STRAIN-SPECIFIC IMMUNE RESPONSES TO
PLASMODIUM FALCIPARUM MERozoITE
SURFACE ANTIGENS

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I declare that the research presented in this thesis is my own work, and that the thesis is my own composition.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of figures</td>
<td>v</td>
</tr>
<tr>
<td>List of tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xiii</td>
</tr>
<tr>
<td>Abstract</td>
<td>xiv</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1- Malaria infection</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1- Life cycle of <em>Plasmodium</em></td>
<td>3</td>
</tr>
<tr>
<td>1.2- Genetic and antigenic polymorphism of <em>Plasmodium</em>, and malaria vaccines</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1- <em>Plasmodium</em> genetics</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2- Stage-specific antigens and malaria vaccines</td>
<td>6</td>
</tr>
<tr>
<td>1.2.2.1- Protein antigens of exoerythrocytic stages</td>
<td>7</td>
</tr>
<tr>
<td>1.2.2.2- Protein antigens of asexual blood stages</td>
<td>9</td>
</tr>
<tr>
<td>A) Merozoite surface protein (MSP)-1</td>
<td>10</td>
</tr>
<tr>
<td>B) Merozoite surface protein (MSP)-2</td>
<td>11</td>
</tr>
<tr>
<td>C) Apical membrane antigen (AMA)-1</td>
<td>13</td>
</tr>
<tr>
<td>D) Rhopty proteins: RAP-1 and RAP-2</td>
<td>14</td>
</tr>
<tr>
<td>1.2.1- Phenotypic antigenic variation in <em>Plasmodium</em></td>
<td>15</td>
</tr>
<tr>
<td>1.2.2- Obstacles to vaccine development</td>
<td>17</td>
</tr>
<tr>
<td>1.2.2.3- Antigenic polymorphism and phenotypic antigenic variation in <em>Plasmodium</em></td>
<td>17</td>
</tr>
<tr>
<td>1.2.2.4- <em>In vitro</em> correlates of protection</td>
<td>19</td>
</tr>
<tr>
<td>1.3- Clinical disease and pathogenesis of malaria</td>
<td>20</td>
</tr>
<tr>
<td>1.3.1- Pathogenesis of cerebral malaria</td>
<td>21</td>
</tr>
<tr>
<td>1.3.1.1- Sequestration of infected erythrocytes in brain tissues</td>
<td>21</td>
</tr>
<tr>
<td>1.3.1.2- Cytokine activation</td>
<td>23</td>
</tr>
<tr>
<td>1.3.3- Severe malarial anaemia</td>
<td>24</td>
</tr>
<tr>
<td>1.3.4- Epidemiology of severe malaria</td>
<td>26</td>
</tr>
<tr>
<td>1.4- Genetic host resistance to parasites and immune responses to <em>Plasmodium</em> infections</td>
<td>27</td>
</tr>
<tr>
<td>1.4.1- Innate natural resistance</td>
<td>27</td>
</tr>
<tr>
<td>1.4.1.1- Genetic resistance to parasites</td>
<td>27</td>
</tr>
<tr>
<td>1.4.1.2- Innate immunity</td>
<td>28</td>
</tr>
<tr>
<td>1.4.2- Naturally acquired immunity</td>
<td>30</td>
</tr>
<tr>
<td>1.4.2.1- Evidence of antibody-mediated immunity to blood stages of human malaria parasites</td>
<td>31</td>
</tr>
<tr>
<td>1.4.2.2- Cell-mediated immunity</td>
<td>32</td>
</tr>
<tr>
<td>1.7- Aims</td>
<td>34</td>
</tr>
<tr>
<td>Chapter 2: Genetic and antigenic diversity of polymorphic <em>Plasmodium falciparum</em> merozoite surface proteins in blood-circulating parasites from Malawian children with malaria</td>
<td>35</td>
</tr>
</tbody>
</table>
2.1- Introduction
   2.1.1- Polymorphism of MSP-1
   2.1.2- Polymorphism of MSP-2
   2.1.3- Methods to study polymorphism
   2.1.4- Aims

2.2- Materials and methods
   2.2.1- Parasite samples
   2.2.2- In vitro culture of *Plasmodium falciparum* isolates
   2.2.3- Indirect immunofluorescent (IFA) serotyping
   2.2.4- DNA extraction
   2.2.5- Polymerase chain reaction (PCR) genotyping
   2.2.6- DNA sequencing
   2.2.7- Statistical analysis
      2.2.7.1- IFA serotyping
      2.2.7.2- PCR genotyping
      2.2.7.3- Association of MSP-1 or MSP-2 alleles with disease severity

2.3- Results
   2.3.1- Mixed *P. falciparum* infections and their relationship with malaria disease severity
      2.3.1.1- Mixed infections detected by IFA serotyping
      2.3.1.2- Mixed infections detected by PCR genotyping
   2.3.2- Antigenic diversity of merozoite surface proteins detected by IFA serotyping
      2.3.2.1- Merozoite surface protein 1
      2.3.2.2- Merozoite surface protein 2 and exporte protein 1
   2.3.3- Genetic polymorphism of merozoite surface protein genes detected by PCR genotyping and sequencing
      2.3.4- Relationship between genetic and antigenic polymorphism
      2.3.5- Relationship between parasite diversity and malaria disease severity
      2.3.5.1- Distribution of MSP-1 genotypes and serotypes
      2.3.5.2- Distribution of MSP-2 and EXP-1 genotypes and/or serotypes

2.4- Discussion
   2.4.1- Relationship between genetic and antigenic diversity of MSP-1 and MSP-2, and comparison between IFA and PCR typing
   2.4.2- Frequencies of MSP-1 and MSP-2 allelic types in different countries
   2.4.3- Mixed infections in relation to transmission and disease severity
   2.4.4- Relationship between MSP-1 and MSP-2 polymorphism and disease severity

Chapter 3: Antigenic diversity of polymorphic surface proteins of *Plasmodium falciparum* in organ-sequestered parasites from Malawian children dying with malaria

3.1- Introduction
   3.1.1- Aims
3.2- Materials and methods
  3.2.1- Collection of tissue samples 92
  3.2.2- Immunofluorescence (IFA) serotyping in tissue specimens 93

3.3- Results
  3.3.1- Preparation of specimens for IFA 96
    3.3.1.1- Tissue homogenates 98
    3.3.1.2- Tissue smears and touches 98
    3.3.1.3- Liquid N$_2$ cryopreserved tissue sections 98
  3.3.2- In situ IFA serotyping of organ-sequestered parasites 104
    3.3.2.1- Complexity of infections in tissues 104
    3.3.2.2- MSP-1, MSP-2 and EXP-1 serotypes in tissues 107
  3.3.3- Comparison between peripheral blood and organ-sequestered parasites 108

3.4- Discussion 111

Chapter 4: Immune responses to *Plasmodium falciparum* merozoite proteins in Malawian children with malaria 117
4.1- Introduction 117
  4.1.1- Human immune responses to *P. falciparum* merozoite proteins 118
    4.1.1.1- Immunity to MSP-1 118
    4.1.1.2- Immunity to MSP-2 121
    4.1.1.3- Immunity to AMA-1 and RAP-1 122
  4.1.2- Immune responses and malarial disease severity 122
  4.1.3- Aims 123
4.2- Materials and methods 124
  4.2.1- Collection of plasma samples 124
  4.2.2- Recombinant proteins 125
    4.2.2.1- MSP-1 constructs 125
    4.2.2.2- MSP-2 constructs 125
    4.2.2.3- AMA-1 and RAP-1 constructs 126
  4.2.3- Enzyme-linked immunoabsorbent assay (ELISA) 126
  4.2.4- Statistical analysis 130
4.3- Results 131
  4.3.1- Antibody responses to MSP-1 in Malawian children 131
  4.3.2- Antibody responses to MSP-2 in Malawian children 133
  4.3.3- Antibody responses to AMA-1 and RAP-1 in Malawian children 134
  4.3.4- Relationship between antibody responses to merozoite surface proteins and malaria disease 135
    4.3.4.1- Antibody responses to MSP-1 137
    4.3.4.2- Antibody responses to MSP-2 141
    4.3.4.3- Antibody responses to AMA-1 and RAP-1 152
4.4- Discussion 152
  4.4.1- Antibody recognition of merozoite proteins in Malawian children 155
  4.4.2- Association between antibody responses and malaria disease 158
Chapter 5: Strain-specific antibody responses to polymorphic merozoite surface proteins of *Plasmodium falciparum* from Malawian children with malaria

5.1- Introduction

5.1.1- Strain-specific antibody recognition of malarial proteins in animal models

5.1.2- Specificity of human immune responses to *P. falciparum* polymorphic proteins

5.1.2.1- Strain-specific responses to MSP-1

5.1.2.2- Strain-specific responses to MSP-2 and other surface proteins

5.1.2.3- Strain-specific responses and malaria disease severity

5.1.3- Aims

5.2- Materials and methods

5.2.1- Statistical analysis

5.3- Results

5.3.1- Specificity of antibody responses to polymorphic block 2 of MSP-1

5.3.2- Specificity of antibody responses to MSP-2

5.3.3- Relationship between strain-specific antibody responses and malaria disease

5.3.3.1- Antibody responses to MSP-1

5.3.3.2- Antibody responses to MSP-2

5.4- Discussion

5.4.1- MSP-1 and MSP-2 are targets of strain-specific immune responses

5.4.2- Strain-specific antibody responses and malaria disease severity

Chapter 6: General discussion and future directions

6.1- Gene polymorphisms, antigenic diversity and strain-specific immune responses

6.1.1- Genetic polymorphism and antigenic diversity

6.1.2- Strain-specific immune responses

6.2- Parasite diversity, acquired immunity and disease severity

6.2.1- Parasite diversity and disease severity

6.2.2- Multiplicity of infections and disease severity

6.2.3- Sequestration and disease severity

6.2.4- Antibody responses and disease severity

Bibliography

Appendix: Publication

Identical alleles of *Plasmodium falciparum* merozoite surface protein 2 found in distant geographic areas and times.
LIST OF FIGURES

Figure 2.1. Schematic representation of the structure of MSP-1 of *P. falciparum* 37
Figure 2.2. Schematic representation of the structure of MSP-2 of *P. falciparum* 38
Figure 2.3. Detection of mixed infections in peripheral blood by double-labelled IFA 52
Figure 2.4. PCR genotyping of MSP-1 and MSP-2 genes 55
   A) DIFS method for detection of MSP-1 dimorphic types 55
   B) Nested PCR for detection of MSP-1 block 2 types 55
   C) DIFS method for detection of MSP-2 dimorphic types 55
Figure 2.5. Associations between epitopes in blocks 2, 3 and 4 of MSP-1 59
Figure 2.6. Alignment of predicted amino acid sequences of MSP-1 block 2 in clinical isolates of *P. falciparum* 61
Figure 2.7. Alignment of predicted amino acid sequences of MSP-2 in clinical isolates of *P. falciparum* 62
   A) MSP-2 group A 62
   B) MSP-2 group B and recombinant 63
Figure 2.8. Distribution of MSP-1 dimorphic (blocks 16) genotypes 74
Figure 2.9. Distribution of MSP-1 polymorphic block 2 genotypes 74
Figure 2.10. Distribution of MSP-2 dimorphic genotypes 78
Figure 2.11. Prevalence of EXP-1 dimorphic serotype 5.1 by IFA 78
Figure 3.1. Detection of parasites by Giemsa staining in brain capillaries from children dying from cerebral malaria 97
Figure 3.2. Detection of parasite antigens in brain capillaries by IFA 99
Figure 3.3. Detection of schizonts in lung by IFA 100
Figure 3.4. Detection of schizonts in spleen by IFA 101
Figure 3.5. Detection of schizonts in liver by IFA 102
Figure 3.6. Detection of parasite antigens in brain cryosections by IFA 103
Figure 3.7. Detection by double-labelled IFA of mixed-clone infection in peripheral blood vs. brain tissue

Figure 4.1. Schematic representation of recombinant proteins of MSP-2
   A) Serogroup A
   B) Serogroup B

Figure 4.2. Age distribution of antibody responses to AMA-1

Figure 4.3. Antibody responses to MSP-1 42kDa

Figure 4.4. Prevalence of antibodies to MSP-1 42kDa

Figure 4.5. Antibody responses to MSP-1 block 2 RO33

Figure 4.6. Duration of antibody responses to MSP-1 42kDa

Figure 4.7. Antibody responses to MSP-2 K1 17/14 (C-terminus)

Figure 4.8. Prevalence of antibodies to MSP-2 C-terminus

Figure 4.9. Antibody responses to MSP-2 C-terminal region

Figure 4.10. Antibody responses to MSP-2 dimorphic regions
   A) MSP-2A
   B) MSP-2B

Figure 4.11. Antibody responses to MSP-2 polymorphic regions
   A) MSP-2A
   B) MSP-2B

Figure 4.12. Antibody responses to MSP-2A full length protein

Figure 4.13. Antibody responses to AMA-1

Figure 4.14. Antibody responses to RAP-1 C2

Figure 5.1. Specificity of antibody responses to MSP-1 K1-type

Figure 5.2. Examples of concordant antibody responses to infecting parasites of MSP-1 block 2 K1 type

Figure 5.3. Examples of discordant antibody to MSP-1 block 2

Figure 5.4. Specificity of antibody responses to MSP-1 MAD20-type

Figure 5.5. Examples of concordant antibody responses to infecting parasites
of MSP-1 block 2 MAD20 type

Figure 5.6. Specificity of antibody responses to MSP-1 RO33-type

Figure 5.7. Examples of concordant antibody responses to infecting parasites of MSP-1 block 2 RO33 type

Figure 5.8. Examples of concordant antibody responses to more than one type of MSP-1 block 2

Figure 5.9. Specificity of antibody responses to MSP-2 group A

Figure 5.10. Examples of concordant antibody responses to infecting parasites of MSP-2 type A

Figure 5.11. Examples of discordant antibody to MSP-2

Figure 5.12. Specificity of antibody responses to MSP-2 group B

Figure 5.13. Examples of concordant antibody responses to infecting parasites of MSP-2 type B

Figure 5.14. Antibody responses to mixed MSP-2 type infections

Figure 5.15. Cross-reactivity between the two MSP-2 types by recognition of conserved determinants

Figure 5.16. Examples of antibody responses to both MSP-2 types
LIST OF TABLES

Table 2.1. Clinical and parasitological data of the patients studied 42
Table 2.2. Antibody reagents for IFA typing 44
   A) MSP-1 44
   B) MSP-2 45
   C) Other antigens 45
Table 2.3. Oligonucleotide primers used for parasite genotyping by PCR amplification and sequencing of MSP-1 and MSP-2 alleles from clinical isolates of P. falciparum 47
Table 2.4. Analysis of mixed infections detected by MSP-1 and MSP-2 IFA serotyping 53
   A) Mean number of clones per isolate 53
   B) Percentages of single- and multiple-clone infections 53
Table 2.5. Analysis of mixed infections detected by MSP-1 and MSP-2 PCR genotyping 57
Table 2.6. Prevalences of MSP-1 marker epitopes detected by IFA 67
Table 2.7. Frequencies of MSP-1 marker epitopes detected by IFA 68
Table 2.8. Prevalences of MSP-2 marker epitopes detected by IFA 70
Table 2.9. Frequencies of MSP-2 marker epitopes detected by IFA 71
Table 2.10. Prevalences of MSP-1 and MSP-2 genotypes detected by PCR 72
Table 2.11. Frequencies of MSP-1 and MSP-2 genotypes detected by PCR 72
Table 2.12. Summary of field studies on genetic and antigenic diversity and multiplicity of P. falciparum infections which used MSP-1 and MSP-2 as markers 81
Table 3.1. Summary of data of fatal cases from whom post-mortem specimens were examined 94
Table 3.2. Organs containing sequestered schizonts typed by IFA 96
Table 3.3. Typing of MSP-1, MSP-2 & EXP-1 in sequestered and circulating parasites from ten fatal CM cases 105
Table 3.4. Typing by PCR of blood-circulating parasites from non-CM fatal controls

Table 4.1. Oligonucleotide primers used in PCR amplification for expression of MSP-2 fusion proteins

Table 4.2. Prevalence of antibodies to distinct regions of MSP-1

Table 4.3. Prevalence of antibodies to block 2 of MSP-1

Table 4.4. Prevalence of antibodies to distinct regions of MSP-2

Table 4.5. Prevalence of antibodies to polymorphic MSP-2 R1 regions

Table 5.1. Concordance between MSP-1 block 2 type and Ab responses

Table 5.2. Concordance between MSP-2 types and Ab responses

Table 5.3. Relationship between concordance of Ab specificity to MSP-2 and disease outcome in all patients
LIST OF ABBREVIATIONS

A: Adenine
aa: amino acid
Ab: Antibody
ADCC: antibody-dependent cell-mediated cytotoxicity
ADCI: antibody-dependent cellular inhibition
AMA: Apical membrane antigen
APCs: Antigen presenting cells
BCS: Blantyre Coma Score
bp: base pair
BSA: Bovine serum albumin
C: Cytosine
°C: degree centigrade
C-: carboxy-
C3: Complement3
CD: Cluster of differentiation
CFA: Freund's complete adjuvant
CK: Cytokine
CM: Cerebral malaria
CRP: C-reactive protein
CSA: Chondroitin sulphate A
CSP: Circumsporozoite protein
CTL: Cytotoxic T lymphocyte
DAPI: 4',6'-diamino-2-phenylindole
DBL: Duffy binding-like
ddH₂O: double distilled water
DIC: Disseminated intravascular coagulation
DIFS: dimorphic-form specific
dl: decilitre
DNA: Deoxyribonucleic acid
dNTP: deoxynucleoside triphosphate
EBA: Erythrocyte binding antigen
EDTA: Ethylenediaminetetraacetic acid
EGF: Epidermal growth factor
EIR: Entomological inoculation rate
ELAM: Endothelial selectin or E-selectin
EPO: Erythropoietin
EXP: Exported protein
FITC: Fluorescein isothiocyanate
g: gram
G: Guanine
GLURP: Glutamic acid-rich protein
GM-CSF: Granulocyte-macrophage colony stimulating factor
GPI: Glycosyl phosphatidyl inositol
GST: Glutathione S-transferase
Hb: Haemoglobin
HLA: Human leukocyte antigen
hsp: heat shock protein
hr: hours
ICAM: Intracellular adhesion molecule
IEM: Immunoelectron microscopy
IFA: Immunofluorescence assay
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
Kb: Kilobases
kDa: kilodalton
LFA: Leukocyte function antigen
LPS: Lipopolysaccharide
mAb: monoclonal antibody
MAP: Multiple-antigen peptide
Mb: Megabases
MHC: Major histocompatibility complex
mM: millimolar
mg: milligram
ml: millilitre
mRNA: messenger ribonucleic acid
MRP: Malaria Research Project and Wellcome Trust Centre
MSP: Merozoite surface protein
min: minutes
µg: microgram
µl: microlitre
N-: amino-
N⁰MMLA: monomethyl-L-arginine
NK: Natural killer
nm: nanometre
NO: Nitric oxide
NOS: Nitric oxide synthase
nt: nucleotides
OD: Optical density
PAGE: Polyacrylamide gel electrophoresis
PBL: Peripheral blood lymphocytes
PBS: Phosphate buffered saline
PCV: Packed red blood cell volume
PCR: Polymerase chain reaction
PECAM: Platelet endothelial cell adhesion molecule
PfEMP: Plasmodium falciparum erythrocyte membrane protein
pmol: picomol
PNG: Papua New Guinea
PRBC: Parasitised red blood cell
PyHEP: Plasmodium yoelii protective hepatocyte erythrocyte protein
PySSP: P. yoelii sporozoite surface protein
RBC: Red blood cell
RAP: Rhoptry-associated protein
RESA: Ring-infected erythrocyte surface antigen
RFLP: Restriction fragment length polymorphisms
RITC: Rhodamine isothiocyanate
RNA: Ribonucleic acid
RNIs: Reactive nitrogen intermediates
ROIs: Reactive oxygen intermediates
RT: Reverse transcriptase PCR
SDS: Sodium dodecyl sulphate
sec: seconds
SM: Severe malaria
SMA: Severe malarial anaemia
SNM: Severe non-malarial disease
STARP: Sporozoite threonine and asparagine rich protein
T: Thymine
Taq: Thermus aquaticus
TBE: Tris borate EDTA
Th: Thymocyte "helper"
TM: Thrombomodulin
TNF: Tumour necrosis factor
TRAP: Thrombospondin-related anonymous protein
TSP: Thrombospondin
U: Units
UM: Uncomplicated malaria
UV: Ultraviolet
V: Volts
VATs: Variant antigenic types
VCAM: Vascular cell adhesion
VSG: Variant surface glycoprotein
WBC: White blood cell
Well: Wellcome Plasmodium falciparum isolate
WHO: World Health Organisation
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ABSTRACT

People living in malaria endemic areas who are exposed to repeated infections with *Plasmodium falciparum* parasites from infancy develop immunity to the disease later in life. The reasons why this is the case are not understood. Natural populations of the parasite are genetically diverse, consisting of genotypes or "strains". These strains differ in their composition of a number of polymorphic molecules, including the merozoite surface antigens MSP-1 and MSP-2. It was suggested that immunity may be essentially strain-specific, and thus a long period could be required to be exposed to a large repertoire of diverse strains. The aim of this thesis is to investigate a hypothesis that these antigens are targets of a strain-specific component of human immunity to malaria.

Dimorphic and polymorphic regions of *MSP-1* and *MSP-2* were genotyped by polymerase chain reaction (PCR) in 379 *P. falciparum* clinical isolates from Malawi. Polymorphisms in the genes were reflected in antigenic diversity of the translated proteins detected by indirect immunofluorescence (IFA) typing. Most *MSP-1* alleles were MAD20 dimorphic and K1 block 2 types, whereas *MSP-2* type A alleles predominated.

The effect of *P. falciparum* genetic polymorphisms on the specificity of immune responses was investigated in children immunised by natural infections typed for *MSP-1* and *MSP-2*. Specific IgG antibodies detected by ELISA were mainly directed to *MSP-1* conserved C-terminus fragments (19kDa and 42kDa), whereas antibodies induced by *MSP-2* predominantly recognised group-specific regions. Overall, naturally induced human antibody responses to *MSP-1* and *MSP-2* were short-lived, type-specific, and correlated with PCR typing of the infecting parasites present.

The wide spectrum of disease manifestations observed in *P. falciparum* infections probably reflects a combination of various host and parasite factors. Children with severe malarial anaemia (SMA, n=50) were distinguished by a higher multiplicity of infections than children with uncomplicated malaria (UM, n=92) or cerebral malaria (CM, n=93). SMA patients had higher prevalence of *MSP-1* Ki/Well and *MSP-2* B dimorphic types, whereas block 2 MAD20-type of *MSP-1* was more common in UM cases. Serotypes of parasites sequestered in tissues detected *in situ* by IFA in autopsy specimens from 10 malaria fatalities did not differ from those found in circulation in the same patients.

Different levels of acquired immunity to *MSP-1*, *MSP-2*, AMA-1 or RAP-1 could not explain the disease severity. However, a differential pattern of antibody responses to defined regions of the proteins was found. Children who developed SMA contained very low levels of antibodies to conserved regions, whereas children with CM had significantly higher levels of antibodies to the conserved regions than children with UM or SMA. In addition, antibody responses to the dimorphic types of *MSP-2* correlated more frequently with the type of the infecting parasite in UM than CM or SMA patients. Thus, CM and SMA differed substantially for all parameters assessed, indicating that they should not be regarded as one severe disease group but as two very distinct syndromes.
Chapter 1: INTRODUCTION

1.1- Malaria infection

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium*. There are four species of plasmodia that infect humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The parasites are transmitted by the female of various species of Anopheline mosquitoes. Malaria occurs throughout the tropical world, where some relevant vectors are: *Anopheles gambiae*, (Africa), *A. farauti* (Papua New Guinea), *A. minimus* (Thailand) and *A. darlingi* (South America). WHO estimates that 4 billion people in approximately 90 countries are at risk of becoming infected and that up to 500 million cases of malaria occur each year (WHO Report, 1996). Malaria results in the deaths of 1-2 million people, mainly children under 5 years of age, but also a significant number of adults including pregnant women. Most of the disease and deaths can be attributed to a single species, *P. falciparum*. *P. vivax* is important in Asia and Latin America.

In the earlier years of this century, some countries in temperate climates also experienced a significant number of cases, though most such countries have now eradicated malaria. Many factors were responsible, including the use of mosquito insecticides such as DDT, changes in the environment to prevent breeding of the mosquitoes, improvements in the standard of living, and anti-malaria drugs, in particular chloroquine, which substituted the traditional quinine treatment. However, as drug and insecticide resistances started to develop during the sixties, attempts to eradicate malaria in the tropics have failed. Furthermore, recent climate and environmental changes are likely to increase malaria incidence, and cases may emerge in places where it had been eradicated. Currently, conditions in most countries where malaria is endemic have made control of the disease increasingly difficult, urging that new strategies are developed.

Prominent achievements in malaria control in recent years include the demonstration of the efficacy of impregnated mosquito bednets (Alonso et al., 1993) and the use of a new family of antimalarials derived from the traditional Chinese medicine
qinghaosu (e.g. artemisinin). However, even new drugs are not always completely effective and there is a danger that new forms of resistance may arise. Available antimalarials used in chloroquine-resistant areas of Africa include mefloquine (as prophylactic), Fansidar® (sulphadoxine/pyrimethamine) and quinine, for the treatment of mild and severe malaria, respectively. Although there is understanding of the molecular mechanisms of resistance to some of these drugs, e.g. antifolates, there is a lack of a commitment of pharmaceutical industry for new drug development, regarded as a very slow, expensive but non-profitable process. At present, vector control tools such as improved mosquito insecticides, repellents and bednets, are still considered the best control measures in areas where drug resistance of the parasites is established.

In this context, a vaccine against malaria is desirable, and much of current malaria research is driven by this need. Advances in techniques, noticeably the optimisation of *P. falciparum* *in vitro* culture (Trager & Jensen, 1976), and the cloning of many parasite antigen genes raised hopes that a malaria vaccine may be obtained. A general approach in malaria vaccine development has been (i) to clone and characterise malaria antigens' genes and their protein products (ii) to test selected antigens in vaccine trials in animal models, where immune responses can be manipulated, and (iii) by field epidemiological studies to investigate human immune responses to these antigens in people naturally infected with the parasite. Our limited understanding of how human immune responses to malaria are regulated and which immune responses lead to resolution of natural infections in man explains why the development of a malaria vaccine is taking so long.

In this introduction, I review areas of malaria research as relevant to this project. The first issue examined is the complexity of the parasite's life cycle, followed by a review on some parasite antigens considered for vaccine development. Second, I focus on the pathological consequences of malaria infection and on the different manifestations of the disease. Third, immune mechanisms of defence are reviewed, with particular emphasis on human immune responses. Finally, questions approached by my project are outlined.
1.1.1- Life cycle of *Plasmodium*

*Plasmodium* is an obligatory parasitic protozoan that requires a vertebrate and an invertebrate host to complete its life-cycle. A blood-feeding mosquito takes up male and female gametocytes of the parasite, contained within red blood cells (RBCs) of an infected vertebrate host. In the mosquito's gut, gametocytes develop to haploid gametes, and fertilisation of the gametes gives rise to diploid zygotes. Meiosis then occurs within a few hours. The zygote develops into a motile ookinete that burrows into the gut's wall to form an oocyst. Each oocyst contains the meiotic products of a single zygote. Immature sporozoites form within the oocyst by mitotic divisions, and then migrate to the salivary glands where they mature, ready to be inoculated into the next vertebrate host on which the infected mosquito feeds.

After inoculation in a skin capillary of a vertebrate host, sporozoites spend less than 30 min in blood circulation before invading hepatocytes or being cleared by other tissues of the body. The phase of the life cycle in hepatocytes is called the exoerythrocytic or pre-erythrocytic stage. In a hepatocyte, over 5-8 days or longer, depending on the species of Plasmodia, a single sporozoite develops into 30,000-40,000 merozoites, each of which, when released, can invade a RBC and there continue the life cycle.

During the next phase, the asexual blood cycle, the parasite develops inside the RBC over a period of 2 days (*P. falciparum*, causing malignant tertian fever; *P. vivax* and *P. ovale*, called tertian fevers) or 3 days (*P. malariae*, quartan fever). The parasite changes its morphology, from so-called ring form to trophozoite and finally schizont or meront. Six to 32 merozoites develop inside each schizont and, following its rupture, each merozoite can continue the blood cycle by invading a fresh RBC. Erythrocyte invasion is not fully understood but it is thought to be a multi-step process in which a number of different parasite ligands are involved. Some of the parasite ligands are believed to be proteins contained in the apical organelles (rhoptries and micronemes) and merozoite surface proteins. As the parasite grows it creates a network of structures within the cell and also alters the surface of the infected RBC. The blood phase includes a period of exponential growth in the numbers of the parasites within the host. However,
given that in a vertebrate the infection starts with only few parasites (typically, <100 sporozoites will be injected, and it is said that most will not enter liver cells), and that the number of RBCs is immense, it takes several cycles of erythrocytic multiplication before parasite numbers increase to levels detectable on a microscopic slide. The asexual blood phase is responsible for all clinical symptoms of malaria, as reviewed in section 1.3.

Finally, some blood parasites undergo sexual differentiation into male and female gametocytes (sexual stage) ready to make the transition from a vertebrate host to the next blood feeding mosquito.

1.2- Genetic and antigenic polymorphism of *Plasmodium*, and malaria vaccines

Within a single species of *Plasmodium*, allelic polymorphisms originate the coexistence of different genotypes, clones or so-called "strains". Allelic polymorphisms give rise to structurally distinct forms of particular proteins in different parasite clones. In particular, it is considered that genetic polymorphisms in certain protein loci lead to the expression of antigenically distinct forms of the proteins.

A different concept is phenotypic antigenic variation. Broadly, the term refers to changes in antigenic phenotype by regulated expression of different genes of a clonal population of parasites over the natural course of an infection. Life-cycle related antigenic changes are one example of this sort of biochemical and antigenic phenotypic variation. However, in parasitology the term is more commonly used to describe a very specific phenomenon reviewed in detail in section 1.2.1.

In the following sections, I first summarise the genetics of *Plasmodium*, including the genetics and polymorphism of certain proteins believed to be important in immunity to malaria. Secondly, relevant stage-specific antigens implicated in vaccine research are reviewed. Thirdly, I describe the phenomenon of phenotypic antigenic variation, and I finish with an outline of some of the obstacles to vaccine development.
Plasmodia are haploid for most of their life cycle. The only diploid stage, the zygote, results from fertilisation of gametes in the mosquito gut where it also undergoes meiosis. The haploid genome of all species of *Plasmodium* so far examined consists of 14 chromosomes that range in size between 0.65-3.4 Mb (Triglia *et al.*, 1992). Two extrachromosomal DNA components, a linear 6 kb element containing certain mitochondrial genes, and a 35 kb circle with chloroplast-like genes (Feagin, 1994), are inherited uniparenterally through the female gamete (Creasey *et al.*, 1993).

Each species of Plasmodia has a number of different polymorphic genes that, in a heterozygote (i.e. zygotes derived from a mating of unlike genotypes), can undergo recombination or reassortment during meiosis. Thus, there is a potential for a very large number of different genotypes to be generated. By crossing experiments in rodent Plasmodia and also in *P. falciparum*, it was demonstrated that mixtures of genetically distinct clones ingested by mosquitoes undergo crossmating (Walliker, 1991). Meiosis in heterozygotes leads to recombination events among parental genes, with the consequent production of novel genotypes in the progeny. Little is known about the precise mechanisms of the recombinations, but they appear to include typical eukaryotic chromosomal reassortments and crossing-over events between linked genes as well as within certain genes.

Phenotypic polymorphisms in *P. falciparum* have been demonstrated using biochemical markers such as isoenzymes (Carter & McGregor, 1973), proteins distinguished by mobility in two-dimensional polyacrylamide gel electrophoresis (PAGE) (Fenton *et al.*, 1985) and drug sensitivity tests (Creasey *et al.*, 1990). More recently, the genetic diversity in natural parasite populations has been investigated by the polymerase chain reaction (PCR), a technique which can be used to analyse genetically parasites from small samples of blood with higher sensitivity than conventional biochemical methods, and makes it feasible to carry out extensive surveys. Further applications of nucleic acid techniques for characterisation of parasites' genes include DNA hybridisation, restriction fragment length polymorphisms (RFLP), and DNA sequencing.
Studies on genetic polymorphisms in *P. falciparum* have expanded our understanding of the structure of natural parasite populations. Based on mating patterns, two main population genetics hypotheses have been proposed and debated. First, a clonality hypothesis proposed that most zygotes result from fusion of genetically identical gametes, i.e. from self-fertilisation (Tibayrenc *et al.*, 1990). This hypothesis is controversial because it was based on a very limited number of isolates, and it contradicts the evidence from most field studies (see chapter 2). The second hypothesis, well supported by direct analysis of oocysts (Ranford-Cartwright *et al.*, 1993; Babiker *et al.*, 1994) and also by observations that infections in single hosts often consist of mixtures of genetically distinct clones, with ample opportunity for genetic exchange in the mosquito, is more consistent with the picture of panmictic or randomly interbreeding populations, whereby many zygotes are formed from gametes drawn at random from those in a population (Walliker, 1991). Indeed, studies attempting to test for linkage equilibrium (i.e. degree of association between unlinked genes) found that the frequencies of multilocus genotypes in natural populations did not differ from those expected under the assumption of random mating (Conway & McBride, 1991).

Although many studies used polymorphic protein genes as markers to examine the genetic diversity of *P. falciparum* in natural parasite populations, very few studies have considered how far polymorphisms in these genes are reflected in antigenic diversity of the parasite.

Understanding of the structure of several malaria antigen genes and some information on their protein products is now available. I review in detail the characteristics of those molecules from the exoerythrocytic and, particularly, asexual blood stages which are relevant for vaccine development.

1.2.2- *Stage-specific antigens and malaria vaccines*

It has been assumed that while the majority of malaria proteins induce immune responses, some of these responses are irrelevant or perhaps even harmful, and that protection would reflect the acquisition of immune responses directed against a small
number of molecules. Research has focused on antigens which have been indicated to be possible targets of protective immunity by different rationales. Firstly, has been reasoned, molecules with critical conserved functions to the parasite should be good targets, since they might be less subject to antigenic diversity, one of the perceived obstacles to vaccine development. Second, focus on surface proteins is based on the rationale that exposed antigens should be vulnerable to immune attack. Third, the identification of antigens and those immune effector mechanisms that contribute to control of natural infections in the natural host.

Likely target molecules have been identified at each phase of malaria parasites life-cycle. The classification of putative malaria vaccines includes vaccines against the exoerythrocytic stages, the asexual blood stages, and the sexual and sporogonic forms in the mosquito (reviewed in Alano, 1991; Kaslow, 1993), with the additional idea of an anti-disease vaccine (see section 1.4.1.2).

1.2.2.1- Protein antigens of exoerythrocytic stages

The initial attentions in malaria vaccine development were directed to the infective sporozoite, in the belief that anti-sporozoite immunity could stop the infection from the beginning, and thus prevent both subsequent clinical disease and transmission. It is possible to induce a complete immunity to sporozoite challenge in rodents, monkeys and human volunteers by vaccination with sporozoites attenuated by X-irradiation. For example, Herrington et al. (1991) confirmed classical experiments conducted in the 1970s (Clyde et al., 1973), showing that protected individuals had high levels of anti-sporozoite Abs and peripheral blood T cells responding to in vitro stimulation with different formulations of a recombinant sporozoite protein. However, technologically it is not feasible to produce any stage of human malaria parasites on a large scale. Safety concerns make it also preferable to identify relevant targets of immunity, and to produce them as recombinant subunit vaccines.

The first generation of subunit vaccines was based on the circumsporozoite protein (CSP). CSP is found on the surface of mature sporozoites and ranges from 40 to
60 kDa in different plasmodial species (reviewed in Nussenzweig & Nussenzweig, 1990). A central region of CSP consists of tandem repeats that are conserved within a species. *P. falciparum* CSP contains a variable number of copies of the amino acid (aa) sequence NANP, and a smaller number of copies of a variant aa repeat, NVDP.

Various functions have been attributed to CSP (reviewed in Nardin & Nussenzweig, 1993). A conserved region in the C-terminus, region II, is thought to act as the ligand in sporozoite-hepatocyte interaction, and sporozoite motility has also been associated with the secretion of CSP. Repeats are targets of neutralising monoclonal antibodies (mAbs), and therefore CSP was considered a target of immunity. Thus, after the identification of the immunodominant B cell epitope (NANP)$_n$, and the demonstration of the inhibitory effect of anti-repeat Abs on sporozoites in animal models, the first vaccine formulations consisted on recombinant or synthetic peptides based on these sequences, coupled to molecules such as tetanus toxoid or R32tet32, with aluminium hydroxide (alum) as adjuvant (Herrington *et al.*, 1987; Ballou *et al.*, 1987). Although the first vaccine trials in human volunteers were only modestly effective, there was enough success to encourage further studies with the goal of inducing high levels of anti-repeat Abs of the right specificity. A new construct of the *P. falciparum* CSP repeat inserted into the hepatitis B surface antigen (HBsAg) with novel adjuvants is currently undergoing clinical trials (Stoute *et al.*, 1997).

Exported protein 1 (EXP-1, also called p23, CRA QF116 or 5.1), is exported into the parasitophorous vacuole membrane and the cytoplasm of PRBCs (Simmons *et al.*, 1987), and can be detected in liver stages by IFA. Recently, the rodent *P. yoelii* homologue of PfEXP-1 has been described as the hepatocyte erythrocyte protein 17 kDa (PyHEP17) (Doolan *et al.*, 1996). PyHEP17 is expressed in infected hepatocytes and RBCs, and it is a target of protective CD8+ T cells (Doolan & Hoffman, 1997). EXP-1 may be immunologically important since it cross-reacts with the (NANP)$_n$ repeat epitope of CSP.

Other vaccine candidates include the liver stage antigens LSA-1 and LSA-2 (Guerin-Marchand *et al.*, 1987). Evidence from epidemiological studies in The Gambia
suggested a link between a human MHC class I molecule (HLA-Bw53) and protection from severe malaria (Hill et al., 1991), and it was speculated that HLA-Bw53 might be involved in the presentation of an LSA-1 cytotoxic T lymphocyte (CTL) epitope.

### 1.2.2.2- Protein antigens of asexual blood stages

There has been a great effort directed towards developing vaccines against asexual blood stages, since the blood infection is wholly responsible for all malaria symptoms, morbidity and mortality (reviewed in section 1.3). Theoretically, each cycle of the asexual parasite replication could boost the immune response to the parasite. This boosting would be advantageous, since the immunity might be continuously enhanced by infection and, as a result, limit the proliferation of the parasite.

Many merozoite antigens have been identified by mAbs that block invasion, or by protection studies in animal models immunised with affinity-purified parasite proteins (reviewed in Anders & Saul, 1994). Most such antigens are expressed on the surface (merozoite surface proteins, apical membrane antigen), or within rhoptry organelles of merozoites involved in RBC invasion. As far as we know, these merozoite proteins are always expressed in parasites from both field isolates and in vitro cultures.

Before the era of cloning malaria antigen genes, it was demonstrated that monkeys could be protected against simian or falciparum malaria by vaccinating the animals with whole parasites emulsified in complete Freund's adjuvant (CFA) (Mitchell et al., 1975; Siddiqui et al., 1977). Immunisation with *P. knowlesi* resulted in robust protection against homologous and heterologous strains. Once malaria antigens were cloned, subunit vaccine trials started in mice and monkeys. A selection of vaccine studies based on four merozoite surface proteins is presented in sections A), B), C) and D).

This thesis will focus on the merozoite surface proteins MSP-1 and MSP-2 (reviewed below). Chapters 2 and 3 concentrate on the genetic and antigenic polymorphism of these proteins, while chapters 4 and 5 investigate human immune responses to them. Recently, genes of other merozoite surface proteins, MSP-3 and MSP-4, have been identified. MSP-3 is a target of cytophilic Abs that promote *P.*
*falciparum* killing by co-operation with blood monocytes in antibody-dependent cellular inhibition assay (ADCI) (Oeufray *et al.*, 1994). MSP-4 is a 40 kDa protein containing a single epidermal growth factor (EGF)-like domain at the C-terminus, and anchored to the merozoite membrane by a glycosyl phosphatidyl inositol (GPI) moiety (Marshall *et al.*, 1997).

**A) Merozoite surface protein 1**

The merozoite surface protein 1 (MSP-1, also known as MSA-1, PMMSA, PSA, p190 or gp195), is one of the best characterised malaria proteins. It was initially discovered in *P. yoelii* with a mAb against Py230 (Freeman *et al.*, 1980), the homologue of *P. falciparum* MSP-1 Although this mAb did not provide protection upon passive transfer to mice, active immunisation with the antigen isolated from PRBCs using the mAb did protect against normally lethal challenge of the virulent YM strain of *P. yoelii* (Holder & Freeman, 1981). Subsequently, mAbs to MSP-1 of *P. yoelii* (Majarian *et al.*, 1984), *P. chabaudi* (Boyle *et al.*, 1982), *P. knowlesi* (Epstein *et al.*, 1981) and *P. falciparum* (Pirson & Perkins, 1986) were demonstrated to inhibit growth of the homologous *Plasmodium* species either *in vivo* or *in vitro*.

MSP-1 is the precursor of major antigens found on the surface of the merozoite (Holder & Freeman, 1984; McBride & Heidrich, 1987). It is encoded by a single copy gene and is synthesised as a protein of 185-220 kDa at the schizont stage. In synchronised *in vitro* cultures of *P. falciparum* the protein is first detected by IFA, immunoelectron microscopy (IEM) or biosynthetic-labelling at the onset of schizogony. Posttranslational proteolytic primary processing during late schizogony generates fragments of 83, 42, 38 and 28-30 kDa, which persist as a noncovalently linked complex on the surface of mature extracellular merozoites (reviewed in Holder, 1988). MSP-1 is anchored into the merozoite membrane through the attachment of a GPI moiety to the C-terminus of the polypeptide (Schofield & Hackett, 1993). Most of the protein is shed around the time of RBC invasion and the only fragment that remains associated with the parasite after invasion is a 19 kDa fragment, MSP-1*19*, a result of a secondary processing...
of the 42 kDa C-terminal fragment MSP-142 (Blackman et al., 1991, 1993). MSP-119 consists of two domains, each with six highly conserved cysteine residues that are characteristic of EGF-like motifs, which are targets of inhibitory Abs (Chappel & Holder, 1993). Major epitopes recognised by human Abs depend on a conformation created by disulphide bonds in the EGF-like motifs (Egan et al., 1995).

MSP-1 is one of the promising vaccine candidates. Evidence supporting the importance of MSP-1 includes inhibitory activities of anti-MSP-1 mAbs, and numerous animal vaccine trials using purified parasite MSP-1, synthetic peptides, or recombinant proteins. In P. falciparum, mAbs specific for either conserved epitopes in the C-terminal MSP-119 (Blackman et al., 1990) or polymorphic epitopes in the N-terminal block 2 region (Locher et al., 1996) inhibit merozoite invasion in vitro.

Aotus monkeys immunised with partially purified MSP-1 of the Palo Alto isolate were completely protected from an otherwise lethal challenge with the homologous P. falciparum isolate (Siddiqui et al., 1987). In addition, vaccination with native and/or processed products of MSP-1 protected Saimiri monkeys from lethal challenge with a heterologous parasite isolate (Etlinger et al., 1991). Other monkey vaccinations with MSP-1 protein fragments either failed (Hall et al., 1984; Pye et al., 1991) or conferred various degrees of protection (Herrera et al., 1992). More recently, vaccine trials have been mainly focused on the C-terminus of MSP-1. Mice vaccinated with E. coli-expressed MSP-119 fusion protein with glutathione S-transferase (GST), were protected against lethal challenge with P. yoelii (Daly & Long, 1995; Ling et al., 1994). A baculovirus-expressed 42 kDa fragment also protected monkeys from challenge with P. falciparum (Chang et al., 1996). However, in monkey studies using a GST-fusion protein of MSP-119 or MSP-142 made in bacteria, no protection against P. falciparum challenge was observed (Kumar et al., 1995; Burghaus et al., 1996).

No robust in vitro correlates of protection were established in these animal models. In mice, some though not all studies suggested that Ab was the means of protection following immunisation. In contrast, in monkeys there was no clear association
between Ab titres or between the ability of sera to inhibit merozoite invasion *in vitro* and protection.

SPf66, a synthetic polypeptide antigen constituted the first malaria vaccine to undergo extensive field trials. SPf66 combined three synthetic peptides (35.1, 55.1 and 83.1), together with the peptide (NANP)$_2$ from CSP. Peptide 83.1 was derived from the N-terminus of MSP-1 whereas the other two peptides were derived from poorly characterised proteins. Initial results from vaccine trials in both *Aotus* monkeys and in humans in South America were very encouraging (Moreno & Patarroyo, 1989). SPf66 was safe, immunogenic and protective against *P. falciparum* in semi-immune Colombians (Amador *et al.*, 1993). However, the nature of the protective immune mechanisms remained unclear; no correlation was established between Ab titres and the incidence of malaria. The first randomised double-blind placebo-controlled trial of SPf66 in a highly endemic area of Tanzania reported a reduced risk of clinical malaria among children, with an estimated efficacy of 31%, but confidence limit ranged from 0-52% (Alonso *et al.*, 1994). These results were not reproduced in further phase III trials in The Gambia (D'Alessandro *et al.*, 1995) and Thailand (Nosten *et al.*, 1996).

**B) Merozoite surface protein 2**

The merozoite surface protein 2 (MSP-2) of *P. falciparum*, also known as MSA-2, antigen 531 or GP3, is a plasma membrane protein of 35-56 kDa, encoded by a single copy gene (Smythe *et al.*, 1988; Clark *et al.*, 1989). Unlike MSP-1, there is no evidence that MSP-2 is proteolytically processed or carried into the RBC on merozoite invasion; like MSP-1, it is anchored to the merozoite membrane by a C-terminal GPI moiety.

Indirect evidence of the vaccine potential of MSP-2 comes from the demonstration that polyclonal and monoclonal Abs to MSP-2 inhibit merozoite invasion *in vitro* (Miettinen-Baumann *et al.*, 1988; Epping *et al.*, 1988; Clark *et al.*, 1989; Saul *et al.*, 1989). Furthermore, mice immunised with synthetic peptides derived from the N- and C-terminal conserved regions of *P. falciparum* MSP-2 were partially protected against challenge with the murine parasite *P. chabaudi* (Saul *et al.*, 1992). In the last study, some
cross-species protection was achieved with near full-length recombinant MSP-2, but the strongest protection was achieved using an octapeptide based in sequences from the conserved N-terminus. However, other vaccination studies with MSP-2 recombinant proteins failed to protect monkeys against malaria (Pye et al., 1991). Vaccine constructs including MSP-2 sequences are currently undergoing clinical trials in Papua New Guinea (Engers et al., 1998).

C) Apical membrane antigen 1

The apical membrane antigen 1 (AMA-1) is an integral protein of 83 kDa which is synthesised late in the development of the asexual blood stages with peak synthesis occurring in mature segmenting schizonts (Peterson et al., 1989). The homologue of AMA-1 in *P. knowlesi*, Pk66, was discovered by the use of mAbs that blocked invasion (Deans et al., 1982). The primary translation product is a ~80 kDa doublet which is processed to a 62 kDa doublet. Prior to schizont rupture AMA-1 appears to be localised in the electron-dense neck of the rhoptries, but is then found on the surface of mature merozoites (Anders et al., 1994).

