Studies on Sequence Diversity and Characterization of the Cysteine Rich Carboxy Terminal Region of Merozoite Surface Protein – 1 (MSP-1) of *P. falciparum* in Indian Isolates

THESIS

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By
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Under the Supervision of
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CERTIFICATE

This is to certify that the thesis entitled **"Studies on Sequence Diversity and Characterization of the Cysteine Rich Carboxy Terminal Region of Merozoite Surface Protein–1 (MSP-1) of *P. falciparum* in Indian Isolates"** submitted by **Vijaya Kumar Singamsetty, ID No. 2000PHXF023** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor

Name in capital block letters: **ASHIS K. DAS**

Designation: Associate Professor

Date: 19/5/2005

Dedicated

To

The Almighty

Shirdi Sai Baba

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Vijaya Kumar Singamsetty

Abbreviations

µm	Micro metre
µg	Micro gram
µl	Micro litre
ab	Antibody
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy ribonucleoside triphosphates
EDTA	Ethylenediamine tetra acetic acid
g	Gram
gp	Glycoprotein
hrs	Hours
IL	Interleukin
kDa	Kilo Dalton
M	Molar
Mab	Monoclonal antibody
Min	Minutes
ml	Milli litre
MSP	Merozoite Surface Protein
ng	Nano gram
nm	Nano metre
°C	Degree Celsius
P.	*Plasmodium*
Pf	*Plasmodium falciparum*
Pv	*Plasmodium vivax*
RBC	Red Blood Cell
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism

Amino Acids

• Alanine	ala	A
• Arginine	arg	R
• Asparagine	asn	N
• Aspartic acid	asp	D
• Cysteine	cys	C
• Glutamine	gln	Q
• Glutamic acid	glu	E
• Glycine	gly	G
• Histidine	his	H
• Isoleucine	ile	I
• Leucine	leu	L
• Lysine	lys	K
• Methionine	met	M
• Phenylalanine	phe	F
• Proline	pro	P
• Serine	ser	S
• Threonine	thr	T
• Tryptophan	trp	W
• Tyrosine	tyr	Y
• Valine	val	V

Nucleotide bases

Adenine	A
Guanine	G
Cytosine	C
Thymine	T
Uracil	U

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Abstract

Malaria parasites exhibit sequence diversity for a number of stage specific antigens. The Merozoite Surface Protein-1 (MSP-1) is a 195-kDa protein present on the surface of the merozoite. Several studies have proved that Merozoite Surface Protein-1 (MSP-1) is an effective target eliciting a protective immune response. The MSP-1_{42} region comprising two EGF like domains is involved in generating protective immune response in humans and other experimental animals. Searching for point mutations in this region is essential in view of vaccine development. We have investigated the sequence variations in *Plasmodium falciparum* Merozoite Surface Protein 1 (MSP-1) carboxy terminal region in field isolates from different regions in India. Our study reveals the presence of eight variant types of MSP-1_{19} in the Indian subcontinent, which comprise of E-TSR-L, Q-TSR-L, E-TSG-L, Q-KNG-L, Q-KNG-F, E-KNG-L, E-KNG-F and E-KYG-F. The last named allele is a novel variant being reported for the first time.

We have studied the antibody response in naturally infected population from different regions of India. Peptides delineating linear epitopes from four different reported MSP-1_{19} alleles were used to analyze the antibody profiles of 35 *P.falciparum* infected patients from eastern, western and southern India. Further, we characterized these peptides by immunizing mice and also did challenging with *P.berghei*. Our initial findings suggest there is a cross reactivity among different alleles in humans and mice. The peptide from EGF 1 appears to be immunodominant. Mice immunized with different allelic peptides, when challenged with *P.berghei* showed delayed and low level parasitemia. This data could be of use in designing effective MSP-1_{19} peptide based vaccine against malaria.

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Chapter 1

Malaria – An Introduction

Chapter 1

Introduction

Malaria is probably one of the oldest diseases known to mankind. Deadly fevers-probably malaria have been recorded since the beginning of the written word (6000-5500 B.C.). The symptoms of malaria were described in ancient Chinese medical writings. In 2700 B.C., several characteristic symptoms of what would later be named as malaria were described in the "Nei Ching", The Canon of Medicine. Details of this disease can be found even in the ancient Indian medical literature like Charaka Samhita and Sushrutha, which even associated these fevers with the bites of the Mosquitoes. However, it was only in the 5th century B.C., that malaria was clinically recognized and described by Hippocrates (Hockmeyer *et al.*, 1988). He classified the fever types as quotidian (daily), tertian (alternate days) and quartan (fever three days a part). It was only at the turn of this century; Laveran and Ross discovered that the disease was caused by a protozoan parasite transmitted by anopheline mosquitoes. Significant control of the disease was accomplished in the 1930,s and 1940,s due to the advent of synthetic antimalarial drugs and modern insecticides. The rise in incidence of drug resistant parasites and insecticide resistant vectors, respectively, has however been responsible for resurgence in malaria cases.

Prevalence of Malaria

According to the WHO Expert Committee on Malaria Twentieth Report (Geneva, 2000), about 100 countries or territories in the world are considered malarious. Large areas of Central and South America (Haiti and the Dominican Republic), Africa, the Indian subcontinent, Southeast Asia, the Middle East, and the Oceania are considered malaria-risk areas. The incidence of malaria world-wide is estimated to be 300-500 million cases each year resulting in 1.5 – 2.7 million deaths, of whom about 1 million are children under the age of five. About 40 % of the world's population is still at risk.

Malaria was a major health related problem in India contributing 75 million cases with 0.8 million deaths every year prior to the launch of the National Malaria Control Programme in 1953. After a significant decline in the 60's, malaria re-emerged as a major health problem of India in the late 70's and presently it poses a major challenge with 2 to 2.5 million cases annually. The malaria parasites responsible for causing the disease in humans are *Plasmodium falciparum* (responsible for most of the mortality); *Plasmodium vivax* (causing much morbidity), *Plasmodium malariae* and *Plasmodium ovale*. *P.falciparum* species is found throughout tropical Africa, Asia and Latin America. *P.vivax* is observed worldwide in tropical and some temperate zones. The *P.ovale* is mainly localized in tropical West Africa where as *P.malariae* has a very patchy distribution worldwide.

P.vivax has been traditionally considered to be the dominant species in India. However, data from intensive studies undertaken in 1930 (Knowles *et al.*, 1930) show an almost equal prevalence of *P.vivax* and *P.falciparum* on the basis of microscopic examination of blood smears. After the control and eradication programmes of the 1950's and 1960's the number of cases of both species went down considerably. However, according to some statistical data published by National Malaria Eradication programme (NMEP) in the year 1997, in India, the incidence of *P.vivax* malaria is 60-70% and that of *P.falciparum* is 30-45%. Surprisingly, the "National Average" of *P.falciparum* malaria has risen to 35.5% from a meager 9.34% in 1972 (NMEP Survey report, 1995). It thus appears that in spite of various control measures the malaria problem in the country persists and has, in fact, become more complex due to the appearance of drug resistant parasite forms and insecticide resistant vectors. This situation is further alarming in the view of reports of incidence of cerebral malaria by *P.falciparum* from different endemic foci in the country.

Life Cycle of *Plasmodium*

The life cycle of *Plasmodium* in mosquito and man is shown in Fig.1.1. Various species of anopheline mosquitoes form definitive hosts for the malaria parasites. A female mosquito may ingest male and female gametocytes along with the blood meal. The male gametocyte matures inside the mosquito resulting in the production of a number of slender spindle shaped microgametes. Simultaneously, the female gametocyte matures to form a macrogamete, which on fertilization forms a zygote. The zygote elongates and becomes active forming an ookinete, which penetrates through the stomach wall and forms an oocyst. The oocyst after further maturation produces a large number of slender threads – like sporozoites. Some of these enter the mosquito salivary glands and may be inoculated into the blood stream of the intermediate host (man) during the next meal.

The sporozoites injected into the blood stream leave the blood vascular system and invade the parenchymal cells of the liver within a very short time. In all the *Plasmodium* sp. Infecting man, asexual multiplication occurs in the liver, resulting in the production of thousands of merozoites. These are released into the circulation and infect the erythrocytes in the blood. A subsequent asexual schizogonic cycle in the red blood cells results in the formation of 4-36 merozoites with in 48-72 hrs. These are released into blood circulation and infect new RBC. A few of these merozoites may subsequently form male and female gametocytes. Rupture of the RBC releases products of metabolism of the parasites as well as the cells into circulation. It is believed that if a large number of infected cells rupture simultaneously, the volume of toxic material thrown into the blood stream may be sufficient to bring about a malarial paroxysm.

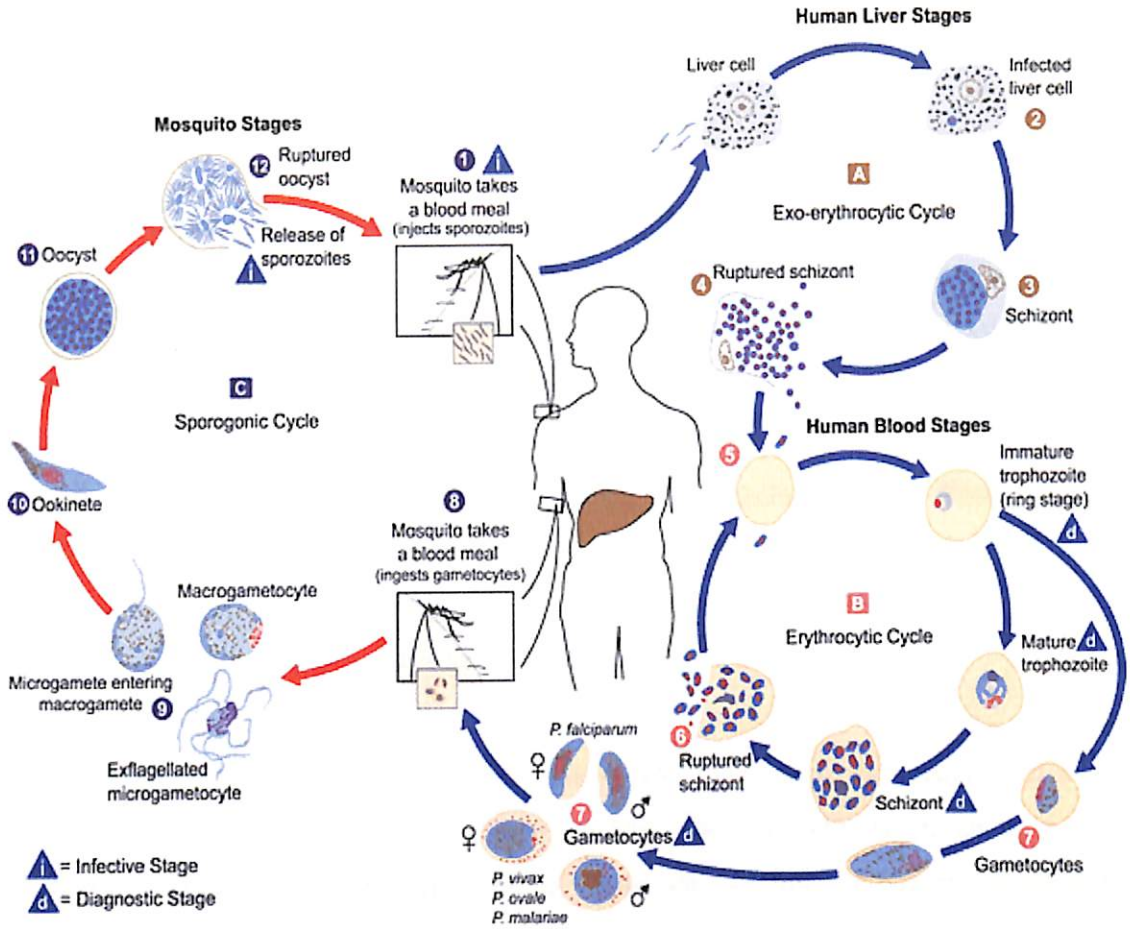


Figure 1.1 Schematic representation of the life cycle of malaria parasite

The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷and mature into schizonts ❸, which rupture and release merozoites ❹. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony Ⓐ), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony Ⓑ). Merozoites infect red blood cells ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle Ⓒ. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites ❶into a new human host perpetuates the malaria life cycle.

Content source: National Center for Infectious Diseases, Division of Parasitic Diseases, CDC, Atlanta.
http://www.cdc.gov/malaria/biology/life_cycle.htm

Erythrocyte Invasion

The interface between the host erythrocytes and the parasite merozoite is an important stage, giving an opportunity for blocking the entry of the parasite into the erythrocytes and, therefore, preventing any onset of clinical disease. However, the invasion is highly complex process, involving several different receptor-ligand interactions, which pose many challenges, yet to be explored. The use of molecular, biochemical, and genetic approaches has yielded valuable insights into this complex process. Parasite proteins involved in these molecular interactions are promising candidates for malaria vaccines. A clear understanding of these interactions is important for a rational design of vaccines, which could attempt to inhibit invasion and prevent malaria.

Invasion of human erythrocytes by *P.vivax* is dependent upon the presence of a single receptor, but *P.falciparum*, as well as some other species, exhibits the ability to utilize multiple alternative invasion pathways i.e.- there are a number of different molecules by which these parasites can enter erythrocytes. These alternative invasion pathways need to be understood at the molecular level in order to develop effective chemotherapeutics or vaccines aimed at blocking this critical event.

Erythrocytic Schizogony Merozoites

The erythrocytic merozoite is an ovoid cell and measures approximately 1.5 μm in length and 1 μm in width (Fig. 1.2). The apical end of the merozoite is a truncated cone-shaped projection demarcated by the polar rings. Three types of membrane bound organelles, namely rhoptries, micronemes, and dense granules are located at the anterior end of the merozoite. The contents of these organelles play a role in the binding and entry of the merozoite into the host cells. Extra cellular merozoites are intrinsically short-lived and must rapidly invade a new host erythrocyte.

Host Cell Entry

Malaria merozoite invasion process is complex (Fig.1.2) and involves a multi-step sequence, which can be divided into four phases: (a) initial recognition and reversible attachment of the merozoite to the erythrocyte membrane; (b) reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of rhoptry-microneme substances with parasitophorous vacuole formation; (c) movement of the junction and invagination of the erythrocyte membrane around the merozoite accompanied by removal of the merozoite's surface coat, and finally (d) re-sealing of the parasitophorous vacuole membrane (PVM) and erythrocyte membrane after completion of merozoite invasion. The initial factor underlying recognition between merozoites and erythrocytes may occur between the merozoite surface coat filament and erythrocyte surface. Multiple different receptor-ligand interactions occur during the merozoite invasion process into an erythrocyte. Merozoite Surface Protein-1, with a glycosylphosphatidylinositol (GPI) anchor, (MSP-1; also called as MSA 1 or gp 195) is involved in the initial recognition of the erythrocyte in a sialic acid- dependent way (Perkins et *al.*, 1988).

During host cell invasion, no surface coat is visible on the portion of the merozoite within the erythrocyte invagination, whereas the surface coat on the portion of the merozoite still outside the erythrocyte appears similar to that seen on the free merozoites. Biochemical studies have demonstrated that a 19-kD fragment is transported into the erythrocyte while other MSP-1 fragments were shed into supernatant during merozoite invasion (Blackman *et al.*, 1990, Holder *et al.*, 1985).

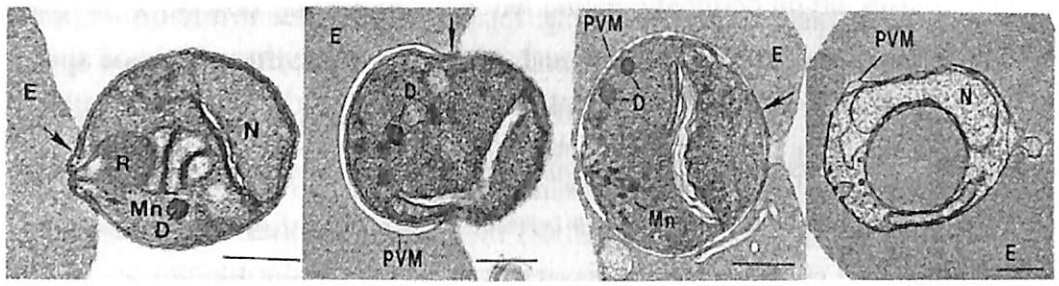


Figure 1.2 Malaria merozoite invasion process

a Apical end of a *P; knowlesi* merozoite attaches to an erythrocyte (E). The erythrocyte membrane becomes thick at the attachment site (arrow). ***b*** Further advanced stage of erythrocyte entry by a *P; knowlesi* merozoite. The junction (arrow), formed between the thickened erythrocyte membrane and the merozoite, is always located at the orifice of the merozoite entry. No surface coat is visible on the portion of the merozoite surface, which has invaginated the erythrocyte membrane, while the surface coat is present behind the junction (arrow) site. ***c*** Erythrocyte entry by a *P; knowlesi* merozoite is almost complete. The junction (arrow) has now moved to the posterior end of the merozoite. An electron-opaque projection connects the merozoite's apical end and erythrocyte membrane. ***d*** A trophozoite (ring form) stage of *P; falciparum* is surrounded by the parasitophorous vacuole membrane (PVM). R = Rhoptry; D = dense granules; Mn = micronemes; E = erythrocyte; N = nucleus. Bars = 0.5 ~m.

Content Source: Malaria Immunology. Karger Publishers, 2002. p.3

Merozoite Surface Protein 1

MSP-1, a 195 kDa protein was the first protein identified on the surface of erythrocytic stage merozoites (Holder *et al.*, 1981). During or at the time of the schizont rupture and release of merozoites, the precursor MSP 1 is processed into at least four distinct fragments (Blackman *et al.*, 1991). Among these fragments the C-terminus 42-kD fragment (MSP-1_{42}) is of particular interest and has been extensively studied. MSP-1_{42} is further processed into a soluble 33-kD fragment and a 19-kD fragment (MSP-1_{19}) that remains attached to the merozoite membrane through a GPI-anchor. MSP-1_{19} contains two epidermal growth factor-like domains that are rich in cysteines residues (Blackman *et al.*, 1991).

Data from several epidemiological and laboratory studies demonstrate that MSP-1 is a target of protective immunity. It has been demonstrated that the presence of antibodies against MSP-1_{19} is associated with protection from clinical *P.falciparum* malaria. The precise mechanism of MSP-1_{19} vaccine induced-immunity is not clear, however, available data suggest that immunity is antibody-mediated (Daly *et al.*, 1995, Hirunpetcharat *et al.*, 1997). In vitro studies show that monoclonal antibodies and polyclonal antibodies specific to the 19- or 42- kD fragments block the entry of merozoites into the erythrocytes (Blackman *et al.*, 1991, Chang *et al.*, 1992). MSP-1 gene sequences obtained by nucleotide sequencing of the gene from various field isolates show extensive polymorphism suggesting that the molecule is under intense immune pressure (Miller *et al.*, 1993).

Aims and Objectives

There have been only a few studies on the cysteine rich carboxy terminal region of MSP-1 of *Plasmodium falciparum* in the Indian context. This is very essential in a malaria endemic country like India, where there is mortality caused by *P.falciparum* malaria. This study was undertaken with the intention of investigating the polymorphism in the cysteine rich carboxy terminal region of *P.falciparum* MSP-1_{42} and MSP-1_{19} in the Indian context. The information from such a study was evaluated experimentally to indicate the effectiveness of this molecule in vaccine-based strategies.

The major objectives of the present investigation were:

1. Investigation of the Genetic diversity of *P.falciparum* C-terminal region of MSP-1 in isolates from Indian sub-continent.
2. Scanning of *P.falciparum* MSP-1_{19} for putative B-cell epitopes from different alleles, based on the information obtained.
3. Evaluation of peptides based on different MSP-1_{19} allelic types, to investigate antibody profiles present in naturally infected individuals.
4. Evaluation of the immunogenicity of the peptides in a rodent model.
5. Protection studies investigating the effect of immunization by these peptides in cross species challenging.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

Study site and *P.falciparum* infected blood sample collection

The two study regions were Rajasthan and West Bengal; in the western and eastern parts of India, about 2000 Km apart. Both are known endemic for *P.falciparum* malaria. Samples were collected from 19 malaria patients with their informed consent. Sixteen patients (providing isolates IR 1 to IR 15 & IR17) live in Rajasthan (Kota, Jaipur, Sikar) and the other three (IW 1, IW 2, IW 3) live in West Bengal. Blood samples (1-5 ml) were collected from each patient and stored at –70°c until DNA extraction.

Parasite infected blood DNA extraction

1) Lysis Buffer A: 10mM Nacl
   50mM Tris
   10mM EDTA
2) Lysis Buffer B: 10mM Nacl
   50mM Tris
   10mM EDTA
   1% SDS
3) 1 X PBS Buffer:
4) Proteinase K
5) Phenol – Tris saturated
6) Sodium chloride solution – 1M
7) 70 % and 100 % Ethanol
8) Chloroform
9) 1 X TE Buffer

The *Plasmodium falciparum* infected frozen blood samples were thawed at 37°C and washed with 1 X PBS to get a clear pellet. The extraction procedure was based on lysis of the cells in a mixture of 10mM NaCl, 50mM Tris, 10mM EDTA in the presence of 1% SDS (Maniatis *et al.*, 1989). The pellet was incubated at 37°C for 1 hr, thoroughly re-suspended by tapping or gentle vortexing at regular intervals. The lysate was thoroughly mixed by inversion and incubated with 100 µg/ml Proteinase K for 1 hr at 50°C. A conventional phenol:chloroform extraction was then performed and the DNA in the aqueous phase precipitated by addition of chilled ethanol in the presence of 0.2 M Nacl. The pellet after precipitation was washed in 70% Ethanol, dried and re-dissolved in 1X TE buffer. A diagnostic PCR was done to confirm *P.falciparum* (Das *et al.*, 1995) (Fig 2.1)

Polymerase Chain Reaction (PCR)

1) Taq Polymerase (Bangalore Genei)
2) 10 X Reaction Buffer
3) 1M $MgCl_2$
4) dNTP 10mM stock Solution in water (Bangalore Genei)
5) Primers

Parasite DNA was extracted from the *P.falciparum* infected blood samples collected from different regions in India (Maniatis *et al.*, 1989). The MSP-1 gene coding for the carboxy terminal region ($MSP\text{-}1_{42}$), was amplified (Fig 2.2) using primers designed based on the flanking regions of the gene. Forward and reverse primers used are (5'GCG GGA TCC GCA ATA TCT GTC ACA ATG 3', and 5' GCG CCA TGG TTA AAT GAA ACT GTA TAA TAT 3') having an additional GCG overhang and BamHI and NcoI sites at their respective ends. 100 ng of parasite DNA was taken as target for each amplification reaction, which utilized standard components for a 50-µl-reaction volume.

Samples were subjected to 35 cycles of amplification. The initial four cycles comprised of denaturation at 94°C for 1.5 min, annealing at 46°C for 2.5 min and extension at 72°C for 2 min. The rest of the 31 cycles consisted of denaturation at 94°C for 1.5 min, annealing at 56 °C for 2.5 min and extension at 72 °C for 2 min.

Standard conditions used for PCR cycles:

The standard PCR cycle used the following parameters

1 - 4 cycles:

Denaturation	- 94°C for 1.5 min
Annealing	- 46 °C for 2.5 min
Extension	- 72 °C for 2 min

5- 31 cycles:

Denaturation	- 94°C for 1.5 min
Annealing	- 56 °C for 2.5 min
Extension	- 72 °C for 2 min.

A standard PCR reaction (50 µl volume) was set up as follows-

Template DNA	-	2 µl (100 ng)
DNTP mix	-	8 µl
Primers	-	20 µl (5 pico moles each)
Taq Buffer	-	5 µl
Taq polymerase	-	1 µl
H_2O	-	14 µl

Agarose Gel Electrophoresis for resolving DNA fragments

Separation of DNA fragments was done by electrophoresis through agarose gels at 70 volts in TBE buffer. 0.5 µg of ethidium bromide was added to the molten agarose just before pouring. The DNA fragments were viewed in a Syngene UV gel documentation system.

