles was shown previously (Smythe et al. 1990). In addition we showed that mice immunized with the recombinant 3D7 repeats antigen elicited antibodies which reacted with FC27 alleles in IFA indicating a shared epitope in these repeats. One of the protective monoclonal antibodies recognized an epitope located in the 32 aa repeats right behind the short homologous sequence to the 3D7 repeats (Epping et al. 1988; Irion et al. 1997). It might be speculated that antibodies recognizing the 3D7 epitope hinder sterically the induction or binding of protective antibodies to the 32 aa repeat as it was shown with an epitope in MSP1

(Guevara Patino et al. 1997). Previously, it was shown in PNG that MSP2 alleles of the FC27 family were strongly associated with malaria morbidity (Engelbrecht et al. 1995). But, individuals with mixed infections of parasites expressing the different allelic families were only as likely to be symptomatic as individuals with parasites of the 3D7 MSP2 genotype. It was concluded that parasites belonging to the 3D7 allelic genotype must have a protective effect observed in adults living in highly endemic areas (Engelbrecht et al. 1995). The finding that the loss of *P. falciparum* infections expressing 3D7 alleles simplifies the establishment of a FC27 expressing infection, supports the hypothesis of protective concomitant infections and the premunition hypothesis (Smith et al 1999). Recently, the cross-protection to infections expressing FC27 alleles mediated by infections expressing 3D7 alleles could not be induced by vaccination with a recombinant 3D7 protein (Genton et al. 2001, Combination B). It might be conjectured that the protective effect of 3D7 alleles bases only on the absorption of these "blocking" antibodies by the repeat structure of present *P. falciparum* infections and not on the induction of protective antibodies. In the vaccine trial in PNG the number of infections expressing FC27 alleles did not increase, but the observed ratio FC27 alleles:3D7 alleles, would support this hypothesis (Genton et al. 2001).

7.3 Dynamics of *P. falciparum* infections

In this thesis it was shown that the dynamics of *P. falciparum* infections expressing different *msp2* allelic families are different in an age-dependent way (paper III). The duration of infections expressing different allelic families reverses when the children getting older. This finding explains an earlier observation of Felger et al. (1994; 1999b), when the distribution of the two allelic families of MSP2 differed significantly between semi-immune adults and children. FC27 alleles were predominantly found in adults, whereas 3D7 alleles were found more frequently in children. This difference in parasitological profiles might be the effect of qualitative differences in the grade of immunity of individuals. And this again is probably influenced by the genotype of the infecting parasite. Infections expressing MSP2 alleles of the 3D7 allelic family show a decreased duration in children older than 18 months whereas infections expressing FC27 alleles persist longer in this age group. The immune response of the host is able to limit *P. falciparum* infections expressing 3D7 alleles, beginning gradually in the first and a half year of life. This difference in duration might indicate the lack of a protective role for antibodies to the conserved regions at the ends of the molecule. The ability of limiting infections expressing FC27 alleles needs probably another process

in which other antigens might also be involved. The negative correlation of the change of antibody titres to 32 aa repeats from the previous to the current sample of one individual with the loss of infections expressing MSP2 of the FC27 allelic family might indicate a short duration of this antibody response, which might need reinfection with *P. falciparum* expressing a FC27 allele for boosting. Additionally, the lowest antibody level measured in the study was that against 32 aa repeats. In addition, in early childhood the chronological sequence of *P. falciparum* infections expressing the different *msp2* allelic families might be important for the development of the immune response, as it was shown in influenza virus infections (Virelizier et al. 1974, Good et al. 1993 `original antigenic sin`).

It was speculated that the two allelic families represent different evolutionary strategies with respect to their repeat regions (Felger et al.1997), which might be supported by the observed difference of the duration of infections.

Hence, further investigations comparing antibody responses of children older than 18 months are needed, since the reported protective effect of infections expressing 3D7 alleles or of concomitant infections are not seen in the younger age-group. Together with the finding of an antibody response with a distracting effect on subsequent infections expressing FC27 alleles it might be allowed to draw a simplified picture of interaction (Figure 2).

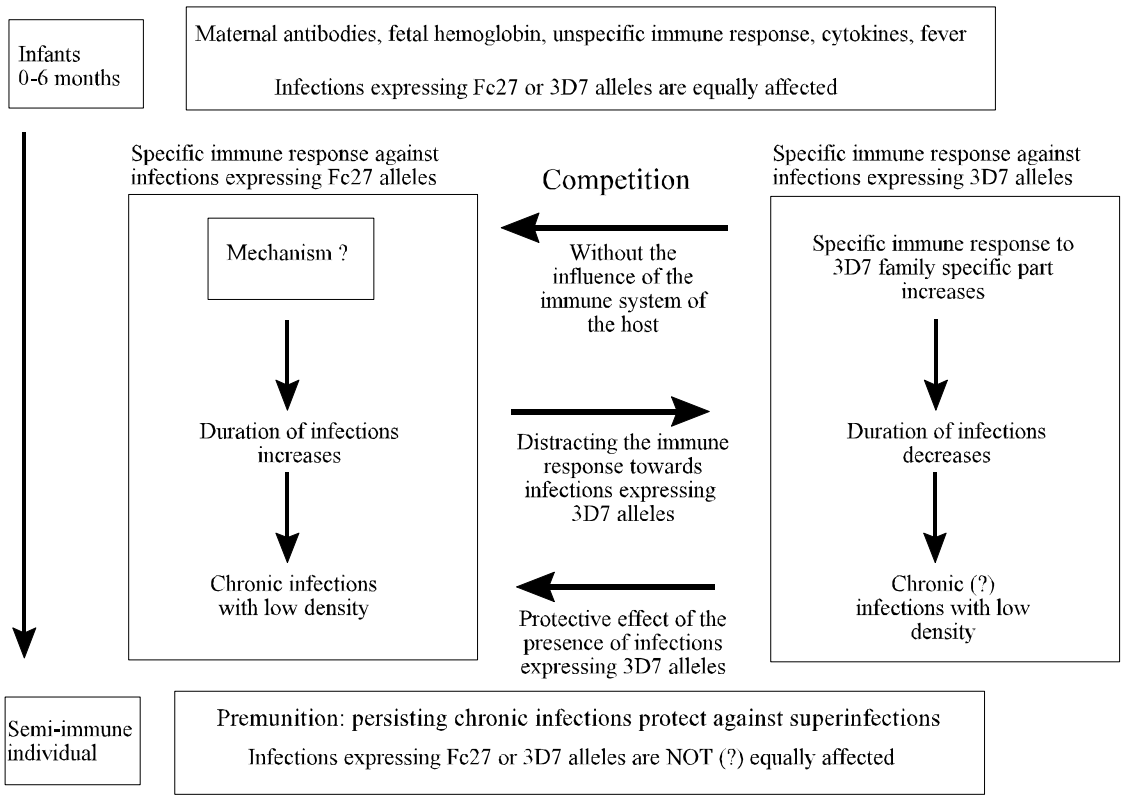

Figure 2: Model of interaction between *Plasmodium falciparum* infections

The competition of different *P. falciparum* infections might be mediated by the immune system of the host. Since the immune response evolves during the life of the host the interaction changes over time and might have different effects on different genotypes of *P. falciparum*.