Comparison of *AMA-1* gene sequences from different *P. falciparum* isolates and rodent and simian species revealed no dimorphism, insertions or deletions, which are commonly found in other polymorphic antigens. Instead, nucleotide point mutations are the main source of variability (Thomas et al., 1990). The *P. falciparum* *AMA-1* sequence differs from that of other species in having a 55-aa insert close to the N-terminus of the mature protein. In all species, the deduced polypeptides have an N-terminal signal for secretion and a presumed stop transfer sequence close to the C-terminus giving, in *P. falciparum*, an N-terminal domain in the mature polypeptide of ~66 kDa and a C-terminal domain of ~6 kDa. Within the presumed external N-terminal domain there are 16 cysteines which are conserved. Thus, the conformation of this domain is likely to be stabilised by intramolecular disulphide bonds. The mutated residues in *P. falciparum* *AMA-1* are not randomly distributed, with most substitutions occurring in a stretch of 45 aa between the first two conserved cysteines (the "hypervariable" loop) and fewer
mutations in the 55 residues of the C-terminal domain, which is generally conserved. Evidence of recurrent mutations and, in common with CSP, a high ratio of nonsynonymous to synonymous mutations in the non-repetitive sequences of AMA-1, indicates that the diversity among AMA-1 alleles may reflect selection pressures (McCutchan et al., 1992).

Immunisation with purified native Pk66, the homologue of AMA-1 in P. knowlesi, protected Rhesus monkeys against homologous challenge (Deans et al., 1988), and the protection appeared to correlate with Ab production. Partial protection was obtained against P. fragile in Saimiri monkeys following immunisation with recombinant P. fragile AMA-1 (Collins et al., 1994). Subsequent challenge with P. falciparum indicated cross-species protection, but this did require prior infection with P. fragile. Such results suggested that some regions of AMA-1 are sufficiently homologous between different strains of plasmodia to give cross-protection in animals and, perhaps, in people (Good et al., 1997). Finally, passive transfer with AMA-1 antisera protected mice against challenge with homologous P. chabaudi parasites (Crewther et al., 1996).

D) Rhoptry proteins

Typical features of protozoa belonging to the phylum Apicomplexa are secretory organelles known as rhoptries and micronemes located in the apex of the asexual invasive forms. The rhoptries are flask-shaped organelles which discharge their contents via an apical pore at the time of merozoite invasion. The majority of P. falciparum antigens located in the body of the rhoptry are two-protein complexes identified by immunoprecipitation with antisera to individual components of the complexes. Work is most advanced with a low molecular weight complex, composed of polypeptides derived from two different gene products known as rhoptry-associated proteins (RAP) 1 and 2.

RAP-1 is initially synthesised as a short-lived 84 kDa polypeptide, later fragmented to 80 and 65 kDa components. The RAP-2 gene encodes a doublet of 42/40 kDa. The deduced structures of RAP-1 and RAP-2 have typical N-terminal signal peptides but no characteristics of integral proteins, since they lack internal or C-terminal
hydrophobic sequences typical of membrane-spanning domains or GPI-attachment signals. RAP-1 sequences from different isolates are highly conserved (only 9 aa substitutions identified from 7 different isolates), and there is only a limited diversity among the RAP-2 genes that have been sequenced (Howard & Peterson, 1996). RAP-2 lacks any sequence of repeats whereas RAP-1 contains five tandem repeats of a serine rich octamer toward the N-terminus (KSSSPS motif).

The first indication that a rhoptry component could be effective as a vaccine was the demonstration that a passive transfer to mice of a mAb to a 235 kDa rhoptry protein of P. yoelii protected against challenge with a virulent strain of this parasite (Freeman et al., 1980). Vaccination of Saimiri monkeys with affinity-purified RAP-1/RAP-2 complex partially protected against P. falciparum challenge (Ridley et al., 1990). The epitopes responsible for this immunity were not determined. In addition, mAbs against a conserved linear epitope of RAP-1 (Harnyuttanakorn et al., 1992), were able to inhibit the growth of parasites in vitro. In several experiments, monkeys immunised with purified RAP-1/RAP-2 complex had substantial protection (reviewed in Anders & Saul, 1994).

1.2.3- Phenotypic antigenic variation in Plasmodium

Antigenic variation in several species of Plasmodium has been shown to be a feature of antigens expressed on the surface of parasitised RBCs (PRBCs). These antigens, described originally in simian malarias, could be detected by Ab-mediated agglutination (Brown & Brown, 1965). During P. knowlesi infection, the parasitaemia oscillated, and each new peak was associated with different antigenic phenotypes. Antibodies present at any time of the infection recognised PRBCs from preceding peaks but not those present at the time serum was drawn or peaks appearing later. For P. falciparum, circumstantial evidence of antigenic variation was first reported from experimentally infected Saimiri monkeys (Hommel et al., 1983). Sera from Gambian children naturally infected with P. falciparum had an extreme degree of agglutinating diversity in phenotypes of the PRBCs (Marsh & Howard, 1986). However, it is not possible to distinguish between antigenic variation and possible polymorphism of antigens.
responsible for the agglutinating phenotypes in such findings. In vitro evidence of antigenic variation in *P. falciparum* was obtained from cloned lines that spontaneously switched their expression of antigenic and cytoadherence types at the surface of PRBCs (Roberts *et al.*, 1992).

The *P. falciparum* genome contains a large family of genes (Su *et al.*, 1995) believed to encode variant antigens, collectively known as the erythrocyte membrane protein 1 (PfEMP-1) (Leech *et al.*, 1984). These large proteins of 200-350 kDa are secreted by the parasite and translocated by an unknown mechanism into the PRBC membrane, where they are concentrated in structures known as knobs (Borst, 1995). It is speculated that the insertion of PfEMP-1 helps to avoid splenic removal of PRBCs from circulation as follows. Some of the PfEMP-1 proteins cause *P. falciparum*-infected RBCs to adhere to vascular endothelial cells in vitro (Magowan *et al.*, 1988). Thus, it is hypothesised, expression of PfEMP-1 on RBCs infected with schizont stages of *P. falciparum* results in their retention in vascular beds with only immature *P. falciparum* forms found in peripheral blood. The ability to attach to endothelial lining of small blood vessels in deep organs is thought to be a crucial factor in the pathogenesis of *P. falciparum*, and will be discussed in greater detail in section 1.3.1.1.

PfEMP-1 genes are believed to be extremely variable, and from this it is speculated that a diversity of PfEMP-1 proteins may reflect a functional diversity. For instance, particular variant antigenic phenotypes (VATs) on the surface of PRBCs may determine adhesion to particular endothelial receptors (Biggs *et al.*, 1992). Likewise, PfEMP-1 seems to be the principal target of variant-specific agglutinating Abs detectable in convalescent serum of malaria patients (Newbold *et al.*, 1992). The high diversity of PfEMP-1 phenotypes is believed to be due, at least in part, to phenotypic variation whereby the expression of different genes changes in a clonal parasite population. Since PfEMP-1 is exposed on the surface of PRBCs and, therefore, may be vulnerable to immune attack, it was proposed that *P. falciparum* uses antigenic variation of PfEMP-1 to evade the host immune responses.
The genes encoding some PfEMP-1s were cloned by three groups using different but complementary approaches. During a chromosome "walk", a number of adjacent genes were identified and called var genes (Su et al., 1995). An antiserum directed against one member of the PfEMP-1 family helped to identify a fragment of the corresponding gene in a cDNA expression library (Baruch et al., 1995). In addition, it was shown that expression of variant cytoadherence and antigenic phenotypes correlated with expression of different var genes (Smith et al., 1995). The authors of these papers concluded that, taken together, the results provided evidence for a differential expression of the members of the var gene family encoding PfEMP-1.

Sequencing of several var genes revealed the presence of two exons, and the structure of an adhesion molecule. The first exon encodes a large variable extracellular domain that includes 2-5 Duffy binding-like (DBL) domains. These domains are cysteine-rich and homologous to domains in merozoite proteins involved in attachment and invasion of RBCs, including P. falciparum EBA-175 and erythrocyte binding proteins of P. vivax and P. knowlesi which bind to the Duffy blood group antigen. The second exon codes for a putatively intracellular domain that is highly conserved, and it also includes a single transmembrane domain.

The var gene family is thought to have between 50-150 members per haploid genome, and the genes are scattered over most chromosomes (Su et al., 1995). Low level of expression of individual var genes, high switching rates (2.4% per generation), extensive homology plus remarkable microheterogeneity, have made it difficult to obtain specific molecular or Ab probes to assess the repertoire of var genes and their products expressed in natural infections.

1.2.4- Obstacles to vaccine development

1.2.4.1- Antigenic polymorphism and phenotypic antigenic variation

Extensive sequence data now available for a number of antigens show that there is a very large number of alleles in loci encoding some of the major vaccine candidates. Different sequences or repeat structures in different alleles of an antigen may give rise to
antigenic differences among parasite isolates, e.g. S-antigens (Bickle et al., 1993), but this hypothesis has not been studied in any detail for the current candidates for vaccine against asexual blood stages. Although anti-repeat Abs can inhibit asexual blood stage development in vitro, it has not been established whether repetitive sequences improve or detract from the efficacy of a particular protective immunogen (Anders & Saul, 1994). A major concern is that vaccination against a particular allelic variant may select surviving parasites which possess different variants, resulting in a reduction in the efficacy of the vaccine over time.

In mice, a mAb specific for one allelic variant of P. yoelii MSP-1 does not recognise other variants of P. yoelii MSP-1 (Burns et al., 1989). T cells specific for P. falciparum CSP epitopes often do not recognise epitopes from other allelic sequences of CSP (Zebering et al., 1994). Also in mice, Abs specific for one allelic form of P. chabaudi AMA-1 can adoptively transfer protection against the homologous, but not a heterologous, strain of this parasite (Crewther et al., 1996).

It is not clear how far the well documented sequence polymorphism in P. falciparum molecules such as MSP-1, MSP-2 or AMA-1 may result in antigenic polymorphism, or whether antigenic polymorphism affects the development of immunity to these proteins in humans. If polymorphic sequences were shown to be immunologically relevant B and/or T cell epitopes, one strategy to overcome the obstacle of antigenic polymorphism would be to combine a range of known allelic sequences in a vaccine. Multiple B and T cell epitopes from different allelic variants could be combined, either encoded in a DNA vaccine, or as synthetic polyvalent immunogens such as multiple-antigen peptides (MAPs). Some putative target antigens are relatively conserved (e.g. C-terminus of MSP-1, AMA-1, RAP-1), presumably due to structural constraints or important functional roles. Thus, a different strategy would be to focus on those sequences that are not polymorphic. The problem is that some conserved sequences appear not to be targeted by the immune system or may be recognised only by ineffective Abs and/or T cells.
A worse problem would be posed by phenotypic antigenic variation. Antisera which specifically agglutinate a particular VAT specifically immunoprecipitate a PfEMP-1 expressed by that VAT. Agglutination studies with field isolates indicate that there are many antigenically distinct forms of PfEMP-1 (Reeder et al., 1994), and this is believed to reflect the size of var gene repertoire. Such diversity may preclude the use of PfEMP-1 in a vaccine. Since PfEMP-1 is involved in cytoadherence and may be important in CM, if there were conserved PfEMP-1 epitopes involved in binding, Abs or specific inhibitors of PfEMP-1 or host receptors might result in the prevention or treatment of the disease.

Of uncertain significance from the vaccine perspective is the failure of some parasite isolates to express certain antigens when grown in vitro. For instance, some P. falciparum isolates maintained in long-term culture do not express the ring-infected erythrocyte surface antigen (RESA), PfEMP-1, and histidine rich proteins necessary for the generation of the knobs on PRBC membranes which have been implicated in adhesion (Anders & Saul, 1994).

1.2.4.2- in vitro correlates of protection

There is no in vitro assay that would reliably correlate with in vivo protection. To date, no malaria vaccine has yet induced good protection against the blood infection in humans and it is not known what type of response is required. The large range of immune responses directed against the diverse antigens of the different stages is the most probable reason why it has not been possible to establish a correlate with clinical immunity to malaria. Antibodies are likely to play an important role in immunity to the blood phase of the infection, and thus assays based on Abs should be explored. Seroepidemiological studies have suggested that RBC agglutination (Marsh & Howard, 1986) and ADCI (Bouharoun-Tayoun et al., 1990) but not merozoite invasion inhibition (Phillips et al., 1972) are apparently associated with in vivo immunity. Naturally acquired immunity, however, takes many years to develop, and the hope is that vaccination will induce immunity more quickly. Whether the assays that correlate with naturally acquired immunity would correlate also with vaccine-induced immunity is not known.
In malaria endemic areas, probably only a small proportion of people who become infected with *P. falciparum* develop clinical disease. The reasons why many infections remain asymptomatic or why just a minority of symptomatic infections evolve to severe illness are not understood.

Clinical symptoms of malaria often commence at about the same time or just before parasites are readily diagnosed in the peripheral blood by microscopy; more sensitive detection by PCR can now detect parasites in the blood for up to 1 week before they can be detected in slides (Cheng *et al.*, 1997). In Africa, it is estimated that the majority of symptomatic infections occur between 6 months and 5 years of age. The commonest clinical presentation of all human malarials is an acutely febrile illness. Other frequent symptoms are headache, anorexia and nausea, myalgia, arthralgia and general prostration, and typical signs are splenomegaly and hepatomegaly (White & Pukrittayakamee, 1993). These are features of mild or uncomplicated malaria (UM).

The manifestations of severe or complicated malaria (SM), as defined by WHO criteria, are highly variable but are associated almost exclusively with *P. falciparum* infections (reviewed in Warrell *et al.*, 1990). The spectrum of severe *P. falciparum* disease includes severe malarial anaemia (SMA), cerebral malaria (CM), acidosis, respiratory distress, shock, apparent disseminated intravascular coagulation (DIC), renal failure and pulmonary oedema. Some manifestations are more common in particular age groups and/or geographical areas. Cerebral malaria is widespread in sub-Saharan Africa and Southeast Asia (and can occur in South America) (reviewed in Mendis & Carter, 1995). In Africa, about 2% of malaria cases between 6 months and 5 years of age are classified as severe, mainly involving CM. A few CM cases also occur in the 5-10 year age group, and are only occasionally reported in adults. In contrast, in Southeast Asia CM typically occurs in adults as well as children. Anaemia is common mainly in African children and pregnant women. Organ failure mainly affects adults in Asia and South America.
Malaria mortality in African children, where the heaviest burden of disease falls, is attributed mostly to CM and SMA. However, life-threatening pulmonary and kidney complications are increasing in areas of multidrug resistant malaria such as in Southeast Asia. Liver pathology may also be a more significant feature of malarial infection than has been realised. In addition, malaria-induced metabolic acidosis in children seems to be an important cause of mortality in East Africa (Marsh & Snow 1997).

Pathogenic mechanisms underlying any form of malaria are poorly understood. However, I will describe existing theories of the pathogenesis of the two main SM syndromes.

1.3.1- Pathogenesis of cerebral malaria

Cerebral malaria is defined in clinical terms as the presence of coma associated with the presence of malaria parasites (Warrell et al., 1990). In Africa, the mortality rate for CM is between 15-30%. In addition, a consistent minority of survivors (9-12%) are discharged with neurological sequelae, though half of these recover fully within 4-6 weeks (Newton et al., 1998).

Two phenomena have been considered in CM pathogenesis. One is the adherence of PRBCs to host cells, either endothelial cells in the post-capillary venules of the brain (cytoadherence) or to circulating non-infected RBCs (rosetting). The other is the induction of host cytokines and their effects on brain tissues via induction of secondary mediators, e.g. nitric oxide (NO) and free oxygen radicals. These phenomena are not mutually exclusive as suspected causes of CM.

1.3.1.1- Sequestration of infected erythrocytes in brain tissues

The histopathology of CM has been based on examination of tissues from people who have died with P. falciparum infection. The most consistent autopsy observation is the presence of PRBCs in brain capillaries and postcapillary venules. The textbook histological picture is that of capillaries full of mature parasites (late trophozoites or schizonts). Though frequently the only lesion seen, such capillaries may also be associated
with small ring haemorrhages indicating a slow leakage of blood, or with punctuate haemorrhages, as if the escape was more severe (Garnham, 1966). Clumping of PRBCs could also result in thrombosis of a vessel, anoxia in the adjacent tissues, and finally necrosis, with interference of the brain function.

This *in vivo* sequestration of PRBCs is believed to be mediated by binding between receptors on the luminal surface of vascular endothelial cells and ligands on PRBCs. Many putative endothelial receptors have been identified by *in vitro* experiments, including thrombospondin (TSP), CD36, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), endothelial selectin (E-selectin or ELAM-1), chondroitin sulphate A (CSA), thrombomodulin (TM), platelet endothelial cell adhesion molecule 1 (PECAM-1) and P-selectin (reviewed in Marsh & Snow, 1997). In particular, a significant co-localisation between site of PRBCs attachment in capillaries and ICAM-1, CD36 and E-selectin expression was found in patients dying of CM (Turner et al., 1994). In addition, the expression of ICAM-1 and E-selectin was upregulated in brain vessels during CM (Turner et al., 1994), possibly by cytokines released during malaria infection (Udeinya & Akogyeram, 1993). Putative ligands on PRBCs include host and/or parasite-encoded components. The human ligands may include a modified band 3 (Sherman et al., 1992) and sequestrin (a putative ligand for CD36) (Ockenhouse et al., 1991). However, consensus of opinion favours the hypothesis that most cytoadherence interactions are mediated by a parasite molecule, PfEMP-1. Recently, a region of PfEMP-1 that mediates adherence to CD36 *in vitro* was identified (Baruch et al., 1997).

There is a great interest in elucidating whether heterogeneity in the degree of adherence or heterogeneity of the site of adherence *in vivo* may lead to different pathological consequences. It has been suggested that CM reflects the unfortunate chance occurrence of the "wrong" parasite in the "wrong" host (Berendt et al., 1989). Using *in vitro* assays and cryopreserved *P. falciparum* isolates from Kenya, most isolates bound to TSP or CD36 and to a lesser extent to other receptors (Newbold et al., 1997). The group reported a trend to higher binding to ICAM-1 by parasites from children with CM than of parasites from those with UM which, they suggested, fitted well with ICAM-1
upregulation in CM. In a marked contrast, a study in Malawi using fresh parasite isolates found a negative association between both CD36 and ICAM-1 binding and disease severity (Rogerson, in press).

Rosetting is another cytoadherence phenomenon whereby PRBCs bind to uninfected RBCs in vitro. A study which examined the ability of P. falciparum-infected RBCs from patients to form rosettes in vitro found a correlation between rosetting and severe malaria (Rowe et al., 1995). This rosetting was mediated by PfEMP-1 and complement receptor 1 (Rowe et al., 1997). However, other studies in Papua New Guinea (Al-Yaman et al., 1995) and Malawi (Rogerson, in press) did not find differences in rosette formation between parasites from different groups of patients. Thus, it is not clear whether rosetting occurs in vivo, whether it could be pathogenic per se, or whether it is a marker for an unknown cytoadherence property.

The sequence of in vivo events following the cytoadherence to brain capillaries is not known. Hypothetically, apart from causing anoxia, sequestration of PRBCs could act as a stimulus to endothelial and/or other surrounding cells for the local release of cytokines, a possibility that links sequestration with the second major hypothesis of CM pathogenesis.

1.3.1.2 Cytokine activation

This hypothesis proposes that a dense sequestration of parasites within brain vessels somehow causes large amounts of tumour necrosis factor (TNF-α) to be released locally, stimulating cerebral endothelium to release massive amounts of NO that can then diffuse into the surrounding tissue, profoundly affecting neurotransmission and subsequently causing the coma (Clark et al., 1991). In support of this hypothesis, it has been observed that children with CM had significantly higher circulating levels of TNF-α than those with UM (Grau et al., 1989, Kwartowski et al., 1990). There are two distinct enzymes involved in NO production, both of which use L-arginine as their initial substrate and give raise to citrulline and NO. One is a Ca-dependent system involving a constitutive
NO synthase (NOS); the other, which is likely to be the more relevant for CM pathogenesis, involves an inducible NOS which is activated by cytokines.

Animal models have provided some support for this hypothesis. The best known experimental model of CM is *P. berghei* ANKA infection in CBA/Ca mice. However, CM in mice is characterised by sequestration of leukocytes rather than of PRBCs. High levels of TNF-α production occur (Grau *et al.*, 1990), and ICAM-1 expression on endothelial cells is upregulated possibly by TNF-α in this mouse model. Anti-TNF-α therapy abrogated the fatal neurological syndrome, and a mAb against leukocyte function antigen 1 (LFA-1) (the ligand for ICAM-1 on circulating leukocytes) had a similar protective effect. Application of the Abs appeared to involve an ability to reverse adhesion of platelets to brain endothelium, suggesting a possible platelet-mediated pathogenesis in the mouse.

The observation that infection with *P. berghei* ANKA resulted in CM in some strains of mice but not in others suggests that genetic differences in TNF-α production and/or responsiveness to TNF-α might determine susceptibility to CM. A polymorphism upstream from the 5' transcriptional start site of the human *TNF-α* locus distinguished two alleles, TNF-1 and TNF-2. Field studies found a strong association between homozygosity for TNF-2 and susceptibility to CM, with a relative risk of 4 for CM and 7 for death or neurological sequelae (McGuire *et al.*, 1994).

Other immunological factors which have been linked to CM include serum levels of IgE. Total IgE and *Plasmodium*-specific IgE levels have been shown to be elevated in most *P. falciparum*-exposed children and adults in several endemic areas. In particular, total plasma IgE levels were more elevated in CM cases than in those with UM (Perlmann *et al.*, 1994). However, levels and specificities of IgE to several tested malaria peptides did not differ between CM and the controls.

1.3.2- Severe malarial anaemia
Severe malarial anaemia is defined as a Hb concentration < 6 g/dl, or a haematocrit (or packed RBC volume, PCV) < 16%, in a patient with a parasitaemia in excess of 10,000 trophozoites/μl of blood, although SMA can develop at lower parasitaemias. In most cases, blood transfusions improve the clinical findings. Overall, mortality rates for children hospitalised with SMA range from 4.7% to 14-16% (Newton et al., 1998).

In addition, SMA contributes significantly to morbidity during pregnancy, and may indirectly increase the fatality rate of post-partum haemorrhage (Menendez, 1995). In areas of high malaria transmission, where acquired immunity in adults is expected to be significant, SMA mainly occurs in primigravidae. The reasons for the susceptibility of this particular group of women are not clear.

Regarding the pathophysiology of SMA, it has generally been considered that there is more destruction of RBCs than can be accounted for by the number of RBCs infected and directly destroyed by parasites. In addition to RBCs lost by lysis when schizonts rupture, various immunopathological mechanisms may contribute to SMA. Parasite antigens, immune complexes containing them, or autoantibodies could bind to unparasitised RBCs and accelerate their clearance by cells of the macrophage/monocyte lineage in the spleen and liver, or by complement-mediated lysis. Non-infected RBCs could also be damaged by disease-induced free oxygen radicals leading to rigidification of the RBC membrane and thus their increased clearance by an hyperactive spleen. At the same time, chronic release of TNF-α or other cytokines in response to the infection could inhibit RBC development from bone marrow stem cells (dyserythropoiesis) and alter the kinetics of RBC turnover. (Clark & Chaudhri, 1988). Alternatively, blocking of bone marrow sinusoids with PRBCs could also cause dyserythropoiesis, which has been described both morphologically and functionally in SMA patients. These findings have led to the idea that SMA may be caused by a chronic process as well as by acute events (Abdalla et al, 1990). However, the question of whether presentations with SMA are essentially acute episodes related primarily to uncontrolled exponential parasite growth,
or whether they are cases of chronically progressive anaemia which finally crosses some critical threshold, remains unsolved.

Studies addressing the role of inhibition of erythropoiesis in the pathogenesis of SMA found that it could not be explained by insufficient erythropoietin (EPO) production; in fact, EPO was markedly elevated and inversely correlated with Hb levels (Burchard et al., 1995). Another study reported that *P. falciparum* infection caused a rapidly reversible suppression of the bone marrow response to EPO in CM, SMA and UM patients, suggesting that the severity of the anaemia would depend upon the degree of peripheral RBC destruction (Kurtzhals et al., 1997). Thus, a rapid destruction of RBCs due to massive infection would cause acute SMA, but, in the presence of continuous inhibition of erythropoiesis, a prolonged destruction of RBCs due to chronic or recurrent malaria would lead to SMA.

Finally, the role of interleukin 10 in SMA has been investigated. IL-10 is an anti-inflammatory cytokine that downregulates the production of TNF-α, stimulates bone marrow function *in vitro*, and counteracts anaemia in mice. Therefore IL-10 might have a role in the hypothesised TNF-α-induced dyserythropoiesis. It was found that patients with SMA had significantly lower plasma concentrations of IL-10 than CM or UM cases (Kurtzhals et al., 1998), concluding that insufficient IL-10 response allowing high TNF-α concentrations may have a central role in SMA.

### 1.3.3- Epidemiology of severe malaria

Epidemiological observations suggest that the incidence of CM and SMA differ with age, and vary with endemicity. In African children, the peak of SMA occurs within the two first years of life, whereas CM occurs later, between 3-4 years of age. This finding has been interpreted in different ways. Firstly, different levels of naturally acquired immunity, which requires repeated exposure over years, could influence the different age-patterns of disease severity. It is possible that children who develop CM may have "less" immunity than children who develop UM. However, studies in Thailand (Tharavanij *et
al., 1984) and The Gambia (Erunkulu et al., 1992) estimating exposure to malaria by presence of Abs did not support this possibility. Alternatively, the gap of several years before an increased probability that a *P. falciparum* infection gives rise to CM is compatible with the idea that a degree of immunological sensitisation by prior malaria infections could predispose an individual to developing CM. Consistent with this view are observations in Thai subjects with CM, whose hospital records indicated that their previous UM infections were more frequent than in such infections in subjects presenting with non-cerebral forms of malaria (Mendis & Carter, 1995). Another possibility is that particular *P. falciparum* strains may differentially predispose to CM, SMA or UM (Gupta et al., 1994) by exhibiting phenotypes associated with varying degrees of "virulence", e.g., ability to invade RBCs, to adhere to endothelial cells, or to stimulate TNF-α response.

1.4- Genetic host resistance to parasites and immune responses to *Plasmodium* infections

1.4.1- Innate natural resistance

It is presumed that in malaria highly endemic areas the high mortality from *P. falciparum* has modified the human genome by selection of genetic variants that reduce the risk of death. I will first review some genetic abnormalities of RBCs that are thought to protect humans from malaria. Next, I will discuss innate immune responses, that may determine whether non-immune individuals survive the first weeks of infection.

1.4.1.1- Genetic resistance to parasites

Malaria is thought to be a major selective force in the evolution of Africans and Melanesians in whom many otherwise deleterious genes are suspected to confer a survival advantage against malaria. Merozoites of *P. vivax* and *P. knowlesi* use a particular blood group on the RBC surface, the Duffy antigen, as a receptor to enter into the RBC. The near absence of this antigen in West African populations renders them
resistant to infection by *P. vivax* (reviewed in Miller, 1996). Other RBC defects which offer some protection against *P. falciparum* are haemoglobinopathies: sickle cell anaemia, α and β-thalassaemias, foetal haemoglobin (Hb) syndrome, and glucose-6-phosphate deficiency. Distributions of these traits correlate geographically with areas of *P. falciparum* endemicity.

Polymorphisms in genes encoding elements of the immune system have also been associated with apparent protection against malaria in mice or humans. For instance, strains of mice carrying certain major histocompatibility complex (MHC) genes are less able to make Abs to certain peptides of CSP because their T cells do not become sensitised (Nardin & Nussenzweig, 1993). Similarly, the possession of the human major histocompatibility complex (HLA) Bw53 allele, widespread in West Africans but rare in Caucasians, appears to correlate with protection against severe malaria (Hill *et al.*, 1991).

### 1.4.1.2. Innate immunity

Innate immune mechanisms can be stimulated by parasite products that induce a cytokine response in non-immune individuals (reviewed in Kwiatkowski, 1992). Effector cells of innate immunity such as tissue macrophages, monocytes and granulocytes may have an intrinsic anti-parasitic activity, e.g. phagocytosis of sporozoites when they enter the body. Although macrophages are normally activated by cytokines secreted by T cells, they can be activated also by T cell-independent mechanisms, e.g. natural killer (NK) cells secrete interferon (IFN)-γ when stimulated by IL-12 produced by macrophages.

It has been proposed that IFN-γ and TNF-α may act by stimulating macrophages and neutrophils to kill parasites via reactive nitrogen intermediates (RNIs), primarily NO (Clark *et al.*, 1991). Immunisation studies in animal models (Herrera *et al.*, 1992) showed that high IFN-γ levels correlated with protection against *Plasmodium* asexual blood stages. However, there is no evidence that either IFN-γ or TNF-α is directly toxic for asexual intraerythrocytic parasites.
TNF-α may have harmful as well as beneficial effects in the infected host, depending upon the amount produced and whether it is free in the circulation or cell-bound. It has been argued that soluble malaria antigens, so-called "exoantigens", released by rupturing schizonts, induce production of cytokines, particularly TNF-α from monocytes and macrophages, which cause much of the pathology of malaria (see 1.3). Thus, the fever, anaemia, diarrhoea and pulmonary changes of acute malaria closely resemble the symptoms of endotoxaemia and are probably caused by TNF-α (Kwiatkowski, 1990). Antisera against exoantigens of *P. yoelii* can inhibit the TNF-α-stimulating activity of exoantigens derived from *P. falciparum* and *P. vivax*, and vice versa, implying the presence of a common TNF-α-stimulating determinant. Phospholipid-containing antigens are candidates for such activity, since the activity is resistant to protease treatment and deglycosylation but destroyed by phospholipase C (Bate *et al*., 1992). The activity is specifically inhibited by inositol-1-monophosphate or phosphatidyl inositol. This suggests that the active moiety contains an inositolphospholipid structure, possibly related to the GPI anchors on MSP-1, MSP-2 and other plasmodial proteins.

In endemic areas, older children and adults frequently have positive parasite smears but no symptoms of the disease, implying the presence of a clinical "anti-toxic" or anti-disease immunity (see below). A vaccine which could induce Abs able to neutralise the toxins before they reach the macrophages and monocytes would therefore be beneficial, at the time when anti-parasite immunity is developing more slowly (Playfair *et al*., 1990).

Periodic fever, produced when large numbers of PRBCs rupture, is the most characteristic feature of malaria. Infected individuals have elevated levels of the endogenous pyrogens TNF-α, IL-1 and IL-6. The fever has been suggested as a possible mode of host defence since febrile temperatures are inhibitory to the growth of intraerythrocytic *P. falciparum in vitro*. It has been argued that the fever may be acting as a density-dependent regulator of parasite growth (Kwiatkowski, 1991).
1.4.2- Naturally acquired immunity

People living in malaria endemic areas who are exposed to repeated *P. falciparum* infections from infancy develop immunity to the disease later in life. Children are more susceptible to clinical malaria and also to the more severe life-threatening forms of the disease, with the highest mortality rates. Adults in such endemic areas, though often harbouring low but detectable numbers of parasites in the blood rarely develop clinical symptoms or die as a result of the infection. This form of acquired immunity to the disease, also referred to as "premunition", "clinical tolerance" or "antitoxic/antidisease immunity", develops slowly over years and is never complete. There is, generally, also a substantial decrease in the parasite load in infected adults, indicating that there is also an "anti-parasite immunity" component malaria in protection which develops with age. Thus, the long period required to achieve protection is probably dependent on age and exposure, but it has not been conclusively explained which component prevails. On one hand, the malaria parasite may be poorly immunogenic at inducing protective responses. On the other hand, if immunity was essentially strain-specific, a long period could be required to be exposed to a large repertoire of diverse strains (see chapter 5). Another hypothesis suggests that immunity to malaria develops differently in adults and children; a more mature immune system would allow an adult to acquire immunity more rapidly than a child under the same exposure (Baird, 1995).

Since it is not possible to carry out *in vivo* experiments into the immunity and immunopathology in human malaria infections, the common recourse has been to approach these problems in animal models, particularly transgenic mice in recent years. However, due to the wide variety of mouse and *Plasmodium* species and strains used in different laboratories, rodent models have not given consistent results, and it is uncertain whether conclusions drawn from these systems are relevant to human malarias. For this reason, in the next two sections I focus mainly on studies that have been carried out in humans, though they are limited to observations of immune responses in naturally infected individuals who live in malaria endemic areas. Such immunoepidemiological surveys may be cross-sectional, examining immune responses of a population usually at
one or few points in time, or longitudinal and prospective studies, following up the
evolution of immune responses in individuals over a period of time. Case-control studies
aim to assess the importance of immunity in the disease outcome. Studies of immune
responses to merozoite proteins are reviewed in chapters 4 and 5.

1.4.2.1- Evidence for antibody-mediated immunity to blood stages of
human malaria parasites

Classical passive transfers of immune IgG (Cohen et al., 1961; McGregor &
Carrington, 1963), reproduced more recently (Bouharoun-Tayoun et al., 1990),
demonstrated that Abs protect against malarial infection. Transfer of pooled γ-globulin
from West African immune adults into East African or Thai children acutely infected with
*P. falciparum* caused a sharp drop in parasitaemia. However, in experimental animal
models protective immunity to malaria does not correlate simply with Ab levels and can
be even induced in their absence (Good et al., 1997). It is generally considered that Abs
would target extracellular parasites and block their capacity to invade new cells, whereas
cell-mediated responses would prevent the development of intracellular forms.

During the asexual blood phase, Abs are thought to be the major effector
mechanisms. In principle, many putative effector mechanisms involving Abs can be
envisaged. Firstly, Abs can cause direct damage to free parasites, either by themselves or
by activating the complement system. Secondly, they can neutralise a function of the
parasite e.g. block its attachment and/or subsequent invasion to a host cell. They can
agglutinate the merozoite at rupture of mature schizonts, and block merozoite invasion of
RBCs. Thirdly, Abs can enhance phagocytosis by macrophages, which could be increased
even more by the presence of complement. Finally, Abs engulfed with the merozoite at
invasion could inactivate the intraerythrocytic parasite, either directly or via antibody-
dependent cell-mediated cytotoxicity (ADCC), as macrophages and neutrophils bearing
Fc receptors may become killer cells in ADCC (Good et al., 1997). An *in vitro* ADCC
(or antibody-dependent cellular inhibition, ADCI, as defined by the authors) assay
correlated with the efficacy of the pooled serum that markedly reduced parasitaemia in
It was thought that if blood monocytes were required for biological function of the Abs, then the isotypes of the Abs might be a relevant variable (Bouharoun-Tayoun et al., 1992). IgG1 and IgG3, both cytophilic isotypes, predominated in protected subjects, whereas subjects experiencing a primary attack of malaria had IgG2 or IgM isotypes (Oeuvray et al., 1994). It was suggested that non-protective Ab isotypes during childhood may block the activity of protective isotypes, and thus explaining the age-related aquirement of immunity. Cytophilic classes co-operated with monocytes in vitro via Fc receptor to produce TNF-α. Competition assays suggested that Ig from non-protected individuals could block the activity of those from immune individuals.

1.4.2.2- Cell-mediated immunity

In malaria, both immunity and immunopathology are associated with T cell activation, but the precise mechanisms contributing to one or the other remain obscure. In some model malaria infections, e.g. P. chabaudi, protection can be conferred experimentally on normal animals by the transfer of T cells from immune animals, whereas in other mouse models, T cell transfers have demonstrated the importance of T cells in severe pathology (Grau et al., 1990).

The products of activated T cells, notably IFN-γ, are potent synergists in the activation of macrophages to produce TNF-α, a putative pathogenic mediator. Thus, it is plausible that immune recognition of malaria parasites by T cell and subsequent secretion of regulatory cytokines such as IFN-γ may play a significant role in pathogenesis (reviewed in Mendis & Carter, 1995), as well as in protection. Most of the available data on the cytokine profiles of Th1 and Th2 cell subsets of CD4+ T cells come from mouse models (reviewed in Fell & Smith, 1998), as few studies have been carried out on humans. Peripheral blood mononuclear cells from donors living in malaria endemic areas stimulated in vitro by peptides based on Plasmodium proteins activated both types of cells, with production of either IFN-γ (Th1 type) or IL-4 (Th2 type) (Troye-Blomberg et al., 1990). In two other studies which measured in vitro production of IFN-γ in response
to recombinant MSP-1, IFN-γ levels increased with age and after malaria transmission season, but no significant differences were found between clinical and asymptomatic patients (Riley, 1992, 1993). Increased levels of circulating soluble markers of T cell activation in acute malaria, e.g. IL-2 receptor, CD4 and CD8 (Jakobsen et al., 1994), have suggested a systemic T cell activation. In addition, the degree of activation correlated with disease severity.

Lymphopenia, i.e. lower numbers of T cells in the peripheral circulation, is a feature of acute falciparum malaria. A likely explanation for this phenomenon is a temporal reallocation of T cells away from the periphery in response to the infection (Hviid et al., 1997). Hviid et al. studied the kinetics of this putative redistribution in children from malaria endemic areas in respect to the clinical manifestations of the disease. They found that anti-malarial treatment rapidly induced re-emergence of sequestered T cells into the circulation, and a correlation between lymphopenia levels and disease severity.

Another feature of acute malaria is a transient inability of peripheral T cells to respond to antigenic stimulation in vitro (Hviid et al., 1994). This could be explained by the reallocation hypothesis or, alternatively, by some immunosuppressive mechanisms. Hviid et al. (1997) reported that peripheral T cells might be functionally compromised due to a down-regulation of the T cell receptor/CD3 complex, which could account for the observed hyporesponsiveness.

Finally, T cells with γδ T cell receptors are increased in the blood during and after malaria infection. Moreover, naive γδ T cells from donors previously unexposed to malaria have been reported to proliferate in vitro in the presence of P. falciparum PRBCs (reviewed in Long, 1993). The significance of these observations is unknown, but it has been speculated that these cells have arisen from crossreacting microbial stimuli, and that their presence could affect responses to vaccination.
1.7- Aims of the project

The general aim of this PhD thesis is to investigate the role of *P. falciparum* diversity in the development of human immunity to malaria and in the pathogenesis of the disease. In particular, I examine whether genetic polymorphism of *P. falciparum* MSP-1 and MSP-2 gives rise to antigenic polymorphism and to specific immune responses to these proteins in Malawian children distinguished by different disease manifestations of *P. falciparum* infection.

The specific objectives are:

- to evaluate the extent of structural diversity in *P. falciparum* MSP-1 and MSP-2 in clinical isolates from Malawi, and to elucidate the relationship between allelic polymorphism and antigenic diversity in these proteins (chapter 2)

- to compare the composition of MSP-1 and MSP-2 types in circulating *P. falciparum* (chapter 2) and in organ-sequestered parasites (chapter 3) from Malawian children with defined clinical malaria presentations, and to test for associations between particular genotypes and/or serotypes and disease manifestations

- to investigate the immune responses to defined polymorphic and conserved regions of *P. falciparum* MSP-1, MSP-2, AMA-1 and RAP-1 in Malawian children with different disease manifestations (chapter 4)

- to test the hypothesis that MSP-1 and MSP-2 are targets of a ‘strain’-specific component of human immunity to malaria (chapter 5)
Chapter 2: GENETIC AND ANTIGENIC DIVERSITY OF POLYMORPHIC PLASMODIUM FALCIPARUM MERozoITE SURFACE PROTEINS IN BLOOD-CIRCULATING PARASITES FROM MALAWIAN CHILDREN WITH MALARIA

2.1- Introduction

The wide spectrum of disease manifestations observed in Plasmodium falciparum infections probably reflects a combination of various host and parasite factors. Parasite heterogeneity is one factor that may play a role in the severity of falciparum malaria, and it has been hypothesised that some strains of P. falciparum might be more virulent than others. Gupta et al. (1994) suggested that a majority of P. falciparum strains may cause uncomplicated malaria (UM), whereas the most severe form, cerebral malaria (CM), might be caused by a few highly virulent strains. Severe malaria anaemia (SMA) might be a complication occurring in a certain proportion of infections with "mild" strains. To date, there is no evidence to support this view, and very few studies (Robert et al., 1996; Kun et al., 1998) have addressed the question whether particular genotypes are differentially distributed in people distinguished by different manifestations of malarial disease.

Due to their extensive polymorphisms, P. falciparum merozoite surface proteins MSP-1 and MSP-2, have been useful markers for studies on the population genetics of malaria parasites. Although polymorphisms in MSP-1 and MSP-2 genes are well documented, speculations rather than evidence prevail about whether these polymorphisms have any effect on the antigenic properties of the proteins which they encode or on the specificity of human immune responses. This is a crucial question to answer to elucidate the role of "strain"-specific immune responses in the acquisition of immunity to malaria (see chapter 5), and to predict the implications of antigenic polymorphism in the development and deployment of malaria vaccines.

In this chapter, I concentrate on the relationships between genetic polymorphism of MSP-1 and MSP-2 and antigenic diversity of P. falciparum, as "strain" markers. I first introduce the subject by reviewing the structure of MSP-1 and
MSP-2, followed by specific aims, experimental approach and results of this chapter, discussed in the context of previous studies.

2.1.1- Polymorphism of MSP-1

MSP-1 is a highly polymorphic protein. Sequence comparisons of MSP-1 alleles indicate that the gene can be divided into 17 blocks that encode conserved, semiconserved and variable regions of the protein (Tanabe et al., 1987; Miller et al., 1993). Dimorphic regions span from block 6 to 16, and define two allelic "families" of MSP-1 (fig 2.1). The families are represented by MSP-1 alleles of the MAD20 and K1/Wellcome (Well) isolates respectively. Block 2 is the most polymorphic region of MSP-1, but all allelic sequences of block 2 fall into one of three main types represented by the RO33, MAD20 and K1 isolates. K1-like and MAD20-like types vary in the sequence, length and number of tandem repeats, whereas RO33 is a non-repetitive sequence with little variation between isolates. The N- and C-terminal regions of the protein, blocks 1 and 17, are almost conserved.

There are three main causes of the variation in the MSP-1 gene: recombination, repeats and mutations. Intragenic recombinations at the 5' end have resulted in exchanges in two types of block 4, and between block 4 and the three types of block 2; the variation in the C-terminal half of MSP-1 is mainly dimorphic (Tanabe et al., 1987; Jongwutiwes et al., 1991). It appears that intragenic recombinants between blocks 2, 3 and 4 are not at equilibrium frequency in natural populations because non-random associations between polymorphic domains were observed (Conway et al., 1991b). Point mutations led to amino acid (aa) changes found in a few positions in the conserved ends of the molecule (Tolle et al., 1995).

2.1.2- Polymorphism of MSP-2

Although the MSP-2 gene is highly polymorphic in natural populations of the parasite, sequences of MSP-2 alleles from a large number of parasite isolates from different geographical locations illustrate an essentially dimorphic structure of the molecule. Thus, two allelic families of MSP-2 exist, usually denoted the IC1/3D7 and the FC27 families, which correspond to A and B serogroups identified by reactivity
Fig. 2.1. Schematic representation of the structure of MSP-1 of *P. falciparum*

The division into 17 blocks is as outlined by Tanabe *et al.* (1987). Dimorphic and polymorphic allelic families are indicated. Shown below the gene is the scheme of the natural processing of the MSP-1 protein. Primer used for PCR genotyping of polymorphic block 2 and dimorphic block 16 (difs) are indicated with arrows.
Fig. 2.2. Schematic representation of the structure of MSP-2 of *P. falciparum*

The division into 5 blocks is as outlined by Thomas *et al.* (1990); the repeat regions are designated as Fenton *et al.* (1991).

Primer used for PCR genotyping of dimorphic regions (difs) and for the sequencing of the full length gene are indicated by arrows.

### Blocks:

1. 16
2. 5
3. R1
4. R2
5. 14

### Dimorphic regions:

**Group A**

- IC1/3D7
  - 4-8 aa tandem repeats
  - poly T repeats
  - difs A

**Group B**

- FC27
  - 32-mer aa repeats
  - 12-mer aa repeats
  - difs B

**Key:**

- White: Conserved
- Dimorphic group-specific shades
- Polymorphic repeats
with monoclonal antibodies (mAbs) (Fenton et al., 1991). In addition, there are forms presumed to have been generated by intragenic recombination between the two families (Marshall et al., 1991; Snewin et al., 1991).

All allelic forms of the protein have a similar structure of conserved N- and C-terminal regions flanking a central variable region t, consisting that differs substantially between the two families (fig 2.2). This family-specific region is composed of non-repetitive sequences surrounding two domains of polymorphic tandem repeats. The FC27 MSP-2 family (serogroup B) has 32- and 12-aa residue motifs one and/or the other of which can be tandemly repeated in different isolates (Smythe et al., 1990). A new repeat unit consisting of 3-aa residues has been recently described upstream of the 32-mer (Irion et al., 1997). Members of the IC1/3D7 (serogroup A) allelic family have shorter repeats of 4-8 aa residues (Marshall et al., 1992), forming so-called R1 and R2 regions. Some alleles of this group are also characterised by the presence or absence of short sequences within the 3' non-repetitive variable blocks (D1 and D2 deletions). In summary, it is proposed that all the protein products of MSP-2 alleles can be divided into five blocks of sequences. The N- and C-terminal regions, blocks 1 and 5 respectively, are conserved. Blocks 2 and 4 consist of non-repetitive predominantly dimorphic regions which best define the two distinct allelic families. Blocks 2 and 4 flank block 3, the region of tandem repeats that vary among alleles in number, length and sequence (Thomas et al., 1990).

2.1.3- Methods to study polymorphism

The first comprehensive study on the extent of polymorphism of MSP-1 and MSP-2 in the field that included significant numbers of clinical P. falciparum isolates was carried out in The Gambia using immunofluorescence microscopy (IFA) (Conway & McBride, 1991). Because blood stages of the parasite are haploid and thus express only a single allele of MSP-1, mAbs specific for particular allelic products can readily detect multiple clone infections by IFA.

At present, DNA typing methods are used more routinely to investigate the diversity and distribution of MSP-1 and MSP-2 allelic variants. For instance, polymerase chain reaction (PCR)-based methods, including Southern blot of genomic
DNA probed with the \textit{MSP-2} gene (Thomas \textit{et al.}, 1990), dot blot hybridisation with family-specific or repeat-specific oligonucleotide probes (Smythe \textit{et al.}, 1991), and multilocus fingerprinting (Prescott \textit{et al.}, 1994), have been used to detect differences in size and internal organisation of \textit{MSP-2} alleles. Genotyping by single PCR (Snewin \textit{et al.}, 1991), PCR-RFLPs (Prescott \textit{et al.}, 1994; Felger \textit{et al.}, 1994) and nested PCR (Ntoumi \textit{et al.}, 1995) allowed further differentiation of alleles within the two allelic families. However, only DNA sequencing provides an absolute means to define the extent of diversity in this gene (Thomas \textit{et al.}, 1990; Fenton \textit{et al.}, 1991; Snewin \textit{et al.}, 1991; Marshall \textit{et al.}, 1994). Direct sequencing of PCR-amplified DNA, is required to avoid some artefacts due to cloning (e.g. deletion of repeat units in \textit{Escherichia coli} hosts) (Prescott \textit{et al.}, 1994).

The present work aimed to compare the genetic and antigenic polymorphism of the merozoite surface proteins and, therefore, both antigen-detection (IFA) and DNA-based methods (PCR genotyping and sequencing) were explored.