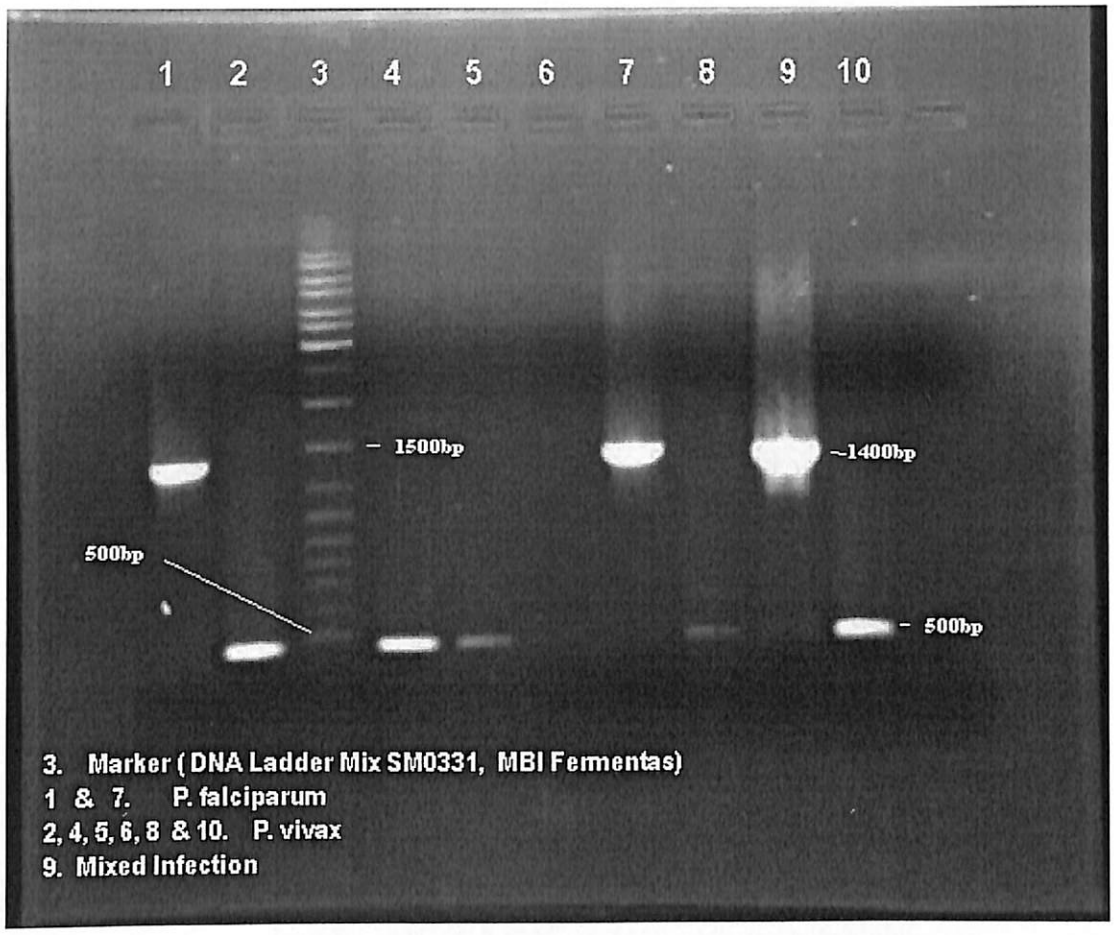


Figure 2.1 Diagnostic PCR showing the differences between *P.falciparum* and *P.vivax*

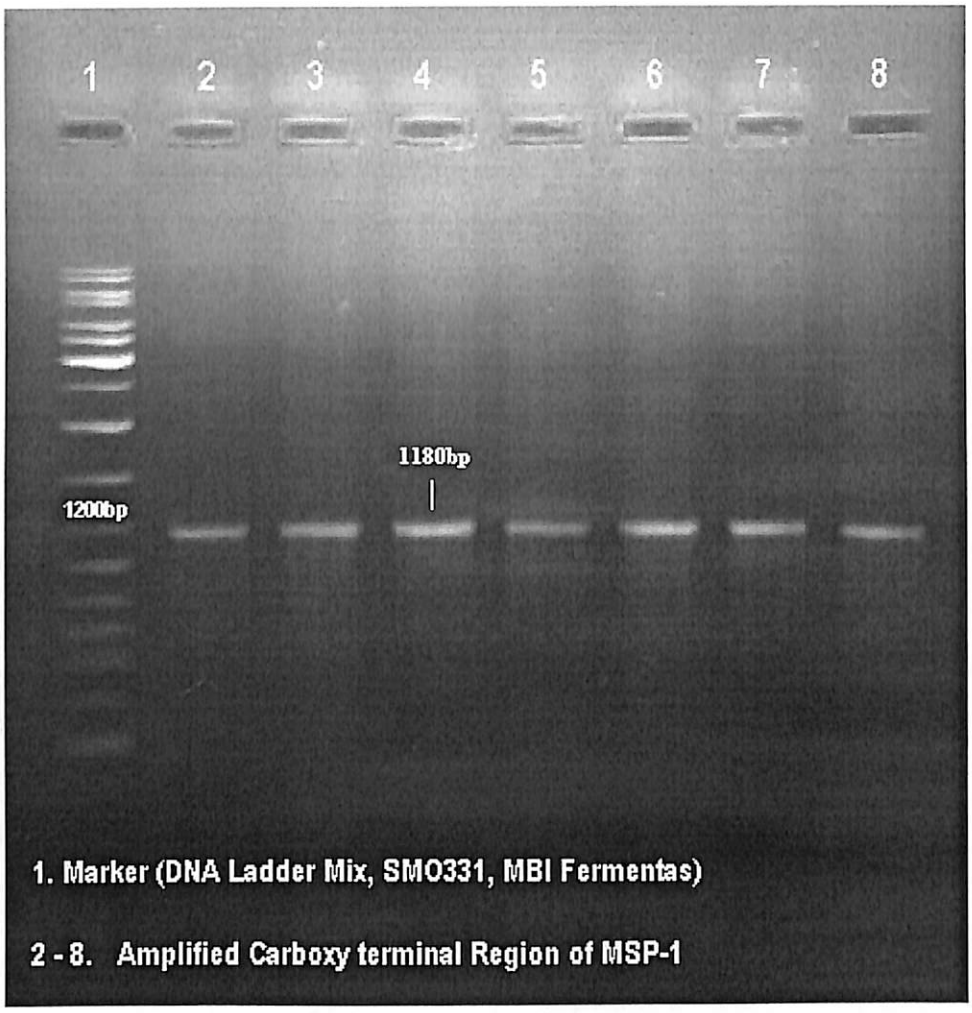


Figure 2.2 Amplification of carboxy terminal region of MSP-1

Cloning of MSP-1_{42} gene in pRSET A vector (Fig 2.3)

BamHI (MBI Fermentas)

NcoI (MBI Fermentas)

Buffer for Restriction Digestion: Y^+ Tango (MBI Fermentas)

T4 DNA Ligase (New England BioLabs)

T4 DNA Ligase Buffer (New England BioLabs)

Restriction digestion of vector and insert

The vector was double digested with BamHI and NcoI enzymes and was purified using Quiagen gel extraction kit. The insert being a PCR product was purified using PCR purification QIAGEN kit and double digested with BamHI and NcoI enzymes (Maniatis *et al.*, 1989). After this, digested insert was gel eluted and quantified for setting up a ligation.

Reaction for restriction digestion of vector

Template DNA	=	2 µl (500ng – 1µg)
Restriction Enzyme	=	2 µl (5 units each of BamHI and NcoI)
Buffer (10x)	=	2 µl
Sterile Millipore water	=	Volume to make 20µl

Reaction was left at 37 °C for 1 – 2 hours.

Reaction for restriction digestion of insert

Template DNA	=	8 µl (1µg)
Restriction Enzyme	=	2 µl (5 units each of BamHI and NcoI)

Buffer (10x)	=	2 µl
Sterile Millipore water	=	Volume to make 20µl

Reaction was left at 37 °C for 1 – 2 hours.

Ligation

The quantities of both vector and insert were calculated by measuring O. D. at 260nm. Based on the quantity calculated a reaction mix was prepared as follows –

Vector	=	1 part
Insert	=	3 parts
T4 DNA Ligase	=	1 unit
Ligase Buffer (10x)	=	1 X (2µl for 20µl reaction)
Sterile Millipore Water	=	Volume to make 20µl

The reaction mixture was left at 16 – 22 °C for 6 – 16 hours depending on the specification mentioned by T4 DNA ligase enzyme supplier and used for transformation.

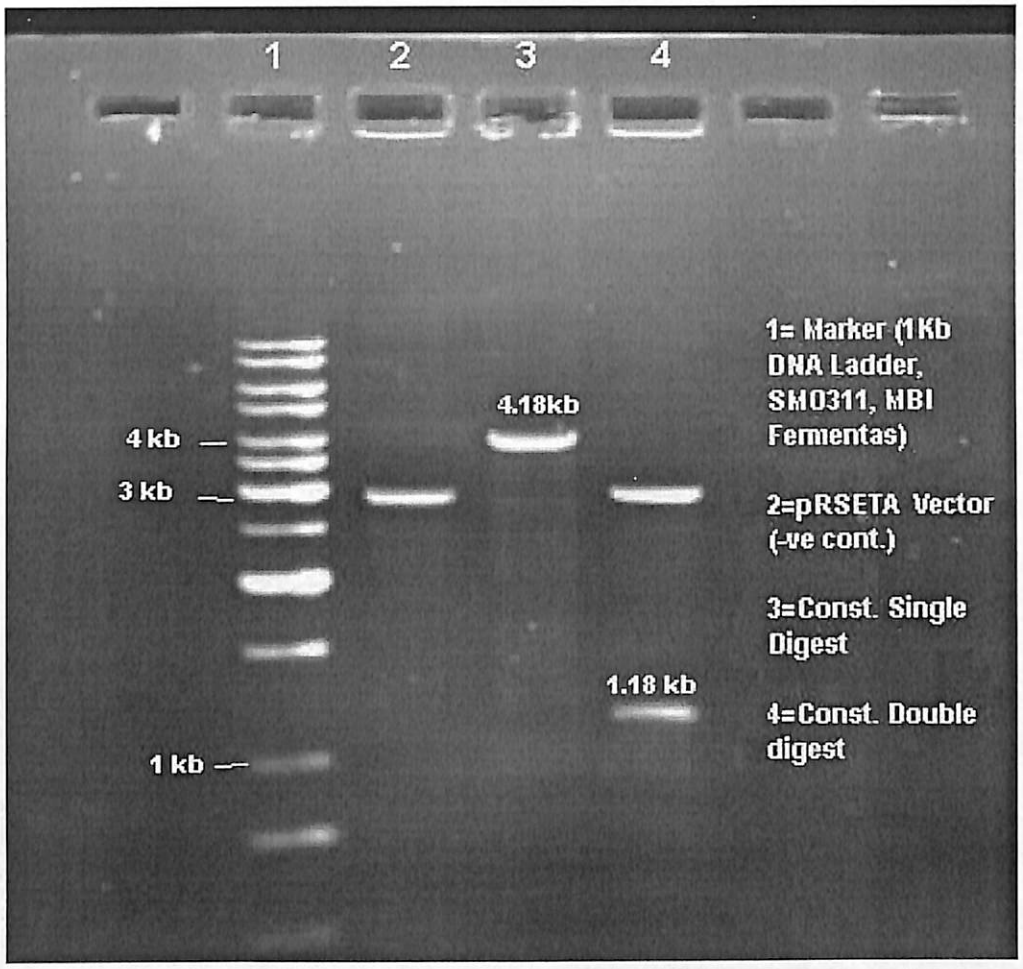


Figure 2.3 Restriction digestion of the MSP-1 $_{42}$ construct

Plasmid extraction procedure

Solution I: 50 mM Glucose
10 mM EDTA (pH 8.0)
25 mM Tris-cl (pH 8.0)

Solution II: 0.2 N NaOH
1 % SDS

Solution III: 5 M Potassium acetate
Glacial Acetic acid
Sterile Distilled Water

Tris saturated Phenol

Chloroform:Isoamyl alcohol

Absolute alcohol

RNAse

1 X TE buffer (pH 8.0)

Cells were pelleted down from overnight cultures and re-suspended in Ice-cold solution I. To this freshly prepared solution II was added and incubated on ice for a couple of minutes with intermittent mixing. Gentle vortexing was done by inverting the tubes after adding Ice-cold solution III. The lysed material was then spun at 12000 rpm for 5 min at 4 °C. A conventional phenol:chloroform extraction was then performed and the DNA in the aqueous phase precipitated by addition of chilled ethanol in the presence of 0.2 M Nacl. The pellet after precipitation was washed in 70% Ethanol, dried and re-dissolved in 1X TE buffer (Maniatis *et al.*, 1989).

Transformation of E. coli with pRSET A

Approximately 100 ng of the plasmid DNA was added to the competent cells and incubated on ice for 45 min. The microfuge tubes were rapidly transferred to a 42^{0}C water bath for a heat shock for 90 sec. These tubes were replaced on ice to which 600 –

900 µl fresh autoclaved LB was added in the hood and incubated at 37^0C for 1 hr. This was plated on to LB agar plates with appropriate antibiotics and incubated over night at 37^0C.

Preparation of competent cells

0.1 M $CaCl_2$

50 % Glycerol in 100mM $CaCl_2$

LB medium

Cells were pelleted out from a culture grown to 0.4 – 0.6 OD, re-suspended in 10 ml of 0.1 M $CaCl_2$ and allowed to stand on ice for 30 –40 min. The cells were spun at 5000 rpm for 5 min and re-suspended in 2 ml of ice cold 0.1 M $CaCl_2$. These were incubated on ice for 60 – 90 min. To this Glycerol was added and aliquoted with immediate snap freezing.

DNA Sequencing

Sequencing of the clones and amplicons was done at CDFD, Hyderabad. All the sequences were submitted to Genbank. Genbank Accession Nos. was as follows: AY776344 to AY776360, AY776362 & AY776363

Epitope scanning and peptide designing

MSP-1_{19} EGF like domains was scanned for B cell epitopes using the online Bcpred server (IMTECH, Chandigarh, India). One epitope from each EGF like domain was selected and designated E1 and E2. E2 was further divided into E2a, E2b, E2c, & E2d corresponding to different alleles (KYG, TSR, TSG, KNG), which differed from each other in one or two amino acids. These 5 peptides were made available by CDC, Atlanta. The sequences were as follows:

E1: NSGCFRHLDEREECKCLLN

E2a: EDSGSYGKKITCECTKPHS

E2b: EDSGSSRKKITCECTKPDS

E2c: EDSGSSGKKITCECTKPDS

E2d: EDSGSNGKKITCECTKPDS

Study area, Human subjects, and sera collection

The three study regions were Andhra Pradesh, Rajasthan and West Bengal, which lie in the southern, western and eastern parts of India respectively, and are separated by several thousand kilometers. 1 – 5 ml of parasitized blood samples were collected randomly at different stages from different areas in these regions, which were known to be endemic for *P. falciparum* malaria. Blood samples were collected from 35 patients in the age group of 20 to 40 years, with informed consent. Twenty-four patients (no. 1-24) were from Rajasthan, five patients from West Bengal (no. 25-29), and six patients from Andhra Pradesh (no. 29-35). Sera was separated from blood samples at the site and stored at –20° C till analysis.

Serum separation from the *P.falciparum* infected blood samples

After blood collection, the blood was allowed to clot for 30 – 60 min at 37° C. The tube was flicked several times to dislodge the blood clot. This clot was then kept at 4 ° C for overnight to allow it to contract. The serum was then separated from the clot by centrifugation at 10,000 rpm for 10 min at 4 ° C. The serum was stored at –20 ° C till the analysis.

ELISA (Enzyme Linked Immunosorbant Assay)

For analyzing human sera

96 well Microtitre Plates (Grenier, Germany)

Peptides

1 X PBS (Phosphate Buffered Saline)

Coating buffer: 1 X Carbonate-Bi carbonate buffer (pH 9.6)

Wash Buffer: PBS-Tween 20

Blocking buffer: 5 % casein in PBS –Tween 20

Conjugate: goat anti-human IgG peroxidase (Bangalore Genei)

Substrate: TMB/H_2O_2 (Bangalore Genei)

Stop Solution: 2N H_2SO_4

Serum samples from 35 patients were analyzed by enzyme-linked immunosorbent assay (ELISA) (Harlow et al., 1988) for total IgG titres to E1, E2a, E2b, E2c, & E2d. Micro titer plates were coated with the appropriate antigen (150 ng of peptide per well, diluted in 100 µl of 1X coating buffer) and incubated at 4°C overnight. Wells were washed thrice with 1X PBS -Tween 20 (0.2 %), blocked with the blocking buffer and incubated at 37^0 C for 2 hrs. After washing with the wash buffer three times, serum diluted (1/4000) in blocking buffer was added (100 µl/well) and incubated at 37^0 C for 2 hrs. After washing thrice with wash buffer, 100 µl of secondary antibody diluted (1/1000) in blocking buffer was added (100 µl/well) and incubated at 37^0 C for 2 hrs. Again after washing, 100 µl of substrate solution was added in dark and left at room temperature for 15 to 30 min, for the color to develop. 100 µl of stop solution was added and the absorbance was read at 450 nm. Threshold of positivity was an OD value of 0.2 based on the mean plus 2 SD of the reactivity of sera from 20 healthy controls who were not exposed to malaria before. All the assays were performed in duplicate. The intra assay standard error, which was calculated, was not significantly different.

Coupling peptides to carrier proteins using gluteraldehyde

1) Synthetic peptides
2) 1X PBS
3) Bovine Serum Albumin (BSA)
4) 0.2 % Gluteraldehyde

A solution of 5 mg/ml of the synthetic peptide in PBS (1 mg of a 10 – mer is approximately 1 μmole) was prepared. To this, the carrier protein (BSA) was added in the correct molar ratio (approximately 1 mole of peptide per 50 amino acids of carrier). This was placed on a magnetic stirrer in a fume hood, with a stir bar added to the solution. An equal volume of the 0.2 % gluteraldehyde in PBS was slowly added to the peptide/protein solution with constant agitation and incubated at room temperature for 1 hr. Un-conjugated peptide was separated from the peptide – protein conjugate by dialysis against PBS (Harlow *et al.*, 1988).

Aluminium hydroxide adjuvant

1) 10 % Aluminum Potassium Sulfate ($AlK(SO_4)_2.12H_2O$)
2) 0.25 N NaOH
3) 0.9 % Saline

10 % Potassium alum (aluminum potassium sulfate) was prepared in distilled water. To 10 ml of 10% Potassium alum, 22.8 ml of 0.25 N NaOH was added drop wise in a 50-ml conical tube. This was incubated at room temperature for 10 min. After centrifuging at 1000g for 10 min, the supernatant was discarded and the pellet ($Al(OH)_3$) was re-suspended in 50 ml distilled water and again centrifuged at 1000 rpm for 10 min. One mg of $Al(OH)_3$ can bind approximately 50 –100 μg of protein antigen. The desired concentration of alum was diluted in 0.9 % saline and then incubated at room temperature for 20 min, along with the antigen. This was then centrifuged at 10,000 rpm for 10 min and the supernatant tested for the presence of the antigen to be certain it was bound to alum (Harlow *et al.*, 1988).

Mice strains and Formulations

Female BALB/c (Inbred), C57BL/6J (Inbred) and Swiss Albino (Out bred) mice of 6 to 8 weeks of age, bred under specific-pathogen free conditions and supplied by National Institute of Nutrition, Hyderabad, India, were used in groups of 5 for each of the alleles. All the peptides were coupled to BSA, using glutaraldehyde as cross-linking agent. Each mouse was give 50 μg of E2 allelic peptide in combination with 50 μg of E1 peptide, adsorbed on the alum and administered sub-cutaneously. Three boosters were given every 21-day after priming. Animals were bled the 9^{th} day after every booster and sera were assayed for antibodies by ELISA.

Collecting sera from a mouse by tail bleed

The mouse was placed in a small cage or a container and heated with an infrared lamp for a few minutes. The heat increases the flow of blood to the tail. The mouse was then placed in a restraining device. A small portion of the tail of about 1.5 – 2 inches from the body was swabbed with alcohol. Using a sterile scalpel, a nick was made on the underside of the tail across one of the lower veins that were visible. The tail was gently stroked to collect the blood with a centrifuge tube kept below to collect the blood.

Immunizations

Subcutaneous injections

The skin on the mouse's back, just below the base of the neck was swabbed with alcohol. The mouse was then held by the left hand by pinching off the skin below the base of the neck, with the thumb and index finger. Using a 23-gauge needle, 100 μl of the antigen was injected under the skin.

Serum preparation from mouse blood

After blood collection, the blood was allowed to clot for 30 – 60 min at 37° C. The tube was flicked several times to dislodge the blood clot. This clot was then kept at 4 ° C for overnight to allow it to contract. The serum was then separated from the clot by centrifugation at 10,000 rpm for 10 min at 4 ° C. The serum was stored at –20 ° C till the analysis (Harlow *et al.*, 1988).

ELISA (Enzyme Linked Immunosorbant Assay)

For analyzing mice sera

96 well Microtitre Plates (Grenier, Germany)

Peptides

1 X PBS (Phosphate Buffered Saline)

Coating buffer: 1 X Carbonate-Bi carbonate buffer (pH 9.6)

Wash Buffer: PBS-Tween 20

Blocking buffer: 5 % casein in PBS –Tween 20

Conjugate: goat anti-mouse IgG peroxidase (Bangalore Genei)

Substrate: TMB/H_2O_2 (Bangalore Genei)

Stop Solution: 2N H_2SO_4

Mouse sera were assayed at regular intervals for anti- MSP-1 $_{19}$ peptide antibodies (total IgG) by enzyme-linked immunosorbent assay (ELISA) (Harlow *et al.*, 1988). Micro titer plates were coated with the appropriate antigen (150 ng of peptide per well, diluted in 100 µl of 1X coating buffer) and incubated at 4°C overnight. Wells were washed thrice with 1X PBS -Tween 20 (0.2 %), blocked with the blocking buffer and incubated at 37^0 C for 2 hrs. After washing with the wash buffer three times, serum diluted (1/100) in blocking buffer was added (100 µl/well) and incubated at 37^0 C for 2 hrs. After washing thrice with wash buffer, 100 µl of secondary antibody diluted (1/1000) in blocking buffer was added (100 µl/well) and incubated at 37^0 C for 2 hrs. Again after washing, 100 µl of substrate solution was added in dark and left at room temperature for 15 to 30 min, for

the color to develop. 100 µl of stop solution was added and the absorbance was read at 450 nm. Threshold of positivity was an OD value of 0.1 based on the mean plus 2 SD of the reactivity of sera from a set of negative controls. Results were represented as ± standard error.

Passive administration of pooled immune sera by intravenous injections

Female Swiss Albino (OB) mice of 6 to 8 weeks of age, bred under specific-pathogen free conditions were used in groups of 5 for each allele. 100 µl of pooled immune sera raised against all the alleles were administered by intravenous injection every 7 days, starting from the day of challenge infection. The mouse was placed in a small cage or a container and heated with an infrared lamp for a few minutes. The heat increases the flow of blood to the tail. The mouse was then placed in a restraining device. A small portion of the tail of about 1.5 – 2 inches from the body was swabbed with alcohol. Using a 1.0 ml syringe fitted with a 26-gauze needle, the needle was slowly guided into one of the caudal veins that were visible. 100 µl of the pooled immune sera was slowly administered into the vein and the mouse was returned to the cage.

Preparation of *P.berghei* inoculums for challenge

P.berghei seed culture supplied by Malaria Research Center, New Delhi was passaged in 4-6 weeks old female Swiss Albino mice. These mice were inoculated intra-peritoneally with 1 X 10^4 parasites diluted in RPMI medium. Parasitemia was monitored daily until it reached 5 %. These mice were bled for the infected erythrocytes and diluted in PBS to yield an inoculum containing 1 X 10^6 infected erythrocytes per 0.1 ml per mouse. The inoculum was kept on ice until injected into all the mice intra-peritoneally.

Intra-peritoneal challenging of mice with *P.berghei* NK-65 *+

*+Oocyst positive in *An.stephensi*

Infective gametocyte producing strain

The mouse was held up side down with the left hand and a small portion of the skin above the peritoneal cavity was swabbed with alcohol. *P.berghei* infected erythrocytes were diluted in PBS in appropriate dilutions and taken in a syringe fitted with 25-gauge needle. 100 µl of the diluted erythrocytes were injected into the peritoneal cavity.

Detection of parasitemia

Giemsa stain (1/1000 dilution)

Methanol

The tip of the mouse-tail was cutoff with a sterile scalpel and a small drop of the blood was taken on a clean glass slide. A thin smear was made using a small glass plate. The smears were dipped in giemsa for 15 to 20 min. Excess stain was washed off by flushing distilled water over the slide. This was then placed for fixing in methanol for about 1 min and examined under oil immersion high power resolution for counting the infected cells.

Vectors and Bacterial strains used

pRSET A (Invitrogen USA)

Bl21DE3 (Invitrogen USA)

Various kits used for this study

Qiagen Gel Extraction Kit (Qiagen USA)

Qiagen PCR Purification (Qiagen USA)

Medium for bacterial culture

LB (Luria Bertani Medium) – 1000 ml

Bactotryptone, 10.0g, Bacto-yeast extract, 10.0g; Nacl 10.0g, pH-7.5

Plating agar:

15.0g of bactoagar were added to LB medium prior to autoclaving.