7.4 The intervention strategies

The question whether ITNs may have a long term benefit to a host is still open. Instead of reducing the toll taken by malaria, their use may merely lead to an increase in mortality and morbidity in the older age groups (Snow et al. 1994; Snow and Marsh 1995; Trape and Rogier 1996). The reason for expecting rebound effects is that partial immunity against malaria develops gradually and is dependent on exposure and the presence and frequency of blood-stage infections. Data from PCR indicated that ITNs had only a small effect on prevalence, but mean parasite densities were significantly reduced in ITNs users (Fraser-Hurt et al. 1999). It was assumed that, in children above one year of age, *P. falciparum* infections, once established, may become chronic and last for a long time at low densities (Smith et al. 1999b). If this is the case, limited reduction in exposure will not have much impact on multiplicity and may not hinder the establishment of premunition (Smith et al. 1999c). However, in this study the use of insecticide-treated bed nets showed a significant decrease in antibody levels to several parts of the MSP2 molecule, which might result in a long-term disadvantage (paper II). In contrast, the antibody response to antigens 5'3'construct, FC27 family specific part, and 6x12 aa repeats are not affected significantly. But as yet we are not able to identify which parts of the molecule are important as protective immune targets (if any). Clearly that proves that it is not possible to conclude from a study with one or two antigens tested that the transmission-reducing interventions may have little effect on antibody levels in such children (Kitua et al 1999), since the nature of the immune responses varies between different antigens even between different parts within one antigen. The findings have to be examined by long-term monitoring of immunological, parasitological and clinical profiles of subjects in areas with different malaria endemicity.

An intervention strategy that prevents either the establishment of *P. falciparum* infection or the onset of malaria morbidity/mortality is needed. The ideal tool is a vaccine that mimics natural immunity without adverse effects of infection. But there is a need to understand the three-way connection between infection, immunity and morbidity. The most successful vaccines of the past were directed at organisms with little antigenic diversity, whereas the antigenic diversity of parasites like *Plasmodium falciparum* raises a number of new questions, that have to be addressed. How will populations of antigenically diverse parasites evolve in response to mass vaccination, which may only target a subset of the antigenic variants, and how will this evolution affect the benefit of the vaccine in reducing total disease? With molecular methods we can

monitor in much more detail and many informations will be added to find answers. Most importantly, these vaccines elicit sterile immunity, which does not represent the nature of anti-malaria immunity in man, where chronicity of *P. falciparum* infections is an important factor of protection.

The reported significant differences in immune responses and dynamics of infections between age groups have to be considered, since, the practice of taking vaccines from phase-IIa trials in non-immune adults to phase-IIb trials in chronically exposed children may impede vaccine development. A vaccine that establishes protection in non-immune adults may not do so in chronically exposed children and *vice versa*. It is an even more difficult step to take a vaccine from older children to very young children or infants. No new vaccine can yet be tested in phase IIb trials in very young children first.

Overall, this thesis gives valuable new insights into antibody responses and infection dynamics which now have to be examined in other age groups in relation to morbidity data. With the increase of the knowledge, the understanding of immune mechanisms of the host and the mechanisms providing evasion of the parasite will improve and a vaccination against malaria becomes viable. But despite all the new and surprising findings, we are still far away from understanding malaria and its interaction with the immune system of the host. With pieces of information gleaned from parts of a multidimensional puzzle it is tempting to try to put the pieces of the puzzle together. It is important to identify those factors which manipulate the disease from both sides, host and parasite, but often it is difficult to distinguish the cause from the effect. Finally, molecular epidemiology might provide us with new insights for developing blood-stage vaccines to reduce the burden of this disease.

8. Conclusions and Outlook

1. It was shown that the methodology of the latent class model can be applied to outputs of biomedical assays, like the ELISA method, which are expected to classify samples into two groups with some extent of overlapping between each other. Using real data sets this application was compared to the commonly used cut-off method and gives more reliable results, especially with variable background levels. Estimation of prevalence of positivity for different populations can be useful in, e.g. understanding of immune mechanisms providing immunity and, consequently, in planning intervention strategies.

2. Genotyping *P. falciparum* infections in field isolates is suitable for various applications. Assessing the multiplicity of infections and, hence in longitudinal surveys, the infection dynamics is only one example. The merozoite surface protein 2 (MSP2) is a useful marker gene, since many different alleles are detected so far, that can be easily differentiated by PCR-RFLP. *msp2* offers the genetic diversity necessary to allow single locus genotyping and the polymorphism is so prominent that a superinfection expressing the same *msp2* allele as the previous one is highly unlikely. Epidemiological studies which involve *msp2* genotyping give additional insights about the biological role of MSP2, e.g., morbidity association or protective potential of an allelic family.

3. Immunisation of mice shows that all recombinant proteins representing various parts of the MSP2 sequence are immunogenic. Elicited antibodies recognize parasite derived MSP2 and IFA positive polyclonal antisera showed a typical MSP2 staining pattern. Antisera and monoclonal antibodies to the 5'3'construct show a recognition of all parasite stages indicating the recognition of a shared epitope. Further investigations with monoclonal antibodies from mice immunised with 5'3'construct will elucidate the nature of this shared epitope(s). Whether these antibodies show an inhibitory effect on *P. falciparum* culture, has to be examined.

4. The present study demonstrates that children aged 5-15 months produce antibodies to different parts of the MSP2 molecule. The frequency of responders is high, although the magnitude of the responses is generally low, but increases with age.