\subsection*{2.1.4- Aims}

The specific questions addressed in this chapter are:

- What is the relationship between allelic polymorphism of \textit{P. falciparum} \textit{MSP-1} and \textit{MSP-2} genes and antigenic diversity?

- What are the allelic distributions of \textit{MSP-1} and \textit{MSP-2} genotypes and serotypes in Blantyre, Malawi, and how do they compare to those in other geographical locations?

- Do parasites associated with different clinical manifestations of \textit{P. falciparum} malaria differ in their composition of these merozoite surface antigens?

- Is there a relationship between multiplicity of clones in \textit{P. falciparum} infection and disease severity?

\section*{2.2- Materials and methods}

\subsection*{2.2.1- Parasite samples}

\textit{P. falciparum} isolates were obtained as infected blood samples from a total of 382 children under 12 years of age suffering from malaria, in Blantyre, Malawi, during
Children with severe malaria (SM) \((n=244)\) were admitted to the Malaria Research Project and Wellcome Trust Centre (MRP), Department of Paediatrics, Queen Elizabeth Central Hospital. A clinical scoring scale, the Blantyre Coma Score (BCS), was used to assess the levels of consciousness in these children by measuring the best motor response to painful stimulus, the best verbal response, and eye movement (Molyneux et al., 1989). 129 of the children with SM were defined as having cerebral malaria (CM), i.e. they had BCS<3 (unable to localise pain) with asexual parasitaemia of any density and no other obvious cause of the clinical syndrome (e.g. meningitis). Patients were excluded from the definition if they improved within 1 hr of a convulsion, or of being restored to a normoglycaemic state (as impaired consciousness may also be due to hypoglycaemia). Severe malarial anaemia (SMA) cases \((n=61)\) were defined as haemoglobin (Hb) \(<5g/dl\) or haematocrit (or packed red cell volume, PCV) \(<16%\). 54 patients were diagnosed as having both CM and SMA.

Control patients with uncomplicated malaria (UM) \((n=104)\) were recruited from three sources: (i) ambulant children screened for enrolment in studies of novel antimalarial therapy at Ndirande Health Centre, Blantyre (ii) patients admitted to the MRP ward with a final diagnosis of UM and (iii) ambulant controls attending the MRP for blood examination for malaria parasites. These controls had no recent history of coma or convulsions, and were fully conscious (BCS of 5). Another group of controls \((n=31)\) was composed of patients presenting to the MRP with coma or severe conditions that could not be attributed to malaria infection (severe non-malarial disease, SNM), e.g. meningitis, septicaemia, bronchopneumonia or local drug intoxication. All patients were asked to come back for a follow up sample after a month, or earlier if they were parasitaemic or had symptoms of malaria. A total of 212 convalescent blood samples were obtained from all groups of patients.

Clinical data including state of consciousness, history of convulsions and history of prior drug treatment, and basic demography were recorded for all patients. An initial blood fingerprick was taken to assess the parasitaemia and haematocrit levels. A thick and a thin blood films were prepared from each patient; at least 200 white blood cells or 500 red blood cells (RBCs), respectively, were counted and the
numbers and stages of parasites recorded. Patients admitted in 1997 constitute the
study population to whom the results presented here refer, unless stated otherwise.
Clinical and parasitological data of patients included in the typing study are
summarised in table 2.1.

**Table 2.1. Clinical and parasitological data of the patients studied**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Arithmetic mean age ± SD</th>
<th>Geometric mean parasitaemia (95% CI)</th>
<th>n</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>Acutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CM d</td>
<td>103</td>
<td>3.62 ± 2.10</td>
<td>28,219 (16,025 - 49,691)</td>
<td>11</td>
<td>87</td>
</tr>
<tr>
<td>SMA</td>
<td>57</td>
<td>2.35 ± 1.41</td>
<td>11,814 (4,693 - 29,738)</td>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>CM+SMA</td>
<td>48</td>
<td>3.09 ± 1.69</td>
<td>56,485 (27,079 - 117,820)</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>UM</td>
<td>76</td>
<td>2.79 ± 1.54</td>
<td>26,738 (15,545 - 45,990)</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>SNM</td>
<td>31</td>
<td>3.63 ± 3.13</td>
<td>11 (2 - 53)</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

a number of patients from whom a RBC sample was obtained on admission to the MRP for parasite typing. Data
on clinical diagnosis, age, parasitaemia and number of deaths refers to these patients.

b Parasitaemia calculated as parasites (ring forms) per microlitre of blood
c number of patients who came back to the MRP for a follow up visit a month after admission. A RBC sample
was also collected for parasite typing

cv Cerebral malaria; SMA=severe malarial anaemia; UM=uncomplicated malaria; SNM=severe non-
malarial disease

Five millilitres of venous blood were taken in lithium heparin or EDTA on
admission at the MRP or at Nidirande Health Centre, centrifuged, and the buffy coat
and plasma removed. Packed red cells including parasitised RBCs (PRBCs) were
washed 3x in sterile phosphate buffered saline (PBS). Samples with parasitaemia (%
ring forms over 500 RBCs) of at least 0.05%, ≥ 0.3 ml RBCs were used to set up
short-term *P. falciparum* in vitro cultures. The rest of RBCs were stored at -70°C for
analysis of parasite DNA.

2.2.2- In vitro culture of *Plasmodium falciparum* isolates

An aliquot of 0.3 ml of washed PRBCs was used for short-term *P. falciparum*
culture as described (Trager & Jensen, 1976; Conway *et al*., 1991a). Briefly, parasites
were incubated at 37°C in RPMI-HEPES medium (Life Technologies) supplemented
with gentamicin 10 µg/ml (Life Technologies), NaHCO₃ 2mg/ml (Sigma) and 10%
Table 2.1. Clinical and parasitological data of the patients studied

<table>
<thead>
<tr>
<th></th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Arithmetic mean age ± SD</th>
<th>Area of residence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Geometric mean parasitaemia (95% CI)</th>
<th>Duration&lt;sup&gt;d&lt;/sup&gt; symptoms</th>
<th>Prior&lt;sup&gt;e&lt;/sup&gt; treatment</th>
<th>n&lt;sup&gt;f&lt;/sup&gt; sequelae</th>
<th>n&lt;sup&gt;f&lt;/sup&gt; deaths</th>
<th>n&lt;sup&gt;f&lt;/sup&gt; conval.</th>
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<tbody>
<tr>
<td>CM&lt;sup&gt;g&lt;/sup&gt;</td>
<td>103</td>
<td>3.62 ± 2.10</td>
<td>40</td>
<td>28,219 (16,025 - 49,691)</td>
<td>56 ± 40.9</td>
<td>37</td>
<td>20</td>
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<td>87</td>
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<tr>
<td>SMA</td>
<td>57</td>
<td>2.35 ± 1.41</td>
<td>16</td>
<td>11,814 (4,693 - 29,738)</td>
<td>83 ± 50.5</td>
<td>19</td>
<td>1</td>
<td>5</td>
<td>47</td>
</tr>
<tr>
<td>CM+SMA</td>
<td>48</td>
<td>3.09 ± 1.69</td>
<td>14</td>
<td>56,485 (27,079 - 117,820)</td>
<td>71 ± 38.6</td>
<td>20</td>
<td>8</td>
<td>8</td>
<td>40</td>
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<tr>
<td>UM</td>
<td>76</td>
<td>2.79 ± 1.54</td>
<td>64</td>
<td>26,738 (15,545 - 45,990)</td>
<td>52 ± 38.1</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>SNM</td>
<td>31</td>
<td>3.63 ± 3.13</td>
<td>12</td>
<td>11 (2 - 53)</td>
<td>68 ± 63.9</td>
<td>15</td>
<td>3</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup> number of patients from whom a RBC sample was obtained on admission to the MRP for parasite typing. Data on clinical diagnosis, age, area of residence, parasitaemia, duration of symptoms, prior treatment and number of sequelae and deaths refers to these patients.

<sup>b</sup> Number of patients whose area of residence was: A. Blantyre town; B. periurban areas; C. villages; D. towns other than Blantyre; E. unknown.

<sup>c</sup> Parasitaemia calculated as parasites (ring forms) per microlitre of blood.

<sup>d</sup> Duration of symptoms (fever, vomit, stop eating, stop drinking/sucking, diarrhoea, convulsions, unconsciousness) in arithmetic mean hours ± SD, as recorded by interviewing parents.

<sup>e</sup> Number of patients with a history of anti-malarial treatment (sulphadoxine/pyrimethamine and/or quinine) in the past week.

<sup>f</sup> number of patients who came back to the MRP for a follow up visit a month after admission. A RBC sample was also collected for parasite typing.

<sup>g</sup> CM=cerebral malaria; SMA=severe malarial anaemia; UM=uncomplicated malaria; SNM=severe non-malarial disease.
human AB serum from non-malaria exposed Australian blood donors, in a gas mixture of 1% O₂, 5% CO₂ and 94% N₂. Parasites were grown for ≈ 48 hr until they matured to schizonts, as judged by thin smear examination.

2.2.3- Indirect immunofluorescent (IFA) serotyping

When cultures contained schizonts, the PRBCs where harvested and washed 2 times in 10 ml PBS to remove all traces of human serum. Cells were resuspended to ≈ 3-5% haematocrit in PBS and 20-25 µl aliquots were placed onto wells of 12-well multispot microbiological slides (Hendley-Essex). The slides were dried in a culture hood, packed and stored at -20°C in self-sealed plastic bags containing silica gel as desiccant.

Type-specific Ab probes can identify distinct allelic forms of MSP-1, MSP-2 and of exported protein EXP-1, and thus the presence of mixed infections in individuals. Serological reagents included mAbs and polyvalent mouse sera specific for *P. falciparum*, MSP-1, MSP-2 and EXP-1 (table 2.2). Three known MSP-1 block 2 types, represented by the RO33, MAD20 and K1 isolates, were identified by specific mAbs (Conway *et al.*, 1991a) and/or polyclonal sera (Cavanagh & McBride, 1997) (fig 2.1). In addition, mAbs were used that detect MSP-1 sequence polymorphisms in blocks 3 and 4b (the C-terminal half of block 4), in dimorphic regions (block 6 to 16) of the MAD20-type or Well/K1-type, and conserved epitopes (block 17) (Conway *et al.*, 1991a). Polymorphisms within and between the two major serogroups of MSP-2, A and B (fig 2.2), were similarly identified by mAbs (Fenton *et al.*, 1991) or mouse sera against recombinant MSP-2.

Working dilutions of Abs were prepared in 1% bovine serum albumin (BSA) in PBS containing 0.01% sodium azide and kept at 4°C (table 2.2), and IFA serotyping was carried out as described (Conway *et al.*, 1991a). Briefly, slides with schizont-infected RBCs were fixed in acetone for 5 min and 25 µl of working dilutions of Abs were placed on separate wells and incubated at room temperature in a wet box. After 30 min, Abs were removed and slides were washed 3× in PBS. After the slides were dried on a warm plate, 15 µl of FITC (fluorescein isothiocyanate)-conjugated anti-mouse immunoglobulin (1/80) were placed in each well, incubated
<table>
<thead>
<tr>
<th>Location</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Isotype</th>
<th>Domain and epitope</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
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<td>17</td>
<td>K1/Well</td>
<td>111.4</td>
<td>500</td>
<td>IgG1/κ</td>
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<tr>
<td></td>
<td>MAD20</td>
<td>6E2/53</td>
<td>100</td>
<td>IgG1/κ</td>
<td>EGF-2, &quot;T-SR&quot;-dependent</td>
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<td>500</td>
<td>IgG2b/κ</td>
<td>EGF-1, 144K/2509</td>
<td>3</td>
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<tr>
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<td>6-16</td>
<td>K1/Well</td>
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<td>IgG1</td>
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<td>500</td>
<td>IgG1</td>
<td>1412IETLY-TKFLS1491</td>
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<td></td>
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<td>17.1-3</td>
<td>1000</td>
<td>IgG1</td>
<td>1412IETLY-TKFLS1491</td>
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<tr>
<td>MAD20</td>
<td>9.2-6-2</td>
<td>2000</td>
<td>IgG1</td>
<td>1078NSLNNPHN-RVSGSG1231</td>
<td>6</td>
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<td>6-16</td>
<td>Conserved</td>
<td>9.8-4-4-2</td>
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<td>IgG1</td>
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<td></td>
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<td>12.4-3-4</td>
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<td><strong>MSP-1</strong></td>
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<td>K1/Well</td>
<td>10-2B</td>
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<tr>
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<td>IgG1</td>
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<tr>
<td></td>
<td>3</td>
<td>K1/Well</td>
<td>13.2-3</td>
<td>1000</td>
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<td>MAD20</td>
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<td>1000</td>
<td>IgG2b/κ</td>
<td>n127Q-Q287n-dependent</td>
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<td>2</td>
<td>K1</td>
<td>12.2-1-1</td>
<td>1000</td>
<td>IgG1</td>
<td>3D7 repeats (SAQ)n</td>
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<tr>
<td>&quot;</td>
<td>123D3</td>
<td>500</td>
<td>IgG2b/κ</td>
<td>Palo Alto repeats (SGT)n</td>
<td>9</td>
</tr>
<tr>
<td>&quot;</td>
<td>CE2.1</td>
<td>60</td>
<td>IgG1/κ</td>
<td>Palo Alto repeats (SGT)nn</td>
<td>10</td>
</tr>
<tr>
<td>&quot;</td>
<td>polyclonal *</td>
<td>1000</td>
<td>-</td>
<td>Palo Alto and 3D7 bl. 2</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>RO33</td>
<td>31.1-5 **</td>
<td>2000</td>
<td>IgG1/κ</td>
<td>65PAPAIVTQ-SDAKEYSL116</td>
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<tr>
<td>&quot;</td>
<td>31.2-2-6 **</td>
<td>2000</td>
<td>IgG1/κ</td>
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<tr>
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<td>-</td>
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<td>11</td>
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<tr>
<td>2</td>
<td>MAD20</td>
<td>polyclonal *</td>
<td>200</td>
<td>-</td>
<td>MAD20 and Well bl. 2</td>
</tr>
</tbody>
</table>


* Sera raised against recombinant MSP-1 fragments in mice
** mAbs raised against recombinant MSP-1
Table 2.2. Antibody reagents for IFA typing (continuation)

### B) MSP-2

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Isotype</th>
<th>Epitope</th>
<th>Refs.</th>
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<tbody>
<tr>
<td>A 4</td>
<td>12.3-2-2</td>
<td>500</td>
<td>IgG1</td>
<td>Group-specific dimorphic</td>
<td>4</td>
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<tr>
<td></td>
<td>12.5-1-2</td>
<td>500</td>
<td>IgG1</td>
<td>Group-specific dimorphic</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>12.7-1-2-4</td>
<td>500</td>
<td>IgG1</td>
<td>Group-specific dimorphic</td>
<td>4</td>
</tr>
<tr>
<td>3,R1</td>
<td>113.1</td>
<td>500</td>
<td>IgG3</td>
<td>Polymorphic repeat GSAGS</td>
<td>13</td>
</tr>
<tr>
<td>&quot;</td>
<td>113.2</td>
<td>100</td>
<td>IgM/κ₇,λ</td>
<td>Polymorphic repeat GSAGS</td>
<td>13</td>
</tr>
<tr>
<td>&quot;</td>
<td>4-4F</td>
<td>200</td>
<td>IgM/κ</td>
<td>Polymorphic repeat GSAGS/GGSA</td>
<td>14</td>
</tr>
<tr>
<td>&quot;</td>
<td>8-5D</td>
<td>200</td>
<td>IgM/κ</td>
<td>Polymorphic repeat GSAGS/GGSA</td>
<td>14</td>
</tr>
<tr>
<td>&quot;</td>
<td>13.4</td>
<td>1000</td>
<td>IgG1</td>
<td>Polymorphic repeat T9/94 GSAGA</td>
<td>15</td>
</tr>
<tr>
<td>&quot; Polyclonal α TTn₅₇ *</td>
<td>300</td>
<td>-</td>
<td>Polymorphic repeat GASGRAGA</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>&quot; Polyclonal α7G8₅₇ *</td>
<td>400</td>
<td>-</td>
<td>Polymorphic repeat GGSGSA</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>&quot; Polyclonal αT9/96₅₇ *</td>
<td>300</td>
<td>-</td>
<td>Polymorphic repeat GAVAGSAGA</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>B 3,R1</td>
<td>8G10/48</td>
<td>1000</td>
<td>IgG2b/κ</td>
<td>Group-specific dimorphic (STNS)</td>
<td>2</td>
</tr>
<tr>
<td>&quot;</td>
<td>Polyclonal αK1₅/₃ *</td>
<td>300</td>
<td>-</td>
<td>Same reactivity as 8G10/48</td>
<td>12</td>
</tr>
<tr>
<td>&quot;</td>
<td>Polyclonal αT9/105 12/6 *</td>
<td>200</td>
<td>-</td>
<td>Group-specific dimorphic</td>
<td>12</td>
</tr>
<tr>
<td>3,R2</td>
<td>8F6/49</td>
<td>50</td>
<td>IgG3/2b</td>
<td>Polymorphic repeat DTPTATE</td>
<td>2</td>
</tr>
<tr>
<td>&quot;</td>
<td>Polyclonal αK1₁₃/₃ *</td>
<td>100</td>
<td>-</td>
<td>Same reactivity as 8F6/49</td>
<td>12</td>
</tr>
<tr>
<td>Conserved</td>
<td>Polyclonal αK1₁₂/14 *</td>
<td>200</td>
<td>-</td>
<td>Conserved</td>
<td>12</td>
</tr>
</tbody>
</table>

### C) Other antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Isotype</th>
<th>Specificity</th>
<th>Refs.</th>
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</thead>
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<tr>
<td>EXP-1</td>
<td>5.1-4</td>
<td>500</td>
<td>IgG1</td>
<td>Dimorphic aa position 136</td>
<td>16</td>
</tr>
<tr>
<td>&quot;Knobs&quot;</td>
<td>18.2-3-3</td>
<td>100</td>
<td>IgG2b</td>
<td>Conserved</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>18.2-4</td>
<td>100</td>
<td>IgG2b</td>
<td>Conserved</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>9.21-4-2</td>
<td>100</td>
<td>IgM</td>
<td>Conserved</td>
<td>12</td>
</tr>
</tbody>
</table>


* Sera raised against recombinant MSP-2 fragments in mice
and washed as above. Parasite DNA was stained in 4',6'-diamino-2-phenylindole (DAPI) (1:100,000) for 5 min, and the blood films counterstained in 0.1% Evans Blue. After 5 min, the slides were rinsed in PBS and mounted under coverslip with Citifluor (City University, London) or 50% glycerol in PBS. Reactions were read at magnification of 315x or 630x and incident light of 450-490 nm for FITC-fluorescence (green) and 390-440 nm for DAPI (blue). For each isolate, the percentage of schizonts giving mAb-specific positive fluorescence was recorded for each mAb. Serological epitopes are referred to by the same code numbers as the mAbs which identify them.

2.2.4- DNA extraction

An aliquot of 20-100 µl PRBCs was used for extraction of *P. falciparum* DNA by the quick boiling method (Foley *et al.*, 1992). Briefly, PRBCs were washed 5x in 0.5 ml of ice-cold 5 mM sodium phosphate pH 8.0 and centrifuged for 10 min. The supernatant was removed, 50 µl of sterile ddH2O were added to the pellet and boiled for 10 min. After a 10 min spin (g=10,000), the pellet was discarded and the supernatant was used as DNA template for PCR.

2.2.5- Polymerase chain reaction (PCR) genotyping

Type-specific oligonucleotide primers (table 2.3 & figs. 2.1, 2.2) were used to identify distinct allelic forms of *MSP-1* and of *MSP-2* and the presence of mixed infections by several approaches. A PCR method using primers specific for the flanking regions of three known types of polymorphic block 2 of *MSP-1* can detect size differences among alleles of each family. In isolates collected in 1996, block 2 was amplified as described (Cavanagh & McBride, 1997). Briefly, 3 sets of primers specific for the K1-like, MAD20-like or RO33 allelic types were used to amplify the block 2 region with the following conditions: 35 cycles of 95°C for 1 min, 50°C for 15 sec and 72°C for 30 sec. For *MSP-2*, combinations of primers 13 and 6 (40 cycles of 94°C for 1 min 30 sec, 60°C for 15 sec and 72°C for 40 sec) or primers 13 and 14 (94°C for 2 min, 30 cycles of 94°C for 25 sec, 42°C for 1 min and 70°C for 2 min, and 70°C for 10 min), were used to obtain almost full length gene.
Table 2.3. Oligonucleotide primers used for parasite genotyping by PCR amplification and sequencing of MSP-1 and MSP-2 alleles from clinical isolates of *P. falciparum*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region</th>
<th>Code</th>
<th>Nucleotide sequence 5' to 3'</th>
<th>Use and specificity</th>
<th>Refs.</th>
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<tbody>
<tr>
<td>MSP-1</td>
<td>Dimorphic</td>
<td>difs 1</td>
<td>GAGAAGTTCCCCATATCACCAC</td>
<td>Genotyping 5' MAD20 dimorphic type</td>
<td>1</td>
</tr>
<tr>
<td>Blocks 6-16</td>
<td>difs 2</td>
<td>GAATTGCTAGTTATCAACAG</td>
<td>Genotyping 5' K1/Well dimorphic type</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>difs 3</td>
<td>CATCTAAATGACTGAAACATCC</td>
<td>Genotyping 3' common primer</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Polymorphic</td>
<td>O1</td>
<td>CACATGAAAGTTATTCAAAAGACTTGTC</td>
<td>Genotyping 5' block 2 outer nested reaction</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Block 2</td>
<td>O2</td>
<td>GTACGTCTAATTCTATTCCACG</td>
<td>Genotyping 3' block 2 outer nested reaction</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K5</td>
<td>TGTGATCAAAGGAAAGAAGATTAC</td>
<td>Genotyping 5' K1-type inner nested reaction</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K3</td>
<td>GGAATTCTTAGTTGCAAGCTGAGG</td>
<td>Genotyping 3' K1-type inner nested reaction</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>TGTGATCAAAGGAAAGAAGATTACT</td>
<td>Genotyping 5' MAD20-type inner nested reaction</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>GGAATTCTAACTTTATGAGAGAAG</td>
<td>Genotyping 3' MAD20-type inner nested reaction</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R5</td>
<td>TGTGATCAAAGGAAAGAAGATTACT</td>
<td>Genotyping 5' RO33-type inner nested reaction</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>GGAATTCTAACTTTATGAGAGAAG</td>
<td>Genotyping 3' RO33-type inner nested reaction</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>MSP-2</td>
<td>Dimorphic</td>
<td>difs A</td>
<td>ACTGCACAACTGAAAGAAG</td>
<td>Genotyping 5' A dimorphic type</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>difs B</td>
<td>CAGACGTTAAGGAAGAAGAAG</td>
<td>Genotyping 5' B dimorphic type</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>difs C</td>
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<td>Genotyping 3' common primer dimorphic type</td>
<td>1</td>
<td></td>
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<tr>
<td>Conserved</td>
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<td>AAGACCTTAAATGAAATATGACACACATTT</td>
<td>Sequencing</td>
<td>4</td>
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<tr>
<td>(full length)</td>
<td>14</td>
<td>TAATATATATTATTATGAGGAATATATGAGAAT</td>
<td>Sequencing</td>
<td>5</td>
<td></td>
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<tr>
<td></td>
<td>5</td>
<td>GCTTATATATAGTAGATATAAAAGAAGAAG</td>
<td>Sequencing</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>CATGCGATATTGCTAGTGGCTAGTCTAAG</td>
<td>Sequencing</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Regarding isolates collected in 1997, a hot start nested PCR reaction was used to amplify block 2 of *MSP-1*. Primers O1 and O2 (Ranford-Cartwright *et al.*, 1993) were used in the outer reaction with the following conditions: 30 cycles of 94°C for 35 sec, 50°C for 35 sec and 68°C for 2 min 30 sec, and 68°C for 10 min. The inner amplification was carried out using 1-2 µl of the previous reaction and the set of block 2 type-specific primers and conditions described above.

Dimorphic regions were typed by the dimorphic-form specific (DIFS) PCR method (Reeder & Marshall, 1994). Briefly, a combination of 3 primers consisting of two 5' family-specific primers and a 3' common primer could distinguish between MAD20 and Well/K1 alleles of *MSP-1* (primers difs 1, 2 and 3) and between IC1/3D7 and FC27 alleles of *MSP-2* (primers difs A, B and C) by differences in the size of bands in a hot start single PCR. The conditions were: 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 45 sec.

In all cases, a 50 µl reaction mixture was prepared, containing 1-10 µl of extracted DNA solution, 5 µl of 2 mM dNTP mix, 0.5 µl of each primer (≥ 25-100 pmol), 5 µl of 10× *Taq* polymerase buffer and 2 U *Taq* polymerase (Boehringer Mannheim). The PCR reactions were run in a Hybaid Omnigene Temperature Cycler. A 10 µl aliquot from each amplification was electrophoresed at 90 V on 2% agarose gels in 1×TBE buffer, stained with 0.5 µg/ml ethidium bromide and visualized by UV transilluminator.

### 2.2.6- DNA sequencing

*P. falciparum* isolates from 1996 which appeared to be single clone infections by initial PCR amplification were selected to carry out sequencing of the block 2 region of *MSP-1*, and almost full length *MSP-2* gene. PCR products were purified using spin UF 100 minicolumns (Costar). Direct automated sequencing was performed with the corresponding PCR primers with ABI PRISM™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). Sequences were analysed by the GCG package.
As it has been shown in many other studies, multiple-clone *P. falciparum* infections were frequently encountered among Malawian patients. Multiplicity of infections was analysed by different methods, depending on the typing technique and the marker used.

### 2.2.7.1- IFA serotyping

By IFA, antigenic diversity of the MSP-1, MSP-2 and EXP-1 proteins is defined by reactivity with a panel of type-specific Abs, and epitopes are referred to by the same code numbers as the Abs which identify them. The prevalence of each marker epitope was measured as the percentage of isolates containing parasites which express the epitope i.e. the denominator is the total number of isolates. An isolate is defined as a sample of parasites taken from a patient on a single occasion. The presence of mixed clone infections by IFA was indicated when (i) only a proportion of parasites expressed certain polymorphic epitopes, and (ii) certain epitopes which are mutually exclusive and never occur together on the same parasites were detected within the isolate. Thus, the number of clones per mixed isolate is estimated by combining the serological reactivities of a panel of type-specific Abs using 3 polymorphic markers. With multiple-clone infections, the epitope prevalences for each alternative polymorphic or dimorphic epitope do not necessarily add up to 100. The relative proportion of each *P. falciparum* clone in a mixed infection can be estimated by IFA, and isolates in which a majority (50 to 100%) of parasites expressed a given epitope were analysed separately from those in which only a minority (1 to 50%) of parasites expressed the epitope.

In addition, double-label IFA (see chapter 3) allows the resolution of the antigenic phenotype of the MSP-1 and MSP-2 proteins in parasites within a mixed isolate (Conway & McBride, 1991). The frequency of a given epitope or their combination within each block of the MSP-1 and MSP-2 proteins was calculated as the number of parasite clones containing the epitope or their combination divided by the total number of clones detected by IFA among all the isolates. In this case, epitopes which occur in parasites which constitute the majority within an isolate (50
to 100%) were also distinguished from those occurring in any parasite >1% detected within an isolate.

2.2.7.2- PCR genotyping

By PCR typing, three polymorphic block 2 regions of *MSP-1*, K1, RO33 and MAD20, and the two dimorphic block 16 regions, MAD20 and K1/Well, can be distinguished. Similarly, for *MSP-2* the two alternative genotypes IC1/3D7 or A, and FC27 or B can be distinguished.

The presence of mixed clone infections in an isolate was detected in the case of (i) simultaneous presence of PCR bands specific for mutually exclusive genotypes in any of these loci, and (ii) size polymorphisms among different block 2 *MSP-1* alleles of the K1 or MAD20 genotypes identified as distinct PCR bands. In this analysis, the number of alleles at each locus is determined by the number of distinct PCR bands amplified for that locus. The number of clones per isolate was estimated by the maximum number of alleles detected by combining the 3 PCR reactions.

As described above for IFA (section 2.2.7.1), the prevalence of a given genotype was measured as the percentage of isolates which were positive for the genotype. The frequency of each genotype at each locus was calculated as the number of alleles of a given genotype divided by the total number of alleles detected by the correspondent PCR reaction in all the isolates (the total number of alleles used in the denominator vary for each PCR method because the power of resolution of the number of alleles depends on the locus).

2.2.7.3- Association of *MSP-1* or *MSP-2* alleles with disease severity

Associations between presence of particular epitopes or genotypes and disease manifestations, and between multiplicity of infections and disease severity were analysed by Chi-squared ($\chi^2$) or Fisher’s exact tests. Yates' continuity corrections were applied when appropriate. Mean numbers of parasite clones per isolate were compared among different groups of patients by $t$-tests. Only isolates from 1997 were included in this analysis because of the inadequate numbers of isolates collected in 1996.

In addition, data on all patients surveyed was introduced into the SAS statistical analysis package (SAS, 1990) and analysed using standard multiple
regression techniques. The relationship between host's disease status as a binary trait and parasite's genotype and multiplicity of infections as explanatory variables was analysed by PROC GENMOD (categorical model), using a binomial distribution for the dependent variable. The model was adjusted for the confounding variables of parasitaemia, age, sex, outcome and date of admission. Significance was defined at the 5% level.

2.3- Results

2.3.1- Mixed *P. falciparum* infections and their relationship with malaria disease severity

2.3.1.1- Mixed infections detected by IFA serotyping

During May 1996, 33 blood samples from children with severe malaria were collected. On the basis of the parasitaemia (thin film >0.5%), 27 *P. falciparum* isolates were selected for *in vitro* culture. 12 isolates matured to schizonts (44% success rate) and were subsequently typed by IFA. In 1997, PRBCs from 173 patients were set up in culture and 134 of the isolates (77.5%) matured to schizonts that were typed by IFA.

Mixed *P. falciparum* infections were common (58.6%) among the 134 isolates cultured in 1997, with a mean number of clones ± SD per isolate of 2.0 ± 1.0. Detection of mixed infections by double-staining IFA is illustrated in fig 2.3. Table 2.4 summarises data on mixed infections in parasites collected from acutely ill patients with various disease symptoms, mean number of clones per isolate (A) and percentages of single and mixed infections (B) were calculated for MSP-1 and MSP-2 separately and combined by selecting the maximum number of different clones resolved by each marker in each isolate.

According to MSP-1 serotyping, the mean number of clones per isolate was significantly lower in CM (n=26, mean ± SD=1.62 ± 0.75) than in UM patients (n=48, mean ± SD=2.15 ± 1.03) (t test, p<0.05). In addition, the proportion of infections having ≥ 3 clones per isolate was significantly higher in UM (n=48, 43.75%) than in
Fig 2.3. Detection of mixed infections in peripheral blood by double-labelled IFA
A) Cultured parasites obtained from peripheral blood from a child infected with a mixture of two *P. falciparum* clones, serotyped for MSP-1 block 2 as K1 (red, 80%) and RO33 (green, 20%) types. Each schizont (S) is positive for either mAb 123D3 (K1-type, isotype IgG2b) + RITC-conjugated αIgG2b, or mAb 31.1 (RO33-type, isotype IgG1) + FITC-conjugated αIgG1 (315× magnification). B) DAPI-stained parasites (blue) at different stages of their development: trophozoites (T) and multinuclei schizont forms (S). C) Same field showing that only schizonts are recognised by either one or the other MSP-1-specific typing mAb. See section 3.2.2, chapter 3, for IFA double-staining methods.
Table 2.4. Analysis of mixed infections detected by MSP-1 and MSP-2 IFA serotyping

The number (n) of clones was resolved by the combined serological reactivities of a panel of 21 MSP-1 and 16 MSP-2 type-specific Abs. These Abs identify alternative polymorphic and dimorphic epitope variants of these merozoite surface antigens, some of which never occur together in the same parasite (see section 2.2.7). The proteins are always expressed in schizonts and are products of single copy genes in the haploid genome of *P. falciparum*, thus allowing *in situ* visualisation of mixed serotype infections. Significant differences in multiplicity of infections between patients' groups are underlined and explained in the text.

### A) Mean number of clones per isolate

<table>
<thead>
<tr>
<th></th>
<th>n isolates</th>
<th>n clones</th>
<th>mean ± SD clones/isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MSP-1</td>
<td>MSP-2</td>
</tr>
<tr>
<td>Total</td>
<td>134</td>
<td>260</td>
<td>257</td>
</tr>
<tr>
<td>CM</td>
<td>26</td>
<td>42</td>
<td>43</td>
</tr>
<tr>
<td>SMA</td>
<td>27</td>
<td>54</td>
<td>56</td>
</tr>
<tr>
<td>CM+SMA</td>
<td>11</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>UM</td>
<td>49</td>
<td>103</td>
<td>99</td>
</tr>
</tbody>
</table>

### B) Percentages of single- and multiple-clone infections

<table>
<thead>
<tr>
<th></th>
<th>1 clone</th>
<th>2 clones</th>
<th>3 clones</th>
<th>≥4 clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSP1</td>
<td>MSP2</td>
<td>both</td>
<td>MSP1</td>
</tr>
<tr>
<td>Total</td>
<td>43.6</td>
<td>43.6</td>
<td>41.4</td>
<td>24.1</td>
</tr>
<tr>
<td>CM</td>
<td>53.8</td>
<td>53.8</td>
<td>53.8</td>
<td>30.8</td>
</tr>
<tr>
<td>SMA</td>
<td>40.7</td>
<td>33.3</td>
<td>33.3</td>
<td>29.6</td>
</tr>
<tr>
<td>CM+SMA</td>
<td>63.6</td>
<td>63.6</td>
<td>63.6</td>
<td>18.2</td>
</tr>
<tr>
<td>UM</td>
<td>37.5</td>
<td>39.6</td>
<td>37.5</td>
<td>18.8</td>
</tr>
</tbody>
</table>
CM (n=26, 15.38%) ($\chi^2$, p<0.025), or CM ± SMA cases (n=37, 16.21%) ($\chi^2$, p<0.01). CM patients also had a significantly lower proportion of multiple infections composed of ≥ 3 clones than non-cerebral cases (n=75, 38.66%) ($\chi^2$, p<0.05 for strictly CM and p<0.025 for CM ± SMA).

According to MSP-2 serotyping, there were no statistically significant differences in the mean number of clones per isolate between CM and UM. However, the proportion of infections having ≥ 3 clones per isolate was again significantly higher in UM (n=48, 29.16%) than in all SM cases (n=64, 14.28%) ($\chi^2$, p<0.05).

Overall, therefore, MSP-1 had a higher power to detect multiple infections than MSP-2, probably due to the larger panel of typing Abs used. When both markers were combined, almost equivalent results to MSP-1 alone were obtained (table 2.4).

In conclusion, IFA serotyping results indicated that children suffering from CM tended to have *P. falciparum* infections composed by less number of clones than those from UM patients.

### 2.3.1.2- Mixed infections detected by PCR genotyping

PCR was a more sensitive method to detect the presence of parasites than microscopic examination of blood smears. All of 255 tested blood samples which were parasite positive by microscopy were also positive by PCR, whereas 91.4% of acute samples which tested positive by PCR had also been diagnosed as positive by blood smear. In contrast, only 39.8% (47/118) of convalescent samples which tested positive by PCR had been diagnosed as slide positive. The convalescent children were mostly asymptomatic, and their parasitaemias were very low. In these cases, nested PCR for amplification of *MSP-1* block 2 was the more reliable method capable of detecting very low parasitaemias. Conceivable reasons for the discrepancy between PCR and microscopy results are that slides from the asymptomatic children were generally examined less thoroughly and by less experienced hospital staff than slides from sick children, and therefore slides with only a few parasites could have been incorrectly diagnosed as negative.

RBCs were available from 466 cases for detection of parasite DNA by PCR. The overall efficiency of PCR amplification for *MSP-1* dimorphic regions by the DIFS
method was 89.2%, lower than that of nested PCR of block 2, which yielded a band in 95.5% of isolates which were shown to contain parasites. The DIFS typing of dimorphic \textit{MSP-2} regions was the least successful (76.6%). Fig 2.4 illustrates how PCR typing detects polymorphisms within the \textit{MSP-1} and \textit{MSP-2} genes and, thus, the presence of mixed infections.

The DIFS typing methods do not detect size differences between PCR bands within an allelic family, and thus the maximum number of different clones that DIFS alone can resolve is 2 in mixtures of the \textit{MSP-1 MAD20} and \textit{K1/Well} dimorphic types, or \textit{MSP-2 IC1/3D7 (A)} and \textit{FC27 (B)} types. In contrast, genotyping of \textit{MSP-1} block 2 is done in 3 separate PCR reactions, with a separate set of primers for each of the types, and detects additional size variations within the K1 and MAD20 types due to repeat sequences. Thus, combination of \textit{MSP-1} block 2 and DIFS typing increases the level of resolution of the PCR.

A higher sensitivity of the PCR resulted in an overall higher mean number of clones per isolate of 2.46 ± 1.00, and in a higher maximum number of distinct clones detected per isolate (up to 6) than those calculated by IFA. Table 2.5 summarises data on the multiplicity of infections combining \textit{MSP-1} and \textit{MSP-2} PCR typing results in each disease group.

---

\textbf{Fig. 2.4. PCR genotyping of \textit{MSP-1} and \textit{MSP-2} genes (figure in next page)}

Detection of mixed \textit{P. falciparum} infections by PCR genotyping visualised by 2% agarose gel electrophoresis

A) The two dimorphic block 16 regions of \textit{MSP-1} were distinguished by differences in the size of the PCR bands amplified: MAD20 (475bp) and K1/Well (204bp). Tracks 9, 10 and 11 correspond to mixed infection of the \textit{MSP-1 MAD20} and \textit{K1/Well} dimorphic types. Tracks 1, 2, 3, 4, 5, 7 and 8 correspond to apparent single infections of the MAD20-type, the predominant in Malawi. Tracks 6 and 12 correspond to aparasitaemic children (PCR negative) B) Three sets of nested PCR reactions were run for each of the three polymorphic block 2 types of \textit{MSP-1}: K1 (lanes 1, 4, 7, 10), MAD20 (lanes 2, 5, 8, 11) and RO33 (lanes 3, 6, 9, 12). Results are shown for four children who contained mixed infections. Patient 1 (tracks 1-3) had a double infection of the K1 and MAD20 block 2 types. Patient 2 (lanes 4-6) had a double infection of the K1 and RO33 types. Patient 3 (lanes 7-8) had a triple infection of the K1, MAD20 and RO33 types. Patient 4 (lanes 10-12) had a quadruple infection of MAD20, RO33 plus two different K1 block 2 alleles distinguished by size polymorphism. C) The two dimorphic regions of \textit{MSP-2} were distinguished by differences in the size of the PCR bands amplified: FC27 or B (306bp) and IC1/3D7 or A (260bp). Tracks 3 and 4 correspond to mixed infection of the \textit{MSP-2} types A and B. Tracks 1, 7 and 11 correspond to apparent single infections with type B parasites, whereas tracks 2, 5, 8, 9, 10 correspond to apparent single infections of type A parasites, the predominant in Malawi. Tracks 6 and 12 correspond to aparasitaemic children (PCR negative). The first lane of each gel contain the DNA marker with the location of the 500bp band indicated.
Fig 2.4 PCR genotyping of *MSP-1* and *MSP-2* genes

**A) DIFS method for detection of *MSP-1* dimorphic types**

DNA marker VI

500 bp

MAD20
475 bp

K1/Well
204 bp

**B) Nested PCR for detection of *MSP-1* block 2 types**

100 bp ladder

500 bp

**C) DIFS method for detection of *MSP-2* dimorphic types**

DNA marker VI

500 bp

FC27 (B)
306 bp

IC1/3D7 (A)
260 bp
The number (n) of clones was determined as the maximum number of distinct PCR bands combining the MSP-1 and MSP-2 loci in each isolate (see section 2.2.7). Significant differences in multiplicity of infections between patient's groups are underlined and explained in the text.

**Table 2.5. Analysis of mixed infections detected by MSP-1 and MSP-2 PCR genotyping**

The number (n) of clones was determined as the maximum number of distinct PCR bands combining the MSP-1 and MSP-2 loci in each isolate (see section 2.2.7). Significant differences in multiplicity of infections between patient's groups are underlined and explained in the text.

<table>
<thead>
<tr>
<th></th>
<th>n isolates</th>
<th>n clones</th>
<th>mean ± SD clones/isolate</th>
<th>1 clone</th>
<th>2 clones</th>
<th>3 clones</th>
<th>&gt;4 clones</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>279</td>
<td>686</td>
<td>2.46 ± 1.00</td>
<td>15.8</td>
<td>40.5</td>
<td>29.0</td>
<td>14.7</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>93</td>
<td>209</td>
<td>2.25 ± 0.89</td>
<td>20.4</td>
<td>43.0</td>
<td>29.0</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>SMA</td>
<td>50</td>
<td>135</td>
<td>2.70 ± 1.20</td>
<td>16.0</td>
<td>32.0</td>
<td>28.0</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>CM+SMA</td>
<td>26</td>
<td>61</td>
<td>2.35 ± 0.80</td>
<td>11.5</td>
<td>50.0</td>
<td>30.8</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>UM controls</td>
<td>92</td>
<td>243</td>
<td>2.64 ± 0.98</td>
<td>8.7</td>
<td>41.3</td>
<td>30.4</td>
<td>19.6</td>
<td></td>
</tr>
<tr>
<td>SNM controls</td>
<td>18</td>
<td>38</td>
<td>2.1 ± 1.02</td>
<td>33.3</td>
<td>33.3</td>
<td>22.2</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>

According to disease severity, the mean number of clones per isolate was significantly lower in CM patients (n=93, mean ± SD=2.25 ± 0.89) than in UM (n=92, mean ± SD=2.64 ± 0.98) or SMA patients (n=50, mean ± SD=2.70 ± 1.20) (t test, p<0.01). This was because single infections were significantly more common in CM (20.4%) than in UM (8.7%) cases (χ², p<0.025). Consistent with the IFA results, multiple infections with ≥ 4 different clones were less frequent in CM (7.5%) than in combined non-cerebral cases (n=160, 20%) (χ², p<0.01), and infections containing ≥ 3 clones were also less common in CM (36.5%) than in UM and SMA cases pooled together (n=142, 50.7%) (χ², p<0.05). All these associations remained statistically significant when the effects of parasitaemia, age, sex and date of admission were accounted for in a multiple regression model. A low complexity of infections was also observed in the SNM control group. However, this result is likely to be biassed by the fact that most SNM patients were infected at very low parasitaemias, probably around the PCR detection threshold, resulting in an underestimation of the number of clones.

In conclusion, a lower multiplicity of *P. falciparum* infections was found in CM compared to UM or SMA patients, consistent with the IFA typing results. In addition, PCR revealed a higher multiplicity of infections among SMA patients than in any other group of patients.
Among all clinical isolates collected in Malawi, the MAD20-type of the major dimorphic regions spanning block 6 to 16, was much more prevalent than the alternative K1/Well-type. In 1997 the prevalences were 96.3% for MAD20-type and 13.4% for K1/Well-type. In 1996, all isolates typed expressed MAD20-type epitopes and only one mixed isolate contained also parasites with MSP-1 of K1/Well-type.

Concerning the polymorphic block 2, the most prevalent type in these patients was consistently the K1-type. In 1997, K1 (78.4%), predominated over RO33 (47.8%) and MAD20 (40.3%). In 1996, all 12 typed isolates contained parasites with the Palo Alto and/or 3D7-specific epitopes (K1-type) at comparable prevalences. 7/12 isolates were double infections and contained also parasites positive for RO33 epitopes; 3/12 isolates were K1 + RO33 + MAD20 triple-clone mixtures.

In block 3, the prevalence of the dimorphic epitope 13.2 (K1/Well) was slightly higher (68.7%) than the prevalence of the alternative MAD20 epitope 9.5 (59.7%) in 1997, and similar in 1996; 13.2- 9.5- was unfrequent.

In 1997, the dimorphic epitope 10-2B epitope (K1/Well) located at the C-terminal half of block 4 (4b) was more much more prevalent (97.7%) than the the alternative 12.1 epitope (MAD20) (33.6%). In 1996, 11/12 isolates had epitope 10-2B, whereas 7/12 isolates expressed 12.1. The frequency of double negatives 10-2B- / 12.1- parasites was extremely low (1 isolate).

Epitopes in block 17 were recognised by mAbs 111.4 (EGF-1, "Q"- dependent) and 6E2/53 (EGF-2, "T-SR"-dependent). In 1997, the prevalence of the epitope 111.4 was 83.6%, roughly double than that of 6E2/53 (39.6%). In 1996, 9/12 (75%) isolates contained epitope 111.4, whereas 6/12 (50%) were positive for 6E2/53. Since these epitopes are located in different domains of block 17, it was possible to analyse the frequency of association of the epitopes. The combination 111.4+ 6E2/53- was the commonest (64.9%), followed by 111.4- 6E2/53+ (23.4%) and by 111.4- 6E2/53- (10.8%); double positives (Q-TSR) were very rare (2 cases).

In isolates which constituted single clone infections, or isolates where 1 clone represented more than 50% of typed parasites, certain combinations of epitopes at
different blocks were observed to occur more frequently than others. As illustrated in fig 2.5, K1 block 2 epitopes (12.2, 123D3, CE2.1) were associated with K1 block 3 epitope (13.2) (p<0.001), which was associated with K1 block 4b epitope (10-2B) (p<0.005). Conversely, RO33 and MAD20 epitopes (31.1, 31.2 and polyclonal sera) occurred more often together with MAD20 block 3 epitope (9.5) (p<0.001) and this with MAD20 block 4b epitope (12.1) (p<0.025).

**Fig 2.5. Associations between epitopes in blocks 2, 3 and 4 of MSP-1.** Epitopes depicted as boxes are joined by lines indicating statistically significant nonrandom associations, determined by $\chi^2$ tests on $2 \times 2$ tables containing the number of parasite clones with both, neither, or one or the other of a given pair of epitopes. A solid line indicates a positive association and a broken line a negative association. P values were < 0.001 except for 13.2 vs. 10-2B and 9.5 vs. 10-2B (p<0.005); and 13.2 vs. 12.1 and 9.5 vs. 12.1 (p<0.025). No lines joining boxes means mutually exclusive epitopes which do not occur together in the same parasite.

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<th>MSP-1 block</th>
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<td>13.2</td>
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<td></td>
<td>9.5</td>
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<tr>
<td>MAD20</td>
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<td>12.1</td>
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2.3.2.2- *Merozoite surface protein 2 and exported protein 1*

Overall, the proportion of isolates in 1997 containing parasite clones expressing MSP-2 A or B serogroups was the same (68% each). When only those parasite clones that constituted the majority population (>50%) in mixed infections were considered, there was a predominance of serogroup A (58.2%) over B (47%). However, among those isolates composed of single clone infections, there was a higher prevalence of serogroup B (detected by mAb 8G10/48, 56.9%) over A (43.1%). In 1996, 9/12 (75%) isolates had MSP-2 type A and 7/12 (38%) had type B.

Regarding EXP-1, the proportion of isolates that reacted positively with the dimorphic mAb 5.1 was very high in both 1997 (82.8%) and 1996 (9/11). Among single-clone isolates, as found with MSP-1 and MSP-2 typing, the prevalence of 5.1+
isolates was 67.24%. Finally, all isolates tested expressed epitopes recognised by mAbs 18.2-3, 18-2.4 and 9.21 in (or under) the membrane of PRBCs, possibly associated with "knobs".