Composition of various buffers and solutions used in the study

TE pH (8.0):

10mM Tris-Cl (pH 8.4)

1mM EDTA (pH 8.0)

Tris Borate EDTA (TBE) 10 X Buffer: (1000 ml)

Tris base 108g

Boric acid 55.0g

0.5M EDTA (pH 8.0) 40 ml.

DNA gel loading buffer (6X):

0.25 % Xylene cyanol,

0.25 % Bromophenol blue

30 % glycerol in water.

Phosphate Buffered Saline pH 7.2 (1X):

137 mM Nacl

2.7 mM Kcl

4.3 mM Na_2HPO_4

1.4 mM KH_2PO_4

Coating Buffer For ELISA (10 X)

Na_2CO_3 1.59 g

$NaHCO_3$ 2.93 g

Double Distilled water 100 ml

Buffers for restriction endonucleases, T4 DNA Ligase and Taq DNA polymerase

The various buffers used were provided by the manufacturers for these enzymes and used as per the instructions provided.

Antibiotics used

Tetracycline (Hi Media)

Ampicillin (Ameresco)

Chapter 3

Investigation of the genetic diversity of *P.falciparum* C-terminal region of MSP-1 in isolates from Indian sub-continent

Chapter 3

Introduction

The malaria parasite *P.falciparum* exhibits sequence heterogeneity for a number of stage specific antigens. The Merozoite surface protein 1 (MSP-1) is a highly polymorphic major asexual blood stage malaria vaccine candidate (Holder *et al*, 1996) and is highly immunogenic in humans. Numerous studies suggest that MSP-1 is an effective target for a protective immune response in humans and experimental animals both at cellular and humoral levels.

Silent nucleotide substitutions which are assumed to be selectively neutral, are very rare or absent in nuclear genes (Rich et *al*, 2000), as well as in the mitochondrial genome of *P.falciparum* (Conway *et al.*, 2000). Despite the fact that single nucleotide polymorphisms (SNP's) are very rarely found in *P.falciparum* introns, there is an extensive polymorphism in most of the genes coding for surface antigens of *P.falciparum*, that are under selective pressure. This diversity in the surface antigens of malaria parasites is generally assumed to be a mechanism for immune evasion. Genetic variation in the *P.falciparum* MSP-1 gene has been extensively investigated in Africa, Latin America and other parts of the world as it may pose an obstacle for the development of malarial vaccines based on MSP-1. The extent and the impact of the allelic diversity on recognition of MSP-1 during the immune response is little understood and has to be further investigated in highly malaria endemic areas like India. The diversity or sequence heterogeneity in the surface antigens of *P. falciparum* arises from multiple point mutations in the AT rich genome or variations in the number, length and sequence of amino acid repeats. This repeat diversity can be because of intragenic recombination, mis-alignment of repeated DNA sequences or complimentary strand slippage (Franks *et al.*, 2003) during DNA replication. This maintenance of variants in the populations has a selective advantage in evasion of the immune responses, leading to the random diversification of the population by selectively neutral mutations. For

example, point mutations cluster in areas immunodominant for T cells (Franks *et al.*, 2003) and can lead to loss of epitope recognition or antagonism of T-cell responses in some genes like CSP (Circumsporozoite protein), although it is not clear that either of these leads to the loss of protective immunity. A characteristic feature of many malarial antigens is the polymorphism caused by variations in the sequence of the short tandem repeats, which often constitute immunodominant regions. Despite these, polymorphisms resulting from point mutations have also been described in several antigens with and without regions of repeats (Bolad *et al.*, 2000) and MSP-1 is the best studied antigen with respect to allelic polymorphism. These antigenic properties in the polymorphic genes constitute major problems in tailoring a vaccine against the disease. To decipher the knowledge about the conserved antigens and conserved regions in polymorphic antigens is considered an essential step towards a rational design of a vaccine against malaria.

Merozoite Surface Protein-1 (MSP-1) of *P.falciparum*, encoded by dimorphic alleles, capable of limited genetic exchange; is the best-studied example of allelic polymorphism. Extensive studies done on MSP-1 of parasites isolated from different parts of the world (Hall *et al*, 1984, Tanabe *et al* 1987) have revealed significant variations in the protein (Mc Bride *et al.*, 1985). Earlier studies done on MSP-1 using a panel of monoclonal antibodies, reported the presence of some epitopes in all the isolates tested, while others occur in only a proportion of strains, indicating that the protein has both constant and variable regions. The partial sequences (Mackay *et al.*, 1985; Weber *et al.*, 1986; Kemp *et al.*, 1986) and southern blots of genomic DNA from different parasite clones also strengthen the fact that there are variable and constant regions in the MSP-1 gene.

Wellcome from West Africa and K1 from Thailand are the two complete sequences of MSP-1 gene that have been reported for the first time from two-parasite isolates (Holder *et al.*, 1985, Mackay *et al.*, 1985). Two major serotypes; out of which one is further divided have been defined with respect to MSP-1 by using Monoclonal Antibodies (McBride *et al.*, 1985). K1 and Wellcome isolates share the same major serotype, with identical nucleotide sequences except for the tripeptide repeats. Another serotype MAD20 from Papua New Guinea has been described (Tanabe *et al.*, 1987) confirming

that two basic versions of the MSP-1 gene exist in nature (Holder *et al*, 1985, Weber *et al.*, 1986 and Kemp *et al.*, 1986). Hybridzation studies (Tanabe *et al.*, 1987) using variable and constant fragments of the gene, provides substantial evidence that there is a high degree of variability in the tripeptide repeat region and that the variable sequences are not sequestered in the genome as silent copies.

Organization of the MAD20 MSP-1 gene

MAD20 allele is predominant in Indian subcontinent (Lalitha *et al.*, 1999 Kumar *et al.*, 2005. In press) and has been described, for the first time in an isolate from Papua New Guinea (Tanabe *et al.*, 1987). The nucleotide sequence of the MAD20 MSP-1 gene is 74.3% A+T rich and has a single ORF corresponding to nucleotides 136-5238. This single ORF codes for a protein of 1701 amino acid residues (194 kDa) and shares most of the basic features of the other sequences like K1 and Wellcome (Mackay *et al.*, 1985 and Holder *et al.*, 1985). The N terminus of MSP-1 has a putative signal peptide, spanning residues 1-19, followed by a group of tandem amino acid repeats spanning the residues 69-104 of the form S-X-X. The C-terminus of MSP-1 has a putative anchor sequence spanning residues 1684-1701 and is considered essential during merozoite invasion into RBC.

Comparison of MSP-1 MAD20 with K1 (Thailand isolate) and WELLCOME (West Africa isolate)

On comparison of MAD20 with K1, Wellcome and CAMP (Weber *et al.*, 1986), MAD20 revealed insertions that account for the larger size of its MSP-1 protein when compared with K1 (188 kDa) and Thai sequence. These insertions occur in the region of tripeptide repeats and at ten other sites (residues 359, 380, 422 to 424, 456 to 458, 738 to 742, 929 to 938, 944 to 958, 1004, 1263 to 1275, 1498 to 1508). Small deletions were also reported at residue positions 635, 1053, 1349, and 1617 to 1618. All the three-showed extensive diversity confined to specific parts of the polypeptide chain, although they are the products of alleles of the same gene.

A major difference between the sequences of MAD20, K1 and Wellcome is that MAD20 does not have the adjacent glutamine and proline rich regions that are the salient features of the K1 and Wellcome sequences (residues 757-806). Instead, in this part of the protein, the sequence T-E-X appears in tandem repeat (residues 723 to 734). Basing on the levels of inter-allele sequence divergence on comparison of different MSP-1 nucleotide sequences, the MSP-1 gene is divided into 17 blocks (Tanabe *et al.*, 1987), with seven variable blocks (2,4,6,8,10,14 and 16) in the gene interspersed with five conserved (1,3,5,12 and 17) and five semi-conserved blocks (7,9,11,13 and 15). In conserved blocks, more than 87% of the amino acids are identical and many of the remaining amino acids are related although different. In contrast, the variable blocks have 10-13% amino acid homology between the isolates.

Blocks 1 and 17 are highly conserved. The polypeptide region coded by block 1 and 17 are structurally and functionally constrained. Block 1 at the N terminus spanning residues 1-56 includes the signal peptide with a peptidase cleavage site (Holder *et al.*, 1985). Block 17 codes for a cysteine rich carboxy terminal domain MSP-1_{19} (residues 1646 to 1744) with a rich hydrophobic amino acid moiety spanning two EGF like domains that are structurally and functionally significant suggesting its role as an anchor sequence. MAD20 and K1 differ from Wellcome by a single base deletion (in Wellcome) giving a frame shift at codon 1716, which is compensated by loss of a base in MAD20 and K1 at codon 1720.

Essentially two versions of each block named after the representative isolates K1 and MAD20 has been reported (Tanabe *et al*, 1987). All the sequences of MSP-1 are grouped into either of these two allelic groups. Block 2 that has highly polymorphic tripeptide repeats is an exception to this dimorphic rule. The third version of the highly variable block 2 was described in the isolate RO33 (Certa *et al.*, 1987) and is represented by one of the three alleles MAD20, K1 and RO33. In addition to block 2, *P.falciparum* MSP-1 also contains several regions in which exceptions to the dimorphic rule occur. These include variations in block 4 and to a lesser extent in blocks 8 and 11 of the RO33 allele.

(Cooper, 1993). Analysis of block 2 nucleotide sequences from the three allelic types reveals a repetitive consensus sequence suggesting that block 2 in RO33 is ancestral to that of the other dimorphic alleles (Jongwutiwes *et al.*, 1992).

Sequence analysis of *P.falciparum* MSP-1 from different geographical regions of the world revealed that allelic variation in MSP-1 is not widely polymorphic, but principally dimorphic represented by K1/Wellcome and MAD20 strains for the two dimorphic types. MSP-1 is a single copy gene per haploid and thus each parasite has one MSP-1 allele at the blood stage. *P.falciparum* MSP-1 gene is an example of how interhelical exchanges of blocks of dimorphic sequences may generate many new alleles (Tanabe *et al.*, 2000, Miller *et al.*, 1993) during sexual (meiotic) recombination (Kerr *et al.*, 1994) at the diploid stage in the mosquito host and provides an evidence of diversifying selection in MSP-1.

This existence of two groups of alleles led to the dimorphic model for MSP-1 and the dimorphism at the amino acid level is strictly maintained in the nucleotide gene sequences. Variation is not random, as only two bases are found at positions where substitutions occur. Most of the allelic diversity is generated by intragenic recombination between the above mentioned representative sequences at the 5' end of the gene with in block 3, a short conserved stretch in variable block 4, block 5 and block 17 at the 3' region (Tanabe *et al.*, 1987). Minor differences do exist between homologous versions of the same variable block, and nucleotide substitutions (most of which are dimorphic) occur in semi-conserved and conserved blocks (Tanabe *et al.*, 1987). Major MSP-1 gene types may be defined as unique combinations of (i) One of the three versions of the block 2 (MAD20, K1 & RO33) (ii) One of four possible versions of block 4, because recombination with in this region generates MAD20/K1 and K1/MAD20 hybrids in addition to the "pure" allelic types MAD20 and K1 (Conway *et al.*, 2000) and (iii) One of the two versions (MAD20 or K1) of the segment between the variable blocks 6 and 16, comprising about 60% of the gene. To date no putative recombination sites or events have been described in this portion of the gene. Apart from the above mentioned types,

several variants or haplotypes or subtypes have been described, because of point mutations, recombination and cross overs.

The 17th block of the MSP-1 gene that codes for the domain MSP-1_{19} is highly conserved with five single amino acid substitutions in the first and second EGF like domains (Jongwutiwes *et al.*, 1993; Kang *et al.*, 1995). Till date several potential variants have been reported in natural isolates from different parts of the world due to point mutations in block 17, leading to five single amino acid substitutions in the first and second EGF like domains (Kaslow *et al.*, 1994; Qari *et al.*, 1998; Lalitha *et al.*, 1999, David *et al.*, 1999; Ferreira *et al.*, 2003; Raj *et al.*, 2004, Kumar *et al.* 2005 In press). Apart from the point mutations described above, several studies reported novel alleles due to recombination and crossing over. The dimorphic amino acids in the MSP-1_{19} domain are positioned at 1644, 1691, 1700, 1701 and 1716 and several alleles of MSP-1 are reported based on the amino acid substitutions at these positions.

From early 80's extensive studies were done on MSP-1 at the molecular level, confirming that the MSP-1 has both constant and variable regions. However the available data was insufficient that time, to give a complete picture of MSP-1, when Tanabe *et al* in 1987, reported an isolate MAD20 from Papua New Guinea and K1 type from Thailand (Thaithong *et al.*, 1981). After that, three different allelic types CAMP (Malaysia isolate described by Weber *et al.*, 1986), Wellcome (An African isolate described by Holder *et al.*, 1985) and MAD20 (Papua New Guiana isolate described by Tanabe *et al.*, 1987) were reported. The two allelic types K1/Wellcome are almost similar and grouped together. These alleles K1/Wellcome and MAD20 are considered to be the prototype alleles. The prototype PNG MAD20 has in the 17 block the dimorphic amino acids E (1644), T (1691), S (1700), R (1701), L (1716) and hence named as E-TSR-L type. Similarly Wellcome allele has the dimorphic amino acids of type Q-KNG-L and K1 has Q-KNG-F. After this, all the other isolates were named after the type of amino acids substituted at the five positions described above in the MSP-1_{19} domain. Initially the MSP-1 variants reported (Tanabe et al., 1987) were restricted to four dimorphic amino acids of the MSP-1_{19} domain, which are E, T, S and R. Apart from the above, the

dimorphic nature of the amino acid L/F at position 1716 has been described (Jongwutiwes *et al.*, 1993, Qari *et al.*, 1998) and hence all the variants reported after are redefined accordingly. Till date many potential variants of MSP-1_{19} have been identified from different parts of the world and these include E-KNG-L (Uganda –PA type by Chang *et al.*, 1988), Q-TSR-L (Indo type by Kang *et al.*, 1995), TSR-F from Brazil (Ferreira *et al.*, 2003), TNG (David *et al.*, 1999) a common African isolate, and also eastern India (Raj *et al.*, 2004). The variant E-TNG-L was also reported in Vietnam (Da Silveira *et al.*, 2001) and represents a new unique combination of SNPs in block 17. This was interpreted as a result of either a point mutation or a single cross over event between the haplotypes E-KNG-L (Uganda) and E-TSR-L (Indo), both of them commonly found in Vietnam. This information clearly indicates that though initially point mutations play a role, it is because of the recombination events new haplotypes are evolved. As the likeliness of occurring a point mutation at the same position in different isolates from different geographical locations is very rare.

Segments within a gene are normally exchanged by crossing over between chromosomes. Such events probably occur in *P.falciparum* during the sexual cycle in the mosquito. Extensive studies done on MSP-1 (Tanabe *et al.*, 1987) concluded that MSP-1 genes are allelic and the MSP-1 protein is encoded through out the parasite population by two dimorphic alleles that are capable of limited recombination and has the documented evidence of intragenic recombination with in the parasite.

Till date all the alleles reported have the same dimorphic amino acid residues, reinforcing the fact novel alleles are emerging because of recombination and crossing over between the existing alleles. The alleles, which have been defined so far, are not confined to different parts of the world, as Wellcome comes from Africa, K1 from Thailand, MAD20 from Papua New Guinea and CAMP from Malaysia. However the available serological data indicates that both types coexist in the parasite population and can even suggest that both have been isolated from a single patient (McBride *et al.*, 1985). Some genotyping studies done on certain regions of *P.falciparum* MSP-1 in parasites from the blood of individuals from malaria endemic areas also strengthens the above fact that *P.falciparum*

infections often consists of multiple clones of antigenically diverse parasite presenting with higher number of different MSP-1 alleles with in the population in high transmission areas.

The frequency of Intragenic recombination, which is considered to be a major mechanism for allelic variation among natural parasite populations in the Merozoite Surface Protein-1, depends on the intensity of transmission in the vector mosquito (Tanabe *et al.*, 2000) and also by the frequency of mixed clone infections, the mean number of clones per person and a repertoire of clones in a local area (Sakihama *et al*, 2001).

Comparison of intra and inter population variances in allele frequencies provides evidence for diversifying selection on a polymorphic region of MSP-1 in *P.falciparum*. Evidence suggests that there exist nonreciprocal recombination events that have homogenized certain gene regions among alleles and reciprocal recombination events that have combined allelic segments with divergent evolutionary histories, there by enhancing allelic diversity. At some gene regions, positive Darwinian selection has acted to diversify alleles at the amino acid level as the rate of non-synonymous nucleotide substitutions significantly exceeds that of synonymous nucleotide substitutions and hence the two major allelic types have been maintained for approximately 35Myr (Hughes *et al.*, 1992).

Based on the above statements it can be concluded that novel alleles are generated by recombination between the existing alleles. This diversity generated by recombination in the MSP-1 gene could be to tackle the immune response of the host. A recent study (Qari *et al.*, 1998) has predicted the existence of additional alleles of MSP-1, using single or double cross over recombination events and reported the presence of three new alleles of the ten predicted alleles, supporting the fact that genetic diversity arise from recombination. According to this study, single or double cross over recombination event between the two prototype alleles PNG-MAD20 (E-TSR-L) and Wellcome (Q-KNG-L), result in the creation of Uganda-PA allele having E-KNG-L and Indo type allele having Q-TSR-L type 19 kDa domain. They confirmed that recombination occurs between the

prototype alleles as all the alleles reported have the blocks 15th and 16th of MAD20 or Wellcome type and Block 17th of different haplotype. The haplotype E-KSR-L (Kenya-2) (Qari *et al.*, 1998) has evolved because of the single cross over recombination event between Uganda (E-KNG-L) and MAD20 (E-TSR-L) or Indo (Q-TSR-L) as depicted below.

E-K*NG-L (Uganda) Q-T*SR-L (Indo) ⟶ E-KSR-L(Kenya 2) + Q-TNG-L

E-K*NG-L (Uganda) E-T*SR-L (MAD20) ⟶ E-KSR-L(Kenya 2) + E-TNG-L

The same E-TNG-L has been reported in field isolates from Vietnam (Da Silveira *et al.*, 2001) confirming that recombination leads to diversity. The haplotype Kenya 1(E-KSG-L) resulted because of single or double cross over between the Uganda-PA (E-KNG-L) and Kenya-2 type (E-KSR-L) or between Uganda PA and MAD20 or Indo. Similarly Kenya-3, is because of cross between Uganda and Thai isolates.

Till date all the alleles identified from different parts of the world has the blocks 15th and 16th of MAD20 or Wellcome type. This is further supported by the sequence data obtained from isolates in highly malaria endemic country like India (Lalitha *et al.*, 1999, Kumar *et al.*, 2005 In press). Of the 16 isolates reported (Lalitha *et al.*, 1999) 15 sequences have the blocks 15th and 16th of MAD20 type and 1 isolate of Wellcome type, with the MSP-1_{19} belonging to the haplotypes of Q-KNG-L (Wellcome type), E-KNG-L (Uganda PA type) and E-TSG-L.

A recent study (Raj *et al.*, 2004) has identified a rare non-synonymous point mutation (V) in MSP-1_{19} domain at amino acid position 1704 in eastern India isolates TSG-V and KNG-V. This mutation has been reported earlier (Lalitha *et al.*, 1999) from central India (E-TSG-V-L) and was also reported in *P.knowlesi* at codon 1704. This conversion of I to V (ATC-GTC) is predominant in most of the eastern India isolates and has not been reported out side. This mutation might have just evolved and trying to withstand the immunological pressure. To confirm the dimorphic nature of this amino acid, more analysis has to be done from different geographical locations.

During the course of evolution, point mutations would have been generated at five different positions in the 17th block of MSP-1 leading to the emergence of dimorphic amino acids to withstand the immunological pressure. This dimorphic amino acid in turn, leads to novel variants because of single and double cross over events.

Origin of MSP-1 alleles

It has been speculated that the product of the two alleles has equivalent function, but different mechanism of action, giving rise to localized diversity in their structure (Tanabe *et al.*, 1987). The two alleles must have originated at different geographical locations at earlier times and when the population fused and interbred, these two alleles came together and generated diversity because of intragenic recombination. An alternative model for the generation of diversity in MSP-1 sequence was hypothesized (Miller *et al.*, 1993). Apart from intragenic recombination, gene conversions or frequent double cross overs that combines fragments from the so-called pseudogenes could also play a major role in generating MSP-1 diversity. The generation of large repertoire of variants because of gene conversions is reported in *Trypanosoma equiperdum* (Roth *et al.*, 1986).

Another report suggests that although there is significant evidence indicating that meiotic recombinational events generate the high allelic diversity in MSP-1 (Kerr *et al.*, 1994), its frequency and role in MSP-1 evolution in natural parasite populations is over estimated. One report (Da Silveira et al., 2001) suggests low meiotic recombination rates occuring in both parasite populations indicating a possible role for non-homologous recombination, such as strand slippage mispairing during mitosis and gene conversions, in generating variation in malarial antigens under strong diversifying selection. If new alleles are frequently generated during the clonal (mitotic) propagation of parasites, antigenic diversity in *P.falciparum* may be less constrained by certain parameters, such as the intensity of malaria transmission and the abundance of genetically distinct clones available for cross mating, than if meiotic recombination were the predominant or sole diversifying selection mechanism.

The dimorphic amino acids were evolved before and after recombination leading to the diversity. The sequence information available so far from different Indian isolates and from the isolates reported out side India indicates that the observed non-synonymous mutations in block 17 are restricted to five allele determining dimorphic amino acid residues at positions 1644, 1691, 1700, 1701 and 1716. These residues are dimorphic because of single point mutations, there by generating new alleles. For example: a single point mutation at first residue in Q-KNG-L (Wellcome type) will generate E-KNG-L (Uganda PA type), and the same Wellcome type Q-KNG-L could generate Q-KNG-F, with a single point mutation at the last residue. Although it is a well-known fact that point mutations in the 17^{th} block are responsible for generating diversity, but on thorough analysis of the sequence data show that only specific mutations are observed on these positions. Like first codon change g/c, a/g and c/t at residues E/Q (1644), R/G (1701) and L/F (1716) respectively; and 2^{nd} codon change c/a and g/a at residues T/K 91691) and S/N (1700) respectively. Instead of point mutations occurring at any of the three positions in codons of these residues. This clearly indicates that point mutations do not seem to be the only source of origin and maintenance of different alleles, which otherwise instead of the specific combinations of these residues the point mutations could generate numerous combinations of these allele determining amino acid residues.

It can be hypothesized that mutations might have occurred at different positions (residues) during the evolution, and due to natural selection (immunological pressure), only these specific mutations are maintained in the populations while others are lost. This maintenance of variants in the population could have a selective advantage in evading the pre existing immune responses. The selectively neutral mutations may lead to random diversification of the population. After these useful mutations are evolved and selected by the nature, these are then shuffled during recombination, leading to allelic diversity. Many studies (Tanabe *et al.*, 1987, Qari *et al.*, 1998) have indicated that the dimorphic nature of allele determining five amino acid residues have independent geographic distribution and there are possibilities that new alleles may exist in other regions of the world.

The current study further explored the sequence diversity of *P.falciparum* MSP -1_{42} and MSP-1_{19} in Indian Isolates.

Results and Discussion

The C-terminal region of *Plasmodium falciparum* MSP-1 gene corresponding to part of 15th (a semi conserved region), 16th (a variable region) and 17th (a conserved region) blocks from 11 Indian isolates (IR1 to IR9 & IR12 from Rajasthan and IW1 from Siliguri, West Bengal) and a part of 16th and whole of 17th blocks from eight isolates (IW2 & IW3 from West Bengal and six isolates from Rajasthan IR10, IR11, IR13, IR14, IR15 & IR17), were analyzed and compared with the corresponding sequences of the two prototypic alleles PfMAD20, PfK1/Wellcome, isolates reported from Uganda (Chang *et al.*, 1988) and Kenya (Qari *et al.*, 1998). The block 15 and 16 of MSP-1 gene of all the isolates analyzed was found to belong to the PfMAD20 allelic family, which is a predominant family in Indian Subcontinent. Analysis of block 17 revealed eight different haplotypes (Table 3.1).

The polymorphism in the 15th block of all the isolates except IR7 is restricted to seven amino acid positions (Fig.3.1), with a significant change observed at position 1391, where isoleucine (I) is substituted by phenylalanine (F), in six of the western India isolates (IR1 –IR5 & IR7), both being hydrophobic non-polar amino acids. This change of I to F has also been reported earlier in the Uganda –PA isolate (Chang *et al*, 1988), two Thai isolates and from Brazil, Papua New Guinea and Kenyan isolates (Qari *et al.*, 1998), but not previously in Indian isolates. In IR7 apart from the above substitution, 5 more non-synonymous changes were observed and these include a stretch of four amino acids NLNL to KVKW at positions 1394-1397 and R to G at position 1408. Also in IR7 at the nucleotide level a 3rd base synonymous change (T to G) at position 1404 was observed. In IW 1, four non-synonymous substitutions were observed: I to M at position 1386, I to L at position 1390, I to F at position 1400 and Y to F at position 1410. Also in IW1, at the nucleotide level a third base non-synonymous change (A to T) at residue 1392 has been observed which has not reported earlier. In IR5 apart from the change of I to F at position

1391, two more non-synonymous changes have been identified which have not been reported before. These include T to P at position 1392 and V to G at position 1414 (Fig. 3.1).