A. High antibody levels to the recombinant construct representing the conserved parts of MSP2 might be due to cross-reactive antibodies elicited due to a ubiquitous antigen. Cross-reactivity might be part of an explanation for the age dependence of semi-immunity to malaria.

B. The antibody response to the recombinant antigen representing 6x12 aa repeats shows no age dependence and might reflect infection history of the child.

C. The antibody response to different repeat structures shows distinct patterns, although their immunogenicity in mice resembles each other. The differences in the MSP2 sequence responsible for the different antibody response in natural settings have to be investigated to assess their relevance for the protective immune response.

D. The antibody responses to both family specific parts differ in background level, probability of positivity, and ability to be induced by *P. falciparum* infections. These differences might reflect those observed in the morbidity association of the different genotypes.

5. *Plasmodium falciparum* infections expressing *msp2* of the FC27 allelic family distract the antibody response to the MSP2 molecule towards antibodies against parts of the 3D7 allelic family. The part of the MSP2 molecule responsible for the induction of 3D7 specific antibodies might be the 32 aa repeat, where some similarities to the 3D7 repeat sequence are observed.

The loss of *P. falciparum* infections expressing 3D7 alleles simplifies the establishment of a FC27 expressing infection, which might be seen indirectly by the boosting of the antibody response to the 5'3'construct.

6. Dynamics of *P. falciparum* infections expressing different *msp2* allelic families are different in an age-dependent way. The duration of infections expressing different allelic families reverses when the children grow older. This difference in parasitological profiles might be the effect of qualitative differences in the grade of immunity of individuals. And this again might be influenced by the genotype of the infecting parasite. Hence, further investigations comparing antibody responses of children older than 18 months are needed, since the protective effect of infections expressing 3D7 alleles or of concomitant infections are not seen in the younger age-group.

7. Since morbidity data from the tested children in our study are missing an association of a specific antibody response and protection cannot be drawn. Therefore, a longitudinal prospective study with children aged 0.5-5 years is needed to assess the impact of the humoral immune response on protection against malaria disease.

8. The use of insecticide-treated bed nets shows a decrease in antibody levels to several parts of the MSP2 molecule. Whether this influence results in a long-term disadvantage, has to be examined by long-term monitoring of immunological, parasitological and clinical profiles of subjects in areas with different malaria endemicity.

A control strategy that prevents either the establishment of *P. falciparum* infection or the onset of malaria morbidity/mortality is needed. The ideal tool is a vaccine that mimics natural immunity without adverse effects of infection. But there is a need to understand the three-way connection between infection, immunity and morbidity. Overall, this thesis gives valuable new insights into antibody responses and infection dynamics which now have to be examined in other age groups in relation to morbidity data. With the increase of the knowledge, the understanding of immune mechanisms of the host and the mechanisms providing evasion of the parasite will improve and a vaccination against malaria becomes viable.

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Appendices

Appendix I: Recombinant *msp2* clones and protein amounts

a. Clones with parts of the sequence deleted:

Clone	*msp2* region	protein amount	ELISA conc.
D40/6	5'3'conserved regions with signal peptide and GPI anchor signal	0.2 mg/litre	
D43/2	16xNAP repeats	5 mg/litre	
D43/5	16xNAP repeats	expression not done	
D44/11	23xNAP repeats	10 mg/litre	10 :g/ml
D46/1	3D7 family specific part	10 mg/litre	0.1 :g/ml
D47/2	4x32 aa repeats	5 mg/litre	1.6 :g/ml
D70/2	4x32 aa repeats	expression not done	
D56/2	6x12 aa repeats	0.1 mg/litre	15 :g/ml
D57/1	5x12 aa repeats	0.1 mg/litre	
D61/9	FC27 family specific part	10 mg/litre	10 :g/ml
D86/2	5'3'conserved regions DHFR fusion protein	0.4 mg/litre	10 :g/ml
D90/2	5'3'conserved regions, 23aa are missing at the 3'end	expression not done	
D99/1	3D7 repeats 25aa	expression not done	
D100/1	3D7 repeats 49aa	10 mg/litre	
D101/1	3D7 repeats 48aa	10 mg/litre	2 :g/ml
D102/4	3D7 repeats 42aa	expression not done	
D109/12	3'conserved region	10 mg/litre	
D110/2	5' conserved region	0.1 mg/litre	
D111/1	5'3'conserved regions without signal peptide	3 mg/litre	
D112/2	5'3'conserved regions without signal peptide DHFR fusion protein	expression not done	
D113/3	5'conserved region without signal peptide	1 mg/litre	
D114/1	K1 full sequence without signal peptide	expression not done	

b. Clones with nested *msp2* sequences:

Clone	*msp2* nested	repeat organisation	expression
D1/9	wos3	1xNAP, 1x32, 3x12	x
D2/1	ifa46	1xNAP, 1x32, 1x12	x
D5/14	K1	1xNAP, 1x32, 5x12	x
D12/6	wos6	1xNAP, 4x32, 0x12	x
D22/15	wos12	1xNAP, 1x32, 4x12	x
D41/10	ifa41	16xNAP, 1x32, 2x12	(x)
D62/10,12 R73 culture	FC27 allele full	seq nd	?
D63/4,5 IFA18 culture	?	seq nd	?
D64/2 MAD20 culture	FC27 allele full	1xNAP, 2x32, at least 1x12	(x)
D65/4,5,6 NF54 culture	3D7 full	seq nd	?
D66/1,2,3 Rfmef culture	3D7 allele	seq nd	?
D67/1,2 7G8 culture	K1 allele	seq nd	?
D68/2,7,8,9,12 W2mef culture	FC27 allele full	seq nd	?
D72/6 NF54 culture	3D7 allele	6xGGSA, 14x Thr	x
D77/7	ifa13	6xNAP, 1x32, 1x12	nd
D78/4	part of the 3D7 family specific region		nd
D79/6,7,8	ifa31	7xNAP, 1x32, 2x12	nd
D80/3,7,8	ifa41	16xNAP, 1x32, 2x12	nd
D81/4	ifa42	recombination	nd
D82/2	ifa45	1xNAP, 4x32, 1x12	nd
D83/1	ifa14	recombination	?
D84/1	3D7 allele	1xGASGSG 1xGASGSA 5x Thr	?
D84/2	3D7 allele	1xGASGSG 2xGASGSA 5x Thr	nd
D84/3	3D7 allele	1xGASGSA 5x Thr	nd
D84/5	3D7 allele	1xGASGSG 2xGASGSA 5x Thr	nd
D85/1 AW755 very short	3D7 allele	1xGASGSA 5x Thr	x