2.3.3- Genetic polymorphism of merozoite surface protein genes detected by PCR genotyping and sequencing

In total agreement with IFA typing, PCR typing also showed that the prevalence of one of the two major MSP-1 dimorphic types i.e. MAD20, was much higher (97% overall) than that of the alternative K1/Well dimorphic type (9.6%). Analysis of MSP-1 block 2 revealed that K1-type was once more the predominant type (81%), whereas MAD20-type was more prevalent (59%) than RO33-type (49%), contrasting with the IFA results (40.3% and 47.8% respectively). A similar distribution of block 2 was recorded amongst the 23 isolates genotyped in 1996 (91% K1, 52% MAD20, 35% RO33). From this set of samples, 6 new block 2 MSP-1 alleles isolated from CM patients were also sequenced. Two alleles belonged to the MAD20 block 2 family, and 4 to the K1 block 2 family (fig 2.6).

Concerning MSP-2, to simplify the terminology in this and the following sections, the two known allelic families IC1/3D7 and FC27 will be designated using the serotypic nomenclature A and B, respectively. MSP-2 group A alleles were more prevalent (73%) than group B (48%) among isolates collected in 1997. In 1996, MSP-2 alleles were not typed by PCR. Instead, 9 new MSP-2 alleles were identified in this year by sequencing: 5 type A, 3 type B and 1 hybrid (fig 2.7). Interestingly, all three type B alleles contained the recently described (NAP)ₙ repeat unit 5' upstream from the 32-mer repeats (Irion et al., 1997) which, in the case of one isolate (MP665) consisted of 12 copies, alternating with a novel (NVP) sequence. Furthermore, the recombinant allele I found in Malawi (MP663) was almost identical to the previously published NIG60 allele (Marshall et al., 1991), differing only in a single nucleotide change (A to G) in the second 12-mer repeat (Dobaño et al., 1997). Amongst six MSP-2A alleles, 3 contained R1 repeat sequences characteristic of the RO33 MSP-2 allele (GASGSAGS)ₙ, one had R1 sequences found in the Thai Tn MSP-2 allele (GASGRAGA)ₙ, whilst MP668 comprised previously unidentified R1 tandem repeats.
Fig. 2. Alignment of predicted amino acid sequences of \textit{MSP-1} block 2 in clinical isolates of \textit{P. falciparum}

MP numbers refer to \textit{P. falciparum} isolates obtained from different patients. In one isolate (MP668), there were two parasite clones with K1 (K) and MAD20 (M) types, both of which were sequenced. Positions of amino acids are numbered in reference to the beginning of the sequence of block 2 of the K1 prototype (Mackay \textit{et al}., 1985). Sequences of the K1 and MAD20 prototype (Tanabe \textit{et al}., 1987) are included for comparison of the characteristic tripeptide repeats. Asterisks indicate stop codons included in the 3' primers. Amino acid positions which could not be predicted due to unclear nucleotide readings in the sequencing gel are indicated by an "x".

\textbf{MAD 20 type}

\textbf{K1 type}

\textbf{MAD 20 type}

\textbf{K1 type}
Fig. 2.7. Alignment of predicted amino acid sequences of MSP-2 in clinical isolates of P. falciparum

A) MSP-2 group A

MP numbers refer to P. falciparum isolates obtained from different patients. Positions of amino acids are numbered in reference to the beginning of allele MP664. The sequences of primers 13 (5') and 6 (3') are underlined. Amino acid positions which could not be predicted due to unclear nucleotide readings in the sequencing gel are indicated by an "x".

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</table>
Fig. 2.7. Alignment of predicted amino acid sequences of MSP-2 in clinical isolates of *P. falciparum* (continuation)

3) MSP-2 group B and recombinant

MP numbers refer to *P. falciparum* isolates obtained from different patients. Positions of amino acids are numbered in reference to the beginning of the recombinant allele MP663. The sequences of primers 13 (5') and 6 (3') are underlined. The sequence from the recently described repeat (NAP/NVP)$_n$ is indicated in italics. Aminoacid positions which could not be predicted due to unclear nucleotide readings in the sequencing gel are indicated by an "x".

<table>
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<th>NESKYSNTFI NNAYNMSIRR SMKESNP... ............ ..PTGAGSGA</th>
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<td>........NTFI NNAYNMRI RR SMANEGSNTK SVGANAPNAP KADTVASVSQ</td>
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<tr>
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<td>MP671c</td>
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<tr>
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2.3.4- Relationship between genetic and antigenic polymorphism

The relationship between genetic and antigenic polymorphism in the MSP-1 and MSP-2 proteins was investigated in 146 isolates which were successfully cultured in vitro and thus serotyped by IFA. Genomic DNA extracted from the same isolates was genotyped by PCR in the two loci (dimorphic and polymorphic regions of MSP-1, and dimorphic region of MSP-2), and thus correlations between genetic and antigenic types could be established for each domain. Results from IFA and PCR typing were in agreement in most isolates from 1997, with 544/611 (89%) concordant paired comparisons between a genetic type detected by PCR and the correspondent antigenic type detected by IFA. In 8% of paired comparisons a genotype identified by PCR was not detected by IFA. This was most probably because it was a minority population in a mixed infection, detected with a higher sensitivity by the PCR technique. Only in 2.9% of all paired comparisons was there a discordance between a positive IFA result which was not reproduced by PCR, as detailed below for each antigen.

The best correlation between PCR and IFA typing was in the dimorphic regions of MSP-1. Practically all 1997 isolates whose MSP-1 protein was typed as MAD20 dimorphic type by IFA (129) were also genotyped as MAD20-type by PCR (128). Similarly, all 18 isolates which were positive for the K1/Well dimorphic type by IFA were also positive for this type by PCR. In four isolates detected to be mixtures of MAD20 and K1/Well types by PCR only MAD20 type was detected by IFA.

Likewise, there was a very good correlation in the identification of the polymorphic block 2 types of MSP-1 by the two methods. All of 105 isolates from 1997 serotyped as K1-type by IFA were also genotyped as K1 positive by nested PCR; only 9 mixed isolates which yielded a K1-type band by PCR were undetected by IFA. RO33 typing agreed in 66 cases, but in 6 isolates IFA failed to detect parasites corresponding to a positive RO33 PCR band. Two isolates which were serotyped as K1+RO33 gave K1+MAD20 bands by PCR. After repeating the two discordant reactions two times with new DNA templates and discarding possible cross-contamination or mislabelling, it was concluded that the two MSP-1 genotypes possibly represented a block 2 hybrid between MAD20 (5') and RO33 (3'). As shown
in table 2.3, the 3' reverse primers specific for MAD20 (M3) and RO33 (R3) are identical, except for one nucleotide change (in bold). Thus, it is possible that the DNA of a MAD20-RO33 recombinant type would be amplified with the MAD20 pair of primers and, at the same time, the translated product would be recognised by RO33-specific Abs due to epitopes at the C-terminal end of block 2. This possibility could be confirmed by combining M5-R3 primers in the same PCR reaction and by sequencing. All of 59 isolates typed as MAD20 by IFA were also genotyped as MAD20 by PCR, but there were 28 isolates which were only positive by PCR and not by IFA. This was probably because there is only polyclonal serum available for typing of MAD20-type, in contrast to the other block 2 types which can be identified by polyclonal as well as monoclonal Abs.

The correspondence between genetic and antigenic MSP-2 types was also reasonably good, particularly in the detection of the dimorphic type A. In isolates from 1997, there was agreement between IFA and PCR in the positivity for MSP-2 type A (IC1/3D7) in 91/97 paired comparisons. In 2 cases IFA failed to detect parasites which were positive for MSP-2 type A by PCR, and 4 isolates which were typed as a mixture of A+B by IFA were typed as single type B by PCR. This last disagreement could be due to the presence of recombinant forms between the two MSP-2 allelic families, which have been described before (see 2.1.2), consisting of alleles with A-like sequences at the 5' end and B-like sequences for most of the 3' end of the gene. These hybrids could be positive by IFA for some A-specific epitopes located at the N-terminus of the protein, but would only be identified as B-like alleles by the DIFS PCR, which amplifies a C-terminal dimorphic part of the gene. In the case of MSP-2 type B (FC27) alleles, all 77 isolates which were genotyped as B by PCR were also positive for B by IFA. However, 11 isolates were typed as A+B mixtures by IFA mAbs but only as single A infections by PCR. Possible explanations for this discrepancy include a hypothetical hybrid MSP-2 form consisting of 5'-B-like sequences and 3'-A-like sequences, yet to be described by sequencing, or it could be due to mutations in the dimorphic region of the MSP-2 genes which would impede the 5' difs B primer from annealing to the DNA template from these isolates.
Concerning isolates collected in 1996, the typing of MSP-1 block 2 agreed completely between IFA and PCR in 20/23 isolates; in 2 isolates PCR failed due to shortage of DNA, and in one instance PCR identified a MAD20-type which had been undetected by IFA. A more accurate analysis was performed in 10 cultured samples for which IFA and sequencing data could be obtained. As expected, parasites whose MSP-1 block 2 sequences revealed MAD20-type repeats (SGG/SVA), and Palo Alto-variant repeats (SGT), (fig 2.6) reacted in IFA positively with anti-MAD20 polyclonal serum or mAbs 123D3 and/or CE2.1, respectively.

Similarly, serological reactivities of MSP-2 proteins reflected the MSP-2 dimorphic sequences. A hybrid allele, composed mostly by B-like sequences (fig 2.7), was positive for group B-specific Abs. Moreover, a relationship was established between aa sequences and epitopes recognised by Abs within regions of polymorphic repeats. In group A, MP664 and MP682 alleles, which had MSP-2 RO33-like sequences in R1 (GASGSAGS), reacted positively with mAbs 113.1, 113.2, 4-4F and 8-5D, specific for these repeats (Table 2.2b). Allele MP669 that had the characteristic MSP-2 Thai Tn-like sequence in R1 (GASGRAGA), was positive for anti-Thai Tn polyvalent sera. In group B, the reactivity of mAb 8F6/49 correlated as expected with the presence of certain aa residues (E +ve, K -ve) in the first 12-mer repeat of four sequenced MSP-2 B alleles.

2.3.5- Relationship between parasite diversity and malaria disease severity

The distribution of the different MSP-1, MSP-2 and EXP-1 genotypes and/or serotypes was analysed in groups of patients distinguished by disease severity. Detailed data on the prevalences and frequencies of epitopes detected by IFA (as defined in section 2.2.7) are shown for MSP-1 (tables 2.6 & 2.7) and MSP-2 (tables 2.8 & 2.9). The prevalences and frequencies of MSP-1 and MSP-2 genotypes detected by PCR (as defined in section 2.2.7) are shown in tables 2.10 & 2.11. Due to the satisfactory correlation between IFA and PCR results as shown above, findings on associations between particular parasite types and disease manifestations using both techniques are combined in the following sections.
Table 2.6. Prevalences of MSP-1 marker epitopes detected by IFA

Percentages of isolates expressing each individual MSP-1 epitope. Mixed isolates in which a majority of parasites (50-100%) expressed a given epitope are distinguished from mixed isolates within which less than 50% of schizonts had the epitope. Data for single-clone isolates (100%) are presented separately. Statistically significant differences in prevalences between patient's groups are underlined. p values are detailed in the text.

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a Epitopes are referred to by the same code numbers as the mAbs which identify them, except for epitopes located within the dimorphic regions (blocks 6 to 16), and polymorphic block 2, which are referred to by the prototype isolate which identifies the allelic type (see table 2.2 for details on Ab specificities). PA=Pollo Alto; Poly=polyclonal serum.

b Total refers to all isolates typed, regardless of the patient's status (acute or convalescent, severe or mild); below, data are presented separately for isolates collected from patients presenting with different forms of acute falciparum malaria, as indicated, and are discussed in section 2.3.5.1. 11 patients had both CM and SMA, but they were too few to be a separate category; they are included in the severe malaria (SM) group, together with patients with CM only and SMA only; c Total refers to all mixed- and single-clone isolates.
Table 2.7. Frequencies of MSP-1 marker epitopes detected by IFA

Values are percentages indicating the frequency of each possible combination of epitopes in each block of the MSP-1 protein. The number (n) of clones was estimated for each isolate by combining the reactivities of a panel of MSP-1-specific Abs (as defined in 2.2.7), and was used in the denominator to calculate the frequency of the serotype in each block. Mixed isolates in which a majority of parasites (50-100%) expressed a given block serotype are distinguished from mixed isolates within which >1% schizonts had the combination of epitopes. Statistically significant differences in prevalences between patient's groups are underlined; p values are detailed in the text.

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Table 2.7. Frequencies of MSP-1 marker epitopes detected by IFA (continuation)

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Table 2.8. Prevalences of MSP-2 marker epitopes detected by IFA

Percentages of isolates expressing each individual MSP-2 epitope. Mixed isolates in which a majority of parasites (50-100%) expressed a given epitope are distinguished from mixed isolates within which >1% schizonts had the epitope. Data for single-clone isolates (100%) are presented separately. Statistically significant differences in prevalences between patient's groups are underlined; p values are detailed in the text.

|                | n isolates | % parasites | 12.7 12.5 113.1 4-4F 8-5D 13.4 Poly Tn Poly 7G8 Poly T9/96 Serogroup A | 8G10/48 Pol K1 Poly T9/105 Serogroup B | Pol K1 5/3 12/6 8F6/49 13/3  
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*Epitopes are referred to by the same code numbers as the mAbs which identify them, except for epitopes located within the dimorphic regions (blocks 6 to 16), and polymorphic block 2, which are referred to by the prototype isolate which identifies the allelic type (see table 2.2 for details on Ab specificities).

bTotal refers to all isolates typed, regardless of the patient's status (acute or convalescent, severe or mild); below, data are presented separately for isolates collected from patients presenting with different forms of acute falciparum malaria, as indicated, and are discussed in section 2.3.5.1. 11 patients had both CM and SMA, but they were too few to be included in this analysis as a separate category; they are included in the SM group, together with patients with CM only and SMA only; cTotal refers to all mixed- and single-clone isolates.
Table 2.9. Frequencies of MSP-2 marker epitopes detected by IFA

Values are percentages indicating the frequency of each possible combination of epitopes in each block of the MSP-2 protein. The number (n) of clones was estimated for each isolate by combining the reactivities of a panel of MSP-2-specific Abs (as defined in 2.2.7), and was used in the denominator to calculate the frequency of the serotype in each block. Mixed isolates in which a majority of parasites (50-100%) expressed a given block serotype are distinguished from mixed isolates within which >1% schizonts had the combination of epitopes. Statistically significant differences in prevalences between patient’s groups are underlined; p values are detailed in the text.

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Table 2.10. Prevalences of MSP-1 and MSP-2 genotypes detected by PCR

Percentages of isolates which either contained only one detectable parasite clone, possessing the indicated genotype (M=MAD20, K=K1, R=RO33, A=IC1/3D7, B=FC27) or mixed isolates that contained more than one parasite genotype. The prevalences in each disease group refer only to acute stages; "Total" includes all acute and convalescent samples tested. Statistically significant differences in prevalences between patient's groups are underlined; p values are detailed in the text.

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Table 2.11. Frequencies of MSP-1 and MSP-2 genotypes detected by PCR

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a Number (n) of isolates which were PCR positive for any of the loci; b The number (n) of alleles results from the total number of bands successfully amplified by PCR for each locus.
In blocks 6-16, the prevalence of parasites of the K1/Well-type was higher in SMA (22.2%) than in CM (3.8%) ($\chi^2$, $p<0.05$) or UM (14.3%) cases, as detected by IFA (table 2.6). However, when Yates' continuity correction was applied to the $\chi^2$ test, the statistical significance was lost, probably due to the small sample size and to the fact that few isolates reacted positively with K1/Well-specific mAbs (18/134). Genotype distributions resolved by PCR benefited from a larger number of samples (tables 2.10 & 2.11). Consistent with the IFA results, the prevalence of K1/Well-type was higher in parasites from SMA patients. When anaemic patients (Hb < 5g/dl, n=74) were compared with non-anaemic (n=190), a significantly higher proportion of the former patients had K1/Well-type parasites (20.3% vs. 8.9%) ($\chi^2$, p<0.025) (fig 2.8). Moreover, there was a higher prevalence of mixed MAD20+K1/Well infections in the anaemic (14.9%) vs. non-anaemic patients (6.3%) ($\chi^2$, p<0.05). The difference in the prevalence of K1/Well-type parasites remained statistically significant between SMA and CM cases when it was analysed by multiple regression controlling for parasitaemia, age, sex and multiplicity of infection (p=0.0276). None of the parasites obtained from children with only CM which appeared as single clone infections, or which constituted the predominant parasite in mixed infections, expressed the K1/Well dimorphic type.

Concerning MSP-1 block 2, the prevalence of MAD20-type epitopes detected by IFA was lower in SM (27.4%) than in UM isolates (55.1%) ($\chi^2$, p<0.005). In single infections, the difference in allelic frequencies of the MAD20 type between SM (12.5%) and UM (38.9%) was statistically significant ($\chi^2$, p<0.05) only if Yates' continuity correction was not applied. An apparently higher proportion of the R033-type in SMA vs. non-anaemic patients was not statistically significant.

By PCR, the distributions of the three block 2 types of MSP-1 appeared similar in all groups of patients (fig 2.9). In contrast to IFA, no significant difference was found between SM and UM regarding the distribution of MAD20 block 2 type when only isolates from 1997 were included in the analysis. This discrepancy might partially be explained by the lower sensitivity of IFA vs. PCR in the detection of
Fig 2.8. Distribution of MSP-1 dimorphic (block 16) types

![Bar chart showing distribution of MSP-1 dimorphic types](chart1)

* p < 0.025  K1;  ** p < 0.05  K1+MAD20

Fig 2.9. Distribution of MSP-1 polymorphic block 2 genotypes

![Bar chart showing distribution of MSP-1 polymorphic genotypes](chart2)
MAD20 block 2 types. However, when all isolates typed in 1996 and 1997 were analysed by multiple regression, a significantly higher prevalence of MAD20 block 2 type parasites was revealed in UM patients compared to CM patients, after adjustment for parasitaemia, age, sex, multiplicity of infections and presence of the K1 and RO33 block 2 types (p=0.0447). This difference might partially be explained by a higher mean number of clones in UM vs. CM (see section 2.3.1), as the significance was lost when only the multiplicity of infection was included in the model as a confounder (p=0.0689). Nevertheless, MAD20-type itself seemed to have an independent effect on disease since neither K1 nor RO33 types had any significant influence in this model.

Certain combinations of mixed infections had different frequencies between groups of patients (table 2.10). When SM (n=168) were compared to UM (n=90) cases, the combination RO33 and MAD20 was almost absent in SM (1.8%), whereas it was less rare in UM (7.8%) ($\chi^2$, p<0.05). The distribution of genotypes was also analysed in relation to the disease outcome (survivors, sequelae or death). When parasite isolates from patients who died were compared to isolates from those who survived, there was an increased prevalence of mixed K1 and RO33 types among fatalities (7/32) than among survivors (28/347) ($\chi^2$, p<0.025); nevertheless, the low frequencies of these combinations of genotypes precludes a meaningful statistical analysis.

In block 3, a trend to a higher prevalence of epitope 9.5 in SMA compared to non-anaemic patients did not achieve statistical significance (results not shown).

Regarding epitopes in block 4, there was a lower prevalence of epitope 12.1 (MAD20) in cerebral compared to non-cerebral malaria cases. The overall prevalence of epitope 12.1 was decreased in CM (7.7%) compared to SMA (44.4%) or to UM (44.9%) cases ($\chi^2$, p<0.005) (table 2.6). When prevalence of 12.1 was computed only for parasites which constituted >50% in a mixed infection, the difference remained significant for CM (3.8%) vs. SMA (25.9%) ($\chi^2$, p<0.05), but it was lost when Yates' continuity correction was applied. However, the frequency of epitope 12.1 was significantly lower in CM than SMA (either if all, $\chi^2$, p<0.05, or only majority alleles, p<0.01, were scored), or UM cases ($\chi^2$, p<0.05) (table 2.7).
In block 17, the prevalence of epitope 6E2/53 was higher in parasites isolated from UM (53.1%) compared to SM patients (30.6%) ($\chi^2$, p<0.01) (table 2.6). This observation could result from a higher proportion of mixed infections detected in UM than in SM cases (see 2.3.1), and may indicate that the 6E2/53 epitope was more prevalent in those parasite clones which constituted a minority within mixed isolates (since no difference was observed when single or dominant clones were compared). The frequency of block 17 double negatives, 111.4- 6E2/53-, was elevated in SM (22.6%) compared to UM cases (7.5%) ($\chi^2$, p<0.05), being notably higher in CM (28.6%) vs. UM cases ($\chi^2$, p<0.025), when only predominant parasite clones were taken into account (table 2.7). This serological combination probably corresponds to the aa sequence E-KNG, denoted as a "recombinant" and found in other natural parasite populations (Qari et al., 1998).

In conclusion, there was an unbalanced distribution of certain MSP-1 allelic types associated with disease manifestations; divergences in prevalences were most noticeable between CM and SMA. In particular, the prevalence of the dimorphic K1-type and block 4 epitope 12.1 (MAD20-type) were higher in parasites from SMA cases, whereas they were very low in those from CM patients. In addition parasites from UM patients were associated with an increased prevalence of MAD20 block 2 type compared to parasites from CM patients.

2.3.6.2- Distribution of MSP-2 and EXP-1 genotypes and/or serotypes

The MSP-2B serotype, defined by reactivity with mAbs 8G10/48 or 8F6/49, was more commonly found than MSP-2A in SMA in contrast to all non-anaemic patients. Conversely, the alternative MSP-2A serotype, defined by reactivity with mAbs 12.3, 12.5 or 12.7, predominated in CM and UM patients. Overall prevalences of group B-specific epitopes recognised by mAb 8F6/49 or by anti-T9/105 polyvalent sera were significantly higher among SMA isolates (70.4% and 63%, respectively) than among CM isolates (38.5% and 34.6%) ($\chi^2$, p<0.05) (table 2.8). The frequency of epitope 8F6/49 in parasites which predominated in mixed infections was significantly higher among SMA (65.2%) than among CM (32%) ($\chi^2$, p<0.025) or non-anaemic patients (31.4%) ($\chi^2$, p<0.005) (table 2.9). The frequency of epitope
8G10/48 (STNS) was higher in SMA (65.2%) than in non-anaemic patients (41.4%) \( (\chi^2, p<0.05, \text{without Yates'continuity correction}) \).

By PCR, the prevalence of parasites containing \( MSP-2 \) B alleles was again higher in anaemic (\( n=69, 66\% \)) compared to non-anaemic patients (\( n=193, 42\% \)) \( (\chi^2, p<0.025) \). Since the prevalence of single clone \( MSP-2 \) B infections was comparable between UM, CM and SMA patients, the above difference may result from the significantly higher proportion of SMA patients having mixed A and B infections (36%) than non anaemic patients (17%) \( (\chi^2, p<0.01) \) (fig 2.10). Conversely, parasites infecting CM patients (\( n=91 \)) had a significantly lower proportion of mixed A and B \( MSP-2 \) types (11%) than isolates from non-cerebral cases (\( n=136, 27\% \)) \( (\chi^2, p<0.005) \). In CM cases harbouring single infections, the prevalence of type A infections was significantly higher (61.5%) than in SMA cases (\( n=44, 34\% \)) \( (\chi^2, p<0.005) \) (table 2.10).

\( MSP-2 \) types were analysed in relation to parasitaemia (geometric mean, parasites/\( \mu l \)). Patients infected with type B parasites had higher parasite densities (18,841) than patients infected with type A parasites (10,270). The difference was significantly higher in mixed A and B (24,495) compared to single A infections \( (p<0.05) \). This effect was independent of disease status, since A+B mixed infections predominated in SMA patients, whose parasite densities were lower than those of CM or UM patients (table 2.1).

The prevalence of EXP-1 epitope 5.1 was lower in SM (\( n=64, 73.4\% \)) than in UM isolates (\( n=47, 91.5\% \)) \( (\chi^2, p<0.025) \). The difference was due to a significantly lower proportion of parasite populations expressing the epitope 5.1 among anaemic patients. Thus, SMA (\( n=27 \)) had a significantly lower total allele prevalence of epitope 5.1 (70.3%) than did UM \( (\chi^2, p<0.025) \) and non-anaemic isolates (\( n=73, 89\% \)) \( (\chi^2, p<0.025) \) (fig 2.11).

Similar to MSP-1, there was a differential distribution of certain MSP-2 and EXP-1 allelic types in parasites obtained from different patients, particularly between CM and SMA. Anaemia symptoms were associated with MSP-2 type B parasites and decreased prevalence of epitope 5.1 of EXP-1, whereas patients with cerebral symptoms were more commonly infected with MSP-2 type A parasites. Thus, CM
Fig 2.10. Distribution of MSP-2 dimorphic genotypes

SMA vs. no-SMA: * p<0.01; ** p<0.025

Fig 2.11. Prevalence of EXP-1 dimorphic serotype 5.1 by IFA

* p<0.025
and SMA significantly correlated with particular MSP-1 or MSP-2 genotypes, and appeared to represent two extreme situations within the spectrum of malarial disease as far as genotype distributions and frequency of mixed-clone infections is concerned.

2.4- Discussion

Genetic polymorphisms of *P. falciparum* MSP-1 and MSP-2 alleles were determined by PCR and sequencing, and were correlated with antigenic diversity of the translated proteins determined by IFA. In addition, parasite polymorphisms were related to clinical manifestations, distinguishing for the first time between CM and SMA disease with significant numbers of patients in each group. To date, this study is the largest to evaluate parasite genotypes and clinical data, and shows some support for the notion that multiplicity of infections and particular parasite types may influence the severity of malarial disease.

2.4.1- Relationship between genetic and antigenic diversity of MSP-1 and MSP-2, and comparison between IFA and PCR typing

This is the first comprehensive study showing that much studied polymorphisms of malaria genes determined at the nucleotide level and defined by aa sequence are reflected in serological diversity at the aa level. Gene polymorphisms correlated well with antigenic polymorphisms of their encoded proteins as recognised by type-specific mouse Abs. To understand the role of the parasite genetic polymorphisms in the acquisition of immunity to malaria it is necessary to study the effect of such polymorphisms in the specificity of human immune responses. This information is required for the development of a malaria vaccine, since most current *P. falciparum* antigen vaccine candidates are products of polymorphic genes. The specificity of antibodies to MSP-1 and MSP-2 antigenic types in people naturally infected with the parasite is examined in chapter 5.

This is also the first study to compare systematically the relative usefulness of PCR and IFA typing techniques in a large number of samples. Despite the valid correlation between IFA and PCR typing shown, both techniques have limitations when used on their own and are more valuable in combination. A major advantage of IFA over PCR is that it allows the quantification of each parasite clone in a mixed
infection, and the resolution of the phenotypes of MSP-1 and MSP-2 proteins (Conway et al., 1991a). However, PCR is more sensitive than IFA and thus able to detect parasite clones at low densities. In particular, the frequencies of MSP-1 block 2 MAD20-type parasites were underestimated by IFA compared to PCR due to a lack of specific mAbs. However, serotyping of MSP-2 allelic proteins was almost as sensitive as PCR genotyping. A few discrepancies between both methods are possibly attributable to the presence of recombinant alleles between MSP-1 block 2 MAD20 and RO33 types, and between the two MSP-2 allelic families. With hybrids, PCR alone may give misleading results which could be resolved by DNA hybridisation with allele-specific probes, RFLP analysis or, ideally, by sequencing. The optimisation of techniques such as in situ PCR or quantitative PCR would be very useful for these kind of studies.

2.4.2.- Frequencies of MSP-1 and MSP-2 allelic types in different countries

I have examined the extent of diversity of P. falciparum merozoite surface proteins from parasites collected in a malaria endemic area of Malawi, South East Africa, where no similar survey has been carried out previously. Previous studies in other locations have shown significant geographical differences in the frequencies of MSP-1 and MSP-2 alleles, as summarised in table 2.12.

In Malawi, the most striking finding concerning MSP-1 was the consistently higher frequency of the MAD20 dimorphic type (93%) over the alternative K1/Well-type (7%) in blocks 6-16, in line with what Conway et al. (1992) found in The Gambia. A predominance of MAD20-type was also found in Brazil (Creasey et al., 1990; Conway et al., 1991b; Ferreira et al., 1998), Nigeria (Conway et al., 1991b), and Sudan (Babiker et al., 1991, 1995). In contrast, the K1/Well-type was almost as common as the MAD20-type in Thailand (Creasey et al., 1990; Jongwutiwes et al., 1991), and Zimbabwe (Creasey et al., 1990). Thus, results from Africa (except Zimbabwe) and South America agree in a dominance of MAD20 dimorphic type, and contrast with the findings in Thailand.
Table 2.12. Summary of field studies on genetic or antigenic diversity and multiplicity of *P. falciparum* infections which used MSP-1 and MSP-2 as markers.

<table>
<thead>
<tr>
<th>Place</th>
<th>Study population</th>
<th>n isolates</th>
<th>Typing method</th>
<th>Multiplicity</th>
<th>Mean dimorphic %</th>
<th>MSP-1 %</th>
<th>MSP-2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MAD 20</td>
<td>K1/Well</td>
<td>K1</td>
</tr>
<tr>
<td>SOUTH AMERICA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MAD K1</td>
<td>Mixed</td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>Patients</td>
<td>20</td>
<td>IFA (8 mAbs)</td>
<td>n.d</td>
<td>30</td>
<td>100 0</td>
<td>n.d</td>
</tr>
<tr>
<td>Rondonia</td>
<td>Patients</td>
<td>54</td>
<td>PCR, sequence</td>
<td>1.42</td>
<td>39</td>
<td>96.1 3.9</td>
<td>48</td>
</tr>
<tr>
<td>Colombia</td>
<td>Patients</td>
<td>31</td>
<td>PCR, hybridis.</td>
<td>2</td>
<td>25.9</td>
<td>n.d n.d</td>
<td>9.6</td>
</tr>
</tbody>
</table>

AUSTRALASIA:

<table>
<thead>
<tr>
<th>Place</th>
<th>Study population</th>
<th>n isolates</th>
<th>Typing method</th>
<th>Multiplicity</th>
<th>Mean dimorphic %</th>
<th>MSP-1 %</th>
<th>MSP-2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand</td>
<td>Patients</td>
<td>20</td>
<td>IFA (8 mAbs)</td>
<td>n.d</td>
<td>50</td>
<td>66 40</td>
<td>n.d</td>
</tr>
<tr>
<td></td>
<td>Patients</td>
<td>18</td>
<td>Hybridisation</td>
<td>n.d</td>
<td>39</td>
<td>66.6 55.5</td>
<td>38.8</td>
</tr>
<tr>
<td>Shoklo</td>
<td>Patients</td>
<td>56</td>
<td>PCR, hybridis.</td>
<td>1.67</td>
<td>70.3</td>
<td>n.d n.d</td>
<td>32.7</td>
</tr>
<tr>
<td>Oksibil</td>
<td>Longitudinal</td>
<td>18</td>
<td>PCR</td>
<td>n.d</td>
<td>38.8</td>
<td>n.d n.d n.d n.d n.d</td>
<td>n.d</td>
</tr>
<tr>
<td></td>
<td>Prospective</td>
<td>135</td>
<td>PCR, hybridis.</td>
<td>n.d</td>
<td>53.3</td>
<td>n.d n.d 46.6 13.5 39.9</td>
<td>49.4</td>
</tr>
<tr>
<td></td>
<td>Madang Villagers</td>
<td>130</td>
<td>PCR, hybridis.</td>
<td>1.8</td>
<td>72</td>
<td>n.d n.d 54 11 35</td>
<td>60</td>
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</tbody>
</table>

Rec. = recombinant; n.d = not determined; hibridis. = hibridisation; A = asymptomatic; S = symptomatic; Underlined values are significant differences in genotype frequencies.
<table>
<thead>
<tr>
<th>Place</th>
<th>Study population</th>
<th>n isolates</th>
<th>Typing method</th>
<th>Multiplicity</th>
<th>MSP-1</th>
<th>MSP-2</th>
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<tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no. clones</td>
<td>MAD K1/20</td>
<td>K1 RO33 MAD20</td>
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<td>AFRICA:</td>
<td></td>
<td></td>
<td></td>
<td>Mixed</td>
<td>Well</td>
<td></td>
</tr>
<tr>
<td>Sudan</td>
<td>Patients</td>
<td>29</td>
<td>IFA (mAbs)</td>
<td>n.d</td>
<td>41</td>
<td>70 30 n.d n.d n.d</td>
</tr>
<tr>
<td>Asar</td>
<td>Patients</td>
<td>114</td>
<td>IFA, PCR, hybris</td>
<td>n.d</td>
<td>33.3</td>
<td>66 33 33.6 25.6 40.7</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Asymptomatic</td>
<td>53(56)</td>
<td>PCR, hybris.</td>
<td>1.30</td>
<td>20</td>
<td>n.d n.d 35 9 56</td>
</tr>
<tr>
<td>Kenya</td>
<td>Case-control</td>
<td>172</td>
<td>PCR, hybris.</td>
<td>1.07</td>
<td>30</td>
<td>n.d n.d 44(51) 30(28) 26(21)</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>Patients</td>
<td>20</td>
<td>IFA (8 mAbs)</td>
<td>n.d</td>
<td>83</td>
<td>66 50 n.d n.d n.d</td>
</tr>
<tr>
<td>Gabon</td>
<td>Asympt child</td>
<td>53</td>
<td>PCR, hybris.</td>
<td>1.73</td>
<td>45.3</td>
<td>n.d n.d 30 39 31</td>
</tr>
<tr>
<td>Diegna</td>
<td>Asymptomatic</td>
<td>AS 40</td>
<td>PCR, hybris.</td>
<td>2.6</td>
<td>87</td>
<td>n.d n.d n.d n.d n.d</td>
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<td>Lambarane</td>
<td>Case-control</td>
<td>SM 100</td>
<td>PCR, hybris.</td>
<td>1.2</td>
<td>23</td>
<td>n.d n.d 62 23 28</td>
</tr>
<tr>
<td></td>
<td>Children</td>
<td>UM 100</td>
<td></td>
<td>1.3</td>
<td>33</td>
<td>n.d n.d 46 35 42</td>
</tr>
<tr>
<td>Nigeria</td>
<td>Patients</td>
<td>60</td>
<td>IFA (19 mAbs)</td>
<td>n.d</td>
<td>96</td>
<td>4 n.d n.d n.d n.d</td>
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<tr>
<td>Senegal</td>
<td>Adult patient</td>
<td>16</td>
<td>PCR, hybris.</td>
<td>n.d</td>
<td>62.5</td>
<td>n.d n.d 56.2 93.7 12.5</td>
</tr>
<tr>
<td>Dielmo</td>
<td>Asymptomatic</td>
<td>51</td>
<td>PCR, hybris.</td>
<td>adult 2</td>
<td>82</td>
<td>n.d n.d n.d n.d n.d</td>
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<tr>
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<td>sickle cell</td>
<td>77</td>
<td>PCR</td>
<td>3.7</td>
<td>92</td>
<td>n.d n.d 45 26 29</td>
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<td></td>
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<td>3.4</td>
<td>90</td>
<td>n.d n.d 59 23 18</td>
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<td></td>
<td>1.6</td>
<td>53</td>
<td>n.d 47 53 2</td>
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<td>Dakar</td>
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<td>PCR, hybris.</td>
<td>2.2</td>
<td>59</td>
<td>n.d n.d 29 54 17</td>
</tr>
<tr>
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<td>UM vs SM</td>
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<td></td>
<td>3.1</td>
<td>77</td>
<td>n.d 33 39 14</td>
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<td>Gambia</td>
<td>Patients</td>
<td>424</td>
<td>IFA (36 mAbs)</td>
<td>1.49</td>
<td>35.6</td>
<td>99 1 53.7 29.6 16.6</td>
</tr>
<tr>
<td></td>
<td>Children</td>
<td>74</td>
<td>Sequencing</td>
<td>n.d</td>
<td>100</td>
<td>53.8 23 23</td>
</tr>
</tbody>
</table>

Rec. = recombinant; n.d = not determined; hibridis. = hybridisation; A = asymptomatic; S = symptomatic; AS = heterozygous sickle-cell trait; AA = normal haemoglobin phenotype; SM = severe malaria, UM = uncomplicated malaria; unpubl. = unpublished. Underlined values are significant differences in genotype frequencies.
Concerning block 2 of MSP-1, in Malawi there was a consistent predominance of K1-type, as has been reported in Tanzania (Babiker et al., 1994, 1997). In contrast, a higher prevalence of the RO33-type was detected in Brazil (Kimura et al., 1990), Colombia (Snewin et al., 1991), Senegal (Scherf et al., 1991) and Gabon (Ntoumi et al., 1996), and MAD20-type predominated in Thailand (Jongwutiwes et al., 1991) and Sudan (Babiker et al., 1995, 1997). Concerning MSP-1 block 3, Malawian isolates resembled Thai isolates in the comparable prevalences of epitopes 13.2 (K1/Well) and 9.5 (MAD20) (Creasey et al., 1990), in contrast to Zimbabwe or Brazil, where either one or the other epitope considerably predominated.

Regarding block 4 of MSP-1, no significant differences were found in the distribution of epitope 12.1 (MAD20), which is present at a lower frequency in Malawi. It is not possible to compare the serotype prevalences of block 4 variants properly because the alternative epitope 10-2B (K1/Well), over-represented in Malawi, was not investigated in that study. Conway et al. analysed the frequency of 10-2B in isolates from The Gambia, Nigeria and Brazil (1991, 1992) and found a predominance of 10-2B vs. 12.1, but not as marked as in Malawi. Moreover, the slightly higher prevalence of epitope 13.5 over 9.5, and the more frequent occurrence of the combination of epitopes 13.2 and 10-2B, and of epitopes 9.5 and 12.1, were in accordance with the findings in Malawi. The data were interpreted in relation to the frequency of intragenic recombination within the MSP-1 gene. In addition, Conway et al. proposed the putative locations of block 3 and 4 variant epitopes on the basis of correlation with sequence polymorphism (1991b). As discussed above, genetic polymorphism reflected well in antigenic polymorphism in other domains of MSP-1. Therefore, it is justifiable to compare the serotypic frequencies of variant block 4b epitopes detected by IFA in this study with the genotypic frequencies of the MAD20 and K1/Well block 4 types defined by DNA typing and/or sequencing in other field studies. In 54 isolates from the Brazilian Amazon, e.g., the K1-type of block 4, which corresponds to the 10-2B epitope in IFA, clearly predominated (82%) over the MAD20-type (Ferreira et al., 1998).

Concerning MSP-1 block 17, only the frequency of epitope 111.4 was assessed as ≤50% in The Gambia (Conway et al., 1991, 1992), somewhat lower than
the prevalence in Malawi. In analogy to block 4, there have been more DNA- than antigen-based studies on the allelic diversity of block 17, and thus the frequencies of epitopes 111.4 (EGF-1, "Q"-dependent) and 6E2/53 (EGF-2, "TSR-dependent") detected by IFA in Malawi can only be compared with isolates from elsewhere whose block 17 have been sequenced. Among 50 Kenyan isolates, alleles which correspond to IFA reactivity 111.4- 6E2/53- (e.g. E-KNG, "recombinant type") were more common (50%) than K1-type (Q-KNG, 34%) or MAD20-type (E-TSR, 16%), with no detection of Q-TSR ("Palo Alto-type") alleles, which have been reported in 5 occasions elsewhere (Qari et al., 1998). This distribution contrasted to some extent with Malawi, where the most and least common combinations appeared to be 111.4+ 6E2/53- (e.g. Q-KNG) and 111.4- 6E2/53-, respectively.

With regard to MSP-2, type A was predominant in Malawi, in agreement with results from Zimbabwe, Thailand, Brazil (Creasey et al., 1990), The Gambia (Conway et al., 1992), Tanzania (Babiker et al., 1994), Solomon Islands (Prescott et al., 1994) and Sudan (Babiker et al., 1997). Genotyping and sequencing of MSP-2 variants from clinical isolates from Colombia (Snewin et al., 1991), Irian Jaya (Marshall et al., 1994), PNG (Felger et al., 1994) and Senegal (Ntoumi et al., 1995) revealed a higher prevalence of MSP-2 type B. Nevertheless, the relative frequencies of both MSP-2 allelic families are overall quite similar and in most places each account for ≈ 50%.

Overall, further evidence for regional differences in the distribution of MSP-1 and MSP-2 genotypes in Malawi compared to other malaria endemic areas was found, supporting the conclusions from previous studies which showed that different parasite clones are found in geographical distant areas. However, the prevalences of polymorphic and dimorphic MSP-1 and MSP-2 allelic types in Malawi generally resembled those found in other African, particularly East African, sites where similar surveys have been carried out.

2.4.3- Mixed infections in relation to transmission and disease severity

Mixed P. falciparum infections were common among the Malawian clinical isolates, as shown in many previous studies elsewhere (table 2.12). The occurrence of multiple infections is subject to regional and temporal variations, probably reflecting
differences in intensity of infection, transmission patterns, human and vector
distributions and parasite population dynamics. Thus, the mean number of clones per
isolate of 2.46 found in Malawi is similar to that reported in areas of hyperendemic
and seasonal malaria transmission (e.g. The Gambia, Kenya), lower than holoendemic
areas (e.g. Tanzania, Dielmo) and higher than mesoendemic (e.g. Sudan) or
hypoendemic areas (e.g. Thailand) (table 2.12). Studies examining mixed infections as
a measure of transmission, assessed by entomological inoculation rates (EIRs), have
been reviewed (Babiker & Walliker, 1997; Arnot, 1998). In PNG (Paul et al., 1995),
multiplicity of genotypes differed from Tanzania (Babiker et al., 1994), where
transmission is 10-fold higher. This suggested that the relationship between
multiplicity and transmission intensity may be nonlinear. The prevalence of mixed
infections was also consistent with the level of endemicity between Tanzania and
Sudan (Babiker et al., 1997). Deviations from the multiplicity of infections predicted
on the basis of EIR have been explained by migration patterns or by prolongation of
the duration of asymptomatic infections due to multidrug resistance (Paul et al.,
1998). To date, there are no data about EIRs in Malawi, and therefore these
comparisons cannot be carried out with our samples. However, areas with more
intense malaria transmission occur south of Lake Malawi, where it would be
interesting to analyse circulating parasite populations to contrast the findings with
Blantyre and other sites.

Multiplicity of infections have also been associated with parasite density and
host age in Senegal (Ntoumi et al., 1995). The proportion of asymptomatic
individuals with >1 PCR band, as well as the prevalence of parasites and parasite
density, decreased with age, from a mean of 4 genotypes in children (under 15 years)
to 2 genotypes in adults. Only a few studies have analysed the relationship between
the number of clones in an infection and the severity of clinical symptoms. This study
is the first to include a significant number of parasites from CM and SMA cases
separately, in contrast to previous studies which grouped them as SM. In these
Malawian patients, there was a significantly lower mean number of parasite clones in
CM compared to SMA or UM patients. A lower proportion of mixed infections was
also found in SM vs. UM patients in Senegal (Robert et al., 1996) and Gabon (Kun et
al., 1998), but no differences were found in The Gambia (Conway et al., 1991a). In addition, a higher mean number of clones per infection was found in asymptomatic vs. symptomatic patients in Senegal (Mercereau-Puijalon, 1996), Tanzania (Beck et al., 1997) and Papua New Guinea (Al-Yaman et al., 1997), but no significant differences were found in Kenya (Kyes et al., 1997) and the opposite was reported in Sudan (Roper et al., 1998). My results support the view that CM would be associated with a limited number of clones multiplying to high parasitaemias, while UM would be caused by a larger number of clones, each one reaching a lower density; these findings are consistent with the hypothesis that CM may be caused by a few *P. falciparum* strains, while a majority of strains may cause UM (Gupta et al., 1994). In addition, if the expansion of a few clones were responsible for the cerebral symptoms, these clones would provide most of the DNA template for the PCR reaction, making it difficult to detect low density co-infections. Moreover, fever and other non-specific anti-parasitic immune responses thought to be associated with the pathology of CM may reduce low-density clones under the PCR detection threshold.

Concerning SMA, high multiplicity of infections was associated with anaemia in Malawi. Kun et al., also found that mixed infections with parasites bearing different alleles were associated with SMA in Gabon (1998). In a experimental mouse model, mixed *P. chabaudi* clone infections were more “virulent” than single-clone infections; virulence was assessed by monitoring anaemia and body weight loss (Taylor et al., 1998). It was suggested that prolonged anaemia might have occurred because genetically diverse infections were less rapidly cleared by hosts. In humans, an association between sickle cell anaemia and multiple infections, was reported in two studies (Ntoumi et al., 1997a: Dielmo; 1997b: Gabon). It was speculated that AS erythrocytes might be more susceptible to parasite infection, resulting in the retention of multiple strains. Although the host Hb type was not determined here, the findings from these studies linking mixed infections and anaemia fit well with my results.

As immune responses to the infecting parasites in these CM, SMA and UM patients are examined later in the thesis (chapters 4 & 5), the interaction between multiplicity of infections, parasite density and strain-specific immunity is especially discussed in chapter 6.
2.4.4. Relationship between MSP-1 and MSP-2 polymorphism and disease severity

Few studies before had focused on the relationship between MSP-1 and MSP-2 genotypes and malaria morbidity or severity, and results have generally been inconclusive. In Malawi, particular genotypes appeared to be increased or decreased in association with anaemia or cerebral symptoms.

Concerning MSP-1, a decreased prevalence of the K1/Well-type of the major dimorphic family (blocks 6-16), and of epitope 12.1 of block 4 (MAD20-type), was characteristic of parasites from CM patients, whilst the prevalence of these types were increased in parasites infecting anaemic patients, both compared to UM patients. Results were consistent by IFA and PCR, and when the effect of multiplicity of infections (higher in SMA than CM patients) was accounted for in the statistical analysis. It may be possible that an increased prevalence of the dimorphic K1/Well type of MSP-1 may relate to a more efficient RBC reinvasion rate, a better parasite growth rate and a more pronounced RBC destruction in these SMA patients. Another possibility is that the decreased presence of K1/Well dimorphic type and, conversely, the striking predominance of the alternative MAD20-type among CM patients indicates a requirement for this particular variant in some step of CM pathogenesis, or that an unknown gene for pathogenicity is closely linked to the MAD20-type alleles in these Malawian isolates. Unfortunately, in the design of this study it is not possible to distinguish between these possibilities. However, the MSP-1 dimorphic K1/Well-type was rare in Malawi, and although the differences in prevalences were statistically significant between CM and SMA, care must be taken when interpreting these results. The high proportion of mixed infections and the low frequencies of these types in Malawi complicated the analysis, resulting in not very striking correlations between genotypes and disease.

Concerning the block 2 of MSP-1, an increased prevalence of the MAD20-type was found among UM compared to CM or SMA patients. This is in agreement with a case-control study in Gabon comparing SM (anaemia or hyperparasitaemia) vs. UM children, where a significant association between UM and MAD20-type of block
2 was found (Kun et al., 1998). The consistency between the two studies suggests that MAD20-type may be a marker for "milder" *P. falciparum* genotypes. Kun et al. also reported an association between the RO33-type and normal Hb levels. In a prospective community study in Papua New Guinea (PNG), infections with parasites of *MSP-1* block 2 RO33-type were associated with reduced disease risk of clinical malaria in children (Al-Yaman et al., 1997). In Malawi, RO33 in combination with MAD20 type was more likely to be found in UM than in SM patients, in line with these two studies. In contrast, in another case-control study in Dakar, Senegal, comparing UM vs. SM patients, there was a trend for a higher frequency of RO33 block 2 type in SM cases but no significant differences in genotype frequencies between both groups (Robert et al., 1996). In addition, Kun et al. reported an association between the K1-type of block 2 and SM, particularly SMA (Hb <6g/dl) (1998). In contrast, K1-type was the most prevalent in all groups of Malawian patients, not being significantly associated with severity. The only trend found in Malawi was an increased prevalence of K1-type in combination with RO33-type in malaria fatalities compared to survivors. Conflicting findings between these and my study may be a reflection of distinct genetic characteristics in parasites and/or hosts in these countries and Malawi.