In block 16, several non-synonymous changes have been identified which have not been reported before (Fig. 3.1). These include Q to E in Isolate IR7 at position 1421, F to P in isolate IR7 & IR12 and F to L in isolate IR4 at position 1422, H to D in isolate IR7 at position 1424, K to Q in isolate IR1 at position 1452, K to T in Isolate IR7 at position 1478, S to R & G in isolates IR4 & IR5 at position 1518, F to S in isolate IR8 at position 1520, L to S in isolate IR4 & IR7 at position 1542, D to A in isolate IR11 at position 1574, N to D in isolate IR5 at position 1583, T to P and K to R in isolates IR11 & IR6 at positions 1596 and 1598. We observed a deletion of residue K (coded by AAG) at position 1496 in all the isolates except IR6 and IR12. This deletion of residue K has been earlier reported (Jongwutiwes *et al.*, 1993; Miller *et al.*, 1993; Qari *et al.*, 1998,) but not in Indian isolates. A change of E to G in isolate IR4 at position 1517 was observed, and is reported earlier in Kenya (Qari *et al.*, 1998). A change of D to N at position 1557 in all the isolates except IR4, IR5, IR8, IR9, IW1, IW2 was observed and was also reported earlier in Kenyan and Indian isolates (Qari *et al.*, 1998; Lalitha *et al.*, 1999). At position 1582 there was a change from T to H in 11 Indian isolates, also reported earlier in Kenyan isolates, Uganda –PA isolates (Qari *et al.*, 1998) and not in India. At the nucleotide level third base non-synonymous changes have been detected in IR4 (T to C), IR5 (A to C), IR6 (A to C) and IR11 (A to G) at positions 1538, 1457, 1457 and 1589.

Novel allele determining amino acid in block 17

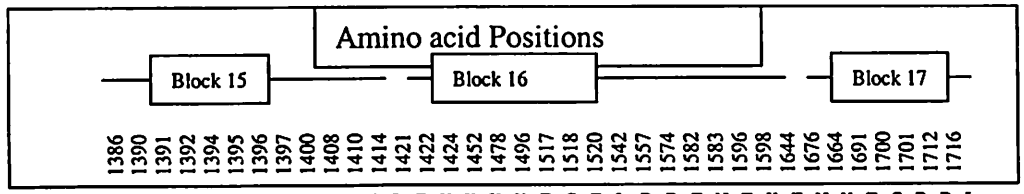
Classification of the allelic types can be done basing on the occurrence of dimorphic amino acid residues at positions 1644, 1691, 1700, 1701 and 1716 (Jongwutiwes *et al.*, 1993, Miller *et al.*, 1993, Kang *et al.*, 1995, Qari *et al.*, 1998). Our data revealed the presence of eight MSP-1 alleles present in the Indian population (Table 3.1). These eight alleles include E-TSR-L (4/19), Q-TSR-L (1/19), E-TSG-L (4/19), Q-KNG-L (1/19), Q-KNG-F (1/19), E-KNG-L (2/19), E-KNG-F (5/19) and E-KYG-F (1/19). Of these Q-

KNG-L, E-KNG-L and E-TSG-L were earlier reported in India (Lalitha *et al.*, 1999) and just the TSR, TSG and KNG without classifying them as E or Q and L or F (residue 1644, 1716) was reported (Raj *et al.*, 2004) from eastern India isolates. The isolates Q-KNG-L, E-KNG-L designated as Wellcome and Uganda –PA were also reported outside India (Holder *et al.*, 1985, Chang *et al.*, 1988). For the first time we are reporting E-KNG-F that was previously reported in Kenya (Qari *et al.*, 1998), Q-KNG-F a Thai isolate, E-TSR-L (PNG-MAD20) and Q-TSR-L (Indo) alleles in the Indian subcontinent. E-TSR and Q-TSR were earlier reported outside India (Tanabe *et al.*, 1987, Kang *et al.*, 1995). Our sequence analysis data reported the presence of a novel allele E-KYG-F that has not been reported elsewhere. The emergence of this allele is not because of the recombination between the prototype alleles as there are no reports on the existence of the residue Tyrosine (Y) at position 1700 in any of the alleles reported to date.

Earlier studies have reported recombination between the existing alleles, leading to the emergence of novel alleles due to crossing over between them (Qari *et al.*, 1998). Seven of the eight alleles reported by us further strengthen the fact that alleles have emerged due to recombination. The same allele determining amino acid residues are observed to be present in different combinations. However, in our novel allele E-KYG, we find Y because of non-synonymous point mutation at AAC changing to TAC in the second EGF like domain. To confirm the trimorphic nature of this amino acid, more analysis has to be done from different geographical locations. From this it can be hypothesized that besides recombination and crossing over, single point mutations lead to the generation of novel alleles.

Apart from the above, three non-synonymous changes have been identified in 17th block not reported earlier in India. These changes are Y to S at position 1664 in isolate IR1, N to D at position 1676 in isolate IR13 reported earlier in Papua New Guinea, in the first EGF like domain and D to H at position 1712 in isolate IR1 in the second EGF like domain. Two 3rd base synonymous changes have been observed in the isolates IR1 (A to C), IR11 (A to C) at positions 1697 and 1715.

Several in vitro studies with the human malaria parasite *P.falciparum* (Blackman *et al.*, 1990, Cooper *et al.*, 1992) demonstrated that monoclonal antibodies to reduction sensitive epitopes in the MSP-1_{42} could inhibit erythrocyte invasion. The point mutation at position 1700 in isolate IR1 lies in the EGF -like domain and spans a potential B cell epitope. It is likely that single point mutations in this region may affect the immunogenicity of the molecule and needs to be further investigated. Several point mutations identified in our study span the 33-kDa region which plays a vital role in proteolytic processing and is also immunologically significant. Our study showed the existence of eight different alleles of MSP-1_{19} from a pool size of 19 isolates and it may not be surprising if more alleles are present in a highly populous, malaria endemic country like India. This has to be further investigated, if MSP-1 is to be incorporated in vaccine strategies for malaria.



	1386	1390	1391	1392	1394	1395	1396	1397	1400	1408	1410	1414	1421	1422	1424	1452	1478	1496	1517	1518	1520	1542	1557	1574	1582	1583	1596	1598	1644	1676	1664	1691	1700	1701	1712	1716
MAD20	I	I	I	T	N	L	N	L	I	R	Y	V	Q	F	H	K	K	K	E	S	F	L	D	D	T	N	T	K	E	N	Y	T	S	R	D	L
IR1	-	-	F	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	*	-	-	-	-	N	-	H	-	-	-	-	-	S	K	Y	G	H	F
IR2	-	-	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	-	-	-	K	N	G	-	F
IR3	-	-	F	-	-	-	-	F	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	-	-	-	K	N	G	-	F
IR4	-	-	F	-	-	-	-	-	-	-	-	-	L	-	-	-	*	G	R	-	S	-	-	-	H	-	-	-	-	-	-	K	N	G	-	-
IR5	-	-	F	P	-	-	-	-	-	-	G	-	-	-	-	-	*	-	G	-	-	-	-	-	-	D	-	-	Q	-	-	K	N	G	-	F
IR6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	N	-	-	-	-	R	-	-	-	-	-	-	-	-
IR7	-	-	F	-	K	V	K	W	-	G	-	-	E	P	D	-	T	*	-	-	-	S	N	-	H	-	-	-	-	-	-	K	N	G	-	F
IR8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
IR9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
IR10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	-	-	-	K	N	G	-	F
IR11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	A	-	-	P	-	-	-	-	-	-	-	-	-
IR12	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	K	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-
IR13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	-	D	-	K	N	G	-	-
IR14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	-	-	-	K	N	G	-	F
IR15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	Q	-	-	K	N	G	-	-
IR17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	G	-	-
IW1	M	L	-	-	-	-	-	-	F	-	F	-	-	-	-	-	-	*	-	-	-	-	-	-	H	-	-	-	Q	-	-	-	-	-	-	-
IW2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-
IW3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	N	-	H	-	-	-	-	-	-	-	-	-	-	-

Legend: '-' indicates the residue conservation
'*' indicates the residue deletion
IR1 to IR15, IR17, IW1 to IW3 (Parasite Isolates)

Figure 3.1 Sequence diversity in the C-terminal region (Blocks 15, 16 and 17) of MSP-1 in Indian *Plasmodium falciparum* isolates

GenBank Accession Nos.

AY776344 to AY776360, AY776362 & AY776363

Allele Type & Isolate	Amino acid and its position					Number of isolates per allele
E-KYG-F (IR1)	1644 E	1691 K	1700 Y	1701 G	1716 F	1
E-KNG-F (IR2, IR3, IR7, IR10, IR14)	1644 E	1691 K	1700 N	1701 G	1716 F	5
E-KNG-L (IR4, IR13)	1644 E	1691 K	1700 N	1701 G	1716 L	2
Q-KNG-F (IR5)	1644 Q	1691 K	1700 N	1701 G	1716 F	1
Q-KNG-L (IR15)	1644 Q	1691 K	1700 N	1701 G	1716 L	1
E-TSG-L (IR8, IR9, IR17, IW2)	1644 E	1691 T	1700 S	1701 G	1716 L	4
E-TSR-L (IR6, IR11, IR12, IW3)	1644 E	1691 T	1700 S	1701 R	1716 L	4
Q-TSR-L (IW1)	1644 Q	1691 T	1700 S	1701 R	1716 L	1

Table 3.1 Classification of allelic types identified in our study based on p19 allelism

Chapter 4

Scanning of MSP-1_{19} for putative B-cell Epitopes

Chapter 4

Introduction

The natural scientific desire to simplify and define the composition of effective vaccines argues that the future of vaccines lies in novel approaches that will discover effective and less expensive components. Peptides whether they are chemically synthesized or produced by living organisms, are an attractive possibility. The use of synthetic peptides as an alternative approach to vaccination and other molecular investigations is currently being pursued, with respect to parasitic diseases. Remarkable progress has occurred towards understanding the structural requirements necessary to stimulate cellular and humoral immune responses, and peptides have been integral in the development of this field. B-cell epitopes must constitute accessible surface regions; as a result it is very essential to analyze the primary structure of an antigen to identify strongly hydrophilic sequences, which most likely correspond to surface regions of an antigen.

Here in this study, the cysteine rich carboxy terminal region of MSP-1 (MSP-1_{19}) corresponding to TSR, TSG, KYG and KNG alleles, was scanned for B-cell epitopes using BcePred Prediction server (Saha *et al.*, 2004).

Analysis revealed the presence of two B-cell epitopes, one in each of the EGF like domains (Fig 4.2, 4.3a, 4.3b, 4.3c & 4.3d). Figure 4.1 represents the structural distribution of E1 and E2 peptides corresponding to EGF I and II domains of *P.falciparum* MSP-1_{19} derived from data based on solution structure (Morgan *et al.*, 1999).

The epitope present in the first EGF like domain is conserved in all the alleles and is designated as

E1: NSGCFRHLDEREECKCLLN

The second epitope detected in the second EGF like domain from the four alleles, has four versions with one or two amino acid substitutions in between. These four has been designated as

E2a : EDSGS**YG**KKITCECTKP**H**S (KYG)

E2b : EDSGS**SR**KKITCECTKP**D**S (TSR)

E2c : EDSGS**SG**KKITCECTKP**D**S (TSG)

E2d : EDSGS**NG**KKITCECTKP**D**S (KNG)

About the Bcepred prediction server

Bcepred can predict continuous B cell epitopes. Identified properties of B cell epitope include hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns. Users can select any physico-chemical property or combination of two or more properties for epitope prediction. It presents the results in graphical and tabular frame. In case of graphical frame, this server plots the residue properties along protein backbone, which assist the users in rapid visulaziation of B-cell epitope on protein. The peak of the amino acid residue segment above the threshold value (default is 2.38) is considered as predicted B-cell epitope. The server is able to predict epitopes with 58.7% accuracy using *Flexibility, Hydrophilicity, Polarity,* and *Surface* properties combined at a threshold of 2.38.

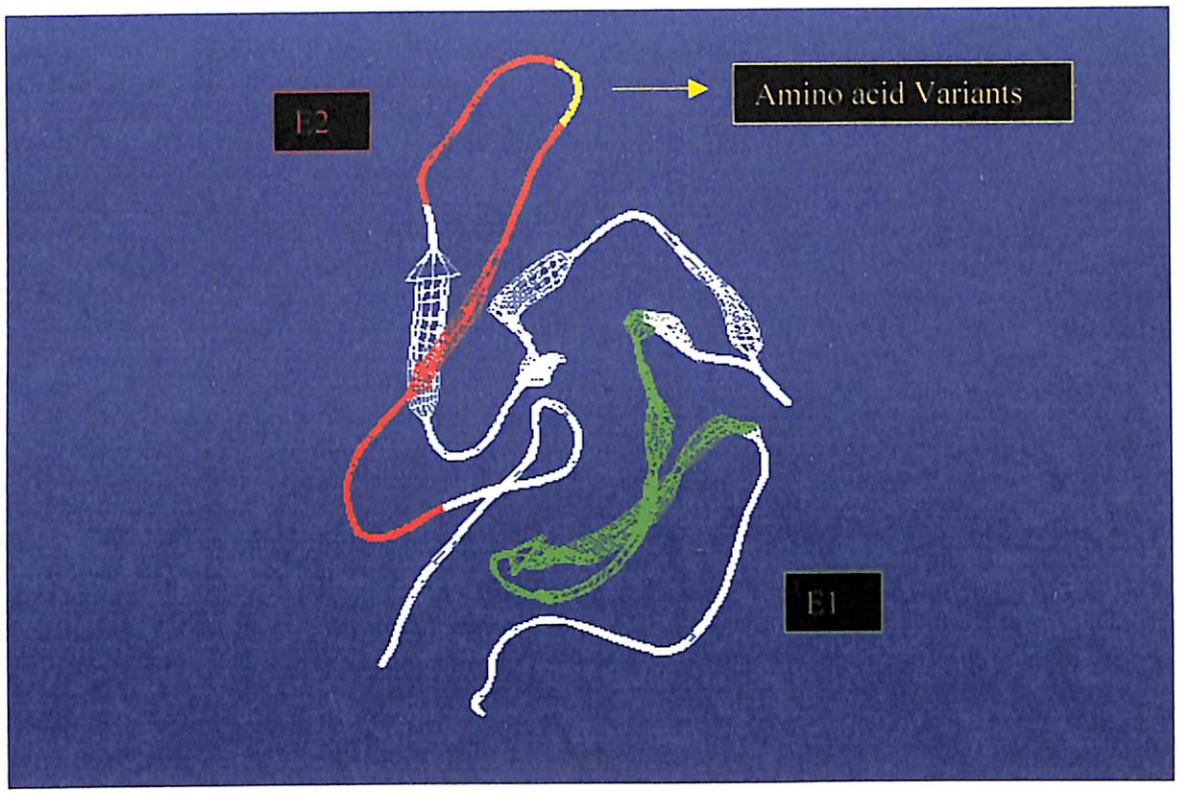


Figure 4.1 The structural distribution of E1 and E2 peptides corresponding to EGF I and II domains of *P.falciparum* MSP-1_{19} derived from data based on solution structure of the same (Morgan *et al.*, 1999)

E1: NSGCFRHLDEREECKCLLN

E2: EDSGSXXSKKITCECTKPXS

E2a:-----YG----------H-

E2b:-----SR----------D-

E2c:-----SG----------D-

E2d:-----NG----------D-

Prediction of B cell epitope regions in MSP-1_{19} of *Plasmodium falciparum* using physico-chemical properties

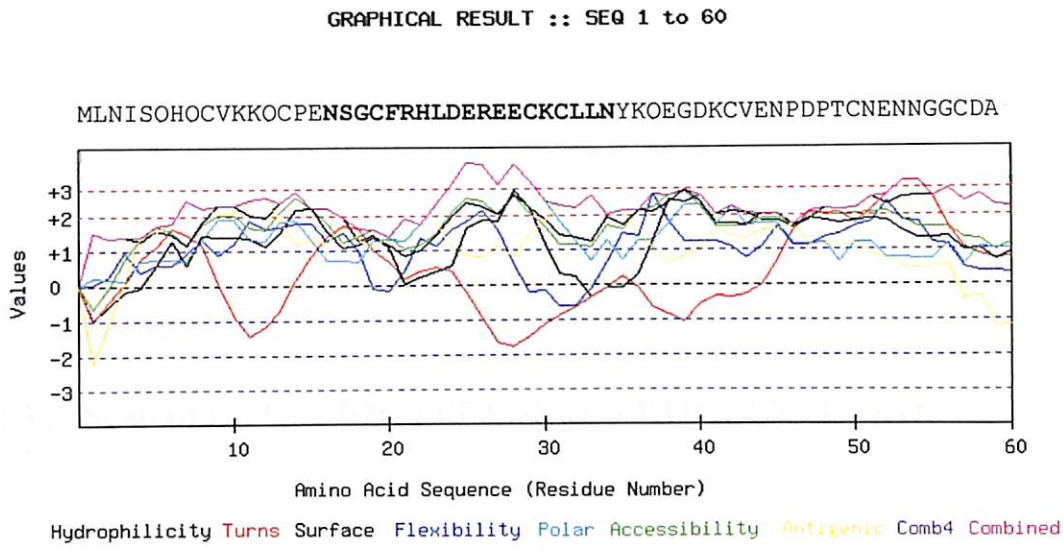


Figure 4.2 A Scan of EGF I Like Domain

E1: NSGCFRHLDEREECKCLLN

A Scan of EGF II Like Domain of different alleles

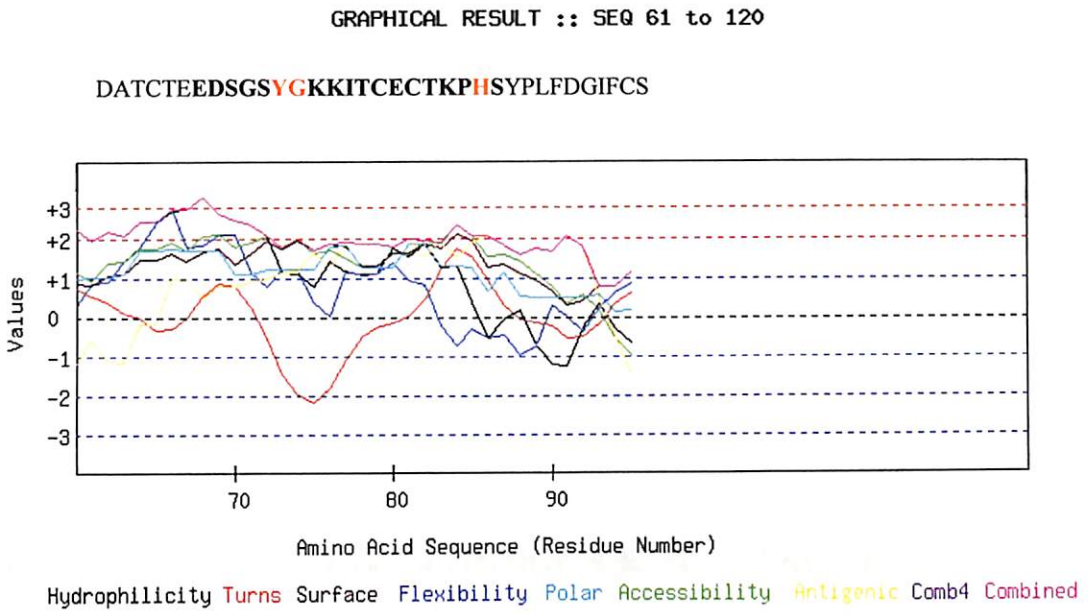


Figure 4.3a Scan of KYG E2a : EDSGSYGKKITCECTKPHS (KYG)

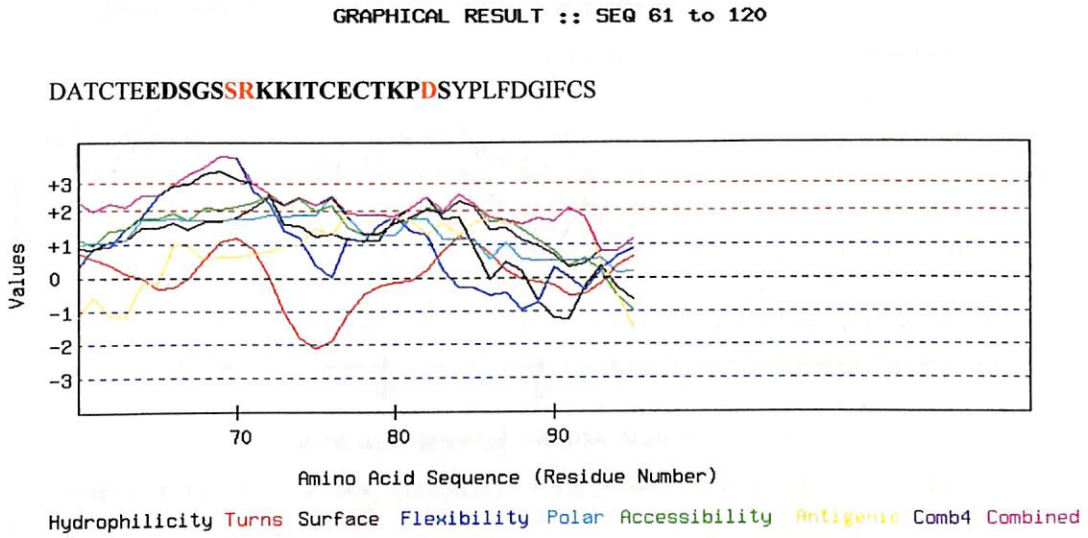


Figure 4.3b Scan of TSR E2b : EDSGSSRKKITCECTKPDS (TSR)

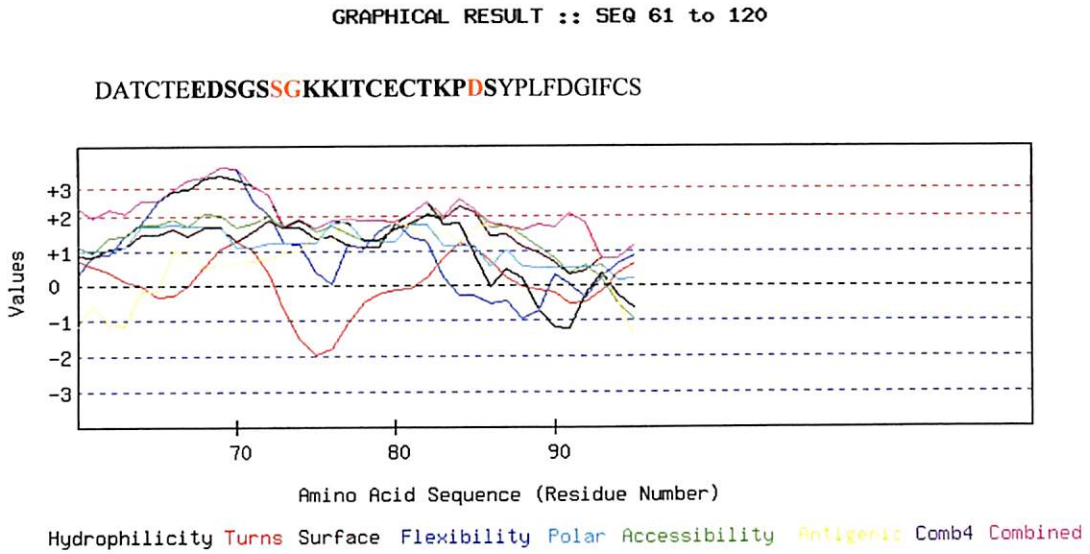


Figure 4.3c Scan of TSG E2c : EDSGSSGKKITCECTKPDS (TSG)

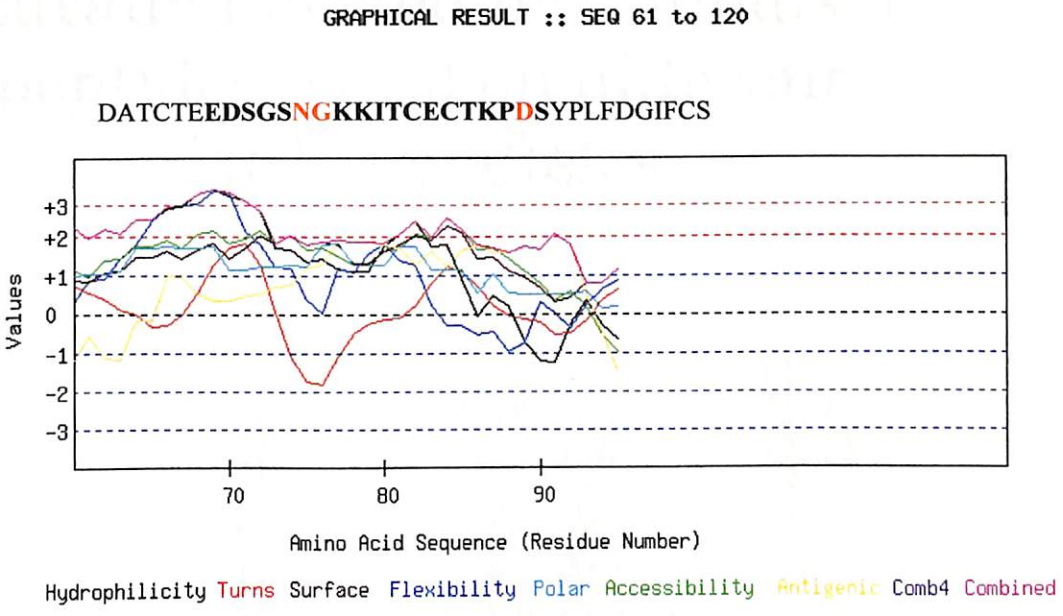


Figure 4.3d Scan of KNG E2d : EDSGSNGKKITCECTKPDS (KNG)

Chapter 5

Evaluation of antibody profiles in naturally infected individuals, to peptides based on different MSP-1_{19} alleles

Chapter 5

Introduction

People naturally exposed to *P.falciparum* develop antibodies against MSP-1, particularly against the conserved epitopes formed by the EGF like domains of MSP-1_{19} (Muller *et al.*, 1989, Riley *et al.*, 1993, Tolle *et al.*, 1993, Udhayakumar *et al.*, 1995, Egan *et al.*, 1995). An association between a naturally acquired immune response to MSP-1 and reduced malaria morbidity has been reported (Riley *et al.*, 1992). A significant correlation between anti MSP-1_{19} antibody titer and reduced malaria morbidity has been reported (Egan *et al.*, 1996). Some studies have demonstrated a statistically significant association between the level of anti MSP-1_{19} antibodies and clinical morbidity in children (Al yaman *et al.*, 1996, Alonso *et al.*, 1998). A study done in Liberia, West Africa has demonstrated that acquisition of high levels of antibodies to MSP-1_{19} from the mother correlates with a reduced probability of an episode of clinical malaria in infants (Hogh *et al.*, 1995). Studies have been done in India (Biswas *et al.*, 1990, Lalitha *et al.*, 1994) to check the immunogenicity of MSP-1 in *P.falciparum* primed individuals. Despite all the extensive studies done, the role of anti MSP-1 antibodies in protection against human malaria under conditions of natural transmission is not clear.