AW755 short	3D7 allele	2xGASGSA 5x Thr	nd
AW755 long	3D7 allele	6xGASGSG 2xGASGSA 5xThr	nd
D93/3	ifa56	2xNAP, 1x32, 1x12	x
D94/3	ifa27	recombination 3x32, 0x12	x
D98/17	K1 w/o GPI anchor signal		x
D104/1	3D7 allele	1xGASGSA 5x Thr	nd
D104/3	3D7 allele	at least 4xGASGSG 2xGASGSA 5x Thr	nd
D104/8	3D7 allele	2xGASGSG 2xGASGSA 5x Thr	nd
mari	ifa17/21	2xNAP, 3x32, 0x12	x
mari	ifa52	15xNAP, 1x32, 1x12	x
D105/1	3D7 allele	8xAGAGGSGS	nd
D105/11	3D7 allele	at least 12xAGAGGTGT	nd
D105/12	3D7 allele	7xAGAGGSGS 8x Thr	nd
D106/3	3D7 allele	2xAGAGGSGS 8x Thr	nd
D106/4	3D7 allele	6xAGAGGSGS	nd
D106/10	3D7 allele	6xAGAGGSGS	nd

nd = expression not done
? = the amount of expressed protein was very small and sometimes doubtful
x = the amount of expressed protein was between 1-10 mg/litre
(x) = the amount of expressed protein was between 0.1-0.5 mg/litre
seq nd = sequencing not done

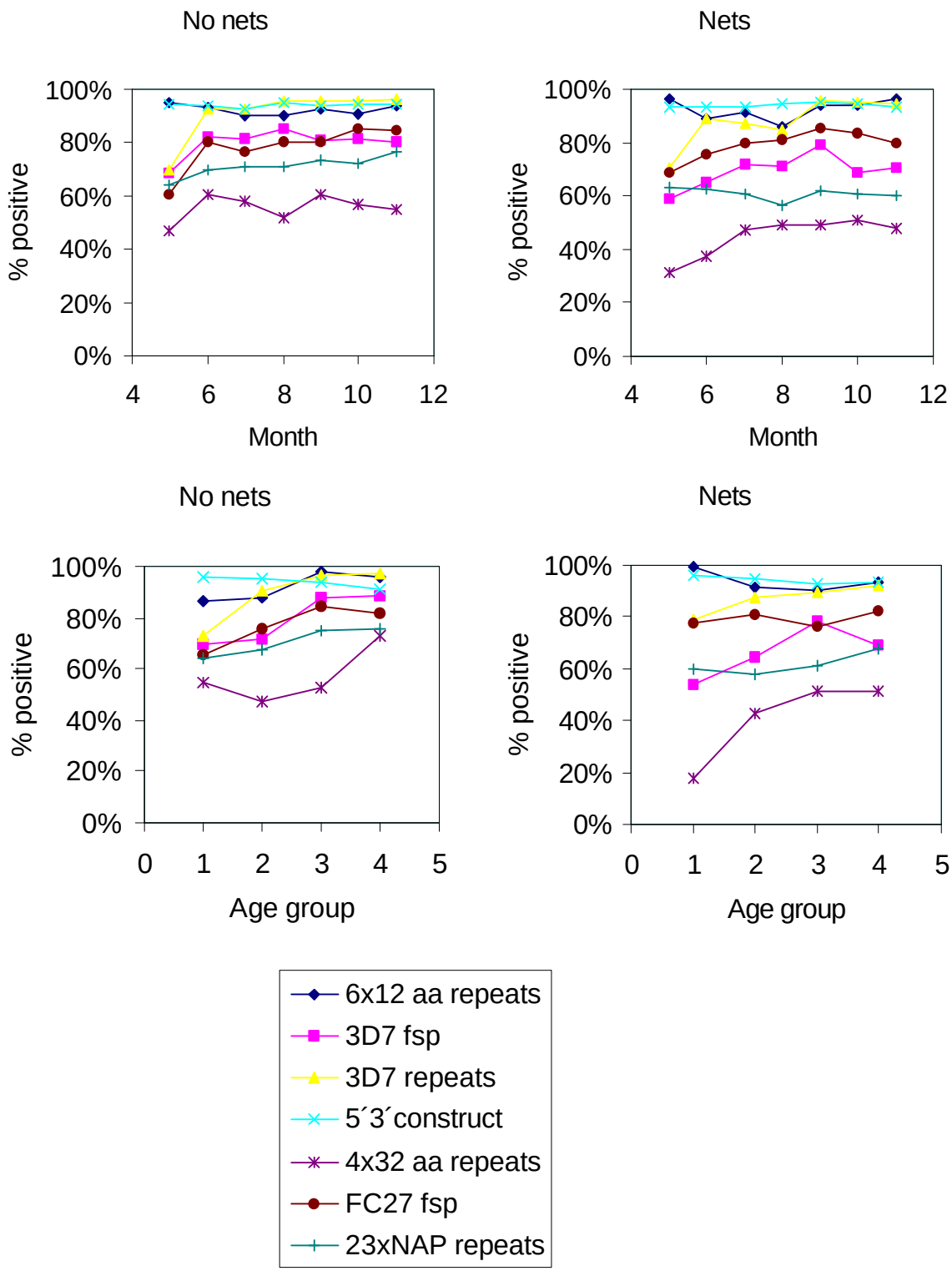
Appendix II: Recovery rate

msp2 genotype of the *P. falciparum* infection		Sensitivity of detection S	recovery probability M	recovery rate :	duration [days]
all	all	0.61	0.25	0.0109	92
3D7	all	0.54	0.26	0.0113	88.5
FC27	all	0.68	0.23	0.01	100
all	no net	0.57	0.23	0.01	100
all	net	0.66	0.28	0.0122	82
all	<18 months	0.6	0.26	0.0113	88.5
all	>18 months	0.62	0.25	0.0109	92
3D7	<18 months	0.49	0.23	0.01	100
3D7	>18 months	0.6	0.29	0.0126	79.4
FC27	<18 months	0.73	0.27	0.0117	85.5
FC27	>18 months	0.64	0.19	0.0083	120.5
3D7	no net	0.53	0.24	0.0104	96
3D7	net	0.55	0.28	0.0122	82
FC27	no net	0.6	0.2	0.0087	115
FC27	net	0.78	0.25	0.0109	92