Concerning *MSP-2* genotypes, parasites having type B or mixed infections containing both types A and B were more prevalent in Malawian anaemic patients, and clinical isolates containing single infections were more likely to contain type B parasites. These results are in accord with those reported in two studies in PNG which aimed to find associations of *MSP-2* genotypes with age, parasite density and malaria morbidity (fever and anaemia). A higher prevalence of *MSP-2* type B was found in adults, whereas in children type A predominated (Felger et al., 1994). In addition, individuals infected with type B parasites had higher densities, as found in Malawi. A case-control study in the same area reported that type B was twice as likely to be found in symptomatic than asymptomatic patients, mostly children (Engelbretch et al., 1995). In addition, mixed type A and B infections had the same morbidity as single A infections. This is the first association between a particular parasite genotype and malaria morbidity reported thus far. However, it was not possible to distinguish
between a protective effect of type A parasites and enhanced virulence of type B parasites. In a later prospective study in PNG, infections with parasites of MSP-2 type A were associated with reduced disease risk of clinical malaria in children (Al-Yaman et al., 1997). Taken together, these findings indicate that MSP-2 type B may be responsible for a higher malaria morbidity due to increased anaemia. Since MSP-2 may be involved in merozoite invasion of RBCs, it is conceivable that type B clones may be more successful during the asexual proliferation in the blood, resulting in the observed higher peripheral parasite densities and, eventually, in anaemia.

The finding of distinct characteristics of parasites from CM and SMA patients emphasise that it is not appropriate to pool CM and SMA as one SM group in studies aiming to identify parasite virulence factors. Thus, virulence indices should be measured in accordance to the different pathogenic mechanisms thought to be responsible for these two distinct severe disease manifestations.

Other studies have failed to find any associations between MSP-1 or MSP-2 genotypes and disease. A longitudinal study in Dielmo examined the genetic diversity of P. falciparum during successive clinical episodes in children (Contamin et al., 1996). Clinical episodes and peak parasitaemias were associated with new alleles not previously found, and some children infected with certain types remained asymptomatic. No “pathogenic type” was identified, but rather clinical attacks were caused by many different genotypes. In addition, in a study in Kenya, comparing asymptomatic vs. clinical malaria cases (Kyes et al., 1997) diversity did not vary with the clinical status; the genotypes present were similar in cases and controls.

In conclusion, differences in genotype distributions found in these Malawian patients point towards potential differences in the genetic composition of P. falciparum infections differing in clinical status. Certainly, more studies of this sort are required elsewhere to confirm the findings reported here, and their significance remain unknown until the biological function of MSP-1 and MSP-2 in P. falciparum infections is established. The apparent effect of particular MSP-1 or MSP-2 types and of multiplicity of infections in disease manifestations encourages the hypothesis that polymorphic and/or dimorphic epitopes of these proteins may be targets of protective immunity, as examined in chapters 4 & 5, rather than simply markers of diversity.
3.1- Introduction

Sequestration of parasitised red blood cells (PRBCs) in deep capillaries is a normal event in *P. falciparum* infection in man. Early studies which followed daily blood parasitaemia of symptomatic patients by microscopic examination revealed a striking and often regular variation in parasite densities in the peripheral blood (Gilles, 1993). Furthermore, while only young asexual blood forms of the parasite (rings and trophozoites) were normally detected in circulating RBCs, post-mortem histopathological examinations were finding organ capillaries filled with the older asexual blood stages (schizonts). Thus it is believed that the alternation of parasite peaks and disappearances from circulation every other day is due to sequestration of mature PRBCs in organs. The fact that *P. falciparum* is the only human malaria species that sequesters, and the only that causes fatal cerebral malaria (CM), strongly suggests a role for sequestration in the pathogenesis of CM.

Autopsies have been essential to the understanding of the disease. Histological studies in *Macaca* monkeys experimentally infected with *P. knowlesi* and *P. falciparum* indicated a differential distribution of sequestered PRBCs between the two species (Miller *et al.*, 1971). Thus, in this model *P. knowlesi* schizonts were sequestered mainly in vessels of the liver and intestine (at low parasitaemias) and in the brain (at high parasitaemias), whereas *P. falciparum* appeared mainly in the heart and adipose tissues. Initial studies which addressed the relationship between sequestration and pathology in humans consisted of quantitative analysis by electron microscopy of sequestered PRBCs in brain sections from adult Thai fatal CM and non-CM malaria cases (MacPherson *et al.*, 1985). It was found that CM was associated with more intense sequestration of PRBCs in the brain than non-CM, though there was no evidence for an inflammatory or immune pathogenesis. It was concluded that, although sequestration was necessary for the development of CM, it was not sufficient in itself, since it also occurred in people who did not die from CM. It
has been hypothesised that a dense sequestration of parasites within brain vessels somehow causes large amounts of cytokine release, inducing cerebral endothelium to produce nitric oxide (NO) which would cause the coma (Clark et al., 1991). Another study showed a correlation between degree of PRBC sequestration in cerebral microvessels and clinical coma score of CM (Riganti et al., 1990). Differences in the sequestration between grey and white matter were assessed in Vietnamese adults (Nagatake et al., 1992). The PRBC sequestration rate as well as the deposition of P. falciparum antigen and IgG were higher in the white than the grey matter. Immunohistochemical studies on tissues of adult Thai fatal cases found evidence for a widespread induction of endothelial activation markers i.e. upregulated expression of the cytoadherence receptors intracellular adhesion molecule (ICAM)-1 and E-selectin in vessels in the brain of CM patients, and suggested a role for ICAM-1 in sequestration in vivo (Turner et al., 1994).

However, most studies of the histopathology of fatal malaria have been conducted in adult patients in areas of unstable malaria, in whom patterns of severe disease may differ from those in children. Previous paediatric autopsy studies (Kean & Smith, 1944; Attah & Ejeckem, 1974) were not complemented by precise clinical data on the patients, making it difficult to distinguish whether the reported features contributed to the pathogenesis of fatal disease or merely accompanied it. Therefore, it remains to be established whether sequestration is critical to the development of CM (and other severe syndromes). In particular, it is not known know whether parasite density, stage or tissue distribution correlate with clinical findings, since anatomical patterns of sequestration may influence patterns of organ specific dysfunction and therefore patterns of clinical disease.

As described in chapter 2, P. falciparum is genetically diverse among, and often within, patients. This has been shown for parasites in the peripheral circulation by immunofluorescence microscopy (IFA) and PCR genotyping using the malaria merozoite surface antigens MSP-1 and MSP-2 and the exported protein EXP-1 as markers of polymorphism. Recent longitudinal studies have pointed out that care is required when analysing P. falciparum diversity using cross-sectional samples, since one blood sample may not represent the entire parasite population infecting an
individual (Daubersies et al., 1996). A longitudinal study that followed the dynamics of *P. falciparum* subpopulations in asymptomatic children in Tanzania by PCR typing (Farnert et al., 1997) found that densities and genotypes changed daily. Different parasite broods fluctuated with a 48 hr periodicity, presumably due to sequestration, and were synchronous in themselves but independent from each other.

It has been proposed that only few *P. falciparum* genotypes or, alternatively, variants may be responsible for CM (Gupta et al., 1994). It is not known whether sequestered parasites are genetically different from those in circulation because to date no study has directly typed *in situ* parasites from autopsy tissue specimens. A related interesting question is whether, in cases of genetically mixed infections, different clones sequester to different or the same tissues or organs.

3.1.1- Aims

The objective of this chapter has been to investigate the role of antigenic diversity of merozoite surface proteins in sequestration by examining the distribution of parasite serotypes in the body. Specifically, I have investigated whether parasites sequestered in organs differ in their composition of MSP-1, MSP-2, EXP-1 and knob antigens, used as “strain” markers, from parasites in the peripheral circulation of the same patient.

3.2- Materials and methods

3.2.1- Collection of tissue samples

Autopsies were carried out at the Malaria Research Project and Wellcome Trust Centre (MRP), Department of Paediatrics, Queen Elizabeth Central Hospital, Blantyre, Malawi during 1996, 1997 and 1998. Autopsies were conducted with consent of parents on bodies of children under 12 years of age. Four categories of patients were included whose clinical manifestations had been characterised: i) CM ii) severe malarial anaemia (SMA) iii) gram-negative septicaemia (without parasitaemia) iv) trauma and non-infective disease (some had incidental parasitaemia). A supra-orbital needle biopsy was obtained from two CM fatalities, an adult (D.N.) and a child (L.S.), in whom full autopsies were not carried out. Details of 16 cases included in the
study are summarised in table 3.1. Venous blood from these patients was obtained on admission.

Thin smears of brain tissue were dried, fixed in methanol, stained with Giemsa's or Field's stain, and examined under the oil immersion lens (x100) to assess the degree and stage of parasite sequestration. Tissues for typing of sequestered PRBCs were prepared in four different ways: i) homogenates ii) smears iii) touches and iv) liquid N\textsubscript{2} cryopreserved sections. The tissue homogenates were prepared by gently grinding a $\geq$ 2x2x1 cm piece of tissue with a pestle and mortar in phosphate buffered saline (PBS) using a rotary movement. The homogenates were washed 2x in PBS, and 20-25 µl of the suspension were placed onto wells of 12-well multispot microbiological slides, dried, packed and stored, as with blood slides. Smears were prepared by placing a piece of tissue $\approx$ 2x2x2 mm in the middle of a glass slide, placing a second slide directly over the first so that the labelled ends were opposite, crushing the tissue firmly between the two slides and massaging the slides together so that the tissue spread out. Finally, slides were pulled away from each other keeping them in line. Touches were prepared by pressing a glass slide against a section of the organ to obtain its imprint. Smears and touches were dried and stored as homogenates. Pieces of tissue of 7x3x3 mm were covered in OCT (a clear jelly-like liquid) and frozen in liquid N\textsubscript{2} in the mortuary. Eight micron sections of cryopreserved tissue placed onto slides were kindly provided by Dr. Georges Grau, Geneva, Switzerland. Slides were stored at -20°C for IFA.

3.2.2- Immunofluorescence (IFA) serotyping in tissue specimens

Parasites sequestered in tissues were typed \textit{in situ} by IFA, essentially as done for parasites obtained from culture (Conway \textit{et al.}, 1991a). Individual (1-colour) IFA is described in chapter 2. For mixed infections, double-labelled (2-colour) IFA was also carried out using combinations of two monoclonal antibodies (mAbs) of different isotypes and epitope specificities. FITC (fluorescein isothiocyanate)- and RITC (rhodamine isothiocyanate)-conjugated anti-mouse immunoglobulins (1:50) specific for the isotype of the typing mAbs were incubated together in the second stage. These slides were not counterstained with Evans Blue. Reactions were read with incident
### Table 3.1. Summary of data of fatal cases from whom post-mortem specimens were examined

<table>
<thead>
<tr>
<th>Autopsy Date</th>
<th>Patient Sex/age</th>
<th>Clinical diagnosis</th>
<th>Parasitaemia on admission</th>
<th>Time between death and autopsy</th>
<th>Autopsy interval</th>
<th>Sequestered stages</th>
<th>IFA in tissue homogenates</th>
<th>IFA in tissue smears or touches</th>
<th>IFA blood</th>
<th>PCR blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP96-6 14/5/96</td>
<td>MP664 F/1y 5m</td>
<td>CM+SMA</td>
<td>5.8% 96,860</td>
<td>1 h</td>
<td>4 h</td>
<td>Schizonts</td>
<td>B1, B2, B11, B12, D1, D2, J2, F4, G1</td>
<td>B (all sites) D2, J2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MP96-7 29/5/96</td>
<td>MP685 F/2y 5m</td>
<td>Anaemia, ?HIV, Encephalitis</td>
<td>n.a</td>
<td>0 h</td>
<td>n.a</td>
<td>-</td>
<td>B1, B2, B11, B12, D1, J2, F4, E1, G1</td>
<td>B1, B11, D1, J2, F4, E1, G1</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>MP97-10 9/3/97</td>
<td>#S753 F/5y 6m</td>
<td>OP toxicity</td>
<td>+ 330</td>
<td>4 h</td>
<td>14 h</td>
<td>-</td>
<td>B1, B2, B11, B12, J2, F4</td>
<td>D1, D2, F4, J2</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>MP97-11 13/3/97</td>
<td>MP770 F/2y 5m</td>
<td>CM+SMA, pneumo., septic.</td>
<td>29.5% 368,750</td>
<td>27 h</td>
<td>13 h</td>
<td>Schizonts</td>
<td>B1, B2, B11, B12, N2, J2, F4</td>
<td>B1, B2, B11, B12, N2, J2, F4, D1, D2</td>
<td>n.a</td>
<td>+</td>
</tr>
<tr>
<td>MP97-12 19/3/97</td>
<td>MP787 M/6m</td>
<td>Septicaemia, anaemia</td>
<td>0 pigment</td>
<td>4 h</td>
<td>12 h</td>
<td>-</td>
<td>J2, F4</td>
<td>B1, B2, B3, B8, N2, D1, D2, F4, J2</td>
<td>n.a</td>
<td>+</td>
</tr>
<tr>
<td>MP97-13 21/3/97</td>
<td>MP792 M/1y 10m</td>
<td>CM</td>
<td>31.3% 156,500</td>
<td>0 h</td>
<td>3 h 51 min</td>
<td>Schizonts</td>
<td>B1, B2, B11, B12, F4, J2</td>
<td>N2, D1, D2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MP97-14 23/3/97</td>
<td>MP795 F/9m</td>
<td>Anaemia, septicmaemia</td>
<td>0 gamet. +</td>
<td>6 h 30 min</td>
<td>14 h 38 min</td>
<td>-</td>
<td>n.a</td>
<td>B1, B11</td>
<td>n.a</td>
<td>+</td>
</tr>
<tr>
<td>MP97-15 25/3/97</td>
<td>MP800 F/8m</td>
<td>CM+SMA</td>
<td>27/200wbc 11,300</td>
<td>10 h</td>
<td>6 h 10 min</td>
<td>Schizonts</td>
<td>B1, B2, B11, B12, F4, J2</td>
<td>N2, J2, F4, D3, D4</td>
<td>n.a</td>
<td>+</td>
</tr>
</tbody>
</table>

*Parasitaemia on admission, expressed as thin film (% ring forms over ~500 RBCs), thick film (no. ring forms over ~200 white blood cells) or parasites per microlitre, as available (see chapter 2); n.a= not available

*Time period between admission to hospital and death

*Parasitaemia recorded shortly (less than 4 h) before death, expressed as thin film (% ring forms over ~500 RBCs), thick film (no. ring forms over ~200 white blood cells) or parasites per microlitre, as available (see chapter 2)

*Time period between death and autopsy

*Tissues from which homogenates were prepared for IFA typing. B1=frontal lobe brain, B2=parietal lobe brain, B11=peripheral cerebellum, B12=dentate nucleus cerebellum, N1=supra-orbital needle biopsy taken shortly after death, N2=supra-orbital needle biopsy taken during the autopsy, D1=right upper lobe lung, D2=right lower lobe lung, D3=left upper lobe lung, D4=left lower lobe lung, E1=heart muscle, F4=liver, G1=kidney, J2=spleen

*Tissues from which smears or touches were prepared for IFA typing. Abbreviations as above.

*Pneumo.= pneumonia; septic=septicemia.
Table 3.1. Summary of data of fatal cases from whom post-mortem specimens were examined (continuation)

<table>
<thead>
<tr>
<th>Autopsy Date</th>
<th>Patient Sex/age</th>
<th>Clinical diagnosis</th>
<th>Parasitaemia on admission</th>
<th>Time to death</th>
<th>Parasitaemia at death</th>
<th>Autopsy interval</th>
<th>Sequestered parasites</th>
<th>IFA in tissue homogenates</th>
<th>IFA in tissue smears or touches</th>
<th>IFA blood</th>
<th>PCR blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/4/97</td>
<td>MP97-16</td>
<td>F/4y 3m</td>
<td>CM</td>
<td>5.5% 125,950</td>
<td>4h 30min</td>
<td>70,900 3h 4min</td>
<td>Trophozoites</td>
<td>B1, B2, B11, B12, F4, J2</td>
<td>N2, J2, F4, D1, D2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7/5/97</td>
<td>MP97-17</td>
<td>F/4y 3m</td>
<td>Reye's Syndrome</td>
<td>0 9h 0</td>
<td>14h</td>
<td>- n.a</td>
<td>n.a</td>
<td>N2</td>
<td>n.a</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3/6/97</td>
<td>MP97-21</td>
<td>F/4y 1m</td>
<td>CM</td>
<td>189/200wbc 9,964</td>
<td>12h 5/200 wbc 7h 47min</td>
<td>Trophozoites</td>
<td>B1, B2, B11, B12 J2, F4</td>
<td>N2, D1, D2, J2, F4</td>
<td>n.a</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>16/3/98</td>
<td>MP98-19</td>
<td>F/2y 6m</td>
<td>CM</td>
<td>29% 7h 30min</td>
<td>15.5% 14h 30min</td>
<td>Schizonts Trophozoites</td>
<td>n.a</td>
<td>B1, B2, B11, B12 D1, D2, J2, F4</td>
<td>n.a</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>30/4/98</td>
<td>MP98-26</td>
<td>M/2y 6m</td>
<td>CM</td>
<td>442,705 3h 45,358</td>
<td>30h 6h 30min</td>
<td>Schizonts Trophozoites</td>
<td>n.a</td>
<td>B1, B2, B11, B12 D1, D2, J2, F4</td>
<td>n.a</td>
<td>n.a</td>
<td></td>
</tr>
<tr>
<td>2/6/98</td>
<td>MP98-27</td>
<td>M/ly 8m</td>
<td>CM</td>
<td>61% 3h 61% 4h</td>
<td>Schizonts</td>
<td>n.a</td>
<td>B1, B2, B11, B12 D1, D2, J2, F4</td>
<td>n.a</td>
<td>n.a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27/3/97</td>
<td>M/11y</td>
<td>CM</td>
<td>+</td>
<td>0h</td>
<td>+</td>
<td>1h</td>
<td>Schizonts</td>
<td>n.a</td>
<td>N1</td>
<td>n.a</td>
<td>+</td>
</tr>
<tr>
<td>16/4/97</td>
<td>M/12y</td>
<td>CM</td>
<td>+</td>
<td>8d 0</td>
<td>1h</td>
<td>Schizonts</td>
<td>n.a</td>
<td>N1</td>
<td>n.a</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

- Parasitaemia on admission, expressed as thin film (% ring forms over ~500 RBCs), thick film (no. ring forms over ~200 white blood cells) or parasites per microlitre, as available (see chapter 2).
- Time period between admission to hospital and death
- Parasitaemia recorded shortly (less than 4 h) before death, expressed as thin film (% ring forms over ~500 RBCs), thick film (no. ring forms over ~200 white blood cells) or parasites per microlitre, as available (see chapter 2)
- Time period between death and autopsy
- Tissues from which homogenates were prepared for IFA typing. B1=frontal lobe brain, B2=parietal lobe brain, B11=peripheral cerebellum, B12=dentate nucleus cerebellum, N1=supra-orbital needle biopsy taken shortly after death, N2=supra-orbital needle biopsy taken during the autopsy, D1=right upper lobe lung, D2=right lower lobe lung, E1=heart muscle, F4=liver, G1=kidney, J2=spleen; n.a=not available
- Tissues from which smears or touches were prepared for IFA typing. Abbreviations as above.
light of 515-560 nm for rhodamine-fluorescence (red). Typing reagents included the same Abs specific for MSP-1, MSP-2, EXP-1 and putative knob epitopes in the surface membrane of PRBCs as specified in chapter 2 (table 2.3).

3.3 Results

Post-mortem specimens were obtained from 17 autopsies and from 2 supra-orbital needle biopsies between 1996 and 1998. In 11 CM cases, mature parasites were observed in brain by light microscopy, as illustrated in fig.3.1, and parasites were subsequently typed by IFA. 5 non-CM fatal cases with no or scanty peripheral blood parasitaemia and no or minimal tissue sequestration were included as negative controls. The clinical diagnosis of these controls were: SMA (case MP96-7), organophosphate toxicity (MP97-10), septicaemia and severe anaemia (MP97-12, MP97-14) and Reye's Syndrome (MP97-17).

3.3.1 Preparation of specimens for IFA

Post-mortem specimens were initially collected from brain (B1-10), cerebellum (B11-12), lung (D), heart (E), spleen (J), liver (F) and kidney (G) of two cases (MP96-6 and MP96-7). Parasites could be detected by Giemsa staining in the brain, cerebellum, spleen, liver and lung but not in heart or kidney, and therefore sampling from later patients was restricted to the former 5 organs. Table 3.2 specifies patients in whom each of these organs had sufficient parasite sequestration for typing purposes.

Table 3.2. Organs containing sequestered schizonts typed by IFA

<table>
<thead>
<tr>
<th>Autopsy</th>
<th>Brain (B1-10)</th>
<th>Cerebellum (B11-12)</th>
<th>Lung (D1-2)</th>
<th>Spleen (J2)</th>
<th>Liver (F4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP96-6</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>MP97-11</td>
<td>+++</td>
<td>+++</td>
<td>+/−</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>MP97-13</td>
<td>+++</td>
<td>++</td>
<td>+/−</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>MP97-15</td>
<td>+++</td>
<td>+++</td>
<td>+/−</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>MP98-23</td>
<td>+++</td>
<td>++</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>MP98-26</td>
<td>+++</td>
<td>+++</td>
<td>n.a</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>MP98-27</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

* +++= intense sequestration; ++=marked sequestration; + = moderate sequestration; +/- = minimal sequestration
Fig. 3.1. Detection of parasites by Giemsa staining in brain capillaries from children dying from cerebral malaria.

Brain capillaries full of sequestered parasites and malaria pigment. A thin smear was prepared from brain homogenates (see M&M), methanol-fixed and Giemsa-stained for examination under the light microscope using 630× magnification. A) Capillary with sequestered trophozoites (T) from autopsy MP96-6. The pink oval bodies are the nuclei (N) of the capillary endothelial cells. B) Capillaries containing mainly segmenter schizonts (S) from autopsy MP97-13.
3.3.1.1 - Tissue homogenates

Homogenates of brain and cerebellum tissues in PBS were adequate for IFA typing. Both grey and white matter from the frontal (B1) and parietal (B2) lobes of the brain (fig. 3.2), and from the peripheral (B11) and dentate nucleous (B12) of the cerebellum were processed from CM autopsies MP96-6, MP97-11, MP97-13, MP97-15, MP97-16, MP97-21 and from non-CM controls. Although the morphology of the organ was disrupted by homogenisation, most capillaries containing PRBCs remained intact. Co-localised fluorescent staining of \textit{P. falciparum} DNA and surface proteins revealed the presence of sequestration only among CM cases. Homogenates were also prepared and tested from the spleen, liver and lung, but they were less satisfactory than brain homogenates because in these organs, sequestered PRBCs were released from the blood vessels by homogenisation. It was not possible to obtain homogenates from fibrous tissue like heart muscle. Homogenates could be easily handled on multi-spot slides, as with cultured parasites, and incubated with multiple typing Ab reagents per slide.

3.3.1.2 - Tissue smears and touches

Tissue smears were prepared from the same areas of brain and cerebellum as homogenates specified above. Touches were more appropriate for lung (right upper, D1, and lower, D2, lobes) (fig. 3.3), spleen (J2) (fig. 3.4), and liver (F4) (fig. 3.5). Thin smears of brain and cerebellum were processed from autopsies MP96-6, MP97-11, MP97-13, MP97-15, MP97-16, MP97-21, MP98-23, MP98-26, MP98-27, supraorbital needle biopsies and from non-CM controls. Viewing of individual parasites and thus estimation of the proportions of mixed-clone infections by IFA was easier with tissue smears, as capillaries did not clump together.

3.3.1.3 - Liquid N\textsubscript{2} cryopreserved tissue sections

Brain tissue sections from autopsy MP96-6 were obtained for comparison with homogenates and smears. Cryosections of brain and cerebellum were as good as smears to carry out parasite counts, and had the added advantage of keeping the histological structure (fig. 3.6).
Fig. 3.2. Detection of parasite antigens in brain capillaries by IFA.
Brain homogenate prepared from autopsy MP96-6 showing capillaries with mature sequestered *P. falciparum* parasites (315× magnification), stained with:

A) FITC stain: Fluorescent schizonts (S) as recognised by mAb 18.2, specific for a putative malaria knob antigen. Note that the lower capillary appears full of parasites while the upper one is almost empty.

B) DAPI stain: Blue dots correspond to the multinuclei schizont forms (S), exactly on the same positions as fluorescence was seen. The blue oval bodies correspond to endothelial cell nuclei (N), which allows identification of the capillary.
Fig 3.3. Detection of schizonts in lung by IFA
Isolated fluorescent trophozoites and one schizont detected in a lung touch from autopsy MP98-27 (630× magnification), stained with:

A) DAPI stain: Scattered blue dots correspond to isolated trophozoites (T). One schizont (S) in the centre is surrounded by lung cells (nuclei, N)

B) FITC stain: FITC-conjugated anti-IgG1 reacting with mAb 111.4, specific for epitopes in block 17 of MSP-1. Both trophozoites (T) and schizonts (S) are recognised by this mAb.

C) RITC stain: RITC-conjugated anti-IgG2b reacting with mAb 123D3, specific for K1-type (Palo Alto variant) of block 2 of MSP-1. Only one schizont (S) is recognised by this mAb.
Fig 3.4. Detection of schizonts in spleen by IFA

Fluorescent schizonts detected in a spleen touch from A-B) autopsy MP96-6 (630× magnification) and C-D) autopsy MP98-27 (315× magnification), stained with:

A) **DAPI stain**: Blue dots in the centre (S) correspond to multinuclei schizonts. The blue round bodies (N) correspond to the nuclei of spleen cells

B) **FITC stain**: FITC-conjugated anti-IgG reacting with mAb 9.8, specific for conserved epitopes of MSP-1

C) **FITC stain**: FITC-conjugated anti-IgG1 reacting with mAb 11.4, specific for epitopes in block 17 of MSP-1. Both trophozoites (T) and schizonts (S) are recognised by this mAb.

D) **RITC stain**: RITC-conjugated anti-IgG2b reacting with mAb 123D3, specific for K1-type (Palo Alto variant) of block 2 of MSP-1. Only schizonts (S) are recognised by this mAb.
Fig. 3.5. Detection of schizonts in liver by IFA

Fluorescent schizonts detected in a liver touch (630× magnification) from A-B) autopsy MP97-13 and C-D) autopsy MP98-27, stained with:

A) DAPI stain: Four parasites are detected with DAPI: two schizonts (S) and two trophozoites (T).

B) FITC stain: FITC-conjugated anti-IgG reacting with mAb 31.1, specific for epitopes of the RO33 block 2 type of MSP-1. Only schizonts are detected by MSP-1 specific mAbs and thus the two trophozoites were negative by FITC fluorescence. Case MP97-13 had sequestered parasites of more than one *P. falciparum* clone (table 3.3), distinguished by different MSP-1 block 2 types. The FITC fluorescent schizont (S) belonged to the RO33 block 2 type, whereas the negative schizont belonged to the K1 block 2 type.

C) FITC stain: FITC-conjugated anti-IgG1 reacting with mAb 111.4, specific for epitopes in block 17 of MSP-1.

D) RITC stain: RITC-conjugated anti-IgG2b reacting with mAb 123D3, specific for K1-type (Palo Alto variant) of block 2 of MSP-1.
Fig. 3.6. Detection of parasite antigens in brain cryosections by IFA
Cross-sectional cuts of brain capillaries containing fluorescent schizonts from autopsy MP96-6 (315× magnification), stained with:
A) **DAPI stain**: Blue round bodies correspond to brain cells nuclei (N) and schizonts (S) sequestered in brain capillaries.
B) **FITC stain**: FITC-conjugated anti-IgG3 reacting with mAb 113.2, specific for MSP-2 group A. Only schizonts (S) are recognised by this mAb.
C) **RITC stain**: RITC-conjugated anti-IgG1 reacting with mAb 5.1 specific for EXP-1. Both schizonts (S) and trophozoites (T) are recognised by this mAb.
3.3.2- In situ IFA serotyping in organ-sequestered parasites

Table 3.3 summarises the results of typing MSP-1, MSP-2 and EXP-1 of *P. falciparum* parasites sequestered in tissue specimens from 9 autopsies and 1 supraorbital biopsy (L. S.). Biopsy D.N. had minimal sequestration and it was excluded. All these CM cases were positive for knob-specific mAbs (18.2-3-3, 18.2-4 and 9.21-4-2) and so these results are omitted from the table. Spleen, liver and lung generally contained fewer parasites than brain and cerebellum (table 3.2), and thus it was not possible to carry out an exhaustive typing of parasites in these tissues. However, in autopsies MP96-6, MP97-13, MP97-15 and MP98-27 it was possible to compare spleen to brain and cerebellum. In these cases, IFA typing results did not vary among different organs. Thus, from this analysis it emerged that all organs examined within a single patient had the same distribution of sequestered *P. falciparum* clones. Therefore, findings are summarised for all tissues in row denoted "IFA-T" (table 3.3).

Mature schizonts were identified in tissues of 9 cases by Giemsa staining, and expression of merozoite surface antigens was shown by IFA using Abs to conserved regions of MSP-1 and MSP-2. Subsequently, antigenic diversity of these sequestered parasites from autopsies MP96-6, MP97-11, MP97-13, MP97-15, MP98-23, MP98-26 and MP98-27 was characterised using a panel of type-specific Abs. Only two thin smears the from brain of L.S. were available, and thus typing was limited to a few Abs.

In addition to schizonts, most cases also had trophozoites and rings sequestered in the tissues, as detected by Giemsa staining and IFA. Thus, sequestered PRBCs were mostly composed of asynchronous parasite broods, with co-existence of parasites of different ages at a each tissue site. In such cases, trophs and rings were positive for DAPI fluorescent staining but negative for most typing mAbs, except for mAbs specific for EXP-1 and knobs antigens, and mAb 111.4 specific for an epitope located in the block 17 of MSP-1, which also react with immature stages.

3.3.2.1- Complexity of infections in tissues

Sequestered parasites belonged to more than one *P. falciparum* clone in most cases; MP98-26 was the only case with apparently single clone infection. MP96-6, MP97-11 and MP98-23 consisted of two-clone infections, whereas MP97-13,
Table 3.3. Typing of MSP-1, MSP-2 & EXP-1 in sequestered and circulating parasites from ten fatal CM cases

<table>
<thead>
<tr>
<th>Antigen Type</th>
<th>Block 17</th>
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<th>Block 3</th>
<th>Block 2</th>
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a MSP-1 types K=K1, M=MAD20; b IFA-T denotes typing in tissues, which refer to a summary of results brain, cerebellum, spleen, liver and lung (see text for details); ++++ very intense, +++ intense, ++ weak, +/- very weak fluorescence; c IFA-B denotes typing in peripheral blood (see chapter 2) in the same patient as the tissue typing was performed; d PCR refers to genotyping of parasites in peripheral blood (see chapter 2), given as +ve or -ve for each locus; the no. of multiple PCR bands is indicated. Polymorphic repeat sequences from MP664 are specified. Shaded boxes mean non available.
Table 3.3. Typing of MSP-1, MSP-2 & EXP-1 in sequestered and circulating parasites from ten fatal CM cases (continuation)

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MSP-1

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K=K1, M=MAD20; *IFA-T denotes typing in tissues, which refer to a summary of results brain, cerebellum, spleen, liver and lung (see text for details); ++++ very intense, +++ intense, ++ weak, +/- very weak fluorescence; *IFA-B denotes typing in peripheral blood (see chapter 2); *PCR refers to genotyping of parasites in peripheral blood (see chapter 2) in the same patient as the tissue typing was performed, given as +ve or -ve for each locus; the no. of multiple PCR bands is indicated. Polymorphic repeat sequences from MP664 are specified; n.t=not tested. Shaded boxes mean non available.
MP97-15, MP98-27 and L.S. were three-clone infections. Double-labelled IFA was
carried out to quantify the proportion of each clone in the mixed infections
(table 3.3).

3.3.2.2- MSP-1, MSP-2 and EXP-1 serotypes in tissues

Frequencies of MSP-1 epitopes were resolved and compared between the
cases. Interestingly, the composition of MSP-1 serotypes in parasites from these fatal
CM cases was quite similar. Thus, all parasite clones detected in this small set of CM
cases belonged to the MAD20 dimorphic family of MSP-1, and most also expressed
10-2B epitope in block 4 of MSP-1. With regard to block 3 of MSP-1, both
alternative epitopes were present at comparable frequencies in tissues; intriguingly,
the combination 13.2-/9.5-, quite uncommon in blood isolates, was present in
sequestered parasites in 3 out of 7 CM cases. Dominant clones from CM patients
tended to contain MSP-1 block 2 K1-types (3 had Palo Alto, two 3D7, and 3 both
variant epitopes). RO33-type was expressed in the dominant parasite clone in case
MP98-26, while it constituted a minority clone in most other cases. MAD20 block 2
MSP-1 type was only detected in L.S. Parasites found in spleen and liver specimens
from cases MP97-16 and MP97-21 were positive for mAb 111.4 in block 17 of MSP-
1. Overall, these MSP-1 epitope prevalences reflected those found in Blantyre and,
particularly, among CM patients (see chapter 2).

Concerning MSP-2, 7 CM cases were composed of type A clones; type B
parasites were found in one case (L.S.). Three cases had parasites positive for the
epitope (GASGSAGS), characteristic of the polymorphic R1 region of MSP-2 of the
RO33 isolate.

Epitope 5.1 of EXP-1 was detected in most cases, except in MP98-27. In
autopsies MP97-16 and MP97-21, tissue-sequestered parasites consisted of mid or
late trophozoites that were negative for most MSP-1 and MSP-2 specific Abs. This is
because merozoite surface proteins are not expressed until late trophozoite or early
schizont stages. Typing these sequestered parasites was restricted to EXP-1, which
distinguished between P. falciparum parasites from these cases: MP97-16 was
positive and MP97-21 was negative for the EXP-1 mAb 5.1.
3.3.3 - Comparison between peripheral blood and organ-sequestered parasites

Out of 19 fatal cases from whom autopsy and/or supra-orbital biopsies were performed, a peripheral blood sample was obtained on admittance and parasitaemias were recorded in 17 cases (two patients died on admission). Four patients did not have asexual parasites detectable by microscopy in the blood on admission. Time of death (hours after admission), parasitemia shortly before death, and time interval between death and autopsy were also recorded (table 2.1). In one case, MP97-15, parasites were not detected in the blood at time of death, but brain, cerebellum and spleen tissues were loaded with sequestered schizonts. PRBCs from 17 fatal cases were used for parasite DNA extraction and PCR analysis, as described in chapter 2. Three parasite isolates were set up in culture for IFA typing.

PCR genotyping in peripheral blood of 14 cases revealed that all, even those diagnosed as negative by slide, were parasitaemic, and mostly composed of mixed infections (table 3.4). Moreover, indirect evidence of *P. falciparum* infection was also obtained by detection of specific Abs (see chapter 5): all controls (i.e. cases with no detectable sequestration of PRBCs) had plasma Abs specific for MSP-1 and/or MSP-2 antigens, except for two meningitis patients.

<table>
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<th>Patient</th>
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<th>MSP-2</th>
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Table 3.4. Typing by PCR of blood-circulating parasites from non-CM fatal controls
In 5 patients, the MSP-1 and MSP-2 serotypes of sequestered parasites detected at autopsy were compared to serotypes of circulating parasites collected on admission. Table 3.3 contains details of IFA (in row designated "IFA-B") and PCR typing of blood parasites together with IFA typing in tissues ("IFA-T") for each patient. A complete comparison between parasites in tissues and in peripheral blood could be done in cases MP96-6 and MP97-13 (fig. 3.7a,b), whereas in cases MP97-11, MP97-15 and L. S. (fig. 3.7c), tissues were typed by IFA and circulating parasites were typed by PCR.

The results of the comparison between organs and circulation in each patient revealed that the multiplicity of clones detected in these infections was consistent in parasites in blood and tissues. Cases MP96-6 and MP97-11 had two-clone infections, and MP97-13 and L. S. three-clone infections in the blood and tissues. MP97-15 was typed as a three-clone infection in tissues, whereas PCR detected a four-clone infection in blood.

Concerning *P. falciparum* serotypes detected for each patient, comparison between blood and tissue typing revealed that all parasites correlated in having the same MSP-1, MSP-2 and EXP-1 dimorphic types. IFA typing of MSP-1 blocks 2, 3, 4 & 17 in sequestered parasites from autopsies MP96-6 and MP97-13 reflected typing of blood parasites from the same patients. With regard to PCR typing in blood vs. IFA typing in tissues, results for MSP-1 block 2 and MSP-2 matched between both techniques in all cases, except for MP97-15, in whom PCR detected parasites with MSP-1 MAD20 block 2 type in circulation which were not found in tissues by IFA. This discrepancy was probably due to a higher sensitivity of PCR, rather than to a differential pattern of sequestration of each clone.

Finally, in cases MP98-23, MP98-26 and MP98-27 only organ-sequestered parasites were typed; in cases MP97-16 and MP97-21, PCR and IFA showed that blood parasites were composed by mixed MSP-1 and MSP-2 types, but unfortunately this could not be related to the sequestered trophozoites. Patient MP832 (autopsy MP97-16) was another mismatch between PCR and IFA typing in blood in the detection of MSP-1 MAD20 block 2 type (table 3.3).
Fig 3.7. Detection by double-labelled IFA of mixed-clone infection in peripheral blood vs. brain tissue

A comparison of MSP-1 serotypes of *P. falciparum* in peripheral blood vs. brain tissue of one patient is shown in panels A) and B):

A) peripheral blood of patient MP792 and B) homogenate from the frontal lobe of the brain (autopsy MP97-13) of the same child. A majority of schizonts (red) react with block 4-specific mAb 10-2B (isotype IgG2a)+RITC-conjugated anti-IgG2a. A minority (green) are positive with mAb 12.1 (isotype IgG1)+FITC-conjugated anti-IgG1. Sequestered parasites did not differ from those in circulation (630× magnification).

C) Thin smear prepared from a supra-orbital needle biopsy from a different CM patient (L.S) showing a mixed infection of two MSP-2 serotypes in a brain capillary. Some schizonts (red) react with MSP-2 B-specific mAb 8G10/48 (isotype IgG2b)+RITC-conjugated αIgG2b, whereas others (green) react with MSP-2 A-specific mAb 12.3 (isotype IgG1)+FITC-conjugated αIgG1 (630× magnification).
For the first time, parasites sequestered in deep organs have been typed *in situ* using Abs recognising the polymorphic *P. falciparum* antigens MSP-1, MSP-2 and EXP-1. IFA is a simple technique that allows resolution of antigenic diversity of parasites sequestered in the brain, cerebellum, spleen, liver and lung, and that is easily carried out in the field. Combinations of mAbs with different isotypes and epitope specificities (double-labelled IFA) were used directly on sequestered parasites in tissue specimens from fatal CM cases to examine the frequencies of MSP-1, MSP-2 and EXP-1 serotypes and to estimate the proportion of each clone in mixed infections.

First, it has been possible to examine the question whether a preferential sequestration of particular parasite clones (serotypes in the case of the chosen markers) in particular vascular beds within the brain and other organs occurs. I found no evidence for a differential sequestration of particular *P. falciparum* serotypes in particular organs. Thus, in those patients in whom it was possible to compare the IFA typing between different organs, sequestered *P. falciparum* parasites had the same composition of MSP-1, MSP-2 and EXP-1 antigens.

Second, a possible differential distribution of *P. falciparum* clones between peripheral blood and organs within the same patient has been investigated. When peripheral parasites could not be typed by IFA, PCR typing was performed. In such cases, DNA typing methods are limited in that they can not quantify multiple-clone infections. The most consistent finding in this study is that clonal multiplicity of infection detected by IFA serotyping did not differ between sequestered and circulating parasites, as shown in 7 out of 8 fatal cases. In only one case, PCR identified parasites (bearing MSP-1 MAD-20 block 2 type) in peripheral blood which were not found in tissues with a specific polyclonal antiserum. This difference may be due to a higher sensitivity of PCR than of IFA in the detection of minority clones (discussed in chapter 2). The high correlation between blood and tissue typing was perhaps surprising considering three related issues: (i) the fact that parasites of different genotypes appear and disappear periodically from peripheral blood independently of each other, shown by longitudinal sampling (ii) the asynchrony of
sequestered parasites stages detected in the tissues at autopsy and (iii) the requirement for mature schizonts at the sites of sequestration for MSP-1 and MSP-1 serotyping.

The first issue emerges in the context of recent longitudinal studies following daily the population dynamics of asymptomatic infections, which have shown that circulating parasites only partially reflect the total parasite burden within the individual (Daubersies et al., 1996; Farnert et al., 1997). This fact was well illustrated here with patient MP800, whose peripheral blood had no parasites at death, while tissues were heavily loaded with sequestered parasites found at post-mortem. In this study, blood parasites were collected on admission, while sequestered parasites were obtained during the autopsy. The time interval between admission and death ranged between 0-30 h, and a time lapse between the death and autopsy ranged between 1-14 hr; overall, the period between the two samples was 1-40 hr in different patients (table 3.1). It is conceivable that over these periods the relative composition of circulating and sequestered parasites could have changed significantly. Ideally, another peripheral blood sample should have been collected on the autopsy to ensure that no clones were missed. However, my findings suggest that the parasites found in the tissues corresponded to those detected in blood some hours earlier.

The second issue relates to the observation that sequestered parasites at different ages of their 48 hr development cycle co-existed within an organ in different capillaries in most post-mortem specimens. It is possible that different ages corresponded to different broods of parasites, which could be genetically distinct. In the complex scenario that emerges from longitudinal studies, it is probable that the presence of different parasite broods or clones overlap at different stages of the life cycle in both blood and tissues, complicating the resolution of the relative frequencies of each parasite serotype in different sites within the individual. Thus, it is possible, e.g., that the one parasite clone detected in blood by PCR but undetected in tissues by IFA represented one of the young stages sequestered in tissues at autopsy. This possibility links to the third issue, i.e. a constraint of IFA serotyping that MSP-1 and MSP-2 need be expressed by schizonts at the sites of sequestration for the parasites to be typeable. Synchronous trophozoites detected in two cases could not be typed with MSP-1 or MSP-2 Abs, and typing was restricted to EXP-1 that is expressed in
trophozoites. Therefore, there is a tendency to underestimate clonal multiplicity inherent to this approach, which did not, however, affect the good correlation between the parasites found in blood and tissues. Alternative methods, such as RT-PCR of MSP-1 and MSP-2 mRNA in cryopreserved tissues, in situ PCR, (or in vitro culture of sequestered parasites from tissue homogenates) could be appropriate for typing of immature sequestered parasites. However, these techniques are more complex and prone to contamination than IFA.

The third interesting finding of this chapter is that mixed P. falciparum infections were common among the fatal CM cases examined. Although CM patients have a lower frequency of multiple-clone infections than other malaria patients, the mean number of clones per individual of 2.25 found in the blood (see chapter 2), is similar to that found in the tissues typed in 8 CM fatal cases (2.37 clones/individual). As discussed above, the extent of diversity of P. falciparum in humans has usually been underestimated in cross-sectional studies, which have been restricted to the analysis of parasites collected from peripheral blood in single samples. Typing of sequestered parasites in parallel with typing of blood parasites in people who die with malaria will describe more accurately the within-host population dynamics of multiple P. falciparum infections. However, this is confined to the few places where autopsies can be carried out. Alternative ways to estimate tissue sequestration without the need of a full autopsy are supra-orbital needle biopsies (Carr et al, in preparation), or skin biopsies, as sequestration in subcutaneous tissue appears to correlate with sequestration in brain (Appleton et al, in preparation).

The fourth finding of this chapter is that, overall, parasites found among the CM fatal cases were quite similar with regard to their merozoite surface antigen composition. Thus, the predominant phenotype which emerged in sequestered parasites was MSP-1 MAD20 dimorphic type, MSP-1 K1 block 2 type, MSP-2 type A and EXP-1 5.1 positive (table 3.3). However, these serotypes were merely a reflection of the local repertoire in Malawi, particularly among CM cases (see chapter 2). Thus, this study provides no evidence to support MSP-1 or MSP-2 being implicated in some step of CM pathogenesis, or that some factor contributing to virulence could be genetically linked to one of these loci, a finding which was not
necessarily expected. This phenotypic composition could then be extrapolated to
parasites in tissues of non-fatal CM cases, which were not examined. However, since
the number of autopsies available was low, I do not rule out that a differential
distribution of parasite clones in different tissues may be found in a larger study. This
approach is a first step in a phenotypic analysis of the parasites responsible for
different clinical presentations of malaria, and must be complemented with
investigation of more specific physiological characters or parasite phenotypes which
have been identified as possible pathogenic markers, as appropriate Ab and DNA
probes are developed.

One of the main hypotheses of CM pathogenesis is that it is caused by the
sequestration of PRBCs in the brain. Sequestration is a specific receptor-mediated
process involving parasite ligands expressed on the knobs of the PRBC surface.
PfEMP-1 is one ligand thought to mediate cytoadherence of PRBCs to vascular
endothelium (reviewed in chapter 1). PfEMP-1 has also been implicated in rosetting,
which was associated with disease severity in African children (Rowe et al., 1995).
PfEMP-1s are highly polymorphic proteins and have been implicated in antigenic
variation (Smith et al., 1995) and immune evasion mechanisms, two other proposed
less-specific pathogenic factors. Thus, a related question is whether sequestered
parasites are composed of more than one clone or antigenic variant. As isolates were
composed of multiple clones, within one clonal population there may be multiple
variant antigenic types (VATs), with different antigenic profiles (Roberts et al., 1992).
Different VATs could be sequentially expressed, potentially changing adhesion
phenotypes and maybe affecting parasite virulence (Biggs et al., 1992). It has been
estimated that the multicopy var gene family encoding PfEMP-1s has between 50-100
copies per haploid genome (Su et al., 1995). It is possible that each sequestered
parasite subpopulation may express a different repertoire of these proteins or, more
unlikely, that different serotypes (as defined by MSP-1 and MSP-2) shared the same
"virulent" set of VATs, which might presumably precipitate events leading to a fatal
outcome. The lack of reagents to detect PfEMP-1 variants is a big obstacle to clarify
these possibilities.
IFA is a simple means to investigate whether parasite sequestration is associated with local upregulation of adhesion molecules. For instance, Turner et al. (1994) used an anti-MSP-1 mAb to identify parasites in conjunction to endothelial receptors. There was a highly significant co-localisation of parasite sequestration with the expression of ICAM-1, CD-36 and E-selectin in cerebral vessels; double staining of MSP-1 and ICAM 1 showed PRBCs adhering to ICAM-1 positive endothelium.

The combined occurrence of host and parasite polymorphisms may have implications in the outcome of the disease (Berendt et al., 1989). Polymorphisms in host cytoadherence receptors, e.g. ICAM-1, might be associated with certain parasite VATs. Thus, a differential ICAM-1 expression in different areas within the tissue capillaries could differentially promote adhesion of the PRBCs. For example, not all capillaries were equally loaded with parasites (fig.3.2). A lack of uniformity in the distribution of parasites in the vascular involvement has been observed before (Miller et al., 1971).