Antibodies play a major role in protective immunity and the role of IgG in the acquisition of antimalarial immunity is still unclear. There are at least two mechanisms by which the protective antibodies can act 1) Opsonizing antibodies that promote phagocytosis of the infected erythrocytes and 2) Cytophilic antibodies of human IgG1 and IgG3 isotypes which act in cooperation with monocytes to inhibit parasite growth (Garraud *et al.*, 1989, Garraud *et al.*, 1994). In Brazil it was shown that the prevalence and the levels of IgG to *P.falciparum* MSP-1_{19} increased with the time of exposure and were positively correlated with the absence of clinical symptoms in parasitemic patients. IgG1 isotype were significantly higher with long-term exposure and in asymptomatic infections, indicating a protective role of IgG1 in naturally acquired immunity (Erika *et al.*, 2002). Affinity

purified human antibodies to the C-Terminus of MSP-1 inhibit parasite growth (Egan *et al.*, 1997).

Several studies conducted in hyper endemic areas showed that MSP-1 $_{19}$ specific IgG1 is associated with clinical protection from *P.falciparum* malaria (Egan *et al.*, 1995, Shi Y P *et al.*, 1996, Cavanagh *et al.*, 2001). In contrast a study (Dodoo *et al.*, 1999) in Ghanian children has demonstrated that antibodies to conserved MSP-1 $_{19}$ are not associated with protection from clinical malaria despite high IgG1 titers. In another similar study (Nguer *et al.*, 1997), it was demonstrated that the responses to the first EGF like domain of MSP-1 $_{19}$ were restricted to IgG1 and IgG3 subclasses, with more of IgG1 (because of high half life of 11-23 days and for IgG3 7-8 days) to achieve parasite clearance in areas where transmission is perennial.

The two EGF-like domains of *P.falciparum* MSP-1$_{19}$ are recognized by human antibodies (Sha *et al.*, 1995) and has been associated with a lowered risk of clinical malaria in immuno-epidemiological studies (Riley *et al.*, 1992, Hogh *et al.*, 1995, Al yaman *et al.*, 1996, Branch *et al.*, 1998). PfMSP-1 $_{19}$ of wellcome type with both EGF like domains recognized by antibodies from 30% of patients sera, Wellcome and MAD20 PfMSP-1 $_{19}$ with only I EGF like domain was recognized by 15%, where as MAD20 type PfMSP-1 $_{19}$ with only second EGF domain was recognized by 4% of the domains (Daniel Dodoo *et al.*, 1999).

Two Murine Mabs, 12.8 (Blackman *et al.*, 1990) and 5B1(Pirson *et al.*, 1985), which are specific for MSP1 and inhibit the growth of *P.falciparum invitro* have been described. In contrast, naturally occurring human antibodies from adults immune to malaria from Ivory Coast, West Africa, that are recognized by the first EGF like domain in the *P.falciparum* MSP1 did not inhibit parasite growth in vitro (Chappel *et al.*, 1994).

Host factors play an important role for the capacity to respond to the C terminal part of *P.falciparum* MSP-1. The difference in frequencies of antigen specific antibody production observed in different studies may be related to the different malaria

transmission rates and consequent difference in exposure to malaria antigens of the two regions (Chang *et al.*, 1989). The low antibody prevalence can be because of the short half-life of anti PfMSP-1 antibodies, which are mainly found shortly after clinical episodes (Fruh *et al.*,1991, Branch *et al.*,1998, Cavanagh *et al.*,1998) and low immunogenicity or lack of adequate T cell help for antibody production (Egan *et al.*, 1997,). Protection by anti-PfMSP-1 antibodies can be because of fine specificity and the balance between antibodies of different subclasses, that are dependent on intensity of the transmission (Cooper *et al.*, 1992, Chappel *et al.*, 1994). Amino acid composition and conformation may also determine the fine specificity of anti PfMSP-1 antibodies, as only some anti PfMSP-1 $_{19}$ Mab,s inhibit the invitro merozoite invasion of erythrocytes (Chappel *et al.*, 1993, Chappel *et al.*, 1994). Naturally acquired PfMSP-1_{19} antibodies may be a mixture of protective and non-protective types.

A recent study (Nwuba *et al.*, 2002) has demonstrated the induction of both antibodies that inhibit the MSP1 secondary processing as well as blocking antibodies, in children from Southwestern Nigeria, by natural infection with *P.falciparum*. The induction of the production of blocking antibodies in natural infection is to abolish the protective effects (Holder *et al.*, 1999) providing an effective means for the malaria parasites to evade the host immune defenses. It has been hypothesized that, after natural infection with *P.falciparum*, both processing inhibitory and blocking antibodies are produced, with the outcome of exposure depending on the kinetics of appearance of these two classes of antibody, their quantities and their relative efficacies (Nwuba *et al.*, 2002). These findings may clearly indicate why no evidence of association between antibody response to MSP1 and clinical protection was observed in a recent study (Dodoo *et al.*, 1999).

Several allele specific sero cross reactivity studies were done from different parts of the world in *P.falciparum* field isolates. Partially purified MSP-1 derived from *P.falciparum* has protected monkeys from artificially induced malaria infection. *Aotus* monkeys immunized wit MSP-1 of the Palo Alto isolate were completely protected from the lethal effects of challenging with the same parasite (Siddiqui *et al.*, 1987). In contrast a lower degree of protection was observed when monkeys were immunized with MSP-1 purified

from K1 and challenged with a heterologous parasite (Hall *et al.*, 1984). Some studies have shown that immunization with MSP-1 of K1 isolate could protect *Saimiri* monkeys from a lethal challenge with the non-homologous Palo Alto isolate of the parasite (Etlinger *et al.*, 1991). These conflicting results can be because of the ambiguity in selecting the parts from the large protein that are important in protective immunity. Naturally acquired human immune responses to MSP-1 have focused on the C-Terminal region (Holder *et al.*, 1996). Plasma from 15 of the 35 patients tested, possessed antibodies, reacting to the epitopes of the conserved MSP-1_{19} (Egan *et al.*, 1997). These ab's bind to both the Wellcome sequence and MAD20 sequence. Eight serum samples recognized the EGF I and one recognized EGF II, which is in close agreement with another study (Egan *et al.*, 1995). This shows that ab's to MSP-1_{19} tend to be cross reactive (Egan *et al.*, 1997).

Sero-epidemiological study using sera from adults living in areas of Kenya where malaria is hyper-endemic demonstrated that 24 (approximately 38%) of the 64 human serum samples tested for binding to the MSP-1_{19} variants, failed to react with one or more of the three variants E-KNG, E-TSR and Q-KNG (Udhaya Kumar *et al.*, 1995). Where as, similar surveys of antibody responses in both children and adults from areas of Philippines where malaria is hyper endemic (Palawan district) as well as hypo endemic (Morong district) showed that 95-97% of the serum samples recognized all of the MSP-1_{19} variants tested (Hui *et al.*, 1996). The differences in the antibody profiles between these two studies are attributed to dissimilarities in transmission characteristics or because of differences in assay methods or conditions (Hui *et al.*, 1996). It has been reported that the prevalence of anti MSP-1_{19} antibodies is low in children (about 20%), but is up to 60% in adults, in Gambia where malaria is endemic (Egan *et al.*, 1995).

A recent phase I trial of recombinant MSP-1_{19} from FVO and 3D7 strains of *P.falciparum*, in non exposed human volunteers, induced high levels of antigen specific Th1 (gamma interferon) and Th2 (interleukin 4 and interleukin 10) response. IL10 is very important in promoting humoral immunity. In this study MSP-1_{19} peptides are used to check the responses of vaccinated volunteers in east (Kenya) and West Africa (The

Gambia) against cytokine-secreting T cells. Novel and conserved allelic T cell epitopes, inducing cross-strain immune responses were identified. It has been suggested that epitope-specific naturally acquired MSP-1_{19} immune responses in endemic populations can be boosted by vaccination. A recent study (Ekala *et al.*, 2002) has investigated *P.falciparum* MSP-1 allele specific humoral responses in residents of Central Africa using peptides from block 2 and demonstrated the relation between antibody specificity and the infecting parasite genotype. When treated against schizont extract, more than 90% of the sera reacted with all K1, Ro33 and MAD20.

Cord blood T and B cell immunity to *P.falciparum* MSP-1 has been examined in infants born in an area of stable malaria transmission in Kenya (Christopher *et al.*, 2002). 6% of the new born had cord blood anti MSP-1_{19} IgM antibodies 18% cord blood samples contained B cells that produced IgG when stimulated with MSP-1_{19} invitro, indicating high fraction of sensitized B cells. A total of 85% of maternal samples and 78% of cord blood samples contained IgG antibodies to MSP-1 $_{19}$ Q-KNG, where as for others are low (E-KNG, Q-TSR, E-TSR). Prenatal sensitization to blood stage antigens occurs in infants born in malaria endemic areas. The allele specific responses to MSP-1_{19} variants demonstrated that IgG antibodies in mothers and their new born recognized the Q-KNG as well as the E-KNG, Q-TSR and E-TSR alleles, although antibodies to the E-TSR variant was less than others. In contrast, cord blood IgM antibodies and invitro B cell IgG reactivities restricted to MSP-1 $_{19}$ Q-KNG. This suggests that MSP-1_{19} antibodies bind to conformational epitopes conserved in the two EGF like domains and binding is not affected by changes in the primary amino acid sequence that do not affect folding through disulfide bonds in cysteine residues (Cooper *et al.*, 1992, Blackman *et al.*, 1993, Hui *et al.*, 1994, Egan *et al.*, 1999, Somner *et al.*, 1999). A similar allele specific sero reactivity study done in natural population (Polley *et al.*, 2003) demonstrated that repeat sequences in Block 2 of *P.falciparum* MSP-1 are targets of antibodies associated with protection from malaria.

The response of different isotypes of IgG antibodies to various analogues of MSP-1_{19} corresponding to Q-KNG, Q-TSR, E-KNG and E-TSR were studied (Diallo *et al.*, 2001).

Anti MSP-1_{19} antibodies in the study population were mostly restricted to IgG1 and IgG3 isotypes and the frequency of IgG1 responses among the 4 variants was not statistically significant. An imbalance in IgG subclass distribution in antibodies to *P.falciparum* blood stage antigens has been reported (Bouharoun *et al.*, 1992), whereas the protection against blood stages are mediated by IgG1 and IgG3 antibodies, whose ratio (IgG1/IgG3) depends on the antigen used in the isotype specific immunoassay (Ferrante *et al.*, 1997). MSP-1_{19} seems to elicit a restricted set of antibody responses in immune population, with elevated IgG1/IgG3.

The immunogenicity of conserved N terminus (block1), the conserved C – terminus and the 3 main types of the major polymorphic region (Block 2) of MSP-1 and the specificity of the antibody responses along with the duration of these responses were demonstrated in Sudamese villagers intermittently exposed to *P.falciparum* infections. Antigens from block 1 were rarely recognized by any donor, responses to C-terminal occurred in the majority of the acutely infected individuals, serving as an indicator of recent infection, whereas responses to block 2 were type specific. This study proves that naturally induced human antibody responses to MSP-1 are short lived (Cavanagh *et al.*, 1998).

It was found that the IgG response to the C-Terminal MSP-1_{19} was significantly higher than that against any of the Block 2 antigens tested, in clinically ill or/and convalescent individuals. It can be assumed that, in a series of malaria infections in one individual, the epitopes present in the conserved C-Terminal region would be presented by all parasites and memory response to MSP-1_{19} would be strong. It was proposed that clinical malaria epitopes and their antigenic challenge are the key determinants of the human responsiveness to MSP-1. "Clonal imprinting" or "Original antigenic sin" can be attributed to non-responsiveness of some individuals to certain malaria antigens. This hypothesis suggests that an individual B cell repertoire against parasite antigens might become fixed by his or her first or early exposure to a particular parasite antigenic variant, thereby preventing the recognition of other variant antigens in subsequent infections.

It was shown that 50% of clinically immune adults posses antibodies which binds to MSP-1_{19} and these antibodies recognize disulfide dependent epitopes formed by the two EGF like domains together and do not differentiate between the two variant sequence of MSP-1_{19} (Egan *et al.*, 1995). The lack of appropriate T-Cell help may be a contributing factor to the low prevalence of antibodies to MSP-1_{19} over time despite persistent re exposure to antigen (Egan *et al.*, 1997).

Peptides derived from 83Kda of MSP-1 coupled to TT and immunized in rabbits, induced antibodies reacting with *P.falciparum* asexual blood stages and with the membrane of the schizonts and merozoites from different geographic locations. Lower antibody titres were obtained with synthetic peptides than with the fusion protein, because of low ability of the synthetic peptides to mimic the epitopes present on the native protein. Some peptides were able to raise high antibody titers, but had few antibodies against the native structure on parasites (Cheung *et al.*, 1986). A synthetic vaccine SPF 66 with peptides derived from the block 1 (YSLFQKEKMVL) was tested (Patarroyo *et al.*, 1988). A synthetic peptide polymer which induces a sequence from the N terminus of *P.falciparum* MSP 1 produced encouraging results in the clinical trial (Valero *et al.*, 1993).

ELISA with peptides from MSP-1_{19}, demonstrated that 8 of 35 donors had Abs to peptides, of which 6 sera recognize peptide, representing a conserved sequence at C-terminal end of EGF II. These did not recognize the recombinant antigens MAD 20/EGF II, indicating the presence of linear epitopes (Egan *et al.*, 1997). According to this study a low level of reactivity of sera with linear peptides was not un expected, as most MSP-1_{19} antibodies in malaria immune sera recognize complex, disulphide bond dependent epitopes (Egan *et al.*, 1995).

The levels and distribution of IgG and IgM antibodies to the recombinant MSP-1_{19}, with the first EGF like domain has been studied in *P.falciparum* immune Senegalese adults (Diallo *et al.*, 2001). This first EGF like domain that is recognized by mouse monoclonal antibody and inhibits erythrocyte invasion in vitro (Chappel *et al.*, 1993), is recognized by anti MSP-1_{19} EGF I IgG and IgM from 74% of the individuals tested by ELISA. The

cellular and molecular immunologial events that regulate *P.falciparum* antigen specific antibody formation are not clearly understood, although it is assumed that protection to asexual blood stage *P.falciparum* is T cell dependent and antibody production is likely to result from appropriate T cell help. The protection is mainly mediated by cytophilic antibodies (IgG1 and IgG3), which bind to Fc receptors on the effector cells and act by both specific and non-specific mechanisms (Bouharoun *et al.*, 1995). Invitro studies at the B-cell level in monkeys and humans have demonstrated that MSP-1_{19} stimulates the production of IL10 by activated B cells. This IL10 regulates the switching from μ to either r1 or r3 heavy chain in human B lymphocytes (Briere *et al.*, 1994). Although there are epitopes recognized by human lymphocytes on MSP-1 EGF-I, there is no clear evidence whether the antibodies are functionally antiparasitic or not, The presence of specific antibodies in *P.falciparum* immune individuals, including those against MSP-1_{19}, varies upon epidemiological conditions before or at a time distant from the highest transmission period, suggesting a dynamic process in antibody production and consumption.

The safety and immunogenicity of two recombinant MSP-1_{19} from 3D7 (E-TSR) and FVO (Q-KNG), adsorbed on to the alum were evaluated in a phase 1 trial (Keitel *et al.*, 2000). The E-TSR was found to be equally reactive with antibodies directed against E-TSR and Q-KNG antigens and multiple doses of MSP-1_{19} vaccine candidates elicited significant immune responses, which was predominantly of IgG1 type. The antibody recognition of polymorphic (block2), dimorphic (block6) conserved (block3) regions of MSP 1 in the Amazonian and clinically immune African patients was compared using a panel of recombinant peptides (Silveira *et al.*, 1999). It was found that all blocks were targeted by naturally acquired cytophilic antibodies of the subclasses of IgG1 and IgG3 and the balance between these depended on the subject's cumulative exposure to malaria. This balance and the duration of antibody responses differed in relation to distinct MSP1 peptides. Previous studies have demonstrated that naturally acquired antibodies react more frequently against variable, rather than conserved MSP blocks (Fruh *et al.*, 1991) and are specific for one of the major versions of each variable block (Fruh *et al.*, 1991,Cavanagh *et al.*, 1998).

Several studies have demonstrated an effective immunity conferred by passive administration of IgG from immune adults, which showed a profound reduction in asexual parasitemia when administered by intravenous inoculations. (Cohen *et al.*, 1961, Sabchareon *et al.*, 1991). Studies have identified multiple distinct immunoglobulin binding sites on MSP- 1_{19} that are clustered or overlapping (Wilson *et al.*, 1987, Chappel *et al.*, 1993) and minor differences in the target epitopes may be critical in the function of the corresponding antibodies. It is assumed that natural infection or immunization with MSP-1 EGF1 may stimulate the production of several different anti-MSP-1 EGF1 idiotypes of which only a subset demonstrate the required biological effects (Chappel *et al.*, 1994).

All the above studies emphasize that human antibodies to the block 17 region of *P. falciparum* MSP-1 are associated with a reduced prospective risk of clinical malaria. However, the immunological nature of MSP-1_{19} epitopes during natural infection and the role of IgG antibodies in the acquisition of antimalarial immunity are still unclear. It is still an enigma how natural protection is acquired and maintained in endemic areas. To address these questions, it is very essential to evaluate the IgG antibodies against MSP-1_{19} in natural malaria infected populations from malaria endemic countries like India.

Results

In this study, we have evaluated the natural antibody response against peptides E1 and E2 taken from EGF I and EGF II like domains of *P.falciparum* MSP–1_{19} in naturally infected population. The E2 in turn has been further tested for different alleles designated as E2a, E2b, E2c and E2d (KYG, TSR, TSG, KNG). E2a is a novel allele reported by us in the Indian subcontinent (Kumar et al., 2005. In press).

Antibody profile in natural populations

Of all the 35-malaria patient's sera tested for total IgG, 88 % had antibodies against E1. Among these, 48 % of the sera had an antibody titer above the average (OD > 0.5). 4 patients were detected as negative for IgG antibodies against E1 (Fig 5.1). Two of them did not respond to any of the epitopes evaluated (E1, E2a, E2b, E2c, E2d). Of all the patients evaluated for antibody against E1, one patient (No. 24) had antibody titer two times the average (OD =1.312) and also responded to other epitopes of all the alleles with varying intensities. Patient no. 24 has been detected for a mixed infection with both *P.falciparum* and *P.vivax.* This value is observed to be the highest of the all patient's, evaluated for all the epitopes including all the alleles (Fig 5.1).

Peptides E2a, E2b, E2c, E2d corresponding to four alleles (KYG, TSR, TSG, KNG) of EGF II like domain, with one or two amino acid substitutions in between have also been analyzed. Sera from the 35 patients tested for E1, has been used for this series. Individual responses are shown in Fig 5.2 – 5.5. The difference in the frequency of IgG responses was not significant among the three analogs E2a, E2b and E2d. The exception was E2c, which showed low titre .The response, was slightly higher for E2b. Of all the patient's evaluated, 24 responded to E2a with an average OD 0.344, 29 responded to E2b with an average OD 0.4695, 10 responded to E2c with an average OD 0.30295 and 26 responded to E2d with an average OD 0.316981. 8 patients responded to all the alleles as well as E1. 28 patients responded to more than one allele evaluated and 4 responded to only one allele. Of these 3 responded to E2b, and 1 responded to E2d. There were 2 (17 & 35) patients who didn't respond to any of the epitopes including E1. Patient no. 6 responded to only E1 and didn't respond to any of the E2 alleles. Of all the 32 patients that have responded to at least 1 linear epitope, 29 responded to E2b.