Appendix III: Positivity and net use

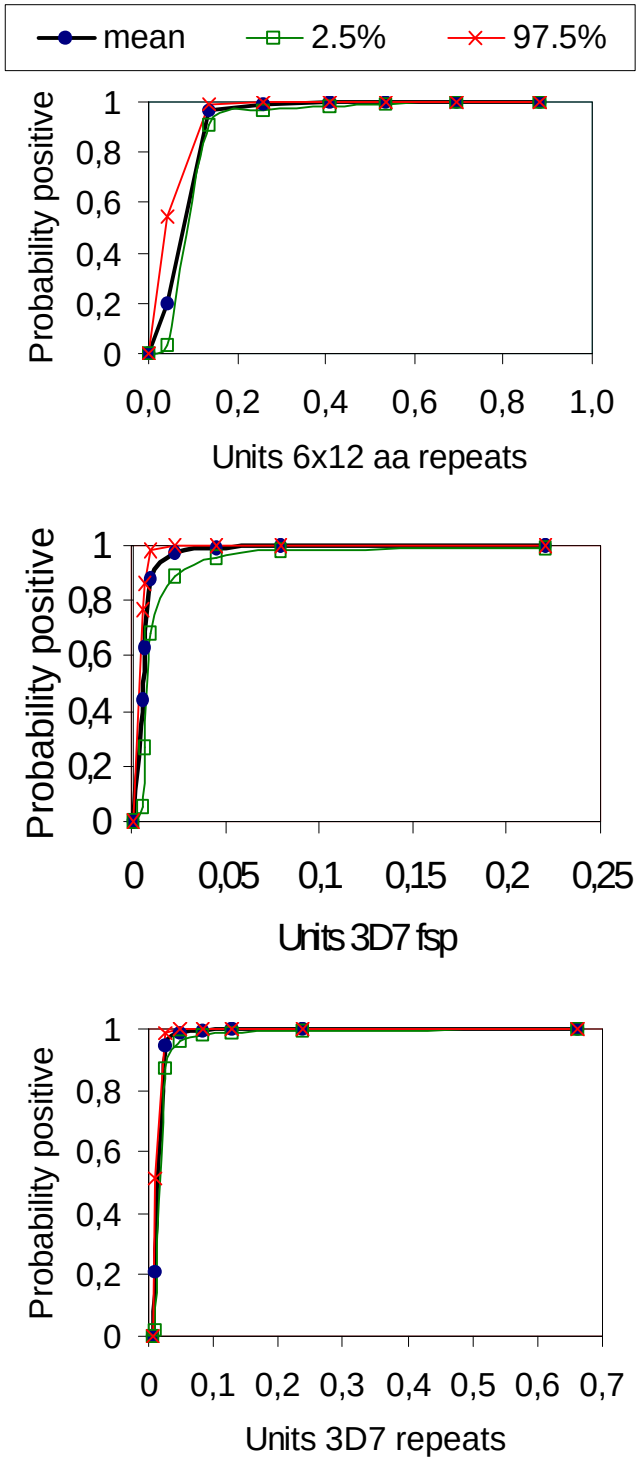
Presented for the investigating period of 7 months and for children divided in four age groups

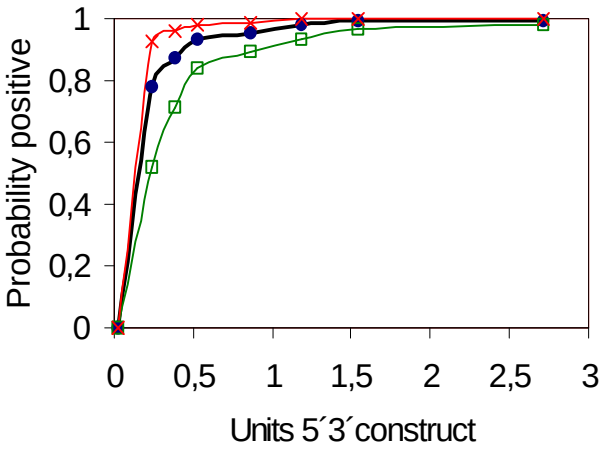
Legend:

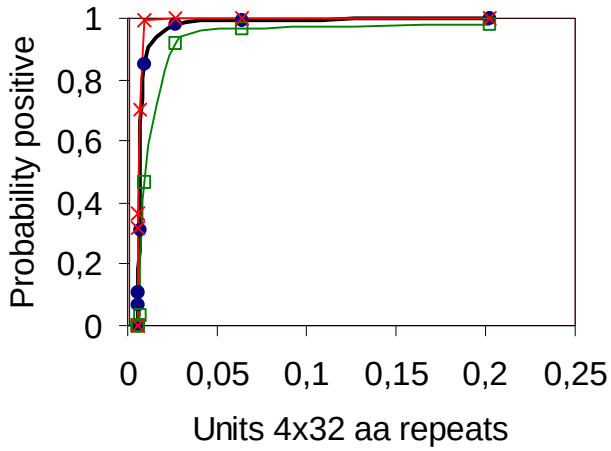


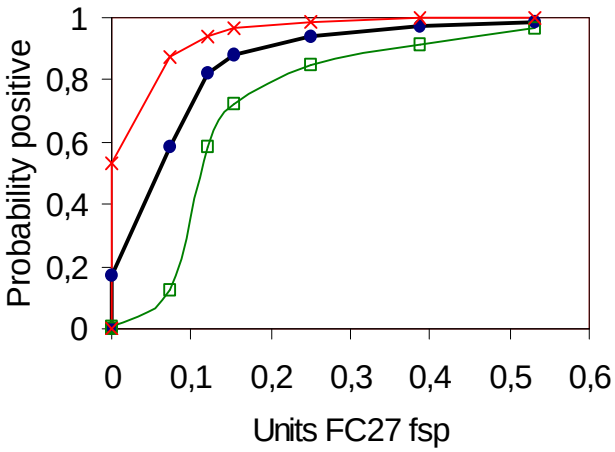
Appendix IV:
Probability of a

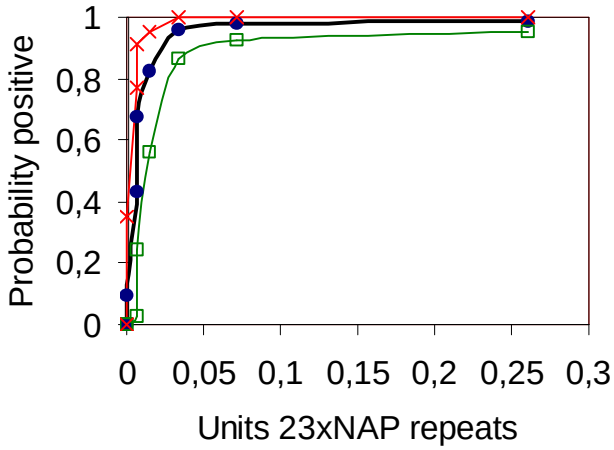
sample being positive, estimated by the latent class model