I have reported here a new application of MSP-1 and MSP-2 IFA typing, \textit{in situ} serotyping of \textit{P. falciparum} sequestered parasites. Potentially, IFA can also complement light microscopy in estimating density and stage of sequestered parasites, for investigating whether these indices may correlate with clinical features of severe malaria. Thus, intensity of sequestration could be quantified by computerised image analysis, and the combined use of Abs recognising stage-specific malaria antigens can assist in the definition of sequestered parasite stages.

In conclusion, using MSP-1, MSP-2 and EXP-1 polymorphic proteins as "strain" markers, parasites sequestered in tissues from fatal CM cases were mainly composed of more than one \textit{P. falciparum} clone, which reflected those found in the blood of the same patients and which did not show a differential distribution among the different tissues examined. Thus, this study does not support a role for these antigens in virulence. It does, however, provide the only practical way to date to assess the global complexity of mixed-infections within a host, information which will be useful to elucidate the role of other polymorphic markers presumably implicated in the pathogenesis of CM.
In the following chapters, the genotyping of blood and tissue parasites is complemented by an analysis of the antibody responses to MSP-1 and MSP-2 antigens in patients with severe and uncomplicated malaria, the aim being to elucidate the relationship between parasite diversity and the development of acquired immunity to malaria.
Chapter 4: IMMUNE RESPONSES TO PLASMODIUM FALCIPARUM MEROZOITE PROTEINS IN MALAWIAN CHILDREN WITH MALARIA

4.1 - Introduction

The merozoite surface proteins MSP-1 and MSP-2, the apical membrane antigen (AMA)-1 and the internal rhoptry-associated protein (RAP)-1 of Plasmodium falciparum are all considered potential malaria vaccine components. The evaluation of these vaccine candidates is assisted by studies of human immune response to natural infection aiming (i) to define T and B cell epitopes which are recognised by human populations, (ii) to correlate a response with apparent clinical status and thus to identify a role for that response, and (iii) by a direct assessment of activity, e.g., an analysis of the effect of affinity-purified human Ig on parasite growth in vitro, to characterise immune defence mechanisms. However, to date it has not been possible to define with any certainty whether these antigens contribute to immunity induced by natural infection.

Naturally acquired immunity to P. falciparum requires apparently a long period to develop. One possible explanation for the age/exposure-related acquisition of immunity could be an intrinsically poor immunogenicity of important antigens of the parasite. It has been argued that low immunogenicity of conserved epitopes in malaria proteins could explain the slow development of immunity, since the host would require a long or repeated exposure to these epitopes to achieve a protection. An alternative hypothesis is that immunity may be essentially "strain"-specific, effective protection being achieved only after immunisation with a succession of parasites representing the main antigenic types transmitted in a community (see chapter 5). Both hypotheses pose the question of the relative immunogenicity of conserved vs. variable P. falciparum antigens. So far, few studies have addressed this question, and thus we do not know what antigens are predominantly responsible for inducing immune responses protective against malaria.

Other suggested explanations for the age-related acquisition of immunity take into account the stage of physiological development of the human host. For example, a mature immune system could allow an adult to acquire immunity more rapidly than a
child under the same levels of exposure (Baird, 1995). In addition, non-protective antibody (Ab) isotypes may be produced during childhood, and may even block the activity of "protective" isotypes (Oeuvray et al., 1994).

Different levels of naturally acquired immunity could influence the wide spectrum of malaria disease manifestations. For instance, a lack of previous immunity or a defect in the humoral or cellular immune response may contribute to the development of severe malaria.

The introduction to this chapter first reviews in detail immuno-epidemiological studies on human immune responses to MSP-1, MSP-2, AMA-1 and RAP-1 (for a review on vaccine studies see section 1.2.2.2.). Second, I consider the possible contribution of acquisition of immunity to the different clinical presentations of *P. falciparum* malaria. Finally, I outline the specific questions approached by my work presented in this chapter.

4.1.1- Human immune responses to *P. falciparum* merozoite proteins

In malaria endemic areas, numerous studies of immune responses to MSP-1 and, to a lesser extent, to MSP-2, AMA-1 and RAP-1, have shown that serum Abs and T cells from people naturally infected with *P. falciparum* recognise these asexual blood stage antigens. A few studies have suggested a "protective" effect for such responses.

4.1.1.1- Immunity to MSP-1

A study in Philippines analysed Ab responses using parasite-derived MSP-1 fragments, expected to contain conformational antigenic determinants that might not be present in recombinant proteins (Kramer & Oberst., 1992). No difference in Ab titres was found between individuals with positive and negative blood films. Anti-MSP-1 Ab titres increased with age, seroprevalence reaching adult levels in the 5-9-year-old age group. Antibody responses to the whole MSP-1 and its natural 83 kDa fragment were higher than to a C-terminal 42kDa recombinant protein, suggesting that, in this setting, the C-terminus might be less immunogenic than the N-terminus. A study in India using synthetic peptides also reported that conserved N-terminal regions were naturally immunogenic (Kabilan *et al.*, 1994).

118
Human B and T cell epitopes were identified within relatively conserved block 3 (Sinigaglia et al., 1988; Crisanti et al., 1988; Rzepczyk et al., 1989) and dimorphic regions of MSP-1 (Rzepczyk et al., 1989). Both polymorphic and dimorphic regions of MSP-1 might play a role in inducing immunity. In Gabon, slide negative individuals had higher titres of Abs detected using a fusion protein containing conserved and polymorphic block 2 sequences of MSP-1, whilst higher parasitaemias correlated with lower IgG titres (Chizzolini et al., 1988). It was suggested that Abs to these regions of MSP-1 might play a role in controlling parasitaemia. In Sudan, Abs to polymorphic block 2 regions occurred in many, although not all individuals (Cavanagh et al., 1998).

Serum Abs from adults living in Burkina Faso were predominantly directed to fusion proteins representing dimorphic regions (Well/K1 and MAD20) of MSP-1, whereas Abs from children did not preferentially recognise any particular region of MSP-1 (Müller et al., 1989). In contrast to Kramer & Oberst’s study in The Philippines (1992), a significantly higher prevalence of anti-MSP-1 Abs was found in parasitised compared to nonparasitised individuals. In Mali, Abs to MSP-1 were also predominantly directed to dimorphic regions (Früh et al., 1991).

Increasing interest has focused on a C-terminal region based on evidence for a protective role of Abs against this region from animal models and in vitro systems. In addition, C-terminal regions are quite conserved and thus considered prime vaccine candidates. There are two major dimorphic types of MSP-142 (blocks 15 and 16), MAD20 and Well, with ≥ 47% identity between them, whilst less than 5% variation occurs among different MSP-119 alleles (block 17). Recombinant proteins representing different allelic forms of MSP-119 were widely recognised by Kenyan adults exposed to *P. falciparum* whereas T cell proliferative responses predominantly targeted epitopes in the dimorphic regions of MSP-142 (Udhayakumar et al., 1995). Using yeast-expressed recombinant proteins representing 3 natural variants of MSP-119, Ab responses of the IgG1 and IgG3 subclasses predominated, and higher levels of IgG1 but not IgG3 correlated with lower parasite densities among young age groups (Shi et al., 1996). In The Gambia, serum Abs from malaria-exposed children and adults recognised conformation-dependent epitopes formed by the two epidermal growth factor (EGF)-like motifs of MSP-119; the Abs were cross-reactive between the two
main allelic sequences, and predominantly of the IgG1 subclass (Egan et al., 1995). In Sudan, MSP-119 Abs occurred mostly in acutely infected individuals, whereas conserved N-terminal regions were rarely recognised by any donor (Cavanagh et al., 1998).

Few studies have assessed the duration of anti-MSP-1 responses. In Mali, Abs to MSP-1 were short-lived, especially in children, who were more susceptible to malaria (Früh et al., 1991). Higher levels were detected at the end of the transmission season. Cavanagh et al. (1998) also found that Ab responses to MSP-1 in Sudanese villagers were short-lived. In The Gambia, lymphoproliferative and IFN-γ responses to recombinant or native MSP-1 fluctuated over time independent of malaria transmission (Riley et al., 1993). In contrast, there was no significant seasonal variation in mean levels of anti-MSP-1 Abs. Malaria infections were more likely to be symptomatic in adults from urban (lower transmission) than rural areas, but there were no significant differences in acquired immune responses to MSP-1 between the two groups.

The relationship between naturally acquired immune responses to conserved and variable regions of MSP-1 and susceptibility to malaria infection and/or clinical disease has been addressed by prospective longitudinal studies. In The Gambia, significant associations were observed between Ab concentration and lymphoproliferative responses to the C-terminal MSP-142 and resistance to episodes of fever associated with high parasitaemia in partially immune children (Riley et al., 1992). In addition, the proportion of children with high levels of Abs to the relatively conserved block 3 was significantly greater among those who had asymptomatic rather than symptomatic infections. Two other studies in different age groups reported conflicting results. In Mali, Abs to polymorphic regions of MSP-1, including block 2 and block 4, correlated with an increased risk of reinfection in adults (Tolle et al., 1993). In adolescents, Abs to dimorphic regions (block 6) of the MAD20 isolate correlated with a 50% reduced risk of reinfection and an increased ability to control parasitaemia. In children, no association between Abs and protection was observed. In contrast, in Papua New Guinea (PNG), a significant association between Ab concentrations against C-terminal MSP-142, but not against N-terminal fragments, and protection
from clinical disease and severe parasitaemia was seen in children (Al-Yaman et al., 1996). An association between Abs to MSP-1\textsubscript{19} and reduced susceptibility to clinical malaria was reported (Egan et al., 1996). Kenyan infants with anti-MSP-1\textsubscript{19} IgG Abs had decreased parasite density, less febrile illness and less infection-related loss of haemoglobin (Hb) (Branch et al., 1998).

Taking together, these results indicate that protective immunity can be directed to proteins derived from either the N- or the C-terminal regions of MSP-1. However, data on the relative immunogenicity of conserved and variable regions of MSP-1, and on the "protective" effects of anti-MSP-1 Abs in humans remains inconclusive and require further investigations in different populations.

4.1.1.2- Immunity to MSP-2

In an analysis of cellular proliferative responses, four synthetic peptides based on MSP-2 (FC27, serogroup B) sequences were recognised by human T cells from donors living in PNG and Solomon Islands (Rzepczyk et al., 1989). Subsequently, most T cell epitopes were mapped to the central polymorphic region of 32-amino acid repeats (Rzepczyk et al., 1990).

Serum from people living in endemic areas contain Abs that react with MSP-2 (Miettinen-Baumann et al., 1988). A survey in PNG assessed the age patterns of anti-MSP-2 Abs prevalence and concentration, and the relationship between Ab response, fever and anaemia (Al-Yaman et al., 1994). Antibodies against nonrepeat regions of MSP-2 IC1/3D7 (A) but not against MSP-2 FC27 (B) allelic family were associated with fewer fever episodes and less anaemia. A prospective longitudinal study in the same area assessed the relationship between Ab responses to MSP-2 and subsequent susceptibility to malaria in children (Al-Yaman et al., 1995). Only Abs against MSP-2 IC1/3D7 (A) allelic type predicted a reduction in incidence rate of episodes of clinical disease. In a cross-sectional study in The Gambia, human Abs predominantly recognised epitopes located in dimorphic and polymorphic regions of MSP-2, and belonged mainly to the cytophilic and complement-fixing subclass IgG3 (Taylor et al., 1995). IgG1 Abs were prevalent in children < 10 years, whereas IgG3 predominated in adolescents and adults; anti-MSP-2 Abs prevalence was age-dependent, reaching a peak during adolescence (Taylor et al., 1998). A negative association between IgG3
Abs to MSP-2 serogroup A and risk of clinical malaria was reported, whereas IgG1 Abs to MSP-2 serogroup B were associated with an increased risk of the disease.

Information on human immune responses to MSP-2 is scarcer than for MSP-1, and the questions about immunogenicity and possible protective role of anti-MSP-2 Abs remain equally unsolved.

4.1.1.3- Immunity to AMA-1 and RAP-1

Field-based studies are required to characterise Ab responses to AMA-1. In one study, seven T cell epitopes of *P. falciparum* AMA-1 were identified by cellular proliferation of lymphocytes from Kenyans who had lifelong exposure to malaria (Lal *et al.*, 1996). Some of the T cell epitopes were localised in highly conserved regions.

Few studies have examined the human immune recognition of RAP-1. In Ghana, *in vitro* cellular proliferation in response to a recombinant protein representing the N-terminal third of RAP-1 was seen in most lymphocyte donors, and this suggested the presence of T cell epitopes in this region (Jakobsen *et al.*, 1993). In addition, sera from these donors contained Abs reactive with the recombinant RAP-1. A larger study in Tanzania using the same protein showed that the proportion of responders increased with age, and found an association between high anti-RAP-1 IgG Ab levels and protection against *P. falciparum* densities in children (Jakobsen *et al.*, 1996). Another survey in PNG confirmed that the recognition of RAP-1 correlated with age (Stowers *et al.*, 1997). A study that compared the relative immunogenicity of different RAP-1 regions found that most Abs were directed against epitopes within the N-terminus (Howard *et al.*, 1993). A set of 8 recombinant RAP-1 proteins was used to evaluate Ab responses in Gambian malaria patients (Fonjungo *et al.*, 1998). Although the sera contained Abs directed to the most N-terminal segment, most Abs were targeted to epitopes outside this region, and were of the IgG1 subclass.

4.1.2- Immune responses and malarial disease severity

Very few studies have addressed the relationship between levels and magnitude of acquired immunity and disease severity. The reasons why only a small proportion of young children infected with *P. falciparum* develop severe or fatal
malaria are unknown. One possibility is that children who develop severe malaria, i.e. cerebral malaria (CM) and severe malarial anaemia (SMA), may have had less previous exposure to malaria infection and therefore less acquired immunity than children who develop uncomplicated malaria (UM). A study in Thailand found that humoral responses in cerebral malaria patients (seronegativity rate and mean Ab titres) were not significantly different from non-cerebral malaria patients and thus did not support this hypothesis (Tharavanij et al., 1984). This was confirmed in The Gambia by estimating malaria exposure by Ab profiles to MSP-1, among other antigens, no significant differences in Ab titres between cases of severe or mild malaria being found (Erunkulu et al., 1992). The Abs measured in these studies were probably a reflection of past exposure to malaria, and not a result of the current infection. This question was addressed to some extent in a study in PNG, where specific Ab responses to MSP-1 and MSP-2 were not significantly different between CM and UM patients (Al-Yaman et al., 1997), in agreement with the two previous reports.

The role of cytokines in the pathogenesis of human malaria has been explored. Several studies have recorded a positive correlation between plasma levels of TNF-α and interleukin (IL)-6 and severity of disease, especially CM, in African children with P. falciparum infections (Grau et al., 1989 & Molyneux et al., 1991, in Malawi). Another pyrogenic cytokine, interleukin (IL)-1, showed a similar picture to that of TNF-α in The Gambia (Kwiatkowski et al., 1990).

4.1.3 Aims

The objectives of work reported in this chapter were:

- to compare the relative antigenicity of conserved, dimorphic and polymorphic regions of the merozoite surface proteins in Malawian children naturally infected with P. falciparum.

- to examine the time course of appearance of antibodies to these proteins after a malaria infection and the duration of these antibodies in convalescent patients.

- to investigate whether antibody responses to these antigens in patients presenting with acute illness may influence the development of severe disease.
4.2- Materials and methods

4.2.1- Collection of plasma samples

A total of 587 plasma samples assessed for Abs were collected during May 1996 (n=56) and over the whole 1997 malaria season (January-June) (n=531). Acute plasma samples were obtained from a total of 348 children under 12 years of age (n=34 in 1996; n=314 in 1997) in Blantyre, Malawi. Children with severe malaria (SM) (n=30 in 1996; n=207 in 1997) were admitted to the Malaria Research Project and Wellcome Trust Centre (MRP), Department of Paediatrics, Queen Elizabeth Central Hospital. A clinical scoring scale, the Blantyre Coma Score (BCS) (Molyneux et al., 1989), was used to assess the levels of consciousness in these children, as detailed in section 2.2.1 (chapter 2). 125 of the children with SM were defined as having cerebral malaria (CM, BCS<3) (n=23 in 1996, n=102 in 1997), 60 as severe malarial anaemia (SMA, Hb < 5g/dl or PCV <16%) (n=3 in 1996; n=57 in 1997), and 51 as both CM and SMA (n=3 in 1996; n=48 in 1997). Control patients with uncomplicated malaria (UM, BCS of 5) (n=77) were recruited as specified in section 2.2.1 (chapter 2). Another group of controls were patients suffering from severe non-malarial disease (SNM) (n=3 in 1996; n=31 in 1997). All patients were asked to come back for a follow up sample after a month, or earlier if they were parasitaemic or had symptoms of malaria. A total of 239 convalescent blood samples (n=22 in 1996; n=217 in 1997) were obtained from all groups of patients.

Five millilitres of venous blood were taken in lithium heparin or EDTA on admission at the MRP or at Nidirande Health Centre, centrifuged, buffy coat depleted, and plasma for serological analysis was stored at -70°C. Clinical and parasitological data including state of consciousness, history of convulsions, history of prior drug treatment, parasitaemia, haematocrit levels and basic demography were recorded for all patients. Patients admitted in 1997 constitute the study population to whom the results presented here refer, unless stated otherwise. Data of patients included in the serological study are summarised in table 2.1 (chapter 2).

Negative control sera were obtained from 50 Scottish adults who had not been exposed to malaria. A pool of sera from African immune individuals was used as a positive control in all tests.
Expression, purification and characterisation of the majority of recombinant proteins used in this study has been reported elsewhere. Briefly, genomic DNA from cultured parasite isolates was used as template for PCR amplification of specific fragments of the genes. PCR products were cloned and expressed in *Escherichia coli* as recombinant proteins fused to the C-terminus of glutathione S-transferase (GST) of *Schistosoma japonicum* using the pGEX-2T vector. GST fusion proteins were affinity purified by adsorption on glutathione agarose beads (Sigma, UK), and their concentration and purity were estimated by the Bradford protein assay kit (Bio-Rad, UK) and Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The GST protein alone was purified from cultures transformed with pGEX-2T vector (without *MSP-1*, *MSP-2* or *RAP-1* inserts) and used as a control antigen in ELISAs. All the proteins included here were shown to reflect the antigenic structure of *P. falciparum*-derived native proteins.

### 4.2.2.1- *MSP-1* constructs

*MSP-1* constructs were derived from both polymorphic and conserved regions of molecule. Proteins based on MSP-1 N-terminal sequences included two block 1 constructs, from the MAD20 and Palo Alto variants, respectively, and five constructs representing the three main block 2 types: K1 (Palo Alto and 3D7 fusion proteins), MAD20 (MAD20 and Well fusion proteins), and RO33 (RO33 fusion protein) (Cavanagh & McBride, 1997). A schematic representation of these proteins and their location is shown in fig 2.1 (chapter 2). In addition, two constructs representing *MSP-1* C-terminal regions were included: a GST fusion protein containing most of block 17 (N<sup>1,631</sup> to N<sup>1,726</sup> of the Wellcome isolate) corresponding to the 19kDa fragment (Burghaus & Holder, 1994), and a baculovirus-expressed recombinant protein corresponding to the 42kDa fragment of the CAMP isolate, donated by Dr. J. Lyon.

### 4.2.2.2- *MSP-2* constructs

*MSP-2* constructs were derived from allelic sequences representing the two major dimorphic types of the protein, IC1/3D7 (13 constructs) and FC27 (9 constructs), designated here as group A and B, respectively. Fig 4.1 shows a
schematic representation of the MSP-2 fusion proteins included in this study; a schematic representation of the structure of the MSP-2 gene is shown in fig 2.2 (chapter 2). Primers used for amplification of MSP-2 DNA and derivation of additional MSP-2 proteins are shown in table 4.1. Eight MSP-2 group A constructs were derived from polymorphic R1 repeat regions of the gene (primers 5/7) of isolates T9/96, ThaiTn, 7G8, T9/102, CH12/12, RO33 and T9/94. Four group A constructs were derived from dimorphic regions of the gene (primers 8/6) of isolates RO33, CH150/9 and T9/102, and one protein represented almost the full length MSP-2 (primers 13/14) of the T9/96 isolate. With regard to MSP-2 group B, 2 constructs were derived from polymorphic R1 regions consisting of 32-mer aa repeats (primers 5/3 or 13/3) of the K1 isolate, 3 constructs were derived from R2 regions consisting of 12-mer aa repeats (primers 12/6) of isolates K1 and T9/105. Two constructs represented almost the full length protein (primers 13/14) of isolates Dd2 and GF88/175, and 2 constructs were derived from conserved regions encoding the N-(primers 13/16) and C-terminus (primers 17/14) of MSP-2 of the K1 isolate.

4.2.2.3- AMA-1 and RAP-1 constructs

Recombinant *P. falciparum* AMA-1 antigen was a kind gift from Dr. R. F. Anders. It consisted of the full ectodomain of the 3D7 form of AMA-1, expressed in *E. coli* as an N-terminal His<sub>6</sub> fusion protein. It was purified by Ni chelate chromatography and refolded *in vitro* as described for the equivalent *P. chabaudi* AMA-1B (Anders *et al.*, 1998). Subsequently, it was further purified by ion-exchange chromatography and RP-HPLC.

The C2 fragment of RAP-1 was produced as a GST fusion protein corresponding to aa 169 to 366 of the antigen (including an inhibitory mAb epitope), as reported (Fonjungo *et al.*, 1998).

4.2.3- Enzyme-linked Immunoabsorbent assay (ELISA)

Wells of microtiter plates (Immunolon-4, Dynatech) were coated overnight at 4°C with 0.05µg/100µl of antigen per well in 0.1 M carbonate (Na<sub>2</sub>CO<sub>3</sub>-NaHCO<sub>3</sub>) buffer (pH 9.6). Three sets of plates containing, respectively, panels of recombinant proteins derived from MSP-1, MSP-2 A and MSP-2 B types were prepared (figs. 2.1
Fig 4.1 a. Schematic representation of recombinant proteins of MSP-2 serogroup A
Fig 4.1 B. Schematic representation of recombinant proteins of MSP-2 serogroup B
**Table 4.1. Oligonucleotide primers used in PCR amplification for expression of MSP-2 fusion proteins**

<table>
<thead>
<tr>
<th>Region</th>
<th>ID</th>
<th>Nucleotide sequence 5' to 3'</th>
<th>Use and specificity</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conserved</td>
<td>13</td>
<td>5' AAAACCTAAATGAAAGTTAATAATAGCAACACATTCC</td>
<td>Expression fusion protein</td>
<td>1</td>
</tr>
<tr>
<td>(full length)</td>
<td>14</td>
<td>5' GCTTAAATAGTAAATGCTAAGG</td>
<td>Expression fusion protein</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5' CCATGCACTATGCTGCCATTGTGCTCTGCTCATT</td>
<td>Expression fusion protein</td>
<td>1</td>
</tr>
<tr>
<td>Polymorphic</td>
<td>7</td>
<td>3' TTCTCTCAGCATCTGCA</td>
<td>Expression R1 type A fusion proteins</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3' GGTTGAATCTTCTTTCTGTAGCTAGG</td>
<td>Expression R1 type B fusion proteins</td>
<td>2</td>
</tr>
<tr>
<td>Dimorphic</td>
<td>8</td>
<td>5' GTCCAAGTACTCCGTC</td>
<td>Expression dimorphic type A fusion proteins</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5' TGATACCCCTACTGCA</td>
<td>Expression dimorphic type B fusion proteins</td>
<td>2</td>
</tr>
<tr>
<td>Conserved</td>
<td>16</td>
<td>3' CATACTTCTCTCTATTACTC</td>
<td>Expression N-terminus fusion proteins</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>3' GCACCAGAGAATAAAGG</td>
<td>Expression C-terminus fusion proteins</td>
<td>2</td>
</tr>
</tbody>
</table>

Refs. = References: (1) Dobaño et al., 1997; (2) McBride, unpublished
In parallel, antigen control wells containing GST or buffer alone were set up in each plate. The plates were washed 3× with PBS-0.05% Tween 20 and blocked for 5 hr at room temperature with 200µl of blocking buffer (1% wt/vol skimmed milk powder in PBS-Tween) per well. At the same time, Malawian tested sera and European control sera were diluted 1/500 in the blocking buffer and incubated at room temperature for 5 hr. Plates were washed as above, 100µl of diluted serum/well was added to duplicate wells, and incubated overnight at 4°C. After washing, IgG bound to the wells was detected with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako Ltd. High Wycombe, UK) at 1/5000 dilution in PBS-Tween for 3 hr at room temperature. After washing, the reactions were developed with 0.012% H₂O₂ as the substrate and o-phenylenediamine (Sigma) as the chromagen (100µl per well). The reactions were stopped after 10 min with 20µl of 2M H₂SO₄ per well. Optical density (OD) was measured at a wavelength of 492nm.

4.2.4- Statistical analysis

Specific reactivity of a serum with a recombinant protein in the ELISA was calculated by subtracting OD values for the GST or buffer controls from the value obtained for the recombinant protein to obtain specific OD values. Positive samples were then defined as those giving a specific OD above the cut-off. The cut-off for each antigen was taken as the mean + 2 standard deviations of 50 European control serum samples.

Associations between the presence of particular Abs in plasma samples collected in 1997 and disease manifestations, were analysed by Chi-squared (χ²) tests; McNemar's χ² tests with Yates' continuity corrections were used for comparison between proportions of responders paired for a case-control study. Antibody levels were compared among different groups of patients by t-tests. Paired t-tests were used to compare antibody levels between acute and convalescent plasma samples.

In addition, data on all patients surveyed in 1996 and 1997 were introduced into the SAS statistical analysis package (SAS, 1990) and analysed using standard multiple regression techniques. The associations between antibody levels, age, parasitaemia and multiplicity of infection were analysed by a generalised linear model.
(PROC GLM), appropriate for continuous dependent variables. The relationship between host's disease status as a binary trait and antibody responses (levels and positivity) as an explanatory variable was analysed by PROC GENMOD (categorical linear model), using a binomial distribution for the dependent variable. The models were adjusted for the confounding variables of parasite density and genotype, multiplicity of infection, age and sex of the child, date of admission and outcome. Five categories were established for the outcome of the disease: death, neurological sequelae (as a result of acute cerebral illness), uncomplicated malaria (recurrent infections accompanied by mild malaria symptoms), asymptomatic (recurrent infection without malaria symptoms) and full recovery (no parasites and no symptoms of malaria disease). Significance was defined at the 5% level in all analysis.

4.3 Results

4.3.1 Antibody responses to MSP-1 in Malawian children

Plasma samples collected from a total of 348 children living in a malaria endemic area of Malawi during 1996 and 1997 were tested by ELISA for the presence of Abs specific for *P. falciparum* MSP-1. Overall, 289/348 (83%) of the acute plasma samples had Abs reacting with at least one MSP-1 recombinant protein. Among 239 convalescent plasma samples obtained, 184 (77%) contained Abs recognising MSP-1. The reactivities of Abs with each of the 3 regions of MSP-1 examined in this study (blocks 1, 2 and C-terminus) are summarised in table 4.2.

<table>
<thead>
<tr>
<th>Table 4.2. Prevalence of antibodies to distinct regions of MSP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Block 1</td>
</tr>
<tr>
<td>Block 2</td>
</tr>
<tr>
<td>C-terminus 19kDa</td>
</tr>
<tr>
<td>C-terminus 42kDa</td>
</tr>
</tbody>
</table>

\(^a\) n refers to the number of individual plasma samples collected on admission (acute) or one month later at a follow up visit (convalescent)
Antibody prevalences to all MSP-1 fusion proteins examined were particularly high in the limited set of plasmas from 1996. However, this finding may be confounded by the fact that all those samples were collected at the end of the malaria season (May), when seropositivity is expected to be high, and mainly from CM patients, who had Ab responses more frequently (see section 4.3.4.1). Most often, detected anti-MSP-1 Abs were directed to conserved regions situated at the C-terminus end of the protein. Thus the recombinant proteins representing the 19kDa and 42kDa fragments of MSP-1 were the most widely recognised by serum Abs from Malawian children. In contrast, only a very small proportion of plasmas contained Abs specific for the conserved block 1 of MSP-1, as determined by recognition of two fusion proteins based on the sequences of the Palo Alto and MAD20 isolates. Antibodies to polymorphic epitopes located in one or another type of block 2 of MSP-1 were detected in 28% of all serum samples tested. Antibodies specific for epitopes of the K1 type MSP-1 block 2 were more prevalent than Abs to the MAD20 or RO33 types (table 4.3).

Table 4.3. Prevalence of antibodies to block 2 of MSP-1

<table>
<thead>
<tr>
<th>Block 2 type</th>
<th>% Acute (n=352)</th>
<th>% Convalescent (n=235)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>17</td>
<td>14.9</td>
</tr>
<tr>
<td>MAD20</td>
<td>6.8</td>
<td>3.4</td>
</tr>
<tr>
<td>RO33</td>
<td>6.8</td>
<td>10.2</td>
</tr>
</tbody>
</table>

$^a$ n refers to the number of individual serum samples collected on admission (acute) or at a follow up visit a month later (convalescent)

Concerning the evolution of Ab responses during a month between acute and convalescent plasma samples, there was a decline in MSP-1 seropositivity. The percentages of positive responses to MSP-1$_{19}$ and MSP-1$_{42}$ were lower a month after the clinical malaria episode (table 4.2). Thus, it appears that Abs produced during *P. falciparum* infection did not last long after recovery from malaria. In contrast, more responders to the block 2 RO33 construct were detected in convalescent than acute samples (table 4.3).
The relationships between parasitaemia, multiplicity of infection, age and sex of the patient, outcome of the disease and Ab responses to MSP-1 were analysed statistically. There was a positive association between Ab levels to the C-terminal region of MSP-1 and higher parasite densities (PROC GLM, 19kDa p=0.0203, 42kDa p=0.0131). Parasite densities also had an influence in the levels of Abs to RO33 block 2 (PROC GLM, p=0.0057, adjusted for diagnosis). A negative correlation was found between multiplicity of infections and levels of Abs to C-terminal fragments (PROC GLM, 19kDa p=0.0363, 42kDa p=0.0489); thus, higher Ab levels appeared to be partially explainable by lower multiplicity. An effect of age in the levels of Abs was only significant for anti-block 2 Palo Alto responses (PROC GLM, p=0.0387). No other significant associations between any of the factors specified above and Ab responses to the rest of MSP-1 fusion proteins were found.

4.3.2- Antibody responses to MSP-2 in Malawian children

All plasma samples were tested for the presence of Abs specific for \textit{P. falciparum} MSP-2. A total of 315/348 (90.5\%) acute and 220/239 (92\%) convalescent serum samples had anti-MSP-2 Abs above cut-off levels. Concerning the two major MSP-2 dimorphic families, 83\% of acute and 77.4\% of convalescent samples had Abs recognising recombinant proteins of the IC1/3D7 family or type A; 79.6\% of acute and 79\% of convalescent sera had Abs reactive with recombinant proteins of the FC27 family or type B. The reactivities of human serum Abs with distinct polymorphic, dimorphic and conserved regions of MSP-2 are summarised in table 4.4.

In the acute stage, most children contained Abs directed to fusion proteins representing dimorphic regions of MSP-2. Antibodies to MSP-2 group A-specific regions were the most prevalent, followed by Abs to MSP-2 group B-specific regions. Half of the acute serum samples contained Abs to polymorphic R1 regions of MSP-2 type A, whereas \( \geq 30\% \) had Abs to the 32-mer repeats (R1) of MSP-2 B. The full length constructs of MSP-2 B were recognised by \( > 70\% \) of individuals, correlating well with reactivities of constructs representing polymorphic and dimorphic regions. However, the seropositivity for the full length MSP-2 A construct was lower than
expected from the prevalences of Abs to dimorphic and polymorphic MSP-2 A proteins. This discrepancy was probably due to the fact that control European sera occasionally reacted with the full length T9/96 13/14 protein, giving a high cut off value (OD=0.94) which translated in lower seropositivity rates among the cases.

Concerning recognition of conserved regions of MSP-2, around 30% of children had Abs to C-terminal regions, whereas Ab responses to the N-terminal fragment were very rare. A decrease in positive Ab responses to the dimorphic regions of MSP-2 was noted from acute to convalescent patients, most markedly in Abs to the conserved C-terminus.

Table 4.4. Prevalence of antibodies to distinct regions of MSP-2

<table>
<thead>
<tr>
<th>MSP-2 family</th>
<th>MSP-2 region</th>
<th>1996</th>
<th>1997</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Acute n=34</td>
<td>% Convalescent n=22</td>
<td>% Acute n=314</td>
</tr>
<tr>
<td>MSP-2 A</td>
<td>R1</td>
<td>55.9</td>
<td>77.3</td>
</tr>
<tr>
<td></td>
<td>dimorphic</td>
<td>91.2</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td>Full length</td>
<td>56</td>
<td>68</td>
</tr>
<tr>
<td>MSP-2 B</td>
<td>R1</td>
<td>50</td>
<td>63.3</td>
</tr>
<tr>
<td></td>
<td>R2 c &amp; dimorphic</td>
<td>82.4</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>Full length</td>
<td>85.3</td>
<td>91</td>
</tr>
<tr>
<td>Conserved</td>
<td>N-terminus</td>
<td>11.7</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>C-terminus</td>
<td>38.2</td>
<td>22.7</td>
</tr>
</tbody>
</table>

a n refers to the number of individual serum samples collected on admission (acute) or at a follow up visit a month later (convalescent)

b R1 refers to polymorphic regions of MSP-2 composed by tandem repeats of 4-8 amino acid (MSP-2 A) or 32 amino acid units (MSP-2 B), as illustrated in fig 2.2 (chapter 2) and fig 4.1a.

c R2 refers to polymorphic regions of MSP-2 B composed of 12-mer amino acid repeats (see fig 4.1b).

No relationship between parasitaemia, multiplicity of infections, age or outcome and Ab responses to MSP-2 could be established.

4.3.3- Antibody responses to AMA-1 and RAP-1 in Malawian children

Serum samples collected during 1997 were tested for the presence of Abs specific for *P. falciparum* AMA-1. A total of 182/314 (57.9%) acute and 121/217 (55.7%) convalescent plasma samples had Abs that reacted positively with the full length AMA-1 recombinant protein. Antibody levels did not vary significantly from
acute to convalescent samples. An effect of age in the levels of Abs to AMA-1 was found to be statistically significant (PROC GLM, p=0.036), in contrast to the other merozoite antigens, in which age did not influence the Ab profiles. The age distribution of anti-AMA-1 Abs, which peaked at 4 years old (fig 4.2), was shown to be a confounder in the analysis of the relationship between Ab levels and disease status (see section 4.3.4.3).

With regard to RAP-1, in 1997 a total of 135/314 (42.9%) acute and 80/217 (36.8%) convalescent serum samples had Abs to the C2 recombinant protein. In 1996, 17/34 (50%) acute and 9/22 (40.9%) convalescent sera contained Abs specific for the C2 fragment of RAP-1.

4.3.4- Relationship between antibody responses to merozoite surface proteins and malaria disease

The antibody responses to *P. falciparum* merozoite surface proteins were compared among groups of children presenting with different malarial disease manifestations. The clinical groups were: cerebral malaria (CM), severe malarial anaemia (SMA), CM with anaemia complications (CM+SMA), uncomplicated malaria (UM) and severe non malaria disease (SNM), as defined in section 4.2.1. Using multiple regression analysis I looked for statistically significant associations between (i) levels of Abs (ii) prevalence of positive Ab responses to distinct regions of the antigens and disease severity. First, antibody responses to MSP-1, MSP-2, AMA-1 and RAP-1 were compared between different clinical forms of malarial disease in the acute stage to test whether a defect in the humoral immune response contributed to the development of severe malaria. Second, the Ab responses were analysed at one-month follow up to assess the ability of children with different malaria symptoms to mount Ab responses to the acute infection, and also to estimate the time-course appearance and duration of these Abs. A differential pattern of Ab responses to conserved and variable regions of these antigens was found in different clinical forms of malarial disease.
Fig 4.2. Age distribution of antibody responses to AMA-1
Columns indicate the mean Ab levels (OD at 492nm) for AMA-1 in plasmas from children presenting with acute malaria illness. The number of children in each age group is indicated by a line. Ages are indicated by the upper limit of the age group (i.e. 0.5 refers to 0 to 0.5 years old). Antibody levels increased steadily from birth until 4 years old, where the highest mean OD values were noted, and decreased in older children. The cut-off value for positive ant-AMA-1 Ab responses (OD=0.27) is indicated with a horizontal line.
4.3.4.1- Antibody responses to MSP-1

Children who presented with SMA were distinguished from CM or UM cases by lower mean levels of Abs to the C-terminal regions of MSP-1 (19kDa and 42 kDa fragments) (fig 4.3). Children admitted with CM had significantly higher mean levels of Abs to these proteins than children with UM (PROC GENEMOD, p=0.0304 19kDa, p<0.05 42kDa), or SMA (p=0.01 19kDa, p<0.01 42kDa) (fig. 4.3). Furthermore, the proportion of children who had Abs against the C-terminus was significantly higher in CM than UM (p=0.0008 19kDa, p=0.0389 42kDa), or SMA cases (p=0.0053 19kDa, p=0.0408 42kDa) (fig 4.4). These effects were not explicable by differences in age, sex, date of admission, outcome, parasitaemia, parasite’s genotype or multiplicity of infections between the disease groups.

These results were reproduced in a case-control study including a subset of malaria patients from 1997 matched according to age (61 pairs CM vs. UM; 48 pairs SMA vs. UM; 44 pairs CM vs. SMA). Thus, the proportion of patients with Abs to MSP-1 C-terminal regions was significantly higher among CM than among UM (McNemar’s χ² tests, p<0.001 19kDa, p<0.05 42 kDa) or among SMA patients (p<0.05 for both 19kDa and 42 kDa). Similarly, mean Ab levels were higher in CM than in UM (paired t-test, p>0.001 19kDa, p>0.005 42 kDa) or in SMA cases (p>0.001 for both 19kDa and 42 kDa). Positive Ab responses to C-terminal regions of MSP-1 also occurred in SNM controls, in whom *P. falciparum* infections were occasionally detected, though normally at low densities (see chapter 2). Mean Ab levels among SNM controls in acute stage were higher than among SMA cases and lower than among CM or UM cases, but did not differ significantly from any disease group (data not shown).

Concerning Ab responses to the polymorphic block 2, the same trend as for the C-terminal region was found for levels and prevalence of Abs specific for the least variable of block 2 types, the RO33 type. Children with CM had significantly higher mean levels of Abs to the RO33 block 2 than children with UM (p=0.0125) or SMA (p=0.0083), taking into account the possible confounder factors of age, sex, date, parasitaemia and multiplicity of infection (fig 4.5). In addition, CM patients had a
Fig. 4.3. Antibody responses to MSP-1 42kDa

Dot plots showing that mean Ab levels (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent) were higher than in uncomplicated malaria (UM, 76 acute, 27 convalescent) or severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=45) for positive sera is indicated by a horizontal line.
Fig. 4.4. Prevalence of antibodies to MSP-1 42kDa

Percentage of patients with cerebral malaria (CM, 102 acute, 87 convalescent), uncomplicated malaria (UM, 76 acute, 27 convalescent) and severe malarial anaemia (SMA, 56 acute, 48 convalescent) who had Abs to the C-terminus 42kDa above cut-off (OD=0.45)
Fig. 4.5. Antibody responses to MSP-1 block 2 RO33

Dot plots showing that mean Ab levels (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent) were higher than in uncomplicated malaria (UM, 76 acute, 27 convalescent) or severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.29) for positive sera is indicated by a horizontal line.
higher proportion of positive responses to this antigen than UM patients (p=0.0458). No significant associations between Ab responses to the other two types of block 2 and disease severity were detected. However, there was a clear trend for higher Ab responses to both K1 and MAD20 types in CM compared to SMA patients.

There was a significant fall in Ab levels to the conserved C-terminal regions of MSP-1 between acute and convalescent stages in patients suffering from any form of severe malaria (73 pairs CM, 39 pairs SMA, 33 pairs CM+SMA) (paired t-test, p<0.001 in all cases) or from SNM (10 pairs, p<0.02 19kDa, p<0.005 42kDa). There was also a significant decrease in Ab responses to MSP-1 block 2 MAD20 and Well (p<0.005) and RO33 (p=0.05) among CM cases. In contrast, Ab levels to the C-terminus were not significantly different in UM patients (15 pairs), and slightly increased for the Well and RO33 proteins. Thus, UM cases seemed to maintain Ab levels better, as illustrated for the 42kDa antigen (fig 4.6).

4.3.4.2- Antibody responses to MSP-2

Children who had SMA were distinguished by almost universally undetectable Abs to the conserved C-terminal region of MSP-2 (K1 17/14 construct). Patients with SMA had significantly lower mean levels of Abs to the C-terminus than children admitted with CM (p=0.0478) or UM (p=0.0114), after controlling by age, sex, date, parasitaemia, genotype, multiplicity of infection and outcome (fig 4.7). CM patients also had higher levels of Abs than UM cases (p=0.056). Moreover, the percentage of children producing Abs to K1 17/14 was significantly higher in CM than SMA patients (p=0.0478), and higher in CM than UM cases (p=0.0532) (fig 4.8). Consistently, Ab responses detectable with a fusion protein containing a short part of the dimorphic region of the T9/105 isolate (MSP-2 B type, fig 4.1), but mostly consisting of sequences from the conserved C-terminus, followed the same trend. Thus, Ab levels detected with the T9/105 12/6 short protein were lower in SMA than UM (p=0.014) or CM patients (p=0.0097), after adjusting by age, sex, date, parasitaemia, genotype, multiplicity of infection and outcome (fig 4.9). The prevalence of Abs to the protein was also higher in CM than UM (p=0.0436) or SMA cases (p=0.0199).
Fig 4.6. Duration of antibody responses to MSP-1 42kDa

Antibody reactivity (OD 492nm) against the C-terminal of MSP-1 in 15 pairs of plasma samples from uncomplicated malaria patients and 33 pairs from patients with cerebral malaria and anaemia at acute and convalescence (one month later) phases. Antibody levels decline significantly among patients with cerebral malaria and anaemia (paired t-test, p<0.001), whereas Ab levels did not vary significantly in uncomplicated malaria cases (p>0.2). Linear regression trend lines for each data series are shown in red.
Fig. 4.7. Antibody responses to MSP-2 K1 17/14 (C-terminus)

Dot plots showing that mean Ab levels (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent) were higher than in uncomplicated malaria (UM, 76 acute, 27 convalescent) or severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.18) for positive sera is indicated by a horizontal line.
Fig. 4.8. Prevalence of antibodies to MSP-2 C-terminus
Percentage of patients with cerebral malaria (CM, 102 acute, 87 convalescent), uncomplicated malaria (UM, 76 acute, 27 convalescent) and severe malarial anaemia (SMA, 56 acute, 48 convalescent) who had Abs to MSP-2 K1 17/14 which were above cut-off (OD=0.18).
Fig. 4.9. Antibody responses to MSP-2 C-terminal region

Dot plots showing the high correlation of Ab levels (OD at 492nm) detected with two MSP-2 proteins based in the C-terminal region of MSP-2. CM (n=102) had higher Ab levels than UM (n=76) or SMA patients (n=56). The cut-off levels for positive sera are indicated for each antigen.
In contrast, there were no statistically significant differences among the different clinical groups in Ab responses to variable regions of MSP-2. SMA patients had similar or slightly higher levels of Abs to dimorphic (fig 4.10) or polymorphic (fig 4.11) regions compared to CM or UM patients. Results obtained with constructs representing polymorphic regions of MSP-2 type A are shown in table 4.5.

| Table 4.5. Prevalence of antibodies to polymorphic MSP-2 A R1 regions |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|                           | % positive responders to 5/7 fusion proteins |
|                           | T9/96 | T9/102 | T9/94 | RO33 | CH12/12 |
| CM                        |       |        |       |      |        |
| Acute                     | 102   | 11.8   | 25.5  | 16.7 | 13.7   | 29.4 | 27.4 | 22.5 |
| Convalescent              | 87    | 9.2    | 20.7  | 12.6 | 8.1    | 24.1 | 20.7 | 16.1 |
| SMA                       |       |        |       |      |        |
| Acute                     | 57    | 7      | 21    | 14   | 14     | 36.8 | 26.3 | 19.3 |
| Convalescent              | 47    | 12.8   | 12.8  | 10.6 | 8.5    | 19.1 | 17   | 6.4  |
| UM                        |       |        |       |      |        |
| Acute                     | 76    | 7.9    | 18.4  | 19.7 | 13.2   | 21   | 17.1 | 13.2 |
| Convalescent              | 27    | 7.4    | 14.8  | 14.8 | 14.8   | 11.1 | 3.7  | 7.4  |

The above results were all confirmed in a case-control study. This analysis also showed a higher prevalence of Abs to the full length MSP-2 A fusion protein (T9/96 13/14) in CM than in SMA patients (McNemar's $\chi^2$ test, $p<0.01$) as well as higher levels of Abs (paired $t$-test, $p<0.005$). UM patients did not differ significantly from the severe disease groups, although the trend existed for lower Ab levels and prevalences than CM and higher than SMA cases. A closer analysis of the Ab responses to T9/96 13/14 showed that the difference was probably largely attributable to Abs directed to the conserved C-terminus of MSP-2, as illustrated in fig 4.12. No trend among the clinical groups was found in Ab responses to the full length MSP-2 B fusion proteins.

With regard to the evolution of Ab responses shown by comparison of acute and convalescent samples, there was a significant decrease in CM patients in Ab levels to all MSP-2 antigens examined (paired $t$-tests, $p$ between 0.05 and 0.001). In contrast, no significant decline between acute and convalescent anti-MSP-2 Ab levels were found in sera from SMA patients. In UM cases, there was a significant decrease in the level of Abs only for the conserved C-terminus ($p<0.01$).
Fig. 4.10a. Antibody responses to MSP-2A dimorphic regions

Dot plots showing that mean Ab (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent), uncomplicated malaria (UM, 76 acute, 27 convalescent) and severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients were similar in the acute stages, whereas Ab levels decrease in convalescence in CM and UM cases. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.21) for positive sera is indicated by a horizontal line.
Dot plots showing that mean Ab levels (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent), uncomplicated malaria (UM, 76 acute, 27 convalescent) and severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients were similar in acute stages, whereas Ab levels decrease in convalescence in CM and UM cases. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.42) for positive sera is indicated by a horizontal line.
Fig. 4.11a. Antibody responses to MSP-2A polymorphic regions

Dot plots showing Ab responses to T9/94 5/3 Long protein in cerebral malaria (CM, 102 acute, 87 convalescent), uncomplicated malaria (UM, 76 acute, 27 convalescent) and severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients.

Ab levels to this construct, representing polymorphic regions of tandem repeats specific for MSP-2A, were the most prevalent in the 3 groups and did not vary significantly according to disease. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.39) for positive sera is indicated by a horizontal line.
Dot plots showing Ab responses to K1 5/3 (32-mer repeats) in cerebral malaria (CM, 102 acute, 87 convalescent), uncomplicated malaria (UM, 76 acute, 27 convalescent) and severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients. Ab levels were not significantly different in the three disease groups. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.28) for positive sera is indicated by a horizontal line.