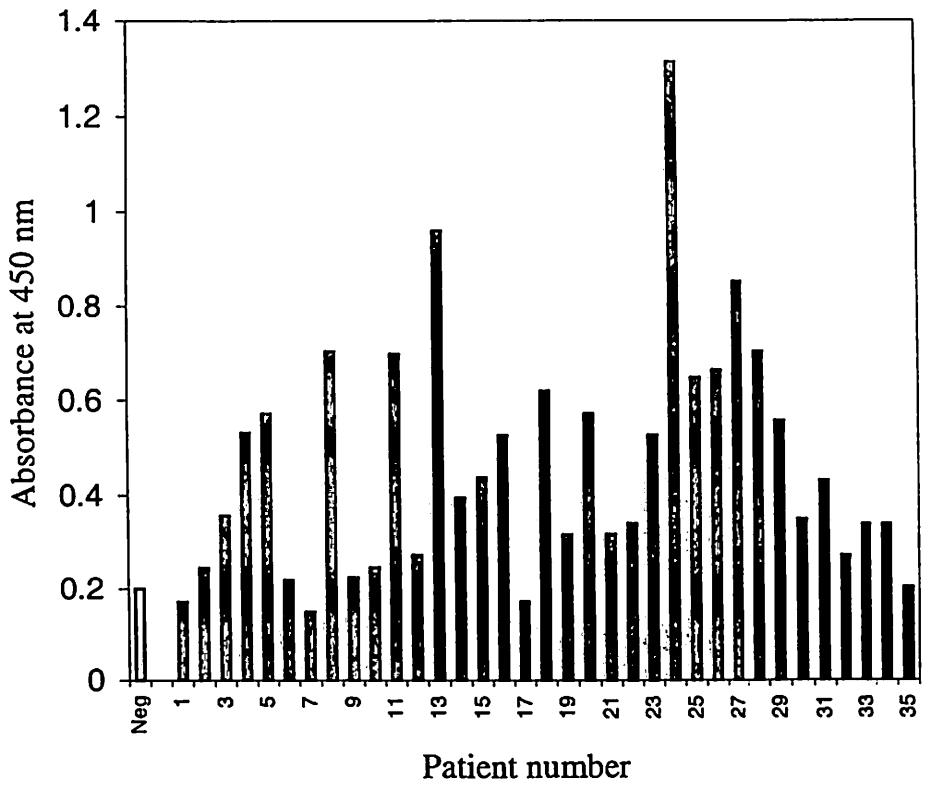


Figure 5.1 Antibody profile against E1

Legend:

Neg- Negative Control

1-35- Patient number

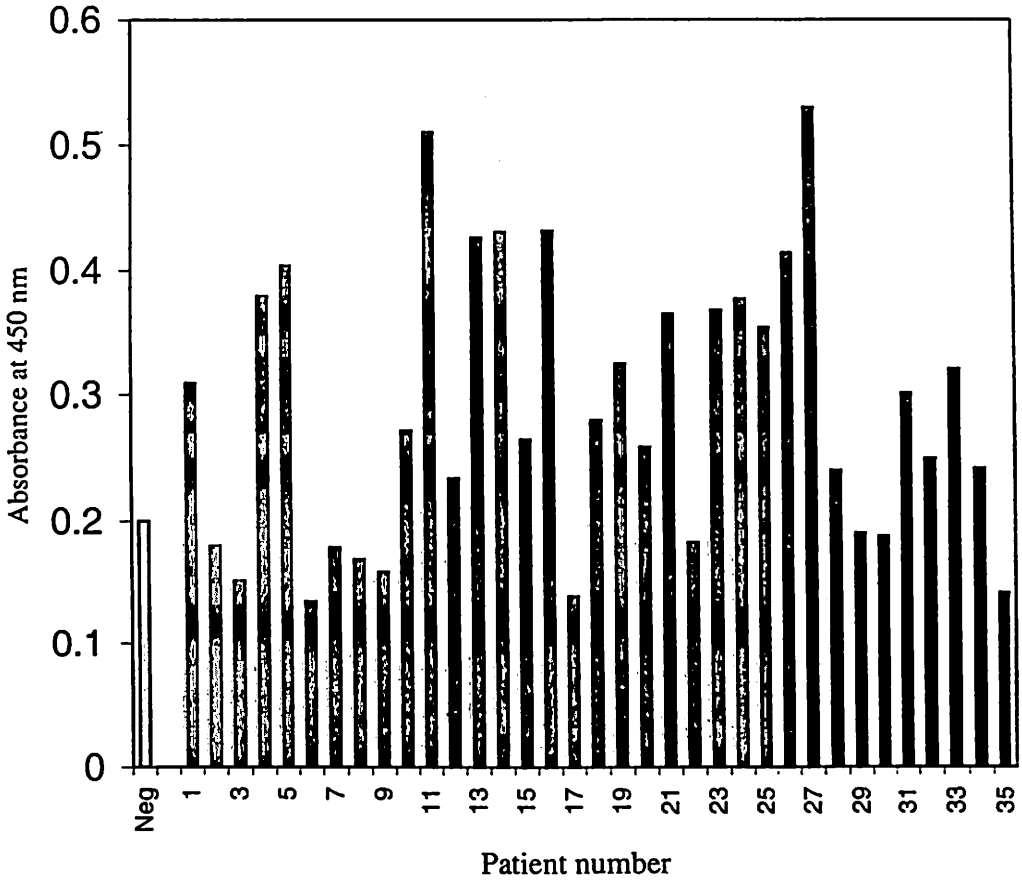


Figure 5.2 Antibody profile against E2a

Legend:

Neg- Negative Control

1-35- Patient number

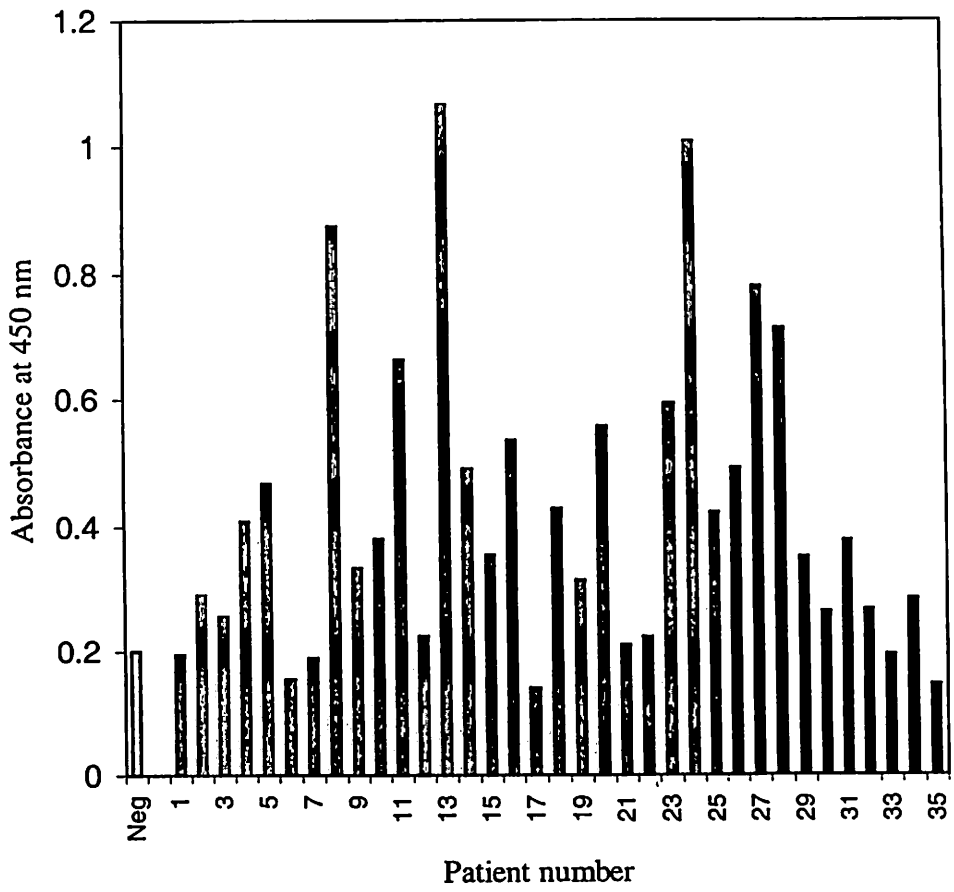


Figure 5.3 Antibody profile against E2b

Legend:

Neg- Negative Control

1-35- Patient number

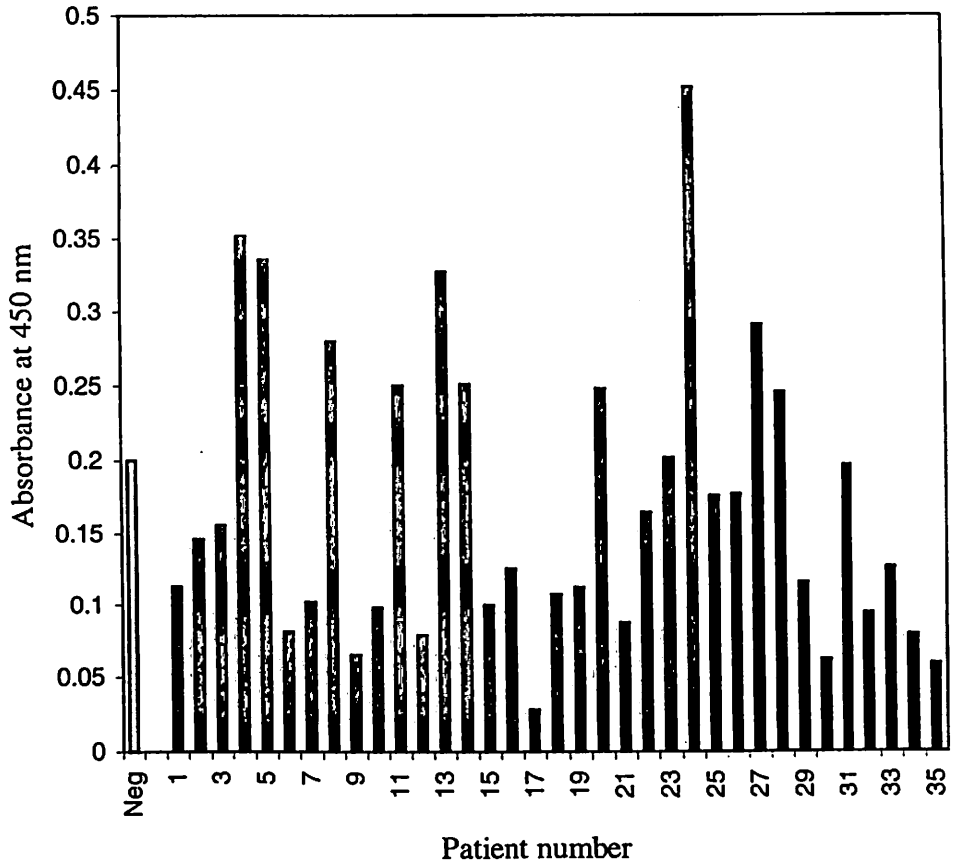


Figure 5.4 Antibody profile against E2c

Legend:

Neg- Negative Control

1-35- Patient number

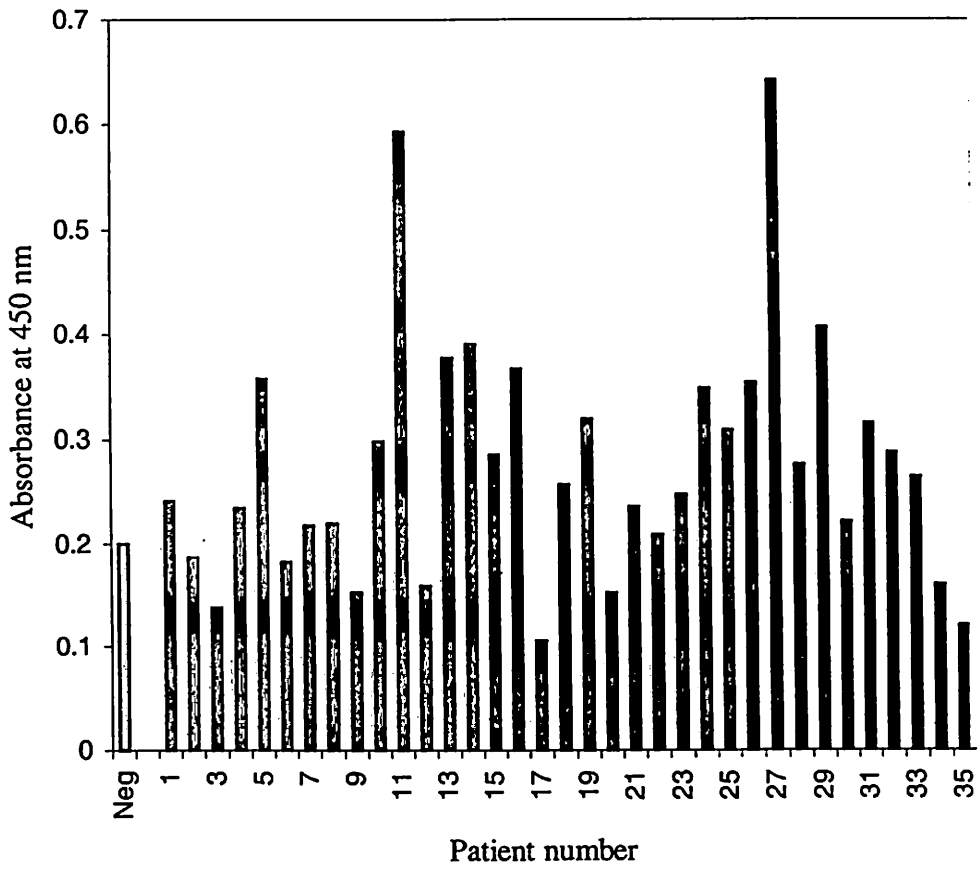


Figure 5.5 Antibody profile against E2d

Legend:

Neg- Negative Control

1-35- Patient number

Discussion

The response to E1 and E2 peptides indicates that linear epitopes may exist in MSP-1_{19} or these peptides best mimic the natural epitopes and are highly immunogenic in natural populations. The sera samples were collected from the patient's at different stages of infection. A major difference was observed between the antibody profiles of E1 and E2 alleles. The response to E1 when compared with that of E2 alleles, demonstrated that E1 is more immuno dominant than E2, as 88 % of the patients responded to E1. Of these, 48% of the patients had an average OD > 0.5 (Fig.5.1). This is much higher than that of all the E2 alleles put together, OD= 0.287001. This first EGF like domain is very significant, as it has been reportedly recognized by a mouse monoclonal antibody and inhibited erythrocyte invasion in vitro (Chappel *et al.*, 1993). It is assumed that natural infection or immunization with MSP-1 EGF1 may stimulate the production of several different anti-MSP-1 EGF1 idiotypes of which only a subset demonstrate the required biological effects (Chappel *et al.*, 1994). Although there are linear epitopes recognized by human lymphocytes on MSP-1 EGF-I, there is no clear evidence to show whether the antibodies are functionally anti parasitic. Whether or not these antibodies against E1 which arise subsequent to natural infection are involved in protection has to be evaluated by further studies.

The high antibody profile against E2b (TSR) could probably indicate that the allele TSR (E2b) is predominant in our samples analyzed or it has the best consensus conformational similarities to all the others as 29 patients responded to E2b from a pool of 32. In contrast, a significant difference was seen in the antibody reactivity profile of E2 alleles between the patients from different areas. More than 73% of the patients from eastern and southern parts of India reacted with all the alleles except E2c, Only 18% of the patients showed reactivity with this allele. This low binding could probably indicate that this allele is less predominant or could be these amino acids that are substituted are probably contact residues. There were several studies outside India reporting that antibodies against different alleles are cross reactive with respect to conformational epitopes on EGF II domain. Our findings clearly demonstrate that antibodies against these putative linear

epitopes of different alleles are also cross-reactive (Fig 5.2 – 5.5). The low antibody prevalence observed, could be because of the short half-life of anti PfMSP-1 antibodies, which are mainly found shortly after clinical episodes (Fruh *et al.*, 1991, Branch *et al.*, 1998, Cavanagh *et al.*, 1998).

Our initial findings appear to suggest that E1 and E2 peptides are mimicking the natural epitopes on EGF like domains of MSP-1_{19} as evident from the antibody titers in naturally infected populations. However, there could also be a possibility that these are linear epitopes existing on MSP-1_{19}, which has to be further investigated. Our data suggest that there is cross reactivity among different alleles, represented by different peptides corresponding to E2 (E2a, E2b, E2c, and E2d). There is also a possibility that the sera collected could be from patients with a mixed parasite population with respect to these alleles. The results of our animal studies, however conclusively show cross reactivity amongst the different E2 alleles, and this information could be extended for the analysis of the human sera. Our findings also suggest that the peptides representing the EGF 1 epitope (E1), has the highest reactivity with all the human sera samples. This indicates that E1 is more immunodominant to E2. The peptide E1 could be taken to represent a linear epitope or could be best mimicked with respect to the native epitope. There could be arguments in favor of conformational bindings because of the presence of three cysteines, two of which are separated by nine amino acids in E1. However since during synthesis, the cysteines added were not modified in any fashion they would have been oxidized immediately after synthesis thereby preventing the cyclisization of the peptides. Whether the antibodies detected in our study are involved in blocking or inhibiting the processing of MSP-1 needs to be further investigated. The different reactivities against peptides representing different potential B-cell epitopes on the MSP-1_{19} could provide information into designing region specific MSP-1 peptide combinations for vaccine strategies.

Chapter 6

Immunogenicity studies with the allele specific peptides in a rodent model

Chapter 6

Introduction

Although repeated exposure to malaria parasites or its antigens can induce immunity to malaria, the efforts to develop a subunit vaccine have not succeeded till date. A number of antigens from different stages of *P.falciparum* with various adjuvants in single and in cocktail have been tested in several systems including humans. Several parasite antigens like CSP, SSP2, LSA1 & LSA2, which are expressed in the sporozoite or EE (Exo-erythrocytic) forms are identified as potential vaccine candidates, which can induce protective immunity. However these vaccines are ineffective once the blood stage infection sets in. During the asexual blood phase, the parasites are released as free merozoites from the protection of intra erythrocytic environment, and exposed to the cells of the immune system. For the protective immunity to be effective at both the liver and blood stages of the malaria life cycle, it is essential to look for the antigens that are expressed at both the stages. Some malaria antigens are expressed at both the EE and blood stages of the parasite (Aley, S.B., *et al.*, 1987). Several studies showed that the precursors and proteolytic products of MSP-1 are expressed in the EE stage; using Mab's specific for MSP-1 (Szarfman *et al.*, 1988, Suhrbier *et al.*, 1989) and this MSP-1 is found in all the malarial parasites. A recent study (Kawabata *et al*, 2002) has indicated MSP-1 specific immune response is also protective against exoerythrocytic forms of *Plasmodium yoelii* and can be induced in animals over a wide range of genetic backgrounds. MSP-1 is synthesized by the intracellular schizont of the asexual blood and liver stages and expressed on the surface of merozoites released from the ruptured schizont (Holder *et al.*, 1988, Diggs *et al.*, 1993).

Successful vaccination experiments with irradiated sporozoites (Clyde *et al.*, 1975), blood stage parasites (Siddiqui *et al.*, 1979) done in 1970's using whole parasites as immunogens demonstrated that *P.falciparum* is vulnerable to immune attack in experimental infections and paved the way further to explore and exploit the parasite

components or antigens to be used as candidate vaccine antigens. It was shown that Mab purified proteins on the merozoite surface of *P.yoelii* were important classes of protective antigens in the murine malaria model (Holder *et al.*, 1981) and this study demonstrated the significance of MSP-1 as an important vaccine candidate. Further, the immunization studies done using the precursor protein and its processing fragments isolated by Mab's (Hall *et al.*, 1984) and with the precursor protein alone isolated by SDS PAGE (Perrin *et al.*, 1984) partially protected *Saimiri* (squirrel) monkeys against heterologous challenge, indicating the involvement of conserved regions of this protein. The merozoite surface coat precursor protein also referred to as P190, gp195 or PSA (polymorphic schizont antigen) is classified into seven serological groups exhibited by 37 strains from different geographical areas of the world, using a panel of monoclonal antibodies (Mc Bride *et al.*, 1985).

MSP-1 is thought to play a major role in the protective response and in particular the C-terminal cysteine rich 19-kDa domain of MSP-1 ($MSP\text{-}1_{19}$) has been identified as protective from several studies. $MSP\text{-}1_{19}$ generated after processing is similar in all allowing the use of various malaria parasites and animal models for the study of $MSP\text{-}1_{19}$ induced protection (Ahlborg *et al.*, 2002). Only limited sequence polymorphism has been identified in *P. falciparum* $MSP\text{-}1_{19}$ (Miller *et al.*, 1993, Lalitha *et al.*, 1999, Qari *et al.*, 1998, Kumar *et al.*, 2005 In press) despite extensive molecular epidemiological investigations. This sequence conservation implies that $MSP\text{-}1_{19}$ is functionally constrained and remains bound to the merozoites as it invades RBC's and thus can be used as potential vaccine candidate. This antigen has been extensively studied in murine, simian and human systems, where active and passive immunizations confers partial to complete protection against asexual blood stage of *P. falciparum.*

A recent study (O'Donnell *et al.*, 2000) has shown that the role of $MSP\text{-}1_{19}$ in red blood cell invasion is conserved across distantly related *Plasmodium* species and the sequence of Pf $MSP\text{-}1_{19}$ is not constrained by the function. In this study the function of most of $PfMSP\text{-}1_{19}$ has been successfully complemented with the corresponding but highly divergent sequence from the rodent parasite *P.chabaudi.*

Several studies were done using native parts of MSP-1, recombinant MSP-1 $_{19}$ (Hall *et al.*, 1984, Perrin *et al.*, 1984, Siddiqui *et al.*, 1987, Holder *et al.*, 1988, Burns Jr. *et al.*, 1989, Etlinger *et al.*, 1991, Daly *et al.*, 1993, Diggs *et al.*, 1993, Ling *et al.*, 1994) or as synthetic peptides (Cheung *et al*, 1986, Patarroyo *et al.*, 1987) which induced complete or partial protection or delayed the progress of infection against homologous or heterologous infections and demonstrated the presence of conformational epitopes on MSP-1_{19}. Several in vitro studies with the human malaria parasite *P.falciparum* (Blackman *et al.*, 1990, Cooper *et al.*, 1992, Chang *et al.*, 1992) and *in vivo* studies with *P.yoelii* (Majarian *et al.*, 1984, Spencer *et al.*, 1998) demonstrated that Mab,s to reduction sensitive epitopes in this MSP-1_{19} can inhibit erythrocyte invasion. Many studies have reported that epitopes present in the EGF like domains are conformational in nature and are structurally constrained (Somner *et al.*, 1999, Spencer *et al.*, 1998). A number of MSP-1 specific Mab,s including the invasion inhibitory antibodies bind to the disulfide constrained epitopes in the first of the EGF-like domains (Chappel *et al.*, 1993). It was shown that, the specificity of a monoclonal antibody which inhibits *P.chabaudi in vivo* is determined by a single amino acid (Mc Kean *et al.*, 1993) and single amino acid differences can affect the binding of individual Mab,s to MSP-1_{19} or to the individual EGF like domains (Benjamin *et al.*, 1999, Tolle *et al.*, 1995). It was demonstrated that Mab 302 which recognized an epitope in the first EGF like domain of *P.yoelii* MSP-1_{19}, protected mice against a lethal parasite challenge (Burns *et al.*, 1989). The first EGF like domain is the target of growth inhibitory antibodies and a single amino acid difference influences the binding of some antibodies to the domain (Chappel *et al.*, 1993, Uthaipibull *et al.*, 2001). The crystal structure of C-terminal merozoite surface protein 1 from *P.cynomolgi* (Chitarra *et al.*, 1999), and *P.falciparum* (Morgan *et al.*, 1999) and the sequence comparison of two EGF –like domains strongly suggest that the conformation is conserved in all *Plasmodium* species including *P.falciparum* and *P.vivax* (Chitarra *et al.*, 1999).

This cysteine rich domain of MSP-1_{19} has many (five) conserved disulfide bonds generating a folded structure with two EGF like domains (Blackman *et al.*, 1991). MSP-

1_{19} has been expressed in E coli in the native folded state as judged by antibody binding (Chappel *et al.*, 1993, Burghaus *et al.*, 1994). The involvement of both the EGF like motifs in MSP-1_{19}, for the induction of a protective immunity has been demonstrated (Ling *et al.*, 1995). In a significant study (Calvo *et al.*, 1996) the efficacy of the individual EGF domains was compared with that of native structure having both the domains. In this study mice were immunized with individual EGF like domain from *P.yoelii.* Although all animals developed some level of antibody only those animals immunized with both EGF-domains produced antibodies, which could recognize the native MSP-1 molecule. Antibodies generated by animals capable of recognizing native MSP-1 protected from challenge infection, although antibodies generated against the individual EGF like domain did cross-react with the double EGF like domain structure. These results suggest that epitopes unique to the double EGF like domain structure may be necessary for generation of protective antibodies. MSP-1_{19} has several well-defined discontinuos epitopes (McBride *et al.*, 1987, Farley *et al.*, 1995, Uthaipibull *et al.*, 2001).

MSP-1_{19} mediated immunity is thought to be antibody dependent. Although both antibodies and effector T cells participate, it was found that the protective immunity induced by vaccination with recombinant MSP-1_{19} is largely mediated by antibodies in rodent and other experimental models (Daly *et al.*, 1995, Hirunpetcharat *et al.*, 1997, Tian *et al.*, 1998,) and the epitopes in this region are the targets of antibodies which inhibit erythrocyte invasion in vitro (Pirson *et al.*, 1985, Blackman *et al*, 1990, Cooper *et al.*, 1992). Several studies have shown that the protection mediated by MSP-1_{19} is by antibodies and not by the T cells (Daly *et al*, 1995, Hirunpetcharat *et al.*, 1997, Tian *et al.*, 1998, Wipasa *et* al., 2002,). Linking additional T cell epitopes in particular when using peptides or subunits can induce protection by enhancing the levels of IgG1 and IgG2a antibodies. This has been demonstrated in monkeys vaccinated with the Pf & Pv MSP-1 $_{19}$ protein linked to T cell epitopes from Tetanus Toxoid (Kumar *et al.*, 1995, Yang *et al.*, 1999).

Several factors are involved in mediating protective immunity against C terminus MSP-1. These include Th1 and Th2 cell subsets (De Souza *et al.*, 1996) together with the IgG1,

IgG2a and IgG2b sub classes along with IFN-r and IL4. Studies in mice has shown that protective immunity induced by MSP-1_{19} is dependent on high titer of specific antibodies present at the time of challenge (Daly *et al.*, 1995, Hirunpetcharat *et al.*, 1998) and an active immune response post challenge that is dependent on B and T cells is critical for MSP-1_{19} induced protective immunity (Hirunpetcharat *et al.*, 1998). It is not clear whether an MSP-1_{19} specific response post infection is sufficient for protection. But in consensus it is clearly demonstrated that high titre of antibodies alone mediate a major role in protective immunity induced by MSP-1 $_{19}$ (Wipasa *et al.*, 2002). The protection mediated by the antibodies is by blocking the processing of the larger mature MSP-1 protein on the merozoite surface (Blackman *et al.*, 1994) or by sterically hindering the merozoite invasion of erythrocytes (Hirunpetcharat *et al.*, 1999). Conserved epitopes are the most desirable, but they are often poorly immunogenic. The binding of an antibody to the carboxy terminal region of MSP-1 on the merozoite surface may not be sufficient to prevent erythrocyte invasion. The interaction of different antibodies with adjacent epitopes within the EGF like domains of MSP-1 may show significant effect (Blackman *et al.*, 1994).