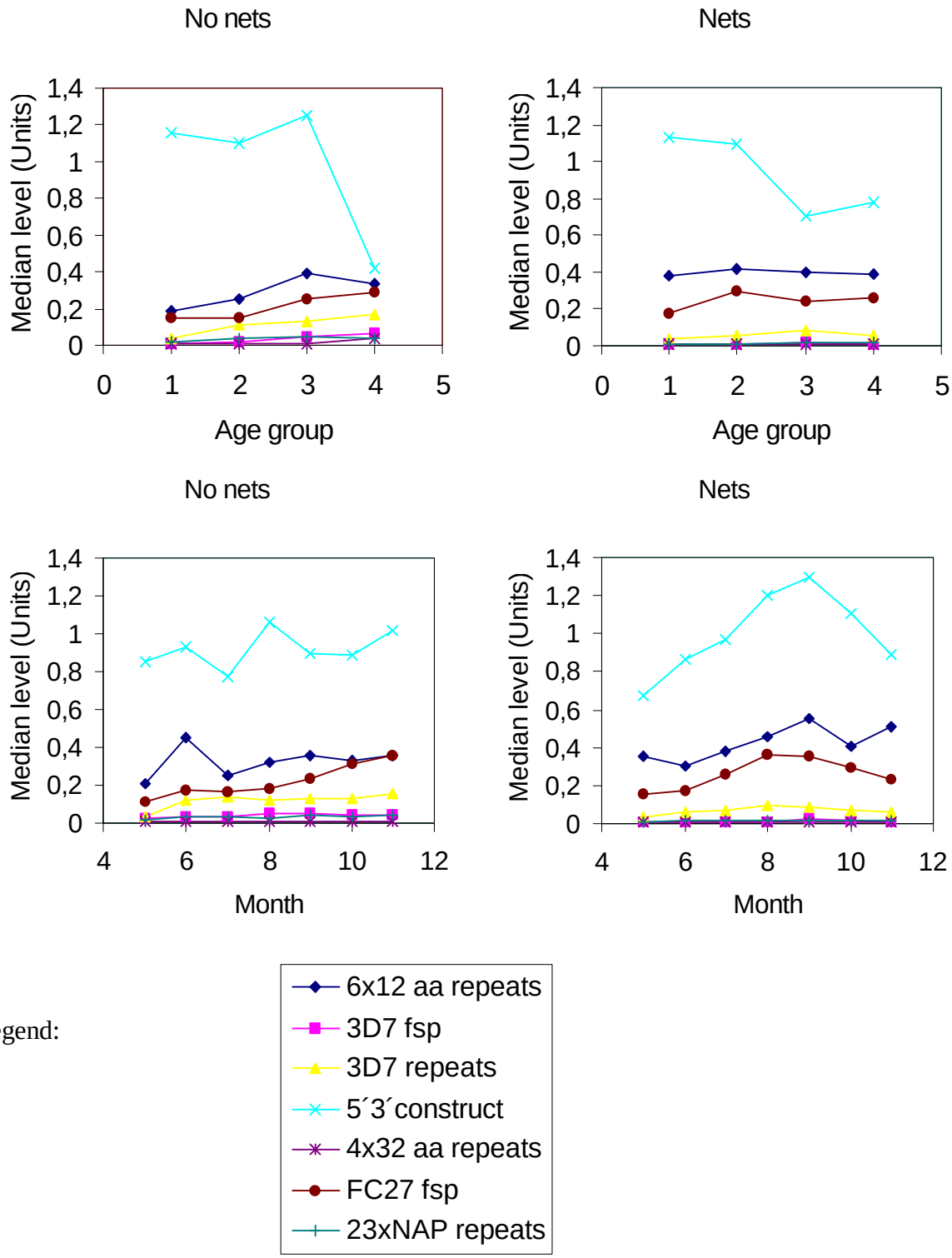

Probability positive
1
0,8
0,6
0,4
0,2
0
0
0,5
1
1,5
2
2,5
3
Units 5´3´construct


Probability positive
1
0,8
0,6
0,4
0,2
0
0
0,05
0,1
0,15
0,2
0,25
Units 4x32 aa repeats