Fig. 4.11b. Antibody responses to MSP-2B polymorphic regions
Fig. 4.12. Antibody responses to MSP-2A full length protein
Dot plots showing the correlation between Ab levels to the MSP-2A full length protein (T9/96 13/14) and to the C-terminal regions of MSP-2 (K1 17/14). CM (n=102) had higher Abs than UM (n=76) or SMA patients (n=56). Antibodies of different specificities can be distinguished: dots positive for both constructs recognise conserved epitopes of the C-terminus, whereas dots only positive for T9/96 13/14 correspond to sera with Abs recognising also dimorphic or polymorphic MSP-2A-specific regions.
4.3.4.3- Antibody responses to AMA-1 and RAP-1

In line with the findings presented above, mean levels of Abs to full length AMA-1 were significantly higher in CM than in SMA cases (p=0.0496), after adjusting by age, sex, parasitaemia, multiplicity of infection and outcome (fig 4.13). The prevalence of positive anti-AMA-1 Ab responses was also higher in CM than in SMA cases (p=0.0382) when controlling by sex, parasitaemia and multiplicity of infections, but the significance was lost when adjusted by age. As shown in 4.3.3 and fig. 4.2, age had an independent effect in the model. The highest levels of anti-AMA-1 Abs were reached at the age of 4, which is closer to the mean age of the CM group (3.6 years) than to the SMA group (2.3 years). This was probably reflected in p values closer to the limit of 5% significance when age was taken into account. However, a case-control study comparing CM vs. UM and CM vs. SMA patients matched by age confirmed that children with CM had higher anti-AMA-1 Ab levels (paired t-test, p<0.005 in both cases) and prevalences (McNemar's \( \chi^2 \) test, p<0.025 in both cases).

With regard to RAP-1, children with CM had higher anti-C2 Ab levels (p=0.0472) and prevalences (McNemar's \( \chi^2 \) test, p<0.025) than children with UlvI after adjusting for the effects of age, sex, parasitaemia, multiplicity of infection and outcome (fig 4.14).

Concerning the evolution of Ab responses during a month between acute disease to convalescence, there was a significant decrease in anti-AMA-1 and anti-RAP-1 Ab levels in CM (paired t-test, p=0.05) and severe non-malarial patients (p<0.02 AMA-1, p<0.005 RAP-1), and in anti-RAP-1 Abs in SMA patients (p<0.001). Antibody levels remained stable in UM cases.

4.4- Discussion

The prevalence and magnitude of serum IgG Abs to defined regions of MSP-1, MSP-2, AMA-1 and RAP-1 were assessed in acute and convalescent plasma samples of paediatric malaria patients from Malawi to determine which regions of these proteins are antigenic in children naturally infected with \textit{P. falciparum}. This has been the first study to include a large panel of recombinant proteins representing conserved, dimorphic and polymorphic regions of MSP-2, and the first study carried
Fig. 4.13. Antibody responses to AMA-1

Dot plots showing that mean Ab levels (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent) were higher than in uncomplicated malaria (UM, 76 acute, 27 convalescent) or severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.27) for positive sera is indicated by a horizontal line.
Fig. 4.14. Antibody responses to RAP-1 C2

Dot plots showing that mean Ab levels (OD at 492nm) in cerebral malaria (CM, 102 acute, 87 convalescent) were higher than in uncomplicated malaria (UM, 76 acute, 27 convalescent) or severe malarial anaemia (SMA, 56 acute, 48 convalescent) patients. The mean OD value for each group is indicated by a horizontal dash. The cut-off level (OD=0.49) for positive sera is indicated by a horizontal line.
out in Malawi with any of the antigens. Moreover, Ab levels and prevalences were compared among groups of patients with different clinical forms of the disease to investigate whether there may be a relationship between Ab responses to these antigens and malarial disease. For the first time, Ab profiles in patients with CM and SMA were analysed separately, and significant differences between the two groups were found.

4.4.1- Antibody recognition of merozoite proteins in Malawian children

Antibody responses to four *P. falciparum* antigens were detected in the majority of Malawian paediatric patients, providing further evidence for the natural immunogenicity of these merozoite proteins.

Most children (≥ 70%) made Abs to the conserved C-terminal region of MSP-1, in accordance with previous studies in Kenya (70-80%, Udhayakumar *et al.*, 1995), The Gambia (≥ 50%, Egan *et al.*, 1996) and Sudan (≥ 90%, Cavanagh *et al.*, 1998). Differences in Ab prevalences reported in the above studies may reflect the levels of malaria endemicity in the countries or differences in the design (cross-sectional or longitudinal, age and disease status of the individuals) or methods (e.g. antigens, serum dilutions tested) of the studies. Here, Ab responses to the two recombinant proteins used, 19kDa and 42kDa, correlated well, indicating that most Abs probably recognised epitopes located in the MSP-1, fragment, a processed product of MSP-142. Antibodies to these proteins are the most reliable indicator of recent clinical infection, as proposed by Cavanagh *et al.* (1998). Consistent with the latter study but in contrast to others (Kramer & Oberst, 1992; Kabilan *et al.*, 1994), Ab responses to the conserved N-terminal block 1 of MSP-1 were rare, implying a poor immunogenicity of this sequence. Concerning block 2, a low proportion of the Malawian plasmas contained Abs to any of the block 2 constructs, possibly due to the high sequence polymorphism of this region of MSP-1. The highest prevalence of Abs to block 2 of the K1 type in patients investigated here correlated well with the predominance of infecting parasites of the K1-type in the same children (see chapter 2). Type-specific Ab responses to block 2 of MSP-1 are the subject of chapter 5. The overall prevalence of anti-block 2 Abs was 28%, lower than in Sudan (≥69%), and
this was possibly attributable to the longitudinal sampling of the latter study (Cavanagh et al., 1998), in contrast to our essentially cross-sectional design.

With regard to MSP-2, Abs predominantly recognised dimorphic regions of the protein, which are conserved within each allelic family, as shown previously (Taylor et al., 1995). Antibodies to MSP-2 A group-specific regions were more prevalent than Abs to MSP-2 B, using both dimorphic and polymorphic constructs. These results correlated well with the higher prevalence of parasites expressing the MSP-2 type A in Malawi (see chapter 2). The conserved C-terminal region was the least immunogenic part of MSP-2, in agreement with previous studies (Thomas et al., 1990; Saul et al., 1992; Taylor et al., 1995), and Abs to this region were also the most short-lived. In contrast to MSP-1, polymorphic regions of MSP-2 were more immunogenic than the conserved C-terminal region of this antigen.

The general fall in the levels of Abs to MSP-1 and MSP-2 within one month was surprising; it was expected that Abs would be higher in convalescence than in acute disease since it takes some time for the host to respond to an acute infection. However, only Abs to the RO33 type of MSP-1 block 2 followed this prediction. Previous studies have reported that Abs to MSP-1 are short-lived, especially in children (Früh et al., 1991; Tolle et al., 1993; Cavanagh et al., 1998). Thus, the results obtained here may reflect a situation in which infected children soon produce Abs which decay after the parasites have been cleared below disease threshold levels.

In contrast to a study by Al-Yaman et al., (1994), no association between Abs to MSP-2 and parasitaemia was found here. However, there was a significant association between high parasitaemias and high levels of Abs to MSP-1 19KDa, 42kDa and block 2 RO33, similar to other findings (Tolle et al., 1993). An infection may rapidly boost the level of Abs to MSP-1, particularly in children (<10 years of age) with a low pre-existing memory, thus establishing the positive association. Al-Yaman et al. (1994) argued that, with age, increased immune competence and more immune priming may result in lower parasite densities being required to boost Ab responses. Nevertheless, the association between parasitaemia and Ab responses to defined MSP-1 regions found here might be confounded by the disease status. When
the model was adjusted for the effect of disease, the significance of the association was lost for Abs against the C-terminal region of MSP-1, as most CM patients (the largest clinical group) had high Ab levels (see 4.4.2) and high parasite densities (shown in table 2.1, chapter 2).

It has been proposed that acquired immunity in adults living in endemic areas not only limits parasite multiplication to a level around the microscopically detectable threshold but also reduces the complexity of the infections (Ntoumi et al., 1995). Compatible with this notion was the finding here of a negative association between multiplicity of infection and levels of Abs to MSP-119. It is arguable that the effect of multiplicity in Ab levels was also confounded by disease status, as CM patients had high levels of Ab and low mean number of parasite clones (shown in table 2.5, chapter 2). However, the significance of this correlation remained when disease was accounted for in the model, suggesting a true association between multiplicity of infections and Abs. Thus, immunologic cross-protection among different genotypes by Abs targeting conserved epitopes of MSP-119 may contribute to the acquisition of an effective anti-parasite immunity. In this context, the findings suggest that Ab responses to merozoite surface proteins are protective rather than a mere indication of the presence of parasites. Therefore, a malaria vaccine based on these antigens may successfully mimic the protection achieved by naturally acquired immunity.

A substantial proportion of children (≥60%) contained Abs to AMA-1 full length protein, indicating that AMA-1 of *P. falciparum* is a good immunogen. As the vaccine potential of AMA-1 is currently being assessed, it will be interesting to carry out similar studies in different populations, and to include a wider range of recombinant AMA-1 proteins, to confirm these results. In contrast to MSP-1 and MSP-2 Ab responses, AMA-1 Abs did not fall significantly in convalescence, suggesting that anti-AMA-1 response might be longer-lived. Antibody profiles varied according to age, with the highest mean Ab levels found in the 4-year old group. This age distribution had an effect in the statistical analysis of the association between anti-AMA-1 levels and disease severity, but it did not completely explain the differential pattern of responses to AMA-1 found among the clinical groups (see 4.4.2).
Concerning the C2 fragment of RAP-1, 41% of children contained Abs of this specificity, a prevalence comparable to 48% reported in a previous study which included 44 Gambian patients of all ages (Fonjungo et al., 1998). Thus, the RAP-1 antigen tested here seemed to be the least immunogenic of the four merozoite proteins.

4.4.2- Association between antibody responses and malarial disease

One major aim of this study was to determine whether humoral response to the different defined malaria antigens was a determinant of development of severe or mild malaria in children. In agreement with previous studies (Tharavanij et al., 1984; Erunkulu et al., 1992; Al-Yaman et al., 1997), this work has shown that the development of severe malaria was not due to a lack of Ab responses in the acute stage.

Immune responses to MSP-2 A have been associated with reduced malaria morbidity (Al-Yaman et al., 1994). Although our study was not designed to identify risk factors of disease, the presence of Abs to MSP-2 A-specific regions did not prevent children from developing CM or SMA. It could be that the Abs detected were directed against epitopes which are not related to essential functional activities of malaria parasites or that Abs were of a "non-protective" IgG isotype. A study in Senegal found that IgG3 activity was associated with a favourable prognosis in patients with severe malaria (Sarthou et al., 1997). It would be very interesting to determine the isotypes of the Abs detected here, as this information may provide some clues about their mechanisms of action. An indication for a possible "protective" effect of these Abs was that levels of Abs remained more stable from acute to convalescent stages in UM cases than in severe malaria cases, although there were too few UM follow-up patients to draw a definite conclusion.

The most striking finding was that children suffering from different severe malaria manifestations were distinguished by a differential pattern of Ab responses to defined regions of the merozoite surface antigens. Importantly, children with CM were distinguished by Ab hyper-responsiveness to all merozoite antigens studied. In
particular, Ab responses to conserved rather than variable regions of these proteins were significantly higher in the CM group than in the UM group, whose responses were higher than in the SMA group. Thus, children with SMA were characterised by a hypo-responsiveness to conserved regions of the merozoite proteins, while responses to polymorphic and dimorphic MSP-2 regions were not defective. These results are compatible with the findings by Tharavanij et al. (1984). In the latter study, children with CM had higher mean initial ELISA titre than UM patients, and a subgroup of CM patients with complications (e.g. anaemia) had reduced humoral responses compared to uncomplicated CM or UM patients.

The first conclusion of these results is thus that SMA emerges as a distinct entity from CM and so, as noted in chapter 2, it is not appropriate to pool these two forms of severe *P. falciparum* malaria together, as some previous studies have done (Tharavanij et al., 1984; Erunkulu et al., 1992). The significant differential pattern of Ab responses found suggests that different immune mechanisms are likely to act in each syndrome.

The second conclusion is the surprising but quite apparent general trend in Ab responsiveness for hyper-reactivity in CM through intermediate responsiveness in UM to hypo-reactivity in SMA. How can these differences be interpreted? Two possibilities can be proposed: 1. The presence or absence of Abs may be a mere indication of other processes, such as (i) different previous exposure to infections or (ii) different cellular immune mechanisms mediating pathogenesis. 2. The Abs may have direct immunopathological consequences in the development of disease.

Epidemiological observations suggest that the incidence of CM and SMA vary with age, and depend on endemicity (Snow et al., 1997). In African children, the peak incidence of CM occurs later in life than the peak of SMA (Brewster et al., 1990; Marsh, 1992). The lapse of several years before an increased probability that a *P. falciparum* infection gives rise to CM is compatible with the idea that a degree of immunological sensitisation by prior malaria infections could predispose an individual to developing CM. Hospital records from Thai patients indicated that previous UM infections were more frequent in subjects with CM than infections in subjects presenting with non-cerebral forms of malaria (Mendis & Carter, 1995). Consistent
with this view are observations from many studies where plasma levels of several immunological factors are raised in CM patients compared to other forms of *P. falciparum* malaria. Elevated levels of IgE, but not IgG, were found in CM compared to UM patients from Africa and Asia (Perlmann *et al.*, 1994). High concentrations of serum TNF-α, IL-1 and IL-6 were also found in severe falciparum malaria patients (Grau *et al.*, 1989; Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990). Thus, the increased levels of Abs and cytokines in children presenting with cerebral acute illness may reflect a history of numerous infections and therefore may reveal individuals with high susceptibility to infection.

However, in children who develop SMA, less previous exposure to malaria infections cannot explain well the apparent hypo-responsiveness to conserved antigens, as levels of Abs to variable regions of the same proteins were high in the anaemic patients. Only Abs to conserved regions of MSP-1 (19kDa and 42kDa constructs), to block 2 RO33 type (whose sequence is conserved among different MSP-1 allelic forms), to conserved the C-terminus of MSP-2 (K1 17/14 and T9/105 12/6 constructs), and to the relatively conserved AMA-1 construct were lower in SMA than in CM or UM patients. It could be that conserved regions such as the C-terminal part of MSP-2 are poorer immunogens than variable regions, and that Abs to conserved epitopes are shorter-lived. Assuming that a child with SMA has suffered less previous episodes of malaria than a child with CM, the former could develop lower levels of Abs to the conserved regions than the latter. Monoclonal Abs to MSP-119 (Blackman *et al.*, 1990), MSP-2 (Epping *et al.*, 1988), RAP-1 (Harnyuttanakorn *et al.*, 1992) and AMA-1 (Deans *et al.*, 1982) inhibit merozoite invasion *in vitro*. Thus, it could be speculated that an absence of Abs to conserved regions of these merozoite proteins would allow more cycles of erythrocyte invasion and destruction, contributing to the development of anaemia. However this hypothesis is difficult to reconcile with the fact that CM patients had higher parasite densities than SMA patients, despite higher Abs concentrations. It may be, then, that the presence of Abs has an anti-disease role i.e. prevention of anaemia, rather than a direct anti-parasite role. As discussed in 4.4.1, the positive association between parasitaemia and Ab levels presumably reflects a boosting of Abs by the current infection. However, if Abs
limit parasite growth, a negative association between Abs, fever and subsequent anaemia would be expected. Thus, a malaria vaccine including conserved regions of these merozoite antigens may be useful in controlling anaemia, but less efficient in preventing cerebral disease.

The reasons for the differential Ab responses in CM and SMA patients may not be explained only by the relative conservation of the target amino acid sequences, but also by the location of the epitopes inducing such Abs. C-terminal regions of MSP-1 and MSP-2 are probably anchored to the merozoite membrane by a GPI moiety (Schofield & Hackett, 1993). GPI structures are putative malaria toxins thought to be primarily responsible for TNF-α induction (see below). Regions containing variable epitopes are shed (in the case of MSP-1) as soluble proteins during erythrocyte invasion. The processing of soluble vs. membrane-bound antigens may involve different pathways, with different antigen presenting cells, lymphocytes or cytokines, each promoting distinct immunopathological processes.

The differential pattern of Ab responses may reflect a different skewing of Th1 and Th2 responses in CM vs. SMA. Stimulation of CD4+ T cells from immune donors with P. falciparum antigen in vitro frequently induced either IFN-γ or IL-4 secretion, indicating the occurrence of distinct Th1- or Th2-like cells in the blood of these individuals (Troye-Blomberg et al., 1990). In human CM, high levels of plasma Abs specific for merozoite antigens may indicated a skewing towards Th2-type responses. Elevated concentrations of serum IgE in patients with malaria, especially in those with CM, reflected an underlying imbalance in favour of Th2 producing cytokines such as IL-4, primarily responsible for isotype switching from IgM to IgE (Perlmann et al., 1997). It was suggested that interaction of IgE complexed with antigen or with anti-IgE IgG with monocytes via FcεR might efficiently induce release of both TNF-α and nitric oxide (NO). An excessive production of TNF-α has been associated with disease severity and fatal outcome (Grau et al., 1989; Kwiatkowski et al., 1990), and NO is thought to contribute to the pathogenesis of CM (Clark et al., 1991).
TNF-α and NO may also be implicated in the pathogenesis of SMA, e.g. by causing dyserythropoiesis and erythrophagocytosis, as shown in experimental rodent malarias (Clark & Chaudhri, 1988). The production of TNF-α is downregulated by anti-inflammatory cytokines such as IL-10. Kurtazhals et al. (1998) found that the ratio of TNF-α to IL-10 was significantly higher in SMA than in CM or UM cases, i.e. patients with SMA had lower plasma concentrations of IL-10. It was proposed that the low IL-10 in SMA reflects an impaired IL-10 response to TNF-α and perhaps other haematopoietic inhibitors (e.g. transforming growth factor β). As IL-10 is also important in B-cell activation, deficient IL-10 production may favour a Th1-type response which could be responsible for the observed Ab hypo-responsiveness to defined regions of *P. falciparum* antigens in SMA. It is possible that children who are susceptible to SMA are unable, because of some genetic or other factor, to develop adequate levels of Abs which may be essential in protection against this severe form of *P. falciparum* infection. For instance, a polymorphism in the *IL-10* gene promoter has been described and some variants have been associated with decreased IL-10 synthesis (Turner et al., 1997).

A different possibility is that the presence of Abs could have a disease-promoting role in itself. For example, immune complex formation and deposition in the cerebral microvessels may contribute to the development of CM (Nagatake et al., 1992). However, no evidence of immune complexes was found in a different study (McPherson et al., 1985). Thus, to date the immunological hypothesis of pathogenesis of acute human CM remains unconvincing.

Concerning SMA, the presence of Abs to variable regions of MSP-2 may also play a role in the development of the disease. It has been proposed that IgG or immune complexes could bind to uninfected erythrocytes and accelerate their clearance by macrophages or complement-mediated lysis, thus contributing to SMA (reviewed in Warrell et al., 1990). Erythrocytes coated with IgG were more rapidly removed from circulation by the spleen in patients with acute falciparum malaria, and this increased clearance persisted for weeks (Lee et al., 1989).
As the pathological mechanisms underlying both syndromes are obscure, interpretations suggested here remain conjectural. Further studies are necessary (i) to elucidate the role of Abs induced by merozoite antigens in the control of parasitaemia and/or disease and (ii) to clarify the implications of the findings reported here in the pathogenesis of malaria, as these antigens are considered prime malaria vaccine candidates.
5.1- Introduction

Immunity to malaria in people living in endemic areas is acquired slowly after years of exposure to the parasite (McGregor et al., 1956). Natural populations of Plasmodium falciparum are genetically diverse, consisting of parasite genotypes or "strains" (Kemp et al., 1990; Walliker, 1991). These strains differ in their composition of a number of polymorphic molecules, including antigens. It was suggested that immunity to P. falciparum may be essentially strain-specific (Wilson & Phillips, 1976), and thus an individual becomes immune after being exposed to a large number of strains circulating in the community; this constitutes the strain-specific immunity hypothesis (Garnham, 1966; Day & Marsh, 1991). Evidence for strain-specific immunity has accumulated from animal studies, basically experimental infections in monkeys (Cadigan & Chaicumpa, 1969) and induced infections in humans, including the treatment of syphilitic patients by inoculations of P. vivax and P. knowlesi (Ciucu et al., 1934). In experimental malaria in man, a primary infection by one parasite strain elicited an immune response protecting against that strain but not against infection by a different strain (Jeffery, 1966).

More recently, longitudinal studies investigating superinfections in asymptomatic individuals have suggested that immunity controlling parasite growth has an important strain-specific component (Daubersies et al., 1996). A study in Dielmo, Senegal, which examined P. falciparum genotypes during successive clinical episodes in children found that the sharp rise in parasitaemia accompanying the disease symptoms was associated with genotypes that had not been previously detected (Contamin et al., 1996). The authors proposed that clinical malaria episodes were induced by recently inoculated parasite genotypes that multiplied rapidly because the children did not have a specific immune response to them. Furthermore, in a study that followed the daily dynamics of P. falciparum subpopulations in children in Tanzania found that numerous parasites of different genotypes were apparently
controlled during asymptomatic infections, suggesting that acquired immunity restricted the growth of a number of distinct parasites strains (Farnert et al., 1997).

Polymorphic proteins that could be targets of such specific immunity acquired in humans include the merozoite surface proteins MSP-1 and MSP-2.

To test the strain-specific immunity hypothesis, first it is required to elucidate the effect that the genetic diversity of MSP-1 and MSP-2 (see chapter 2) has in the specificity of the human immune response. It is necessary to show whether variable regions of these antigens are responsible for inducing strain-specific immune responses following natural *P. falciparum* infections (see chapter 4). Second, it is necessary to define whether such responses are "protective". It is generally considered that there are two forms of naturally acquired immunity to malaria which develop with age: an anti-disease and an anti-parasite component. It is not known whether strain-specific immune responses to MSP-1 and/or MSP-2 contribute to the regulation of disease symptoms (morbidity and/or severity) and/or to the control of the parasite load. Thus far, very few studies have approached these questions in humans.

### 5.1.1 Strain specific antibody recognition of polymorphic malarial proteins in animal models

The significance of allelic dimorphism of MSP-1 in the specificity of immune responses to *P. falciparum* was investigated in rabbits. Immunisation with affinity purified MAD20 and Well/K1 allelic forms of *P. falciparum*-derived MSP-1 raised antisera that were largely cross-reactive in competitive ELISA, IFA and inhibition of parasite growth *in vitro*, probably due to recognition of conserved regions (Hui et al., 1992). However, type-specific antibodies (Abs) to either allelic protein of MSP-1 were also detected. Antisera to the C-terminal MSP-1\textsubscript{142} fragment also cross-reacted with four different allelic forms of the protein, and a major proportion of Abs was specific for conserved determinants (Hui et al., 1993).

In *Aotus* monkeys, immunisation with MSP-1 of the K1 isolate before a challenge with a heterologous isolate of *P. falciparum* resulted in low levels of protection (Hall et al., 1984), in contrast to the complete absence of parasitaemia observed in a different study where monkeys were challenged with the homologous
isolate (Siddiqui et al., 1987). However, further studies showed that vaccination with MSP-1 of the K1 isolate could protect Saimiri monkeys from lethal challenge with a heterologous Palo Alto isolate, representing the alternative MSP-1 dimorphic (MAD20) type (Etlinger et al., 1991). Recombinant vaccinia viruses expressing several P. falciparum antigens including MSP-1 and MSP-2 did not protect Saimiri monkeys against malaria (Pye et al., 1991). This failure could be partially due to the fact that the immunisation involved an allelic form of MSP-2 (FC27 or group B) different from that present in the isolate used for challenge (IC1/3D7 or group A).

Variant-specific antigenic determinants on the surface of red blood cells (RBCs) infected with P. falciparum were targets of vaccine-induce protective immunity in Saimiri monkeys (Fandeur et al., 1995). Passive transfer of variant-specific Abs did not cross-protect against challenge with an heterologous parasite line. In addition, strain-specific responses to MSP-1 and MSP-2 were shown to be an important component of anti-parasite acquired immunity in this monkey model (Fandeur & Chalvet, 1998).

The antigenicity of the polymorphic block 2 of MSP-1 has been assessed in mice (Cavanagh & McBride, 1997). Immunisation with fusion proteins representing block 2 types, K1, MAD20 and RO33, produced polyclonal Abs which specifically recognised parasites' MSP-1 of each type in IFA and Western blot. In addition, the antigens reacted appropriately in ELISA with mouse mAbs specific for variant block 2 epitopes. This indicated that different types of block 2 of MSP-1 are antigenically distinct and induce type-specific Abs in mice.

The impact of diversity of P. chabaudi adami AMA-1 on vaccine efficacy was investigated in mice (Crewther et al., 1996). Immunisation with a recombinant AMA-1 failed to protect against challenge with an avirulent heterologous strain of P. chabaudi. In addition, passive transfer of rabbit anti-AMA-1 Abs into mice had little effect on heterologous challenge but reduced peak parasitaemia and mortality due to infection with the homologous parasite, suggesting that protective immune responses induced by AMA-1 were strain-specific.
5.1.2- Specificity of human immune responses to *P. falciparum* polymorphic proteins

5.1.2.1- Strain-specific responses to MSP-1

A study in Mali analysed the specificity of Abs acquired upon natural infections with *P. falciparum* in relation to dimorphic types of MSP-1 present during the infection (Früh *et al.*, 1991). The presence of specific Abs against the MAD20 dimorphic regions of MSP-1 correlated with the higher prevalence of MAD20 dimorphic type over the alternative K1/Well-type in the infecting parasite populations, as detected by IFA. However, this conclusion was weak being derived from the comparison between the typing of only 8 parasite isolates and the serology of 94 individuals.

In Gabon, prevalence and concentration of specific Abs to MSP-1 increased with age (Chizzolini *et al.*, 1988). Likewise, in a cross-sectional survey in The Gambia, levels of Abs to all regions of MSP-1 increased with age, with the highest prevalence of Abs against conserved regions (Riley *et al.*, 1992). The fact that in these and many other studies immune responses to polymorphic antigens appear to be acquired in an age-dependent manner with peak prevalence, or maximum intensity of response, occurring in late childhood or early adolescence, suggested to the authors that repeated exposure to infection is required to induce responses in the majority of the population and may reflect cumulative exposure to different polymorphic forms. Moreover, IFN-γ production and *in vitro* lymphoproliferative responses induced by recombinant proteins representing polymorphic regions of MSP-1 also increased with age, whereas proliferative responses to some parts of MSP-1, including some conserved sequences, decreased with age (Riley *et al.*, 1992). It was suggested that Ab-mediated specific epitope suppression may contribute to that desensitisation. Age-dependent immunity to malaria has been recently assessed in Javanese transmigrants moving from an area of very low transmission to Irian Jaya, where malaria transmission is high (Andersen *et al.*, 1997). The risk of acquiring *P. falciparum* during the first 14 months of exposure was the same for children and adults, but after that period, children had significantly higher parasite densities. However, in this
situation no age-dependent difference in Ab titres to MSP-1\textsubscript{19} induced by exposure to malaria was observed.

Although the C-terminal MSP-1\textsubscript{19} is highly conserved compared to other regions of MSP-1, changes in four amino acid (aa) residues exist in laboratory and field isolates. The effect of this polymorphism on the specificity of human responses to MSP-1\textsubscript{19} was studied in Kenya. T cell proliferative responses to three natural variants of MSP-1\textsubscript{19} (referred to as E-KNG, Q-KNG, E-TSR) were considerably variable in different individuals, suggesting that MSP-1\textsubscript{19} may contain strain-specific T epitopes (Shi \textit{et al.}, 1996). Moreover, although natural anti-MSP-1\textsubscript{19} Abs cross-reacted with all three variants, IgG3 Abs recognised only two variants (E-KNG and Q-KNG), suggesting that the fine specificity of IgG3 Abs might differentiate among strain-specific natural B determinants in the second EGF-like domain (KNG or TSR) of MSP-1\textsubscript{19}.

Concerning the three main types of block 2, sera from malaria-exposed Gambians contained IgG Abs that recognised very specifically one or another type, showing that different block 2 types are immunogenic, antigenically distinct and distinguishable in natural infections (Cavanagh & McBride, 1997). Furthermore, in a longitudinal study of Ab responses against MSP-1 during natural infections in Sudan, type-specific Abs to block 2 correlated with PCR typing of parasites present at the time of infection (Cavanagh \textit{et al.}, 1998).

5.1.2.2- Strain-specific responses to MSP-2 and other surface proteins

Strain-specificity of Abs to the two MSP-2 dimorphic families were examined using sera from Gambian adults (Taylor \textit{et al.}, 1995). Human Abs to serogroups A and B did not cross-react. Another study investigated the Ab recognition of recombinant MSP-2 B proteins which differed in the number of 12-mer aa repeats present (Ranford-Cartwright \textit{et al.}, 1996). High Ab levels were detected in most sera using a protein with 5× 12-mer repeats, whereas low or no reactivity were measured with proteins containing 1 or no 12-mer repeat. However, specificity of Ab responses against polymorphic regions has not yet been investigated in detail during natural infections. There is a need for studies in the field including probes that can discriminate a higher range of polymorphic MSP-2 variants.
Human agglutinating Ab responses to antigens expressed on the infected RBC surface were found to be predominantly variant-specific (Marsh & Howard, 1986). In a study of agglutinating reactivities of Gambian field isolates and laboratory clones, Abs which cross-reacted between different serovariants were rare and reacted only with a subset of parasite-derived antigens on the infected RBC (Newbold et al., 1992). However, in a study including field isolates from distant geographic regions, Aguiar et al. (1992) identified antigenic determinants for agglutinating Abs that were cross-reactive. A larger study in Papua New Guinea (PNG) failed to identify any two *P. falciparum* isolates with the same agglutinating phenotype (Reeder et al., 1994).

The putative target of agglutinating Abs is the erythrocyte membrane protein (PfEMP)-1 (Leech et al., 1984). PfEMP-1 is also thought to mediate cytoadherence and rosetting, both proposed pathogenic factors of severe malaria (Newbold, 1997). The observed variant specificity of immune responses to PfEMP-1 is probably the result of phenotypic antigenic variation (Borst et al., 1995). It appears that antigens on the RBC surface are targets of naturally acquired immunity to malaria (Bull et al., 1998).

### 5.1.2.3- Strain-specific immune responses and malaria disease severity

A recent study in PNG investigated the relationship between acquired immune responses to MSP-1 and MSP-2 and disease severity (Al-Yaman et al., 1997). Previous studies (Tharavanij et al., 1984; Erunkulu et al., 1992) found a similar degree of previous exposure to malaria in mild and severe disease, but they did not assess Abs for specificity against the particular parasite clone(s) comprising the individual infecting parasite. Al-Yaman et al. genotyped parasites from cerebral malaria (CM) and uncomplicated malaria (UM) patients for MSP-1 and MSP-2 and measured prevalence and levels of specific Ab responses to these proteins in the same patients. They found higher levels of Abs to antigens matching the infecting parasite genotype than to heterologous genotypes, but this was only significant for Abs against one recombinant MSP-2 A protein in children infected with the MSP-2 A type. In addition, Ab levels to MSP-2 A were significantly lower among those CM patients who died than among survivors of CM. No difference between CM and UM patients was found. However, a parallel detection of the MSP-2 type of parasites and the Ab
response of hosts was only done in about 25% of the children. Patients with severe malarial anaemia (SMA), who may respond differently from CM or UM patients were not studied. Therefore, more studies are required to establish the role of strain-specific Abs in protection against severe forms of malaria.

5.1.3- Aims

Strain-specific Ab responses to MSP-1 and MSP-2 were assessed in acute and convalescent samples of children distinguished by different malaria disease manifestations to test:

- whether MSP-1 and MSP-2 are targets of strain-specific immune responses in children naturally infected with *P. falciparum*.

- whether children with UM are more likely, in the acute stage, to have Abs specific for the infecting parasite than children with CM or SMA.

5.2- Materials and methods

Details of samples and procedures used for parasite typing and ELISA serology were described in chapters 2 and 4, respectively.

5.2.1- Statistical analysis

Data on all patients surveyed was introduced into the SAS statistical analysis package (SAS, 1990) to analyse correlations between the MSP-1 or MSP-2 types of the infecting *P. falciparum* and the specificity of the Ab responses mounted by the patients. A positive Ab response to MSP-2 A (IC1/3D7 type) was defined as the presence in a child of Abs recognising one or more than one of 13 fusion proteins based on MSP-2 A alleles (see chapter 4, M & M). Likewise, a positive Ab response to MSP-2 B (FC27 type) was defined as the presence of Abs recognising any of 7 constructs representing MSP-2 B-specific sequences. A trait called here “concordance” was defined as a match between the type-specificity of positive Ab responses and any parasites detected in the acute or convalescent stages of the illness. Thus, an Ab response was defined as concordant when the specificity of the Ab matched the type of the infecting parasite found in the blood of the same patient; a
response was defined as discordant when the specificity of the Ab detected did not correlate with the type of any parasite present. Children who had no detectable Ab were defined as non-responders and were excluded from the analysis. The relationship between concordance, disease status and outcome was analysed by PROC GENMOD (categorical linear model), using a binomial distribution for the dependent variable. Significance was defined at the 5% level.

5.3- Results

The MSP-1 and MSP-2 types of the infecting parasites, detected by PCR, were matched to the specificity of serum Abs, assuming that polymorphisms in these genes are reflected in antigenic diversity of the expressed proteins, as shown in chapter 2.

5.3.1- Specificity of antibody responses to polymorphic block 2 of MSP-1

The correlation between the block 2 type of MSP-1 in the infecting parasite and the specificity of the Ab response to block 2 was analysed in those children whose plasma contained detectable Abs directed to fusion proteins representing one of the three polymorphic types of block 2: K1, MAD20 and RO33. In the acute stage of the disease, parasite typing and serological analysis were carried out using blood samples from 345 children. 53 (15.4%) plasma samples had Abs specific for the K1 type of MSP-1, i.e. reacted positively with either Palo Alto and/or 3D7 fusion proteins (proteins described in M&M chapter 4 and fig. 2.1). Abs specific for the K1 type matched the presence of an infecting parasite of the K1-type in 44 serum samples (83% concordance), whereas a mismatch was found in 9 samples (17%) (figs. 5.1, 5.2 & 5.3). 25/350 (7.14%) serum samples contained Abs specific for the MAD20 type, i.e. reacted positively with MAD20 and/or Well fusion proteins. Among those 25 samples, the presence of MAD20-specific Abs correlated with the presence of an infecting parasite of the MAD20 type in 17 children (68% concordance), and a mismatch was observed in 8 patients (32%) (figs. 5.4, 5.5 & 5.3). Finally, 20/348 serum samples (5.7%) contained Abs reacting with the RO33 fusion protein; 15
Fig 5.1. Specificity of antibody responses to MSP-1 K1-type infections

Correlation plots showing Ab responses (OD 492nm) against homologous K1-type parasites (along X axis) and against heterologous MAD20 (A & B) and RO33 (C & D) types (along Y axis), in children infected with parasites of the MSP-1 block 2 K1-type. Acute (A & C) and convalescent (B & D) plasma samples are distinguished. Most children had Abs that were specific for the K1-type of block 2. However, a high proportion of these children had concurrent infections with the MAD20 and/or RO33 types and thus explaining some Ab reactivities with these other two types. This shows that most children infected with multiple clones who responded to block 2 polymorphic antigens had Abs specific for either one or another of the three types.
Antibody reactivities (OD levels at 492nm) to fusion proteins of K1-type block 2 sequences in acute (A) and convalescent (C) patients infected with parasites of the K1 type. Patient MP886 had a single K1 (K) infection and Abs to K1 proteins in acute stage, which decreased after one-month convalescence. Patient MP812 had a mixed infection with K1 and MAD20 (M) parasites, but produced Abs only to the K1 type. Patient MP874 had an infection consisting of three parasite clones of the K1, MAD20 and RO33 (R) types but produced Abs only to the K1-type which increased from acute to convalescent stages. The diagnosis on admission is indicated for each patient, as well as the clinical status one month later. CM=cerebral malaria, SMA=severe malarial anaemia, Healthy=fully recovered, SQ=neurological sequelae. Antibody responses to the conserved C-terminal fragment of 19kDa are indicated for comparison.

### Antibody Reactivities to K1-type Block 2 Sequences

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<th>Parasite Type</th>
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<tr>
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<tr>
<td>MP874 C K</td>
<td>SQ</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Fig 5.2. Examples of concordant antibody responses to infecting parasites of MSP-1 block 2 K1 type**

- **K1 block 2**
- **MAD20 block 2**
- **RO33 block 2**
- **C-terminus 19kDa**
Fig 5.3. Examples of discordant responses to MSP-1 block 2

Antibody reactivities (OD levels at 492nm) to fusion proteins of MSP-1 block 2 types in acute (A) and convalescent (C) patients infected with parasites of the indicated type. Patient MP769 had a single K1 (K) infection, but Abs to MAD20 proteins in the acute stage, which declined at one-month convalescence. Similarly, patient MP857 had a single infection with K1 parasites, but contained Abs to the RO33 type which remained high after one month. Patient MP789 was infected with a parasite of the RO33 (R) type, but contained Abs to the K1 type which decreased in convalescence. The diagnosis on admission is indicated for each patient, as well as the clinical status one month later. CM=cerebral malaria, SMA=severe malarial anaemia, Well=fully recovered, n.t=not known; - = PCR negative. Antibody responses to the conserved C-terminal fragment of 19kDa are indicated for comparison.
children had RO33-type parasites (75% concordance), and 5 children did not (25%) (figs. 5.6, 5.7 & 5.3).

Overall, despite the low prevalences of Abs specific for any one type of block 2, strain-specific Ab responses were detected in 77.5% of children who responded to block 2 at acute stage. Although mixed infections were very common in these patients (see 2.3.1, chapter 2), Ab responses to more than one type of block 2 were very rare (examples in fig. 5.8).

A total of 190 children came for a follow up visit one month later, and additional follow up blood samples were obtained from 31, 11 and 4 children after two, three and four months, respectively, from the date of admission. From these visits, 154/218 (71%) blood samples were parasite positive, whereas 64/218 (29%) had no detectable parasites. 97 (63%) of the positive samples were obtained from a child with no malaria symptoms, 44 (28.6%) from a child with UM, 10 (6.5%) from a child with persisting neurological sequelae and 3 (2%) from a child readmitted to hospital with SMA. 79/97 (81.4%) asymptomatic infections were undetected by microscopic examination but became apparent by PCR typing, as explained in section 2.3.1.2 (chapter 2). In these infected cases, block 2 Abs detected in convalescent sera were compared to the parasites found during acute infection and also to the parasites found in convalescence. 34/234 convalescent sera (14.5%) had K1-specific Abs, and this Ab specificity matched the presence of an infecting parasite of the K1-type in 32/34 cases (94% concordance). A mismatch was found in only 2 samples. 11/235 convalescent serum samples (4.7%) contained MAD20-specific Abs. The presence of MAD20-specific Abs correlated with the presence of an infecting parasite of the MAD20 type in 8/11 of the convalescent children (73% concordance), whereas a mismatch was observed in 3 patients. Finally, 22/233 convalescent sera (9.4%) contained Abs reacting with the RO33 fusion protein. In 21/22 children (95.5% concordance) a parasite of the RO33 type had been detected in acute and/or convalescent phase, whereas in only 1 sample there was a mismatch between parasite type and Ab.

In summary, Ab responses specific for the MSP-1 block 2 genotype(s) present in acute and/or convalescent stages were detected in the majority (91%) of
Fig 5.4. Specificity of antibody responses to MSP-1 MAD20-type infections
Correlation plots showing Ab responses (OD 492nm) against homologous MAD20-type parasites (along X axis) and against heterologous K1 (A & B) and RO33 (C & D) types (along Y axis), in children infected with parasites of the MSP-1 block 2 MAD20-type. Acute (A & C) and convalescent (B & D) plasma samples are distinguished. Most children had concurrent infections with the K1 and/or RO33 types and responded specifically to either one or another of the three block 2 types.
Fig 5.5. Examples of concordant antibody responses to infecting parasites of MSP-1 block 2 MAD20 type

Antibody reactivities (OD levels at 492nm) to fusion proteins of MAD20 type block 2 sequences in acute (A) and convalescent (C) patients infected with parasites of the MAD20 type. Patient MP829 had a single MAD20 (M) infection and Abs to MAD20 proteins in acute stage, which decreased at one-month convalescence. Patient MP916 had a mixed infection with K1 (K) and MAD20 parasites, but produced Abs only to the MAD20 type. Patient MP727 had an single infection consisting of a MAD20 type and produced Abs only to the MAD20 fusion proteins, which increased from acute to convalescent stages. The diagnosis on admission is indicated for each patient, as well as the clinical status one month later. CM=cerebral malaria, SMA=severe malarial anaemia, Asymptomatic=parasite positive but no malaria symptoms, Healthy=fully recovered. Antibody responses to the conserved C-terminal fragment of 19kDa are included for comparison.
**MSP-1 block 2 RO33-type infections**

Fig 5.6. Specificity of antibody responses to MSP-1 RO33-type

Correlation plots showing Ab responses (OD 492nm) against homologous RO33-type parasites (along X axis) and against heterologous K1 (A & B) and MAD20 (C &D) types (along Y axis), in children infected with parasites of the MSP-1 block 2 RO33-type. Acute (A & C) and convalescent (B & D) plasma samples are distinguished. Most children had Abs that were specific for the RO33-type of block 2. However, a high proportion of these children had concurrent infections with K1-type parasites, the predominant in Malawi, and some children also had MAD20-type parasites. In these cases, Ab responses were directed to either one or another of the three block 2 types present in the blood.
Antibody reactivities (OD levels at 492nm) to fusion proteins of RO33 type block 2 sequences in acute (A) and convalescent (C) patients infected with parasites of the RO33 type. Patients MP919, MP922 and MP714 had a mixed infection containing parasites of the K1 (K), MAD20 (M) and RO33 (R) types, but produced Abs only to the RO33 type. Antibody levels declined from acute to convalescent stages.

The diagnosis on admission is indicated for each patient, as well as the clinical status one month later.

CM=cerebral malaria, Healthy=fully recovered, Asymptomatic= parasite positive but no malaria symptoms; n.t= not known

Antibody responses to the conserved C-terminal fragment of 19kDa are included for comparison.
Figs 5.8. Examples of concordant antibody responses to more than one type of MSP-1 block 2

Antibody reactivities (OD levels at 492nm) to fusion proteins of MSP-1 block 2 types in acute (A) and convalescent (C) patients infected with parasites of the indicated type. These patients had Abs to more than one type of block 2 type, quite uncommon in these children. Patient MP733 was infected with three P. falciparum clones of the K1 (K), MAD20 (M) and RO33 (R) types on admission, and had positive Abs to K1 and MAD20 proteins, which gradually declined at one-month (C) and two-month (C2) convalescence, when these two parasites were not detected in the blood. Patient MP753 had detectable parasites of the three MSP-1 types over a period of four months and produced high Ab levels to K1-type; low Ab levels to MAD20 were detected after four months, coinciding with the first detection of MAD20 parasites. The diagnosis on admission is indicated for each patient, as well as the clinical status one month later. CM= cerebral malaria, SMA= severe malarial anaemia, UM= uncomplicated malaria, Asymptomatic= parasite positive but no malaria symptoms, SQ= neurological sequelae.
convalescent responders. In the asymptomatic individuals, a number of genotypes were controlled below the disease threshold level in the presence of MSP-1 type-specific Abs produced in response to concurrent infection(s). These results indicate that the polymorphic block 2 of MSP-1 is a target of naturally acquired immune responses in children.

5.3.2- Specificity of antibody responses to MSP-2

Correlations between two dimorphic types of MSP-2 in the infecting parasite and Ab responses mounted by the hosts were analysed as above. In 279 children, parasite genotypes were compared to Ab responses in acute stage. Among those, 219 (78.5%) contained Abs to MSP-2 A. In 181 of these children (82.6%), there was a match between presence of anti-MSP-2 A Abs and presence of a parasite of the MSP-2 A type, but in 38 children (17.4%) there was a mismatch (fig 5.9, 5.10 & 5.11). 208 children (73.5%) had Abs to MSP-2 B in acute disease. Abs matched the parasite in 108 patients (52%) and did not in 100 patients (48%) (figs. 5.12, 5.13 & 5.11).

Genotyping and serology were also compared in 192 convalescent children. 142 children (74%) had Abs to MSP-2 A. In 130 (91.5%) there was a match between presence of anti-MSP-2 Abs and presence in acute and/or convalescent stage of a parasite of the MSP-2A type, whereas in 12 children there was a mismatch (8.5%). 136 children (72%) had Abs to MSP-2 B; in 100 patients (73.5%) there was a concordant response, whereas in 36 patients (26.5%) there was a discordant response. Convalescent sera were more likely to contain Abs matching an infecting parasite of the MSP-2 B type than acute samples (p<0.001).

Antibody responses to both MSP-2 A and MSP-2 B types were common in individual children, as mixed infections containing both types were frequently encountered in these patients (fig. 5.14). Some of the double positive responses were explained by cross-reactive Abs recognising C-terminal conserved sequences of MSP-2 (fig 5.15), but in most cases Abs were directed to group-specific regions that are conserved within each allelic family (example in fig 5.16).

Overall, 68% of acute and 83% of convalescent patients made specific Abs matching the MSP-2 type(s) of the infecting parasite(s). The high prevalence of
Fig 5.8. Specificity of antibody responses to MSP-2 group A
Correlation plots showing Ab responses (OD 492nm) against homologous MSP-2 type A parasites (along X axis) and against heterologous MSP-2 type B parasites (along Y axis), in children infected with single infections of the MSP-2 type A. RO33 8/6 is a fusion protein containing dimorphic group A-specific regions, whereas K1 5/3 is a fusion protein containing polymorphic group B tandem repeat sequences. Most children infected with MSP-2 A parasites had Abs against group A specific regions, most remarkably in convalescence. Some Abs also reacted with MSP-2 B epitopes, especially in acute stage. These Abs may persist from previous infections with parasites of type B.
Antibody reactivities (OD levels at 492nm) to fusion proteins of MSP-2 type A sequences in acute (A) and convalescent (C) stages in patients infected with a single parasite clone of the MSP-2 type A. Patients MP805 and MP848 had Abs to proteins representing a polymorphic region (T9/102 5/7) and the dimorphic region (R033 8/6), which persisted in acute and convalescent stages, but decreased in levels. Patient MP690 had Abs only to the dimorphic region of MSP-2 type A. Sera from all three patients reacted with the full length MSP-2 A protein (T9/96 13/14). Antibody responses to the conserved C-terminal fragment of MSP-2 (K1 17/14) were absent in these patients.

Diagnosis on admission is indicated for each patient, as well as the clinical status one month later.