Several parameters are considered essential to enhance the protection with recombinant proteins. These include the optimized immunization protocols as well as the use of different adjuvants. Adjuvants are considered to play a major role in conferring protective immunity in mice and monkey models, when immunized with MSP-1_{19}. Several studies have shown that the choice of adjuvant is crucial in determining the immunogenicity of blood stage malaria vaccine. Blood stages of parasites of *P. yoelii* were tried with different adjuvants like Saponin, *Bordetella pertusis*, Co polymer p1004, detoxified preparation of lipopolysachariide. Various adjuvants have been tested in several systems including alum-based vaccines, which were poorly immonogenic in humans (Patarroyo *et al.*, 1988), whereas in monkeys and rabbits the most effective vaccines required the use of FCA (Patarroyo *et al.*, 1987, Gozalo *et al.*, 1998, Kumar *et al.*, 2000, Angov *et al*, 2003), which is not acceptable in humans. Along with the adjuvant, route of antigen administration also plays a major role in inducing immunity. Blood stage *P.yoelii* with saponin as the adjuvant was very effective when given intrpertoneally but less effective

when given subcutaneously (Hui *et al.*, 1998). A recent study has demonstrated (Angov *et al.*, 2003) that MSP-1 $_{42}$ along with adjuvants like alum and others like SGK were safe and immunogenic in mice. Functional antibodies from rabbits vaccinated with MSP-1_{42} with Freund's adjuvant inhibited parasite growth *in vitro* and also inhibited secondary processing of MSP-1_{42} to MSP-1_{33} and MSP-1_{19}. Some studies suggest that animal species variability in responses to vaccine adjuvants is due to differences in the utilization of immune system pathways by an adjuvant among animal hosts (Hui *et al.*, 1998) and studies on animals immunized with malaria vaccine antigens, shows that adjuvants influence the specificities of immune responses induced, the MHC regulated responsiveness (Hui *et al.*, 1991, Daly *et al.*, 1996, de Souza *et al.*, 1996) and also vary with the animal species or sub species.

A first report of complete protection by infecting *P. falciparum* merozoite surface coat protein in *Aotus* monkeys with FCA demonstrated that adjuvant plays a major role. When the formulation with a different synthetic adjuvant (MDP) was used, there was no protection though antibodies were elicited. This study hypothesizes that there is a possibility that protective antibody in nature is directed at weakly immunogenic or antigenically diverse epitopes and levels adequate for complete protection might be induced only after repeated infections or with strong adjuvants (Siddiqui *et al.*, 1986).

Several studies were done using synthetic peptide vaccines of MSP-1. Patarroya *et al.*, 1987 showed the induction of protective immunity against experimental infections with malaria using synthetic peptides. Partial protection was observed in *Saimiri* monkeys immunized with a peptide comprising residues 24-67 of blocks 1 and 2 of MSP-1 (Cheung *et al.*, 1986) and in a similar experiment, immunization with a peptide comprising residues 43-53 delayed the onset of disease in *Aotus* monkeys (Patarroyo *et al.*, 1987). In one study (Lew *et al.*, 1989) BALB/c mice when immunized with *P.chabaudi adami* IC preparations and challenged, a protective monoclonal antibody recognized a linear epitope in the precursor of MSP of *P chabaudi adami*. Mice passively transferred with these antibodies were protected. This epitope is at the start of block 8,

which is a variable block. To further check this, the peptide was synthesized and tested with the monoclonal antibody.

A synthetic gene was constructed encoding 12 B cell, 6 T cell proliferative and 3 cytotoxic T lymphocyte epitopes obtained from stage specific, *P.falciparum* antigens corresponding to the sporozite, liver, erythrocyteic asexual and sexual stages (Shi *et al.*, 1999). This gene termed as CDC/NIIMALVAC-1 was expressed in baculovirus system. Rabbits immunized with this elicited antibody responses, recognizing vaccine anitigens, linear peptides contained in the vaccine and all the stages of *P.falciparum*. Though the antibody levels obtained to MSP-1_{19} Bcell epitopes are very low, they are effective. These MSP-1_{19} B cell epitopes are naturally immunogenic and have been implicated in protection by *in vitro* studies. Though these epitopes are said to be confirmative, results obtained from the study clearly indicates linear epitopes also play a vital role, though not that effective when compared to that of the native protein MSP-1_{19} (Shi et *al.*, 1999).

P.falciparum MSP-1 peptides from 19 kDa. interacted with human RBC's and inhibited merozoites invasion of RBC's (Urquiza *et al.*, 1996). MSP-1_{19} has been shown to demonstrate high activity peptide binding to human red blood cells (MLNISQHQCVKKQCPQNS). This study has demonstrated that by altering a few amino acids induced immunogenicity and protectivity against experimental malaria was induced (Torres *et al.*, 2003).

MSP-1_{19} specific monoclonal antibodies and antiserum passively protected mice against challenge infection with homologous blood stage parasites, in studies using the rodent malaria models *P.yoelii* and *P.chabaudi* (Boyle *et al.*, 1982, Majarian *et al.*, 1984, Lew *et al.*, 1989, Daly *et al.*, 1995, Spensor *et al.*, 1998). These antibodies are shown to be reactive with MSP-1_{19} (Chang *et al.*, 1996) and EGF like domains are the targets for these protective antibodies. MSP-1 is also synthesized by *P.berghei* (New bold *et al.*, 1982). Passive injection of human antimalarial antibodies inhibits the proliferation of asexual blood stages of *P. falciparum in vivo* (Cohen *et al.*, 1961). These antibodies seem

to act against merozoites, which are the invasive forms of the parasite during the asexual cycle.

A vaccine should stimulate an immune response in individuals of diverse genetic make up, to be effective. If widespread, genetically controlled non-responsiveness is a common characteristic of malaria antigens, the feasibility of the subunit vaccine strategy will be severely compromised. The genetic control of immune responsiveness to most of the malarial antigens of the sexual stages of the parasite life cycle has been demonstrated. The effect of the MHC genes or responsiveness to asexual blood stage antigens are to be still explored. It is very essential to determine whether immune responsiveness to gp195 is restricted to certain MHC haplotypes. Individuals of diverse major MHC, are capable of recognizing the gp 195 antigen, the recognition of B and T –cell epitopes being/may be under the control of MHC (Chang *et al.*, 1989).

Cross Reactivity Studies

Although the region(s) responsible for this immunological and biological cross reactivities was not clearly explored, the above studies suggest that a blood stage vaccine based on MSP-1 may be effective against heterologous infections and in particular which is essential in a malaria endemic country like India where different alleles of MSP-1 are reported (Raj *et al.*, 2004, Lalitha *et al.*, 1999, Kumar *et al.*, 2005 In Press). The sequence analysis of MSP-1 gene and Mab epitope analysis revealed two regions within conserved block 17 where amino acid changes gives rise to variant B-Cell epitopes recognized by Mab's (Cooper *et al.*, 1992). The amino acid substitutions in 17^{th} block of p42 includes GLU to GLN at position 1654, LYS 1691, ASN 1700 and GLY 1701 to THR, SER and ARG (the above variant B cell epitope recognized by Mab 4H4/34 by Cooper *et al.*, 1992). This study shows that Major Cross- reactive and inhibitory B-Cell epitopes of MSP1 lies within the conserved regions of the p42 fragment.

Till date many studies were done to check the immunological cross reactivity of MSP-1_{19} (Etlinger *et al.*, 1991, Kumar Sanjai *et al.*, 2000, Stowers *et al.*, 2001, Singh *et al.*, 2003) demonstrating that antibodies cross-reacted extensively with heterologous MSP-1 alleles, suggesting that determinants within conserved block 17 are dominant B cell epitopes and antibodies were equally effective in inhibiting the *in vitro* growth and the parasite carrying homologous and heterologous MSP1 alleles. However there were also studies where immunization with a recombinant fusion protein containing the two *P.yoelii* EGF like domains protected mice against a homologous but not a heterologous challenge (Benjamin *et al.*, 1999) and rabbits immunized with one allelic form (Q-KNG) mount an antibody response with various degrees of specificity, which in some cases can entirely be allele specific (Stowers *et al.*, 2001). That such specific responses can be mounted should come as no surprise now that the 3D structure of MSP-1_{19} is known (Chitarra *et al.*, 1999, Morgan *et al.*, 1999). The most surface exposed portion of the molecule is the KN – TS loop. So it is easy to imagine this being a prominent and entirely specific B-cell epitope. Indeed, although the studies mentioned above using polyclonal antiserum demonstrated no allele specificity, it has been previously noted that monoclonal antibodies differentiate epitopes defined by both E-to-Q and the TSR-to-KNG variations (Tolle *et al.*, 1995).

The ability of the recombinant protein Vs native protein to protect against a heterologous challenge with *P.falciparum* was tested (Etlinger *et al.*, 1991). In this study monkeys were immunized with recombinant antigens and native gp190 from *P.falciparum* K1, Thai isolate and challenged with Palo Alto (from Uganda, Africa). Results indicated that native protein/processed product provided excellent protection against heterologous challenge and antibodies mediated this protection.

The ability of antigens to Prime host for memory responses, upon natural challenge infections is of prime concern designing any vaccine using these antigens. Some experiments showed that immunizations with one allelic form of MSP-1 could prime for antibody response upon secondary challenge with a heterologous MSP-1 protein (Hui *et al.*, 1993). Very little work is done on allele specific immunogenecity among the isolates

in Indian subcontinent. In one such study (Lalitha *et al.*, 1999) the MSP-1_{19} protein from two of the isolates representing Q-KNG and E-TSG, respectively were expressed in *E.coli* and screened for reactivity with Mab's polyclonal antisera specific for MSP-1_{19} and sera collected from *P.falciparum* infected patients from central and northern India to assess their antigenicity and conformational integrity. Mab 111.4 for the Q variant at positions 1644 (Chappel *et al.*, 1993) strongly reacted in western blot with Q-KNG variant but not with the E-TSG variant where as Mab 1e1 reacted only with the Q-KNG variant this differential reactivity is because of the changes in amino acids sequence and suggested the presence of the native immunodominant conformational epitopes, which is in accordance with earlier reports (Burghaus *et al.*, 1994; Kaslow *et al.*, 1994). It has been shown that (Ahlborg *et al.*, 2002) when mice vaccinated with MSP-1_{33} and MSP-1_{19} of *P.yoelii* were subsequently challenged with the lethal *P.yoelii* blood stage, mice immunized with MSP-1_{19} were protected where as the other group failed, demonstrating that the protective immune responses for the 42- Kilo Dalton (kDa) region of *P.yoelii* MSP-1 are induced by the C-terminal 19Kda region but not by the adjacent 33Kda region.

A.nancymai monkeys when immunized with MSP-1_{19} of *P.falciparum* identical to FVO and challenged with FVO (homologous) and CAMP (MAD20) MSP-1_{19} failed to induces protection against the challenge, though the sera partially blocked the binding to MSP-1_{19} of Mab's that inhibit erythrocyte invasion in vitro (Burghaus *et al.*, 1996). These results also indicate that animals developed at least some antibodies, recognizing epitopes on MSP-1_{19}, however these antibodies were present in low titres as high concentration of serum were required in the blocking assay. In particular with Mab that binds to these epitopes this and other studies demonstrate that high concentration of antibody to MSP-1_{19} can prevent erythrocyte invasion (Burghaus *et al*, 1996). The concentration of the antibody against MSP-1 $_{19}$ plays a major role in controlling the infection (Gozalo *et al.*, 1998).

It is very essential to determine whether or not sequence variation may results in antigenic differences, which enables parasites with one sequence to evade the immune

responses to another. The MSP-1_{19} B cell epitopes, which harbor single amino acid substitutions, are thought to be associated with the parasite immune evasion and represent one major obstacle to malaria subunit vaccine development. It is also of prime importance to understand how conserved an epitope has to be to serve as a suitable vaccine candidate. Although several studies were done in murine models, little work has been done for *P.falciparum* alleles using synthetic peptides from MSP-1_{19}. To address these questions, we analyzed different allelic peptides representing EGF I and EGF II like domains of PfMSP-1_{19} from Indian subcontinent, where infection with parasites having one or more allelic forms of MSP-1 are common. We have evaluated the immunogenicity and allele specific sero cross reactivity of these peptides in a murine model.

Results

Immunization of BALB/c, C57BL/6J and Swiss Albino mice with peptides corresponding to B-cell epitopes of MSP-1 $_{19}$ alleles

Four experimental sets of five mice each for BALB/c, C57BL/6J and Swiss Albino mice were used for this study. Four different allelic peptides from EGF II like domain of PfMSP- 1_{19}, corresponding to KYG, TSR, TSG and KNG alleles along with E1 from EGF I like domain were used for immunizations. Each mouse was give 50 μg of E2 allelic peptide in combination with 50 μg of E1 peptide, adsorbed on the alum and administered sub-cutaneously. Three boosters were given every 21-day after priming. Animals were bled the 9^{th} day after every booster and sera (at 1/100 dilution) were assayed for antibodies by ELISA. There was no major difference in the antibody profile of E1 and E2 alleles. The antibody response for E1 as well as E2 alleles were high after the last booster. Difference in the antibody profile was observed with in the different strains of mice having different genetic make up (Fig 6.1 & 6.2).

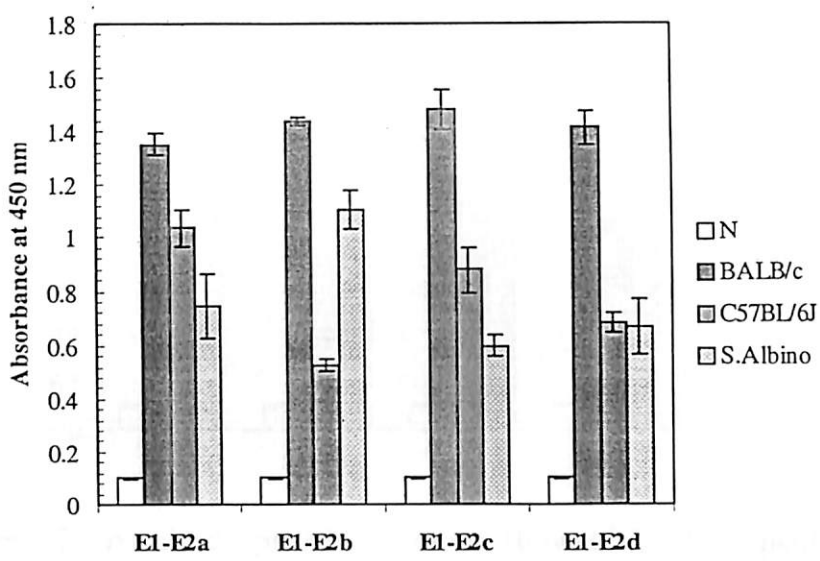


Figure 6.1 Antibody profile against E1 in different strains of mice immunized with E1 along with E2 allelic peptides

All the Values represent ± 1 SE

Legend:

N-negative Control
S.Albino-Swiss Albino

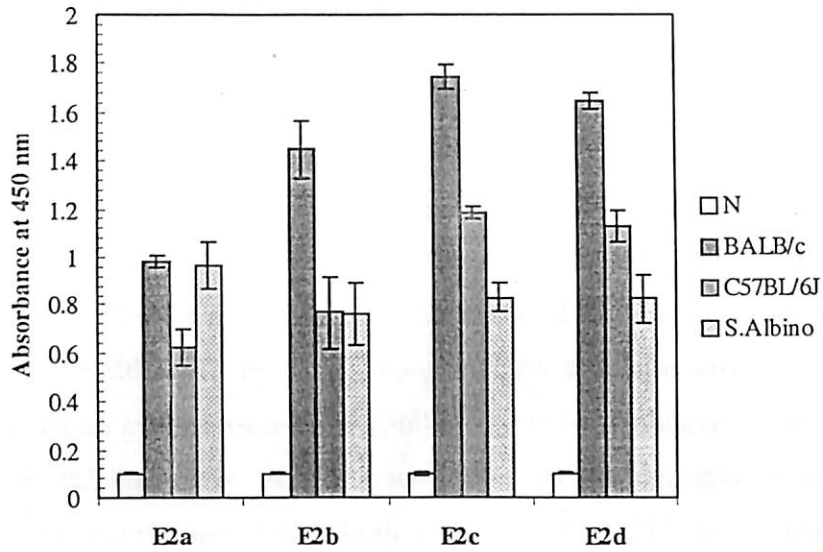


Figure 6.2 Antibody profile against different E2 allelic peptides

All the Values represent ± 1 SE

Legend:

N-negative Control
S.Albino-Swiss Albino

Antibody responses to different allelic variants of MSP-1_{19}

Sera from mice vaccinated with E2a (KYG) were assayed for binding to E2a (KYG), E2b (TSR), E2c (TSG) and E2d (KNG). All the assays were performed in duplicate at 1/100 dilution. All the four peptides differ with each other with one or two amino acid substitutions in between. Sera reacted to different extent with all the alleles. Similar findings were obtained when sera from E2b, E2c and E2d were assayed for binding to all the other allelic peptides. There is no strain specific response observed and all the epitopes of E2 alleles are cross-reactive. Similar results were obtained with all the three BALB/c, C57BL/6J and Swiss Albino mice. Since the immunizations were done in cocktail with epitopes corresponding to both the EGF 1 and EGF 2 like domains for each allele, sero cross reactivity studies were done by eliminating E1 peptides during ELISA, to avoid false positives (Fig 6.3a, 6.3b, 6.3c, 6.3d).

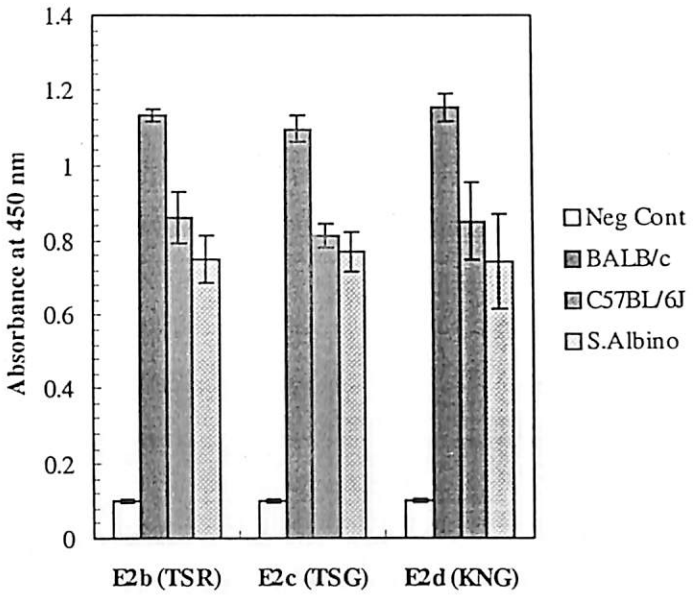


Figure 6.3a Sera of E2b, E2c, E2d tested against E2a (KYG)

All the Values represent ± 1 SE

Legend:

N-negative Control
S.Albino-Swiss Albino

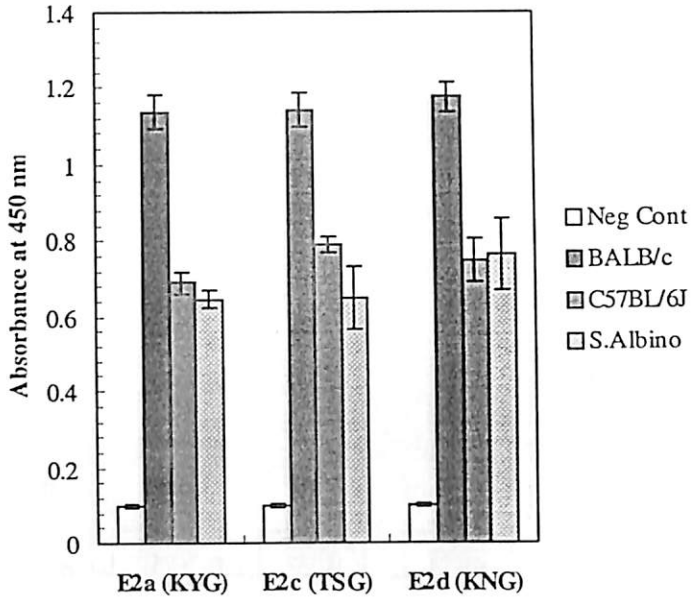


Figure 6.3b Sera of E2a, E2c, E2d tested against E2b (TSR)

All the Values represent ± 1 SE

Legend:

N-negative Control
S.Albino-Swiss Albino

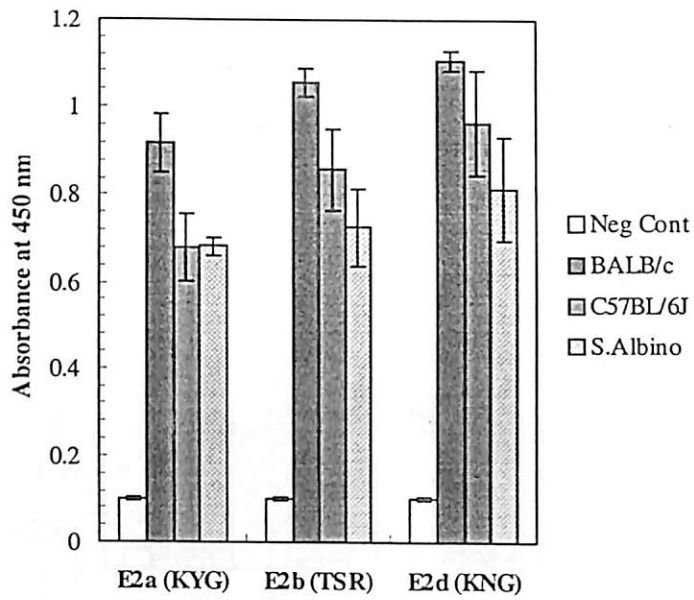


Figure 6.3c Sera of E2a, E2b, E2d tested against E2c (TSG)

All the Values represent ± 1 SE

Legend:

N-negative Control
S.Albino-Swiss Albino

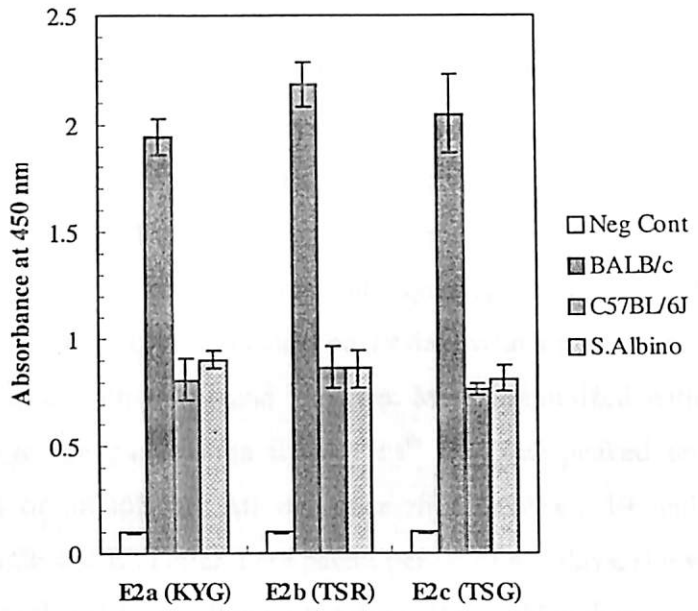


Figure 6.3d Sera of E2a, E2b, E2c tested against E2d (KNG)

All the Values represent ± 1 SE

Legend:

N-negative Control
S.Albino-Swiss Albino

Heterologous *P.berghei* challenge in mice immunized with peptides from PfMSP-1 $_{19}$ variants

To check the efficacy of the peptides derived from the several PfMSP-1_{19} alleles in controlling the infection and to see whether there is any cross species responses in protection, mice immunized with different allelic peptides of PfMSP-1_{19}, were challenged with 10^6 lethal *P.berghei* parasite administered intra peritoneally. BALB/c, C57BL/6J and Swiss Albino mice previously immunized with E2a, E2b, E2c and E2d along with E1 were infected along with a set of naïve control mice. Following challenge, the pre patent periods were 4-5 days for all the experimental sets followed by a low parasitemia till the 9th day for all the animals. The parasitemia subsequently increased rapidly as depicted in the Figure 6.4, for the controls and peaked on 19 day, with a parasitemia of 65.93 %. All the control mice died with in 17 and 21 days. Mice immunized with E2a, showed a significant delay in the parasitemia till the 13th day and peaked on 21 day with a parasitemia level of 38.363 %. All the mice died between 19 and 21 days. Mice immunized with E2b and E2d after a pre patent period of 4-5 days, showed a delayed and low parasitemia levels till 11 and 9 days and died. This sudden death could be because of several reasons and needs further investigation. However, a significant pattern was observed for E2c. In these a brief persistent low-level parasitemia was observed till 9th day, after a prepatent period of 4-5 days. This was followed by a zig zag profile with the parasitemia reaching 48.2% on the 19th day after initial challenging and a sudden drop to 25.4% on 21 day. This again peaked on 27 day with the parasitemia at 81%. 50% of the E2c immunized mice died in between 17 and 19 day, where as the rest died when they reached the peak parasitemia (Fig 6.4).