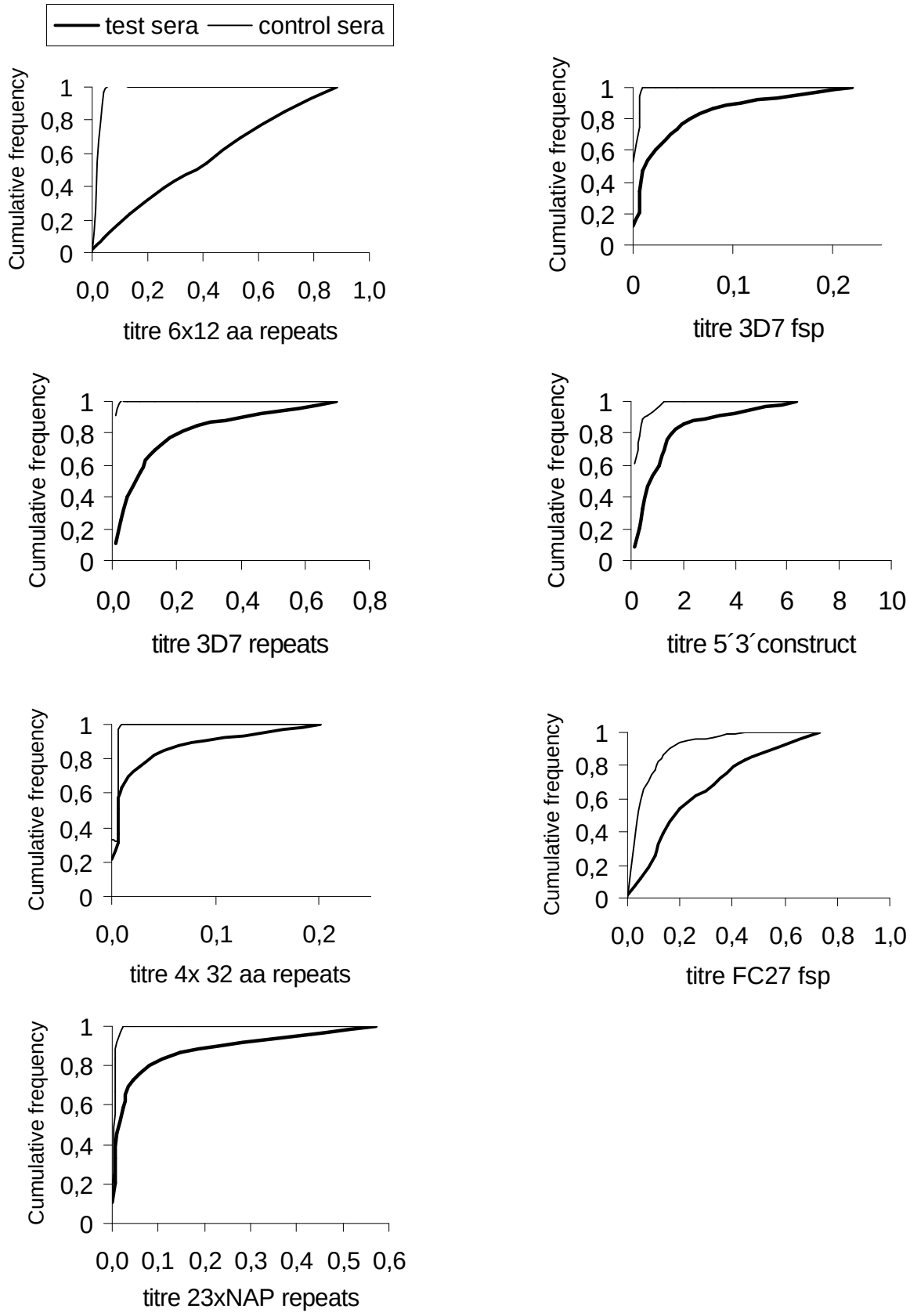

Probability positive
1
0,8
0,6
0,4
0,2
0
0
0,1
0,2
0,3
0,4
0,5
0,6
Units FC27 fsp


Probability positive
1
0,8
0,6
0,4
0,2
0
0
0,05
0,1
0,15
0,2
0,25
0,3
Units 23xNAP repeats

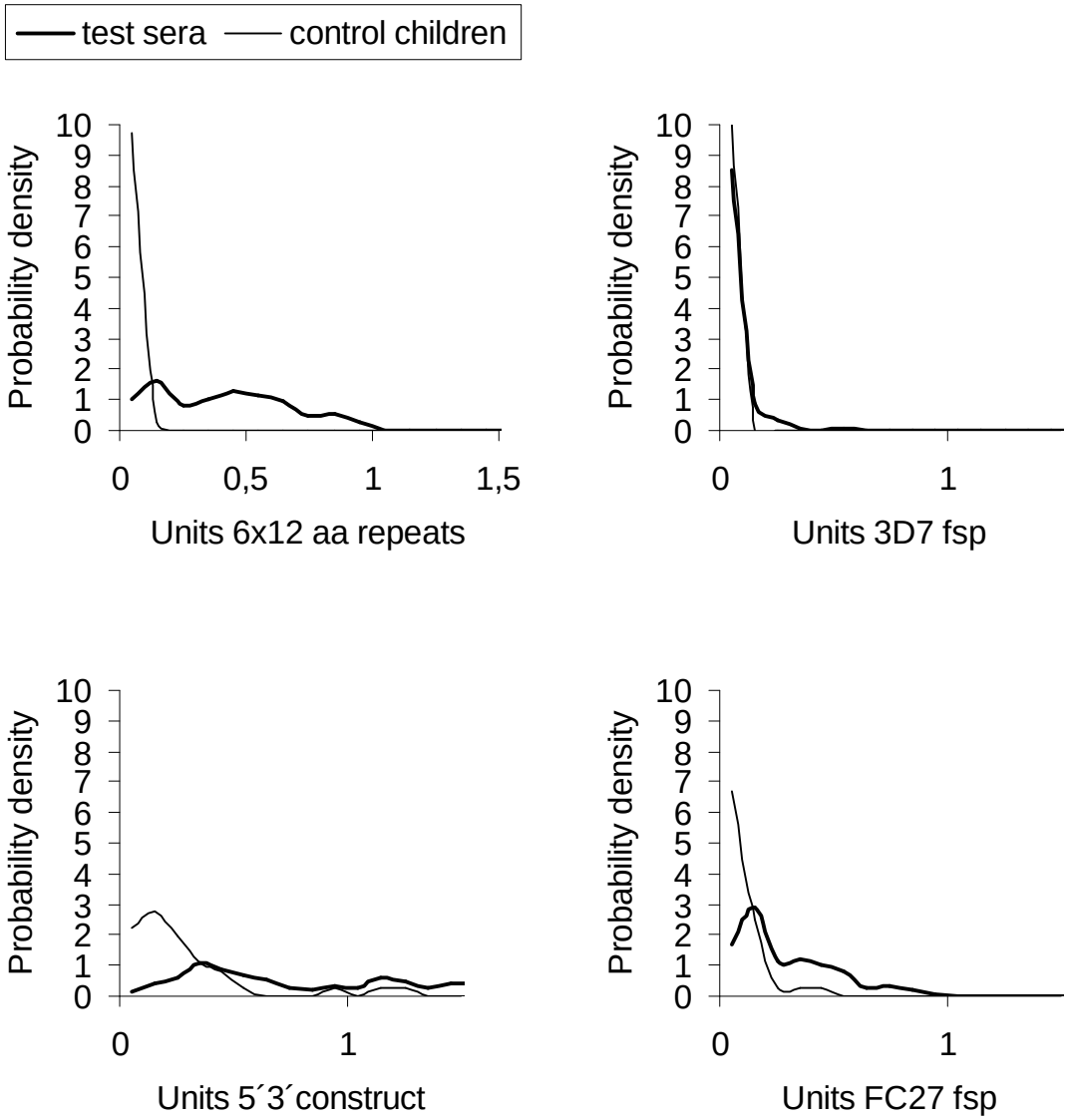
Appendix V: Median antibody levels and net use
Presented for the investigating period of 7 months and for children divided in four age groups