**CM**=cerebral malaria **SMA**=severe malarial anaemia, **SQ**=neurological sequelae, **Healthy**=fully recovered, **n.t.**=not known
Fig 5.11. Examples of discordant antibody to MSP-2 types
Antibody reactivities (OD levels at 492nm) to fusion proteins of MSP-2 types in acute (A) and convalescent (C) patients whose specificity did not correlate with the infecting parasite type. Patient MP723 had a single infection with a parasite of the MSP-2 B type but had Abs specific for MSP-2 type A proteins (polymorphic: T9/94 5/7; dimorphic RO33 8/6; full length T9/96 13/14). Patient MP474 was infected with a parasite of the MSP-2 A type but the serum contained Abs recognising MSP-2 B proteins (polymorphic K1 5/3; dimorphic K1 12/6; full length (Dd2 13/14). In both patients, Ab levels were lower in convalescent stages, indicating that the discordance might be explainable by Abs persisting from a previous infection with a different parasite type. Antibody responses to the conserved C-terminal fragment were absent in these patients. The diagnosis on admission is indicated for each patient, as well as the clinical status one month later.
CM=cerebral malaria, Asymptomatic=parasite positive but no malaria symptoms, Healthy=fully recovered, SQ=neurological sequelae; - = PCR negative
Single MSP-2 type B infections

Fig 5.12. Specificity of antibody responses to MSP-2 group B
Correlation plots showing Ab responses (OD 492nm) against homologous MSP-2 type B parasites (along X axis) and against heterologous MSP-2 type A parasites (along Y axis), in children infected with single infections of the MSP-2 type B. RO33 8/6 is a fusion protein containing dimorphic group A-specific regions, whereas K1 5/3 is a fusion protein containing polymorphic group B tandem repeat sequences. Children infected with MSP-2 B parasites had Abs against the homologous type less frequently than children infected with MSP-2 A parasites (see fig 5.9).
Antibody reactivities (OD levels at 492nm) to fusion proteins of MSP-2 type B  in acute (A) and convalescent (C) stages in patients infected with a single parasite clone of the MSP-2 type B. Patient MP778 had Abs to fusion proteins representing polymorphic (K1 5/3, including 32-mer repeats) and the dimorphic region (K1 12/6, including 12-mer repeats) which persisted in acute and convalescent stages. Patient MP810 had Abs predominantly directed to the dimorphic region of MSP-2 B, whereas patient MP821 had Abs which predominantly recognised polymorphic regions of MSP-2 type B. All three patients reacted well with the full length MSP-2 B protein (Dd2 13/14).

Antibody responses to the conserved C-terminal fragment of MSP-2 (K1 17/14) were absent or very low in these patients. Diagnosis on admission is indicated for each patient, as well as the clinical status one month later.

SMA=severe malarial anaemia, UM=uncomplicated malaria, Asymptomatic=parasite positive but no malaria symptoms, Healthy=fully recovered.
Fig 5.14. Antibody responses to mixed MSP-2 type infections
Correlation plots showing Ab responses (OD 492nm) against MSP-2 type A RO33 8/6 (along X axis) and against MSP-2 type B K1 5/3 (along Y axis).
Children infected with mixed MSP-2 A+B infections contained Abs against both types.
Fig. 5.16 Cross-reactivity between the two MSP-2 types by recognition of conserved determinants

Correlation plots of serum samples containing Abs that reacted with conserved regions of MSP-2. A) and B) correspond to children infected with single infections of MSP-2 A parasites but who reacted with MSP-2 B proteins. Some discrepancies may be explained by cross-reactivity between the two allelic families by recognition of conserved epitopes. A) Ab reactivities (OD at 492nm) against the two full length proteins of type A (T9/96 13/14) and B (Dd2 13/14). B) Some of the Abs contained in these children react with the conserved C-terminal region of MSP-2 (K1 17/14). Similarly, C) and D) correspond to children infected with single infections of MSP-2 B that reacted with MSP-2 A proteins. Some of these reactivities are due to cross-reactivity between the two types by recognition of the conserved C-terminal region of MSP-2.
### Fig 6.16. Examples of concordant antibody responses to both MSP-2 types

Antibody reactivities (OD levels at 492nm) to fusion proteins of MSP-2 types in acute (A) and convalescent (C) patients infected with parasites of the indicated type. These children had persistent infections with mixtures of parasites of the two types of MSP-2 and produced Abs of both specificities. Patient MP809 was infected with a parasite of the MSP-2 A type in the acute stage, and had Abs to dimorphic (RO33 8/6) and polymorphic (T9/94 5/7) regions of MSP-2 A. A month later, a parasite of the MSP-2 B type was detected, and Abs to the polymorphic (K1 5/3) regions of MSP-2 B appeared. After two months, only Abs to polymorphic MSP-2 A persisted. Patient MP753 was infected with a mixture of MSP-2 A and B parasites, and produced Abs directed to proteins representing dimorphic regions of each protein (K1 12/6 in the case of type B).

Diagnosis on admission is indicated for each patient, as well as the clinical status one month later. CM = cerebral malaria, SMA = severe malarial anaemia, UM = uncomplicated malaria, Asymptomatic = parasite positive but no malaria symptoms.

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<td>MP753 C3</td>
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Abs to MSP-2 detected at convalescence in response to natural infections indicates that MSP-2 is a target of strain-specific immune responses to *P. falciparum* in humans.

### 5.3.3- Relationship between strain-specific antibody responses and malaria disease

To test whether the presence or absence of Abs specific for the infecting parasite in acute infection had an influence in the severity of malarial disease, Ab responses to infecting parasite type(s) were compared among groups of children presenting with different clinical forms of the disease. In addition, time-course and duration of type-specific Ab responses between acute and convalescent phases were compared among different groups of patients.

#### 5.3.3.1- Antibody responses to MSP-1

The concordances between the presence of a parasite bearing a particular MSP-1 block 2 type and the presence of Abs of the corresponding specificity for each disease group are shown in table 5.1.

The low numbers of seropositive sera in each disease group impede meaningful statistical analysis. However, there were no apparent differences in the ability to mount strain-specific immune responses to the polymorphic block 2 of MSP-1 among these groups of patients. In all groups, most of the few responding patients (between 6-15% of total) recognised the infecting parasite and produced Abs of the appropriate specificity in the acute and convalescent stages.

#### 5.3.3.2- Antibody responses to MSP-2

The concordances between the MSP-2 type of the infection and the specificity of the Ab response for each disease group are shown in table 5.2.

The statistical analysis of the relationship between concordance of Ab-parasite specificity and disease status revealed some differences among groups of patients. Among children infected with parasites bearing MSP-2 type B, matching anti-MSP-2 B Abs were significantly less prevalent during acute disease in children who developed CM than in those with SMA (χ² test, p<0.005) or UM (χ² test, p<0.025).
Table 8.1. Concordance between MSP-1 block 2 type and Ab responses

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a n is the number of patients whose sera were positive for Ab to a recombinant protein of the type indicated, and in whom the block 2 type of MSP-1 of the infecting parasite(s) was determined.

b Percentage of patients in whom there was a concordance between the MSP-1 block 2 type of the infecting parasite and the specificity of the Ab response to block 2.

c CM=cerebral malaria, SMA=severe malarial anaemia, UM=uncomplicated malaria, SNM=severe non malarial disease.

d Ab specificity of convalescent sera was compared to the MSP-1 type of the parasite(s) infecting the same patients in acute (admission) and/or convalescent stages.

CM patients with anaemia followed a similar trend as children with CM alone i.e. lower percentage of matches for MSP-2 B than SMA alone (p<0.05). Among children infected with parasites of the MSP-2 type A at admission, those who developed SMA had anti-MSP-2 A Abs in acute and convalescent stages less frequently than those who developed UM (p=0.06) or CM, but the differences were not statistically significant. The few SNM control patients examined (asymptomatic for malaria but suffering from other severe diseases) behaved similarly to UM controls, i.e. high percentage of matches for MSP-2 A and B.
### Table 5.2. Concordance between MSP-2 types and Ab responses

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<td>84</td>
<td>31</td>
<td>84</td>
</tr>
<tr>
<td>CM+SMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>38</td>
<td>81.6</td>
<td>33</td>
<td>45.4</td>
</tr>
<tr>
<td>Convalescent</td>
<td>31</td>
<td>97</td>
<td>26</td>
<td>77</td>
</tr>
<tr>
<td>UM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>55</td>
<td>87.3</td>
<td>53</td>
<td>62.3</td>
</tr>
<tr>
<td>Convalescent</td>
<td>13</td>
<td>92.3</td>
<td>17</td>
<td>76.5</td>
</tr>
<tr>
<td>SNM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>8</td>
<td>100</td>
<td>8</td>
<td>75</td>
</tr>
<tr>
<td>Convalescent</td>
<td>4</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

*a* n is the number of patients whose sera were positive for Ab to a recombinant protein of the indicated type, and in whom the dimorphic type of MSP-2 in the infecting parasite(s) was determined.

*b* Percentage of patients in whom there was a concordance between the MSP-2 type of the infecting parasite and the specificity of the Ab response to MSP-2.

*CM=cerebral malaria, SMA=severe malarial anaemia, UM=uncomplicated malaria, SNM=severe non malarial disease.

*Ab specificity of convalescent sera was compared to the MSP-2 type of the parasite(s) infecting the same patient in acute (admission) and/or convalescent stages.

CM patients infected with MSP-2 B parasites had lower percentage of Ab-genotype matches than UM (p<0.025) or SMA (p<0.005) cases; CM+SMA patients also differed significantly from SMA patients (p<0.05).

In summary, patients who had mild malarial disease appeared to have a higher proportion of matching Ab responses to MSP-2 than those who developed severe symptoms. Children who were infected with parasites of the MSP-2 type B and developed CM, produced Abs that were the least concordant to MSP-2 B parasites present at the acute stage. Children infected with parasites of the MSP-2 type A who developed SMA produced Abs that were the least concordant to MSP-2 A parasites in acute and convalescent stages.

The relationship between concordance and disease outcome in convalescent patients who survived from malaria or other diseases is shown in table 5.3. Children who suffered neurological sequelae, as a result of CM, were more likely to have Ab responses of the wrong specificity than children who recovered from any form of disease. However, there were too few patients with sequelae and the differences were not significant.
Table 5.3. Relationship between concordance of Ab specificity to MSP-2 and disease outcome in all patients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>MSP2A</th>
<th></th>
<th>MSP2B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Full recovery b</td>
<td>48</td>
<td>81.25</td>
<td>43</td>
<td>53.5</td>
</tr>
<tr>
<td>Asymptomatic c</td>
<td>63</td>
<td>76.2</td>
<td>67</td>
<td>61.2</td>
</tr>
<tr>
<td>Sequelae d</td>
<td>8</td>
<td>50</td>
<td>7</td>
<td>28.6</td>
</tr>
</tbody>
</table>

a Percentage of children who had Abs matching the infecting parasite of the indicated MSP-2 genotype.
b Children who had no parasitaemia and no symptoms of malaria disease at follow up.
c Children who had parasitaemia (slide and/or PCR positive) but no symptoms of malaria disease at follow up.
d Children who suffered from neurological sequelae cerebral malaria.

5.4- Discussion

In this chapter, I have investigated the relationship between antigenic diversity in *P. falciparum* and the specificity of the Ab response in the human host. First, I have tested whether the polymorphic merozoite surface proteins MSP-1 and MSP-2 are targets of a strain-specific component of the immune system. Second, I have tested the hypothesis that a lack of Abs specific for the infecting parasite in the acute stage may predispose a child to develop severe malaria. This is the first study to systematically compare strain-specific Ab responses to MSP-1 and MSP-2 and typing of the infecting parasite in significant numbers of patients with distinct forms of severe *P. falciparum* malaria.

5.4.1- MSP-1 and MSP-2 are targets of strain-specific immune responses

Children naturally infected with *P. falciparum* produced Ab responses which were specific for the parasite strains present in their blood. In most patients, the specificity of serum Abs to variable regions of MSP-1 and MSP-2 matched the PCR type of the infecting parasite. Discordances found in some acute and/or convalescent samples might be due to the persistence of Abs produced in previous infections with different parasites. Although levels of Abs tended to decrease soon after parasites had been cleared by drugs (see chapter 4), patients did not normally become seronegative after one month. In addition, as parasite clones appear and disappear from the
peripheral blood every 48 hr (Farnert et al., 1997) some discordances may be due to
sequestration of particular genotypes in asynchronised mixed infections.

The overall concordance of Ab responses to block 2 of MSP-1 was between
70-87%. This is in agreement with a recent longitudinal study of block 2 type-specific
Ab responses in Sudan (Cavanagh et al., 1998). To complement this work, it would
be very interesting to test also the specificity of Ab responses to the dimorphic regions
of MSP-1 (blocks 6-16), using recombinant proteins representing the two major
families of MSP-1 (MAD20 and Well/K1) in analogy to the study by Früh et al.

A good correlation of MSP-2 type-specific Ab responses was found, with
concordances of 77% for MSP-2 A and 52% for MSP-2 B. Compared to MSP-1, this
is overall lower concordance. It may be partially explainable by a lower sensitivity of
detection of the PCR typing used for the MSP-2 marker than for block 2 of MSP-1
(see chapter 2), which might have underestimated parasite genotypes present in blood
at low densities. In particular, infections with the MSP-2 type B induced Abs of the
right specificity apparently less frequently than infections with the MSP-2 type A. It is
possible that parasites bearing MSP-2 type A or B vary in their ability to induce
immune responses. The observations that MSP-2 genotype B was more common in
adults than in children (Felger et al., 1994), and also more common in symptomatic
than asymptomatic individuals (Engelbrecht et al., 1995), led to the suggestion that
parasites with MSP-2 type B must be more virulent than those with type A. It was
argued that clones with MSP-2 type B may be more successful in evading the immune
system of immune individuals than clones with type A. In addition, Abs specific for
dimorphic regions of MSP-2 type A were associated with lower malaria morbidity
(Al-Yaman et al., 1994). In the view of my results, it is possible that type A parasites
do not survive as well as type B parasites due to a more efficient strain-specific
acquired immunity. Data presented here indicated that the immune system may less
efficiently respond specifically to type B antigens during the acute phase, leading to
more parasite proliferation and morbidity. Indeed, MSP-2 B infections were
associated with high parasitemias in patients studied here (see chapter 2). A putative
immunological advantage of type B parasites might be related to distinct structural
features of type A and B proteins. A less diverse repeat structure in MSP-2 type B compared to type A probably reflects separate evolutionary strategies of the two allelic families (Felger et al., 1997). The conservation of a general structural organization in MSP-2 B alleles could be explained by functional constraints, e.g. involvement in receptor-ligand interactions with the host.

Strain-specific Ab responses were detected in a high proportion of children in acute phase of malaria, suggesting that Abs to current infection appear early in the course of the disease. Antibody levels at the time of patients' presentation can reflect both past and present infections. It was expected that only Abs specific for the current infection, and thus concordance, would increase during convalescence. As expected, Ab responses detected at a follow up matched the types of the infecting parasite(s) better than Abs detected in acute infections, particularly for MSP-2 B infections. Perhaps because of this, no evidence for an anti-parasite role of strain-specific Abs was apparent in that the presence of type-specific Ab responses did not prevent many patients (70%) from being infected. However, children with persistent infections at convalescence had very low parasite densities, detectable only by PCR and not by microscopy, and mild (30%) or no symptoms (63%) of malaria. This suggested that type-specific Abs may in fact have some anti-parasite role at controlling densities below the disease threshold level. A feature of acquired immunity to malaria is that it is never complete or sterile. Thus, the results do not contradict the strain-specific immunity hypothesis. The finding of the apparently controlled infections in the presence of these Ab responses may suggest that the children were in the process of acquiring the status of premunition rather than these Abs just being an indication of exposure to the previous P. falciparum infections. This is in agreement with recent longitudinal studies suggesting that acquired immunity restricts the growth of a large number of parasite genotypes during asymptomatic infections (Daubersies et al., 1996; Farnert et al., 1997).
5.4.2 Strain-specific immune responses and malaria disease severity

No difference between disease groups were detected regarding Ab responses to block 2 of MSP-1, largely due to the low prevalence of Ab responses to any given type of block 2. Concerning Ab responses to MSP-2, children who developed severe malaria symptoms differed as follows from children with UM in their ability to mount specific responses to the infecting parasites in the acute phase of the disease.

A decreased ability to respond to parasites of type B may predispose a child to develop CM. As a whole, the CM group had very high prevalences of anti-MSP-2 B Abs (80%). Therefore the low frequency of responders to type B was a characteristic of CM patients infected with parasites of this type.

Similarly, a decreased ability to respond to infecting parasites of type A may partially contribute to SMA. The lower frequency of anti-MSP-2 A positive responses among SMA patients was apparent only in those who were infected with parasites of type A, since 88% of sera from the SMA group as a whole had Abs to MSP-2 A. This observation fits with conclusions from a study in Papua New Guinea which found that Abs specific for MSP-2 type A were associated with less anaemia (Al-Yaman et al., 1994). The majority of SMA patients had Abs matching MSP-2 type B infecting parasites. Thus, high levels of Abs against MSP-2 B do not seem to confer any protection against anaemia, as pointed out by Al-Yaman et al. (1997).

No differences in the specificity of Ab responses in relation to the outcome of the disease were found by comparison between those who died and those who survived (data not shown). This was in contrast to Al-Yaman et al. (1997), who reported lower anti-MSP-2A Ab levels among fatalities. In this study, patients who survived and suffered from neurological sequelae after CM had less concordant Ab responses to MSP-2 than patients who had a full recovery or who were asymptomatic. Despite this trend, there were too few patients with sequelae to draw a clear conclusion.

In conclusion, a lack of Abs to one or the other allelic family of MSP-2 might differently predispose a child to develop one or the other severe forms of malaria, but the reasons why this might be the case remain unknown. As suggested in chapter 4, it
is conceivable that different regions and/or allelic forms of the merozoite surface antigens are processed and presented to the immune system by different pathways and, perhaps, result in divergent immunopathological processes. For instance, it is possible that parasites bearing MSP-2 type A or B vary in their ability to induce inflammatory cytokines (e.g. TNF) implicated in causing severe pathology. The possibility of a differential Th1/Th2 pattern of immune responses induced by each MSP-2 dimorphic type merits investigation as it may have important implications for malaria vaccine development and deployment.
The development of naturally acquired immunity to *Plasmodium falciparum* infections is not understood. The long period of time required to achieve protection and the wide spectrum of disease manifestations observed in *P. falciparum* infections indicates that the acquisition of immunity is a very complex process. Understanding the interaction between the various host and parasite factors involved in this process is crucial for the development of appropriate vaccines, as the malaria parasite remains a major cause of morbidity and mortality in the world.

The fact that natural populations of *P. falciparum* are genetically diverse, consisting of different genotypes or "strains", suggested that immunity to this parasite could be "strain"-specific, effective protection being achieved only after immunisation by a succession of parasites representing the main strains circulating in a community. These strains differ in their composition of a number of polymorphic molecules, including the merozoite surface proteins MSP-1 and MSP-2, that are among major candidates for a malaria vaccine.

This thesis aimed to test the hypothesis that MSP-1 and MSP-2 are targets of a strain-specific component of human immunity to malaria. Many studies have shown that the genes encoding these proteins are polymorphic, but none has investigated the effect of these polymorphisms on antigenic diversity or on the specificity of human immune responses. Therefore, the objectives of this project were (i) to assess the extent of *MSP-1* and *MSP-2* polymorphisms (ii) to study the relationship between allelic polymorphisms and antigenic diversity and (iii) to investigate the relationship between parasite diversity and the specificity of human immune responses.

The study population consisted of Malawian paediatric patients distinguished by clinical forms of *P. falciparum* infection. For the first time, significant numbers of patients suffering from cerebral malaria (CM), severe malarial anaemia (SMA) and uncomplicated malaria (UM) could be compared to test (i) whether parasites associated with different disease manifestations differed in their composition of MSP-1 and MSP-2 (ii) whether strain-specific and/or multi-specific immune responses to MSP-1, MSP-2, RAP-1 and AMA-1 contributed to the severity of the disease.
6.1- Gene polymorphisms, antigenic diversity and strain-specific immune responses

PCR genotyping of \(MSP-1\) and \(MSP-2\) is a simple method to assess allelic polymorphisms of \(P. falciparum\). Because of that, a considerable amount of data is becoming available from a number of studies with parasite isolates from Africa, Australasia and South America (see table 2.12). Fewer studies have investigated the antigenic diversity of the encoded proteins by the use of monoclonal antibodies (mAbs) in IFA. Most of this research aims to understand the relevance of parasite heterogeneity in the acquisition of immunity and the implications for the development of a vaccine. However, these studies have only partially addressed the issue, since none has investigated simultaneously the genetic basis of the serological diversity of the proteins and their recognition by human Abs. This has been done in this thesis by (i) correlating the genotypes of \(MSP-1\) and \(MSP-2\) with the serotypes of the expressed proteins in the same parasites and (ii) correlating the genotypes of the parasites with the specificity of the Ab responses in the host.

6.1.1- Genetic polymorphism and antigenic diversity

First, polymorphisms in the \(MSP-1\) and \(MSP-2\) genes were determined in 279 \(P. falciparum\) clinical isolates from a malaria endemic area of Malawi. Dimorphic (block 16) and polymorphic (block 2) regions of \(MSP-1\) and dimorphic regions of \(MSP-2\) were genotyped by PCR. The frequencies of some \(MSP-1\) and \(MSP-2\) genotypes differed from those found in distant geographical locations, as shown in many previous studies. By sequencing, 9 new \(MSP-2\) alleles and 6 new block 2 \(MSP-1\) alleles were identified. However, in Malawi, where no similar survey had been carried out previously, the distribution of genotypes was similar to that found in other African countries. There was a striking predominance of the MAD20 dimorphic type of \(MSP-1\) (93%) over the alternative K1/Well-type (7%), as detected in The Gambia (Conway et al., 1991), and a higher prevalence of the K1 block 2 type of \(MSP-1\) (48%) over the MAD20 (30%) or the RO33 (22%) types, as in Tanzania (Babiker et al., 1994, 1997). With regard to \(MSP-2\), type A (60%) predominated over the alternative type B (40%), as reported in most countries (table 2.12), the relative
frequencies of both dimorphic families are quite similar. In addition, mixed infections of different *P. falciparum* genotypes were common among these isolates, with a mean number of clones per patient of 2.5, similar to that found in areas of hyperendemic and seasonal malaria transmission (table 2.12).

In parallel, 134 *P. falciparum* isolates were successfully cultured to schizont stages, and their expressed MSP-1, MSP-2 and EXP-1 antigens were serotyped by IFA. This allowed, for the first time, a detailed comparison between the PCR and IFA typing techniques. Both methods have limitations when used on their own and are more valuable in combination. PCR is more sensitive than IFA and thus able to detect parasite clones at low density. However, IFA provides a means to resolve the phenotypes of MSP-1 and MSP-2 and to quantify each clone in a mixed infection. The relative density of each parasite genotype is probably an important factor in the induction of specific Ab responses in the host. Overall, PCR and IFA typing results correlate well, indicating that polymorphisms of malaria genes determined at the nucleotide level are reflected in serological diversity at the amino acid level, as recognised by type-specific mouse Abs.

6.1.2- Strain-specific immune responses

Second, I investigated whether MSP-1 and MSP-2 are targets of type-specific Abs responses in children naturally infected with *P. falciparum*. Panels of recombinant proteins representing polymorphic, dimorphic and conserved regions of the antigens were used in ELISA (i) to assess the relative antigenicity of defined regions of MSP-1, MSP-2 RAP-1 and AMA-1 and (ii) to correlate the MSP-1 and MSP-2 genotypes in the parasite with the specificity of the Ab response in the host.

IgG responses to the four antigens examined were detected in the majority of children, but different regions of the proteins varied in their relative immunogenicity. Antibodies to the C-terminal fragments of MSP-1 (19kDa and 42kDa) were very prevalent (70%), being the most reliable indicator of recent clinical infection. This prevalence was comparable to other African populations (Udhayakumar *et al.*, 1995; Egan *et al.*, 1996; Cavanagh *et al.*, 1998). In contrast, the N-terminal block 1 of MSP-1 was poorly immunogenic. Only few children (7%) contained any detectable
Ab to this region, suggesting that Ab responses induced by a vaccine containing block 1 sequences, e.g. Spf66 (Moreno & Patarroyo, 1989), may not be boosted by natural *P. falciparum* infections.

Antibody responses to the polymorphic block 2 of MSP-1 were detected in 28% children, less frequently than in Sudan (Cavanagh et al., 1998). Importantly, the highest prevalence of Abs to block 2 of the K1-type reflected the highest prevalence of parasites of the K1-type in the same children. This strongly suggested that immune responses to polymorphic regions of MSP-1 are strain-specific. To confirm this, a more detailed analysis of the concordance between the MSP-1 block 2 type of the infection and the specificity of the Ab response in each patient was done in chapter 5. The result was that between 70-87% of children made Abs that matched with the block 2 type of the infecting parasite, indicating that MSP-1 is indeed a target of strain-specific Ab responses.

Antibody responses to MSP-2 in these children were predominantly directed to dimorphic (75%) or polymorphic (50%) regions of the protein, as previously found in Gambian donors (Taylor et al., 1995). The conserved C-terminal regions were recognised by a significant proportion of patients (30%), whereas Ab responses to the conserved N-terminal region were very rare (4%). Concerning the specific recognition of each of the two MSP-2 allelic families, Ab responses to type A were more prevalent than Ab responses to type B. This finding correlated well with the higher prevalence of parasites expressing type A in Malawi. The comparison between parasite genotypes and Ab specificities in each patient also revealed that Ab responses to MSP-2 are essentially strain-specific.

Less is known about the natural immunogenicity of the other two merozoite proteins, AMA-1 and RAP-1. In contrast to MSP-1 and MSP-2, they are relatively conserved among different *P. falciparum* isolates, an advantageous quality in a vaccine candidate. A substantial proportion of children produced Abs to AMA-1 (60%) and the C2 fragment of RAP-1 (41%), indicating that these antigens are immunogenic and thus Ab responses induced by a vaccine may be boosted by natural *P. falciparum* infections.
6.2- Parasite diversity, acquired immunity and disease severity

Another aspect of interest in the research on parasite diversity concerns the possible relationship between parasite heterogeneity and disease. It is not know why only a small proportion of children infected with *P. falciparum* develop a severe disease while the majority have mild or no symptoms of malaria. Several host and parasite factors are likely to determine the severity of the disease manifestations. In this thesis, I have examined three possibilities: (i) particular parasite genotypes are associated with clinical forms of malaria (ii) the number of distinct genotypes in an infection influences the disease severity (iii) a lack of previous immunity to the parasite predisposes a child to develop severe disease.

6.2.1- Parasite diversity and disease severity

First, the frequencies of the *MSP-1* and *MSP-2* genotypes of blood-circulating parasites were compared among groups of patients with different clinical presentations. Certain genotypes appeared to be associated with anaemia, cerebral or mild malaria symptoms.

The K1/Well dimorphic type of *MSP-1*, present in a minority of patients, was significantly over-represented in parasites from SMA patients and under-represented in parasites from CM patients, both compared to UM patients. However, it was not possible to distinguish between a putative enhanced virulence of K1/Well-type parasites causing SMA or a requirement for the MAD20-type in parasites causing CM. The low proportion of parasites of the K1/Well dimorphic type advices that this finding must be interpreted with caution and requires to be confirmed in other studies.

Concerning block 2 of *MSP-1*, MAD20-type parasites were more prevalent in UM patients than in CM or SMA patients, suggesting that this block 2 genotype may be a marker for "milder" *P. falciparum* clones. This result agreed with one study (Kun *et al.*, 1998), but was not found in another study (Robert *et al.*, 1996).

With regard to *MSP-2*, type B parasites were associated with SMA. In Papua New Guinea, Felger *et al.* (1994) found a higher prevalence of *MSP-2* type B in adults, whereas type A was more frequent in children. In addition, *MSP-2* B was twice as likely to be found in symptomatic than asymptomatic individuals
The association between the MSP-2 B genotype and a higher malaria morbidity, estimated by fever and anaemia, led to the conclusion that MSP-2 B parasites are more virulent.

Virulence may be a consequence of the way in which the immune response of the host interacts with the parasites. Felger et al. (1994) suggested that parasites expressing MSP-2 type A might not survive as well in adults due to acquired immunity, whilst type B parasites might be more successful in evading the immune system of immune individuals. An association was found between Abs to dimorphic regions of MSP-2 A and less anaemia (Al Yaman et al., 1994). In Malawi, there was a lower frequency of anti-MSP-2 A positive responses among SMA patients infected with type A parasites than among CM or UM patients. Thus, a decreased ability to respond to parasites of type A might partially contribute to SMA.

Higher parasite densities were associated with type B infections in Malawi and in the study by Felger et al. (1994). Hyperparasitaemia is generally a feature of CM (Warrell et al., 1990). A decreased ability to respond to parasites of type B may result in high parasite densities and may predispose a child to develop CM. The finding here of a significantly lower proportion of responders to type B infections among CM patients than among UM or SMA patients would conform with this possibility.

6.2.2- Multiplicity of infections and disease severity

Increasing interest is currently focused on the relationship between multiplicity of infections and naturally acquired immunity. So far, few studies have analysed the relationship between complexity of infections (i.e. mean number of clones per patient and percentage of mixed infections) and severity of clinical symptoms, and none has separated CM from SMA patients. In this study, CM patients were distinguished by lower mean number of parasite clones than UM or SMA patients. This is in agreement with studies in Senegal (Robert et al., 1996) and Gabon (Kun et al., 1998). Robert et al. (1996) suggested that CM would be associated with high parasitaemia, resulting from overwhelming multiplication of a limited number of genotypes, while UM would be caused by a large number of genotypes reaching lower parasitaemias. This scenario would be compatible with the hypothesis that CM may be caused by a reduced
number of more virulent strains (Gupta et al., 1994). Higher multiplicity of infections were associated with asymptomatic rather than symptomatic individuals in Senegal (Mercereau-Puijalon, 1996), Tanzania (Beck et al., 1997) and Papua New Guinea (Al-Yaman et al., 1997). The opposite trend was reported in one survey in a hypoendemic area of Sudan (Roper et al., 1998), and no significant differences were found in The Gambia (Conway et al., 1991a) or Kenya (Kyes et al., 1997). Taken together, however, these findings seem to reflect a continuity from higher clonal multiplicity in the mildest forms of malaria to lower multiplicity in the most severe (cerebral) manifestations.

High multiplicity in UM compared to CM suggests a protective role for mixed infections and is concordant with the strain-specific hypothesis. There is evidence from the field that clinical malaria in children in endemic areas occurs as a result of an infection with a genotype to which they do not yet have immunity (Contamin et al., 1996). As a child ages, the repertoire of genotypes to which the immune system has been exposed increases, and thus the child becomes more likely to be protected against any new infections (Marsh, 1992). Interestingly, an age-dependent decrease in carriage of multiple MSP-2 alleles was found (Ntoumi et al., 1995). The authors suggested that acquiring efficient anti-parasite immunity with age results in decreasing the complexity of the infections as well as the parasite load. The protective effect of mixed infections indicates that what determines the specificity of effective immune mechanisms against new infections is not only memory from previous infections but also the repertoire of concurrent infections. This protection seems to depend on short-lived immune responses to coexisting infections. The observation that the process of acquisition of premunition varies with malaria endemicity may explain the only association between high multiplicity of infection and disease found in Sudan, where transmission is unstable and immunity against previously experienced genotypes has time to wane (Roper et al., 1998). As shown here for MSP-2, responses to concurrent infections are partially type-specific but also entail some cross-protection against related genotypes. Cross-protection between different genotypes by Abs directed to conserved antigenic determinants may also increase the aquisition of immunity. In accord with this idea, I found a significant negative correlation between multiplicity
and levels of Abs to conserved C-terminal regions of MSP-1 (19kDa and 42kDa), providing some more indirect evidence for the vaccine potential of these proteins.

High multiplicity of infections, however, was also associated with SMA in Malawi and in Gabon (Kun et al., 1998). Thus, alternative explanations have to be sought for the apparently detrimental effect of multiple infections in SMA. It is not clear whether SMA represents the extreme of a continuum of pathology or constitutes a separate disease entity. In *P. chabaudi*, mixed-clone infections were more virulent than single-clone infections, producing more anaemia in mice (Taylor et al., 1998). This suggested that, in this mouse model, genetically diverse infections are less rapidly cleared by the host and thus prolonging the anaemia. Diverse parasite populations could stimulate proliferation of a larger number of T- or B-cell clones, and hence a greater immune cascade causing the destructions of more RBCs, or higher production of cytokines (e.g. TNF-α) which may be involved in the pathogenesis of SMA. Alternatively, the high frequency of mixed infections might also be explained by some host genetic factors, e.g. the sickle cell anaemia trait has been associated with higher multiplicity of infections (Ntoumi et al., 1997a, 1997b). It will be very interesting to determine the Hb type in the Malawian patients to test this possibility.

Mixed infections of *MSP-2* types A+B had the same morbidity as type B single infections in Papua New Guinea (Engelbretch et al., 1995). The authors proposed that the 32-mer repeats of *MSP-2* B may be more immunogenic but not protective ("smokescreen" hypothesis), while Abs involved in controlling the parasites would be directed to dimorphic *MSP-2* A regions. If Abs specific for *MSP-2* B were more immunogenic and stable, there could be a lack of boosting of Abs to type A in those SMA patients in whom mixed A+B infections are common. However, I found no evidence to support the hypothesis that 32-mer repeats are more immunogenic or longer-lived than Abs to other regions of *MSP-2*. Moreover, patients appeared to have a "delayed" response to type B compared to type A, as the proportion of children with Abs matching type B parasites was high only at convalescence.

From my results, it seems more likely that SMA is a distinct entity and cannot be grouped with CM. Studies exploring the relationship between polymorphisms and malaria morbidity are still in their infancy and results remain inconclusive. More field
studies are required to identify more consistent associations between genotypes and
disease in different populations. On balance, though, the data suggest that strain-
specific immunity is important in developing resistance to malaria, and that multiple
infections probably stimulate protective immunity against a greater range of
genotypes.

6.2.3- Sequestration and disease severity

Genotyping of circulating parasites taken from single blood samples only
partially reflect the complexity of an infection within an individual. It has been shown
that genotypes in the peripheral blood fluctuate daily with a 48 hr periodicity,
presumably due to sequestration (Farnert et al., 1997). Autopsy specimens from 16
fatal cases were collected in Malawi to investigate the composition of MSP-1, MSP-2
and EXP-1 in sequestered parasites, and to compare parasites in the peripheral
circulation with parasites in organs of the same patients. For the first time, the
antigenic diversity of these proteins was resolved in situ in parasites sequestered in
brain, cerebellum, lung, spleen and liver using IFA microscopy. In this study, clonal
multiplicity of infection did not differ between sequestered and circulating parasites in
7 out of 8 fatal cases. In addition, I found no evidence for a differential sequestration
of particular P. falciparum serotypes in particular organs. The serotypes found in
organs were merely a reflection of the local repertoire in Malawi. Thus, in this limited
set of samples, my results do not support MSP-1 or MSP-2 being directly implicated
in the pathogenesis of CM, as no "pathogenic" type could be identified. However, this
work provides the only available way thus far to examine multiplicity of infections in
the sites of parasite sequestration.

In addition, Ab responses to MSP-1 and MSP-2 were analysed in all the
patients who died to test whether a defective Ab response to the infecting parasites
could account for the fatal outcome. A study by Al-Yaman et al. (1997) reported that
Ab levels to MSP-2 type A were significantly lower among those who died than
among survivors, although only 13 CM fatal cases were included in the comparison.
In addition, the latter study found that children who died were more likely to be
infected with parasites of the MSP-2 type B, which appears to be more virulent, as
discussed above. In contrast, I did not detect significant differences in the levels or prevalences of type-specific or multi-specific Abs between patients who recovered and those who died. Thus, death from malaria could not be attributable to the presence of particular parasite genotypes or to the absence of an appropriate Ab response to the current infection.

6.2.4 - Antibody responses and disease severity

Antibody responses to MSP-1, MSP-2, AMA-1 and RAP-1 were analysed in patients presenting with acute illness to test whether differences in previous exposure to infection could explain the development of severe or mild disease. I found that the development of CM or SMA was not due to a lack of Ab responses in the acute stage. However, children with different severe malaria manifestations were distinguished by a surprising differential pattern of Ab responses to defined regions of the merozoite antigens. There was a quite apparent general trend in Ab reactivity to conserved antigenic determinants that ranged from hyper-responsiveness in CM through intermediate responsiveness in UM to hypo-responsiveness in SMA. In contrast, Ab responses to variable regions of MSP-1 and MSP-2 were quite comparable among the different clinical groups.

It becomes clearer that what determines the clinical severity of *P. falciparum* infections must be multifactorial. Almost all variables measured in both parasite (genotype, density, multiplicity) and host (mean age, Ab responses) differed significantly between CM and SMA patients, usually with intermediate values for UM or CM+SMA patients. Different mechanisms may even act in patients with CM+SMA and in patients with CM or SMA only. The findings reported here may shed some light on ways to approach the investigation of these mechanisms in the future, with special attention to the possibility of a different skewing of Th1 and Th2 responses in CM vs. SMA. The interpretations suggested in section 4.4.2 (chapter 4) need to be fully explored, especially to clarify whether immunology contributes to the pathogenesis of severe forms of *P. falciparum* infection and, if it does, to elucidate the immunological mechanisms involved, as these will have important implications for malaria vaccine development.


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APPENDIX:

Publication generated during the period of my studies
Identical alleles of *Plasmodium falciparum* merozoite surface protein 2 found in distant geographic areas and times

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The merozoite surface protein 2 (MSP-2) of *Plasmodium falciparum* is a 35–56 kDa antigen located on the merozoite plasma membrane [1,2]. Although the protein is highly polymorphic in natural populations of the parasite, sequencing of MSP-2 alleles from a large number of parasite isolates from different geographical locations has shown an essentially dimorphic structure of the molecule [2–8]. Thus, two allelic families exist, the IC1/3D7 and the FC27 families [3], which correspond to the A and B serogroups defined by reactivity with monoclonal antibodies [4]. In addition, forms presumably generated by intragenic recombinations between these two families have been described [5,6]. All variants of the protein have a similar overall design, consisting of conserved N- and C-terminal regions flanking a long central region that differs substantially between the two families. These family-specific regions are composed of non-repetitive sequences surrounding two domains of highly polymorphic tandem repeats.

All variants of MSP-2 sequenced to date [2–8] appear to be built up from a limited number of basic sequence-blocks present in alleles from geographically distant parasite populations. Thus we would expect that despite the apparent overall diversity, there may be a limited repertoire of alleles distributed worldwide. Identical MSP-2 alleles have been found occasionally only in patients presenting at the same location at one time [6–8] but, so far, identical alleles have not been reported from distant parasite populations.

To define the extent of MSP-2 polymorphism, we have sequenced the gene from 33 clinical *P. falciparum* isolates and five laboratory-established
cultures. During this analysis we have come across identical MSP-2 sequences in parasites isolated in distant areas at different times. Here we report the identity between the MSP-2 alleles of isolates V306 from The Gambia and RO33 from Ghana, and an almost complete identity between alleles of MP663 from Malawi and NIG60 from Nigeria [5].

V306 was collected in Fajara, a malaria endemic area in The Gambia, West Africa in 1992. MP663 was collected in Blantyre, Malawi, South East Africa in 1996. Genomic DNA was extracted as described [9]. Synthetic oligonucleotide primers (underlined in Fig. 1a) corresponding to the 5' and 3' conserved regions of the MSP-2 sequence [4], were used in polymerase chain reaction (PCR) amplification. The V306 PCR product was cloned (TA cloning, Invitrogen), purified (Wizard Minipreps, Promega) and double-stranded sequenced by the dideoxynucleotide chain termination method (USB Sequenase Version 2). Direct automated sequencing was carried out with the purified MP663 PCR product (ABI DyeDeoxy™ Terminator Cycle Sequencing Kit). RO33 was isolated in 1987 from blood of a Swiss tourist who had visited Ghana [10]. RO33 genomic DNA was amplified by PCR and sequenced as described [4]. MSP-2 sequence of the isolate NIG60, collected in Nigeria in 1989, was reported elsewhere [5].

We have found a complete identity between MSP-2 alleles of the isolates V306 (The Gambia, 1992) and RO33 (Ghana, 1987) (Fig. 1a). In the IC1/3D7 family to which this allele belongs, the majority of nucleotide changes are found within two polymorphic regions, the R1 (152–205 nt) and R2 (251–295 nt) tandem repeats, that vary in sequence, length and number among different alleles. In the V306/RO33 allele, the R1 region is the shortest within the IC1/3D7 family. It is composed of one copy of the sequence GASGSA, followed by three copies of GS, and the sequence GAVASA, motifs present variably in some other alleles of the family. R2 is composed of the sequence TPATPA followed by nine threonine residues. Both these repetitive regions and also all of 19 other positions where point mutations are known to occur in the IC1/3D7 family (printed in bold in Fig. 1a) are conserved between V306 and RO33. Although the 3' end of the V306 allele has not been fully sequenced, nucleotides at this end are conserved in all known members of this family.

To ascertain that the identical MSP-2 allele obtained from the V306 and RO33 isolates belonged to genetically different parasites and thus to exclude any possibility that the finding could be due to a cross-contamination between these isolates in the laboratory, we have sequenced also a part of MSP-1, another polymorphic gene, from V306. The polymorphic block 2 of the MSP-1 gene from V306 was amplified by PCR [11]. XL1 Blue competent cells were transformed with recombinant TA vector containing the PCR product. DNA was purified by Qiagen Plasmid Mini Kit and sequenced using ABI PRISM™ Ready Reaction Kit (Perkin Elmer). The V306 and the RO33 isolates were clearly distinguished by their MSP-1 genetic marker (Fig. 2), confirming that they were indeed different parasites. The new V306 allele of MSP-1 is similar though not identical to the Palo Alto/FUP allele [12,13], with characteristic tripeptide repeats (SGX) present in V306 in 20 copies, a higher number than in any other documented MSP-1 allele. In contrast, the RO33 MSP-1 allele is characterised by the absence of any amino acid repeats in its block 2 [10].

Only a single nucleotide difference distinguishes MSP-2 alleles of isolates NIG60 (Nigeria, 1989) and MP663 (Malawi, 1996). Both these alleles are recombinant forms between the IC1/3D7 and the FC27 families [5]. They have the N-terminus and a R1 amino acid repeat (AGSGAVAS)_4 from the IC1/3D7 family, followed by R1 and R2 repeats characteristic of the FC27 family (a partial copy of a 32-mer and two copies of a 12-mer amino acid repeat) (Fig. 1b). There is only one amino acid difference between the two isolates, K to E, due to a single base difference AAA to GAA in the second 12-mer of R2 repeats (Fig. 1b). To exclude a PCR error, we have confirmed this difference in the MP663 allele by direct sequencing in both directions of PCR fragments amplified in two independent experiments. In addition, this particular nucleotide and the consequent change of the second amino
Fig. 1. Alignments of nucleotide sequences and predicted amino acid sequences of MSP-2 allelic variants. (a) R033 (Ghana, 1987) and V306 (The Gambia, 1992). Outer underlined DNA sequences correspond to primers 1 and 4 used for PCR amplification of R033 genomic DNA; inner underlined sequences correspond to primers 13 and 6 used for PCR amplification of V306 genomic DNA. N- and C-terminal regions are conserved and the central region is polymorphic. The central region includes two blocks of variable tandem repeats (designated R1 and R2). Positions where mutations are commonly found in different MSP-2 alleles within the IC1/3 D7 family are printed in bold. Both sequences are identical throughout the polymorphic regions. (b) MP663 (Malawi, 1996) and NIG60 (Nigeria, 1989). Sequence of the second 12-amino acid repeat from these alleles contains the only nonsynonymous point mutation that distinguishes them (printed in bold). This nucleotide change is common in 12-mer amino acid repeats of many other alleles of the FC27 family [6-8,15,16]. The rest of the MP663 sequence between the conserved regions (nt 132-630, not shown) is identical to that published for NIG60 [5].
Fig. 2. MSP-1 (Block 2) alleles distinguish the isolates R033 and V306. Nucleotide sequence of block 2 of the MSP-1 gene from V306 isolate, and alignment of the predicted amino acid sequence from V306, R033 and Palo Alto. The reference MSP-1 sequence is Palo Alto/FUP between amino acid residues M 5' and S 145 [12]. Sequences underlined in V306 correspond to primers used for PCR amplification of MSP-1 block 2 [11]. The first amino acid of each repeat is printed in bold, and differences between Palo Alto and V306 are outlined. The new V306 sequence with twenty tripeptide repeats (SGX) is somewhat similar to the Palo Alto/FUP allele, but longer than any reported MSP-1 allele. In contrast, block 2 of the R033 MSP-1 allele is characterised by the absence of any amino acid repeats [10]. The sequencing results match IFA serotyping of block 2 and other markers on MSP.1 (data not shown) [13], confirming that R033 and V306 are genetically distinct isolates.

Our finding of identity between MSP-2 alleles isolated from human hosts geographically and chronologically separated, is so far unique in the context of MSP-2 diversity. It is consistent with the idea that the evolution of MSP-2 variants may be relatively slow. A similar conclusion was reached in a study of the diversity of a merozoite surface protein of Theileria, alleles of which did not cluster according to geographic origin [14]. It was proposed either that there had been a rapid spread of novel sequences of the Theileria gene across geographic regions, or that the gene was evolving at a slow rate since the parasite's populations had become separated.

To date, nothing suggests that contemporary MSP-2 sequences found in Africa ([5] and this report), Australasia [2–4,7,8,15–17] or South America [6,16] are distinct between these continents. Flow of P. falciparum genes with parasites (carried in infected human hosts) migrating from one population to another and leaving successful offspring could partially account for the observed lack of geographical clustering. From the evolutionary point of view, two main theories have been proposed to explain similarities between alleles. First, identity is achieved if alleles derived from the same common ancestor persist unchanged for a time [18]. Alternatively, some kind of convergent evolution could repeatedly select allelic variants which confer particularly successful phenotypes. We favour the first explanation for MSP-2, since it seems unlikely that exactly the same multiple point mutations would have occurred in both the R033 and V306 alleles if they arose through separate evolutionary lineages. This hypothesis does not rule out the possible influence of homoplasly (similarity or identity not di-
rectly attributable to common ancestry) in the generation of local microheterogeneity. As proposed by McCutchan et al. for the circumsporozoite protein (CSP) gene [19], homoplasy centering on focal points within a gene and resulting from non-synonymous mutations indicates extensive selective pressure on such positions. That is, there exists only a limited repertoire of successful versions which could be generated by multiple convergent point mutations.

Analysis by Hughes and Hughes [20] indicated a much higher rate of non-synonymous vs. synonymous mutations in *P. falciparum* surface proteins as evidence for positive Darwinian selection acting on these molecules. Furthermore, it was argued that the high rate of amino acid changes seen in polymorphic regions is indicative of immune selection acting to promote antigenic diversity. However, there are apparent restrictions to the diversification of polymorphic antigens of *P. falciparum*. For example, CSP repeats are generally conserved, which suggests that they are under considerable unifying selection, presumably because they have a function other than interacting with the immune system [21]. Thus, the conservation of a general structural organization could be explained by functional constraints, e.g. involvement in receptor–ligand interactions with the host [22].

The finding of the same MSP-2 allele in distant infections suggests that the antigen which it encodes may confer a selective advantage to the parasite, and that natural selection acts to maintain it. The V306 allele was found in only one isolate among 14 that contained alleles of the IC1/3D7 family in The Gambia in October 1992 (unpublished data); the allele itself was thus quite rare in the population at that time. However, though not identical, six of these other IC1/3D7-family alleles had R1 sequences similar to that of RO33/V306, this R1 sequence-type thus being the most frequent at that time. If repeats evolve by duplication events involving flanking regions [2,4,15–17,23], the simplest sequence of the RO33/V306 MSP-2 allele may represent an ancestor of the more complex repetitive motifs. Conservation of this ancestral form would seem to support the proposed MSP-2 structural stability. The identity reported here may not be unique, and it will be of great interest to compare sequences from a larger number of clinical field isolates of *P. falciparum*.

The wide distribution of very similar MSP-2 sequence types which include identical forms in different countries, indicates slow evolution of this molecule and thus supports the idea that a malaria vaccine including the MSP-2 antigen could have worldwide applicability.

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