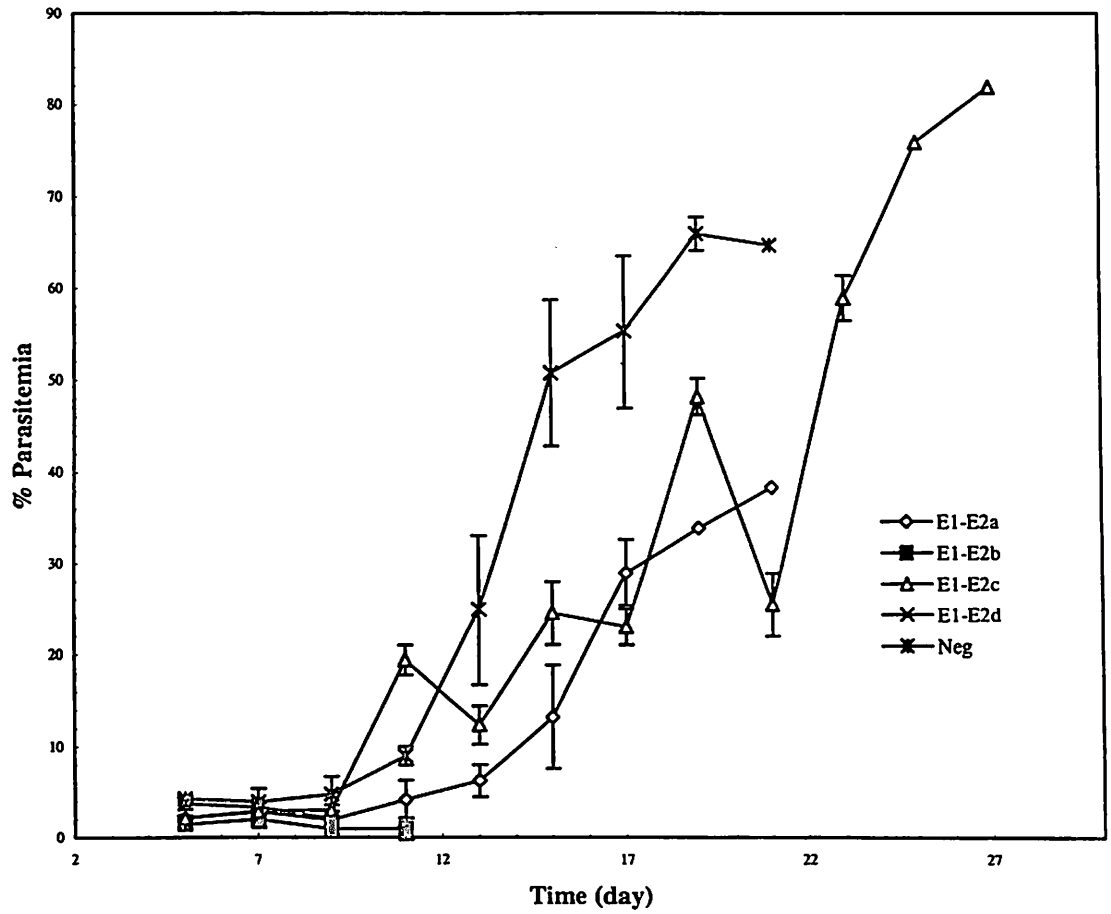


Figure 6.4 Parasitemia profile of Swiss Albino mice challenged with *P.berghei*

All the Values represent ± 1 SE

Points with out error bars indicates one or two animals alive and intra-assay errors were very low

Pre and Post Challenging Antibody measurements

To check if the *P.berghei* can boost and stimulate the antibody response in mice immunized or primed with PfMSP-1 $_{19}$ allelic peptides, the challenging with *P.berghei* was done when the antibody levels were depleted as evident from the figure 6.5 & 6.6, in Swiss Albino mice immunized with different alleles. After initial challenging, ELISA was performed in duplicate with the sera taken on the 9th day for all the experimental sets (E1 + E2a, E1 + E2b, E1 + E2c, E1 + E2d). The sera was tested for the antibodies against different peptides E1, E2a, E2b, E2c, and E2d and compared with that of the sera taken from challenged, but un-immunized mice serving as negative controls. Results indicate that there was an increase in the antibody response post challenge, and is high for E2b (TSR) and E1 as were shown in the fig 6.5 & 6.6.

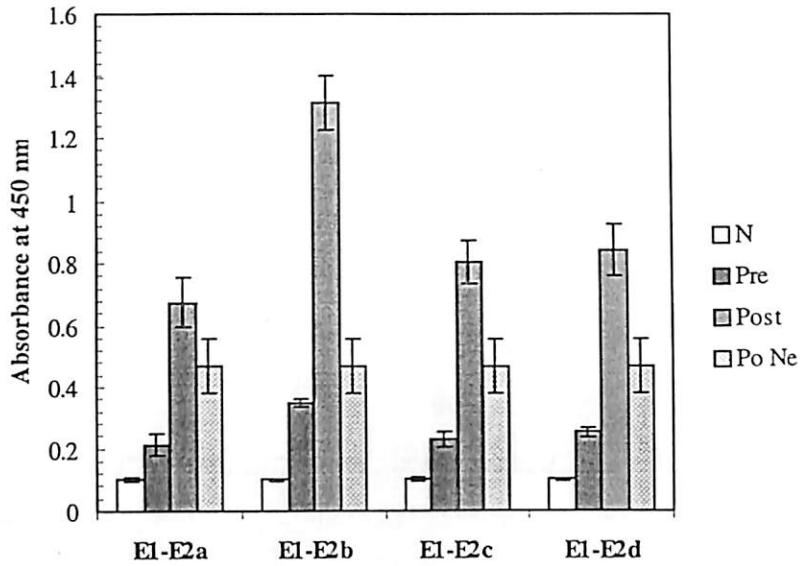


Figure 6.5 Comparison of pre and post challenging antibody profile against E1

All the Values represent ± 1 SE

Legend:

N- Naïve Negative Control
Pre-Pre challenging antibody
Post-Post challenging antibody
Po Ne- Negative controls (Challenged but un-immunized)

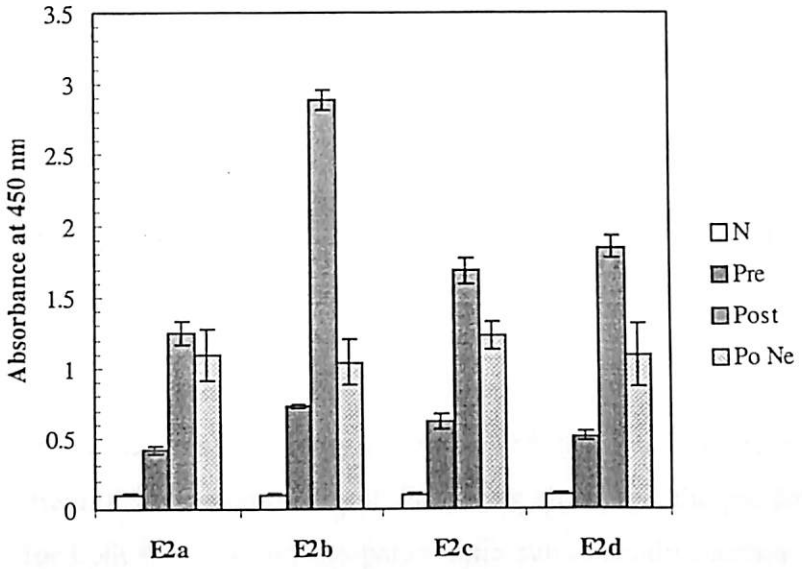


Figure 6.6 Comparison of pre and post challenging antibody profile against different E2 alleles

All the Values represent ± 1 SE

Legend:

N- Naïve Negative Control
Pre-Pre challenging antibody
Post-Post challenging antibody
Po Ne- Negative controls (Challenged but un-immunized)

Passive immunization suppressed and delayed parasitemia

Two sets of mice (Swiss Albino) of five in each were used for this experiment. One set was kept as negative control and the other set was administered intravenously from day 0, with the pooled hyper immune sera, every 7 days till 28th day. Both the sets were infected with the lethal strain of *P.berghei* on day 0. Following challenge, the pre patent periods were 4-5 days for both the sets and the parasitemia subsequently increased rapidly as depicted in the figure (6.7), for the controls and peaked on the 19th day, with a parasitemia of 65.93 %. All the control mice died within 17 to 21 days. However, for the passively protected mice, the patency periods were markedly prolonged and all the mice developed persistent but low-level parasitemia until day 17. The parasitemia peaked on day 29 of initial challenging with 49.787%, much less when compared with that of the controls. All these mice were alive till the parasitemia peaked and died between 29th and 33rd days (Fig 6.7).

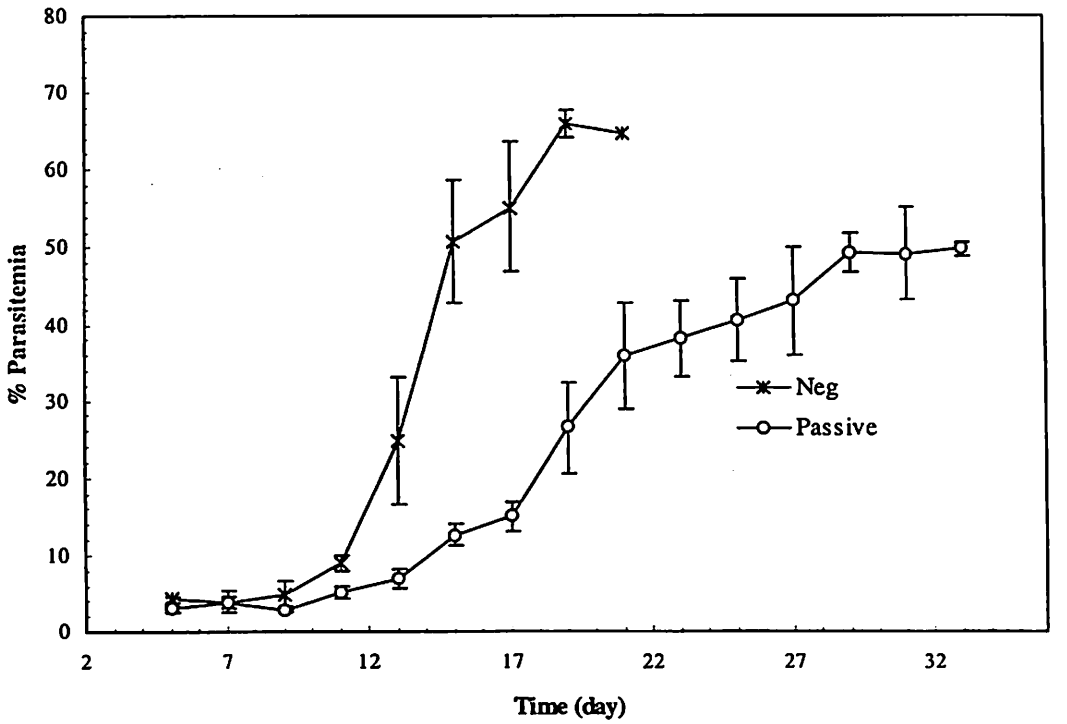


Figure 6.7 Parasitemia profile of passively immunized mice

All the Values represent ± 1 SE

Legend:

Neg- Negative controls (Challenged but un-immunized mice)
Passive- passively immunized and challenged mice

Discussion

The C-terminal 19-kDa region of MSP-1_{19} has long been regarded as an attractive vaccine candidate. Several studies have demonstrated the presence of conformational epitopes on EGF I and EGF II domains of PfMSP-1_{19}, and their role in protective immune response. The presence of conformational epitopes and the significance of correctly folded MSP-1_{19} in generating protective immune responses have been demonstrated by many experiments as described above.

All the peptides elicited a strong antibody response in mice, only after the repeated immunizations. There was no major difference in the antibody profile of E1 and E2 alleles. The antibody response for E1 as well as E2 alleles were high after the last booster, indicating that these peptides were poor immunogens and needs repeated exposure to elicit a sufficient antibody response. In this study BALB/c responded more efficiently with E1 as well as the E2 alleles as expected because of Th_2 bias, when compared with the other strains. The next best response was observed in Swiss Albino strains. A vaccine should stimulate an immune response in individuals of diverse genetic make up, to be effective. Genetically controlled non-responsiveness is a common characteristic of malaria antigens, and if widespread, could compromise subunit vaccine strategies. The genetic control of immune responsiveness to most of the malarial antigens of the sexual stages of the parasite life cycle has been demonstrated. The effect of the MHC genes or responsiveness to asexual blood stage antigens is still to be explored.

Studies have identified multiple distinct Ig binding sites on MSP-1_{19} that are clustered or overlapping (Wilson *et al.*, 1987, Chappel *et al.*, 1993). Minor differences in the target epitopes have been considered critical in the function of the corresponding antibodies. Previous studies using Mab's have defined two variant B-cell epitopes with in the epidermal growth factor like domains of MSP-1_{19} (Blackman *et al.*, 1990, Cooper *et al.*, 1992). These are KNG versus TSR in the second EGF like domain and the Q versus E variant in the first EGF like domain. After this, several other variants were identified with

respect to EGF II domains. However, the sero cross reactivity studies done by us, by assaying the sera raised against one allele with the other allelic peptide, demonstrated that there is no allele specific immune responses and all the epitopes are cross reactive. E2a, considered to be a novel allele reported by us in the Indian sub-continent has two amino acid substitutions Y and H, and still didn't affect the antibody binding, which was evident from our cross reactivity studies (Fig.6.3a). Previous studies have found a consistent serological cross reactivity to different allelic forms like Q-KNG and E-TSR in both infected naïve monkeys (Hui *et al.*, 1996), and actively immunized animals (Hui *et al.*, 1994, Kaslow *et al.*, 1994) However, there are also studies (Stowers *et al.*, 2001) negating this, demonstrating that rabbits immunized with one allelic form (Q-KNG) mount an antibody response with various degrees of specificity, which in some cases can entirely be allele specific. Although the studies mentioned above using polyclonal antiserum demonstrated no allele specificity, it has been previously noted that monoclonal antibodies can differentiate epitopes defined by both E-to-Q and the TSR-to-KNG variations (Tolle *et al.*, 1995).

In the present study it was observed that the challenging with *P.berghei* boosted the immune response in all the mice immunized with different allelic peptides, suggesting a cross species reactivity as was evident from the fig. 6.5 & 6.6. This cross species reactivity has been demonstrated by several studies in the animals immunized with recombinant proteins as well as the purified parasite components of MSP-1. Post challenging sera when assayed for antibodies against all the alleles, E1 (EGF I) showed a major difference in the antibody titre against E2 (fig 6.5 & 6.6). After the initial challenging, there was persistent low-level parasitemia in the mice immunized with E2a + E1 and E2c + E1 and peak parasitemia reached only on 21 and 27 days. This clearly indicates the protective role of antibodies in controlling the parasitemia. However, this protectivity could be either because of anti E1 antibodies or anti E2 allele antibodies and has to be further evaluated. It has been demonstrated that the concentration of serum antibodies at the time of challenge also plays a major role in controlling the infection (Gozalo *et al.*, 1998). An important observation made from this study was that, the antibody profile against E2 allelic peptides was high after challenging with *P.berghei*. This could be because of the sequence similarity of E2 peptides with that of *P.berghei*.

Whether or not these natural antibodies against E1 or E2 are involved in protection has to be evaluated by further studies.

Antibodies play a vital role in protection against intra erythrocytic stages and in particular when immunized against MSP-1. Several studies were done in which antibodies from immune serum were passively transferred and observed some protection against rodent, simian, and human malarias. Mice when passively transferred with hyper immune sera and Mab,s and when challenged with *P. chabaudi*, the immune sera reduced the rate of increase in parasitemia, decreased peak parasitemia, but did not have marked effect on clearance (Boyle *et al.*, 1982). In the present study, test animals when given the pooled immune sera raised against all the alleles, a persistent low-level parasitemia was maintained till the day 17^{th}, and peaked only on 29 to 33 day. When compared with the controls that were dead between 17 and 21 days with a parasitemia of 65.93 %, the parasitemia of the test animals was very low. This suggests that antibodies do play a major role in controlling the infection. However, it is not clear whether antibodies against EGF I or the EGF II are helping in controlling the infection and is to be further evaluated. There are several studies demonstrating that high dose of antibodies could protect the mice from death. Several studies demonstrated that immune sera passively transfer protection in a dose-dependent manner (Hirunpetcharat et al., 1999). Since it was only 100 µl of immune sera that was administered passively every 7 days, from the data obtained in this study, it can be hypothesized that higher concentration of antibodies could definitely help in controlling the infection to a great extent and could also protect the animals from death. Also one reason for the death of all the animals could be the lethal *P.berghei* strain that was used for infection. This study hypothesizes that there is a possibility that protective antibody in nature is directed at weakly immunogenic or antigenically diverse epitopes and levels adequate for complete protection might be induced only after repeated infections or with strong adjuvants (Siddiqui et al., 1986).

Our initial findings appear to suggest that E1 and E2 peptides are mimicking the natural epitopes on EGF like domains of $MSP\text{-}1_{19}$. However, there could also be a possibility that these are linear epitopes existing on $MSP\text{-}1_{19}$, which has to be further investigated. Our data suggests that there is cross reactivity among different E2 alleles, in animals and

data suggests that there is cross reactivity among different E2 alleles, in animals and human subjects. There is also a possibility that the sera collected could be from patients with a mixed parasite population with respect to these alleles. The results of our animal studies, however conclusively show cross reactivity amongst the different E2 alleles, and this information could be extended for the analysis of the human sera. Our findings also suggest that the peptides representing the EGF I epitope (E1) has the highest reactivity with all the human sera and with the mice sera prior to *P.berghei* challenging. This indicates that E1 is more immunodominant to E2. The peptide E1 could be taken to represent a linear epitope or could be best mimicked with respect to the native epitope. There could be arguments in favor of conformational bindings because of the presence of three cysteines, two of which are separated by nine amino acids in E1. However since during synthesis, the cysteines added were not modified in any fashion and they have been oxidized immediately after synthesis thereby preventing the cyclisization of the peptides. Although there were some differences in the cross reactivity profiles of mice and humans, the difference in the amino acids of different alleles did not influence the linear nature or sequential nature of these peptides. This was evident in our cross-reactive studies in humans and mice. This work clearly demonstrates that these peptides evaluated, elicited a partial protective immune response and may need other components of the same or a different protein for a complete protection. However, the precise role of these antibodies in protection needs to be further investigated. The different reactivities against peptides representing different potential B-cell epitopes on the MSP-1_{19} could provide information into designing region specific MSP-1 peptide combinations for vaccine strategies.

Chapter 7

Conclusions

Chapter 7

Conclusions

1) This is the first study in India; investigating diversity in the MSP-1_{42} sequence from naturally obtained parasites. We have found significant differences in the sequences we have obtained, on comparison with the sequences reported in literature.

2) Due to the absence of data indicating the discrete B-cell epitopes in this region (out side the MSP-1_{19}), we cannot comment on the significance of the changes we have observed. However our data indicates that this region is less conserved as reported earlier.

3) The analysis of MSP-1_{19} region revealed the presence of a novel allele (E-KYG-F). In addition to this novel allele, we also found the presence of alleles reported in literature. These include E-TSR-L, Q-TSR-L, E-TSG-L, Q-KNG-L, Q-KNG-F, E-KNG-L, and E-KNG-F.

4) Peptides based on the EGF I and EGF II like domains of MSP-1_{19} revealed differing levels of natural antibody responses in the infected individuals.
    a) 88 % of the infected population tested had antibodies against E1.
    b) A major difference was observed between the antibody profiles of E1 and E2 alleles. E1 is more immuno dominant than E2.
    c) The difference in the frequency of IgG responses was not significant among the three analogs E2a, E2b and E2d.
    d) Of the 35 patients tested, 28 patients responded to more than one E2 allele evaluated and 4 responded to only one.
    e) Of the different E2 alleles evaluated, 29 patients responded to E2b allele from a pool of 32 patients.

5) We found antibody cross reactivity against different EGF II allelic peptides.

6) On immunization of different strains of mice, these peptides showed high antibody response.

7) As seen in the naturally infected populations, these peptides also showed extensive cross reactivity in the animal model.

8) Challenging with *P.berghei* after the antibody titre in the immunized animals had reduced to normal levels, showed in some cases that delayed and suppressed parasitemia resulted as a consequence of the antibody response elicited.

9) Passive immunization studies with the pooled sera-containing antibody raised against all the alleles, showed a significant delay in onset of parasitemia and lethality in the Swiss Albino mice.

10) Although different alleles of MSP-1_{19} are present in natural populations, significant cross reactivity among some of these can reduce the allelic types of MSP-1_{19}, which has to be incorporated in a vaccine. The EGF I and EGF II like domains also contains possibly linear B-cell epitopes which are effective in controlling parasitemia, seen in cross species challenging studies.

Future Perspectives

Till date, most of the studies pertaining to MSP-1_{19} have dealt with recombinant antigens emphasizing more on conformational epitopes. However, this study demonstrates that peptides corresponding to EGF I and EGF II like domains of MSP-1_{19}, can also elicit a strong protective immune responses in experimental animals with different genetic backgrounds. Also these peptides corresponding to different alleles

of MSP-1_{19} were well recognized by the antibodies in the naturally infected population as observed in this study. This study indicates that these peptides can be used in cocktail with other antigens in vaccine strategies against malaria. Further studies will be required to determine which of the peptides could be eliminated from such a vaccine cocktail and also to delineate protective epitopes of the MSP-1_{33} region.

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Appendix

List of Publications

1. **Kumar, S. V.**, Ranjan, S., Saxena, V., Rajesh, V., Roy, S. K., Ranjan, A. and Das, A. (2005) *Plasmodium falciparum*: Genetic Diversity of C-terminal region of MSP-1 in isolates from Indian Sub-Continent. (In Press, **Experimental Parasitology**).

2. Kochar, D., Saxena, V., Singh, N., Kochar, S. K., **Kumar, S. V.** and Das A. (2005). *Plasmodium vivax* Malaria. **Emerging Infectious Diseases**. Vol.11, No. 1. P.132 – 134.

3. **Kumar, S. V.**, Saxena, V., Garg, S., Roy, S. K., Kochar, D., Albin, A., Pareekh, R. P. and Das, Ashis (2005) *Plasmodium falciparum* merozoite surface protein 1_{19} (MSP-1_{19}) – Humoral responses to allele specific variants from India. (Communicated).

Papers in Conferences/Symposiums

1. **Kumar, S. V.**, Ashis K. Das. Presented "Polymorphism in MSP-1, a leading vaccine candidate antigen of malarial parasite", 3rd EMBL PhD student Symposium "Life within Boundaries: Membranes and Compartments in Biology", EMBL, Heidelberg, Germany (14th – 16th Nov., 2002). P. 72.

2. Das, A., **Kumar, S. V.** "Polymorphism in MSP1, a leading vaccine candidate antigen of *Plasmodium falciparum*", "International Symposium and CME on Modern Trends in Malaria" All India Institute of Medical Sciences, New Delhi, January, 2003. Abstracts p. 38

Biography of Dr. Ashis K. Das

Dr. Ashis K. Das is presently working as an Associate Professor at Biological Sciences Group, Birla Institute of Technology and Sciences, Pilani. He received his Ph.D. degree in 1993 from National Institute of Immunology, Jawaharlal Nehru University, New Delhi. Worked as Post Doctoral Fellow in the Department of Molecular Biology and Immunology, SHPH, Johns Hopkins University, Baltimore, USA and WHO Fellow at the Malaria Branch, Center for Disease Control, Atlanta, Georgia USA. He has been involved in teaching and research for 20 years. His areas of interest include Molecular and Immuno – Parasitology, Molecular Diagnostics and Industrially oriented projects dealing with different aspects of vector design, cloning and expression and bioinformatics. He has published various research articles in peer reviewed international journals. He is currently working on various projects that are funded by CSIR, Dabur and UGC Major.

Biography of Mr. Vijaya Kumar Singamsetty

Mr. Vijaya Kumar Singamsetty completed his M.Sc. in Biotechnology, in the year 1999 from Nagarjuna University, Andhra Pradesh. He has received several fellowships like BCIL fellowship, Ministry of cooperation fellowship from Govt. of Luxembourg. His area of interest is Molecular Immunology. Mr. Vijaya Kumar has been a research fellow working for his Ph.D. at BITS, Pilani from 2000-2005. He has published research articles in well renowned international journals and presented papers in various International and National conferences/symposiums.