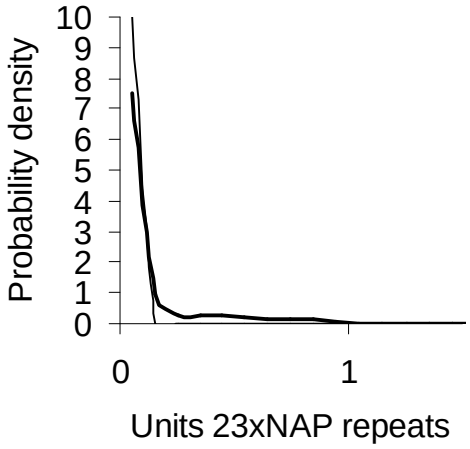


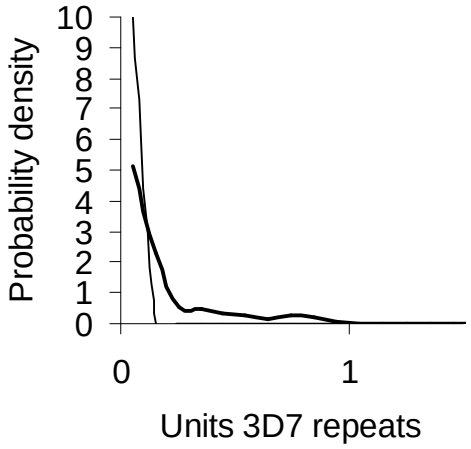
Appendix VI: Cumulative frequency of antibody titres

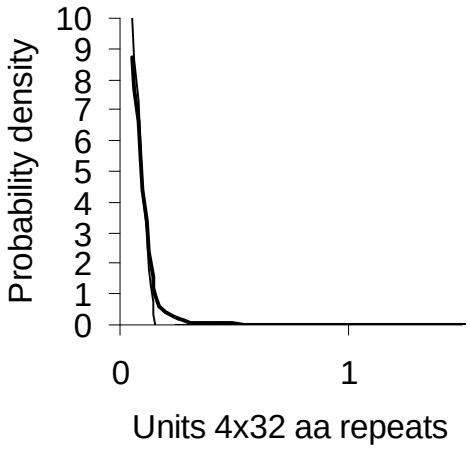


Appendix VII: Distribution of antibody units in negative control sera and test sera
Positive control serum pool is defined as one antibody unit.




Probability density
10
9
8
7
6
5
4
3
2
1
0
0
1
Units 23xNAP repeats


Probability density
10
9
8
7
6
5
4
3
2
1
0
0
1
Units 3D7 repeats


Probability density
10
9
8
7
6
5
4
3
2
1
0
0
1
Units 4x32 aa repeats

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Oct.1991-May1997	Studies in Biology at Albert-Ludwigs-Universität, Freiburg, Federal Republic of Germany
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Publications:

Irion, A., Beck, H.-P., Felger, I. (1997). New repeat unit and hot spot of recombination in FC27-type alleles of the *Plasmodium falciparum* surface antigen MSP2. *Molecular and Biochemical Parasitology* 90:367-370.

Irion, A., Felger, I., Smith, T., Mull, R., Tanner, M., Hatz, C., Beck, H.-P. (1998). Distinction of recrudescences from new infections by PCR-RFLP analysis in a comparative trial of CGP 56697 and chloroquine in tanzanian children. *Tropical Medicine and International Health* 3:490-497.

Irion, A., Felger, I., Smith, T., Beck, H.-P. (Submitted for publication). Longitudinal study of antibody responses to distinct regions of the polymorphic merozoite surface protein 2 of *Plasmodium falciparum* in infants from malaria-endemic area. *Infection and Immunity*.

Irion, A., Beck, H.-P., Smith, T.(Submitted for publication). Assessment of positivity in immuno assays with high levels of background: a new approach applied to antibody response to Plasmodium falciparum MSP2. *Journal of Immunological Methods*.

Irion, A., Felger, I., Smith, T., Beck, H.-P. (In preparation). Dynamics of *Plasmodium falciparum* infections and anti-MSP2 immune response in young children.

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Smith, T. Beck, H.-P., Kitua, A., Mwankusye, S., Felger, I., Fraser-Hurt, N., Irion, A., Alonso, P., Teuscher, T., Tanner, M . (1999). The epidemiology of multiple *Plasmodium falciparum* infections. 4. Age dependence of multiplicity of infection with *Plasmodium falciparum* and other malarilogical indices in an area of high endemicity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93:S1/15-S1/20.

Schwemmle, M., Kaspers, B., Irion, A., Staeheli, P., Schultz, U. (1996). Chicken Guanylate-binding Protein. Conservation of GTPase activity and induction by cytokines. *The Journal of Biological Chemistry* 17:10304-10308.

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A comparative trial of GCP 56697 and chloroquine in Tanzanian children with subsequent PCR analysis of reoccurring parasites. Irion, A., Abdullah, S., Felger, I., Mull, R., Hatz, C., Beck, H.-P. 9th Malaria Meeting of the British Society for Parasitology 1997 (oral).

PCR-RFLP genotyping of *Plasmodium falciparum* and its use in longitudinal studies with malaria patients. Felger, I., Irion, A., Smith, T., Beck, H.-P. 10th Malaria Meeting of the British Society for Parasitology 1998 (poster).

Molecular diversity and immunological properties of the merozoite surface protein 2 of *Plasmodium falciparum*. Irion, A., Felger, I., Beck, H.-P. 10th Malaria Meeting of the British Society for Parasitology 1998 (poster).

Molecular diversity and immunological properties of the merozoite surface protein 2 of *Plasmodium falciparum*. Irion, A., Beck, H.-P. 11th Malaria Meeting of the British Society for Parasitology 1999 (oral).