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Expression, purification and characterization of allelic variants of MSP-1_{42} from Indian *Plasmodium falciparum* isolates☆

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ABSTRACT

The C-terminal 19 and 42 kDa fragments of *Plasmodium falciparum* merozoite surface protein 1 (MSP-1) have shown to be protective in animals against lethal parasite challenge. The MSP-1_{19} being highly conserved may lack sufficient number of T-cell epitopes in order to elicit a broader response in genetically diverse populations. The inclusion of additional epitopes from the N-terminal MSP-1_{42} has shown to enhance the protective efficacy of MSP-1_{19} vaccine. In an attempt to examine the strain specific immunogenicity to MSP-1, we have cloned and expressed three diverse allelic variants of MSP-1_{42} from Indian *P. falciparum* isolates in bacteria. Among three alleles, one was extremely rare and not been found before. These purified and refolded recombinant products were recognized by conformation specific monoclonal antibodies and hyper-immune sera. Immunization of mice and rabbits with the purified proteins generated high titer biologically active polyclonal antibodies supporting further development of this vaccine candidate antigen.

1. Introduction

Plasmodium falciparum is the major cause of malaria morbidity and mortality. The pathology associated with malaria is related largely to the asexual blood stages of the parasite [1]. People living in malaria endemic areas get repeated infection and develop immunity to severe disease [2,3] mediated partly by erythrocyte stage parasite specific antibodies [4]. Thus the antigens expressed during the blood stage of *P. falciparum* infection are targeted for malaria vaccine development [1]. Merozoite surface protein (MSP-1) of *P. falciparum* is the most extensively studied erythrocyte vaccine candidate antigen under consideration [5]. Antibodies to MSP-1 are shown to block parasite invasion of red blood cells in vitro [6–8]. MSP-1 is a 195 kDa antigen and undergoes proteolytic cleavage into smaller fragments [9]. The C-terminal fragment MSP-1_{42} undergoes further cleavage producing 33 and 19 kDa fragments. During invasion the MSP-1_{19} is carried on to the newly invaded erythrocyte [10]. MSP-1 sequence polymorphism has been analyzed for *P. falciparum* isolates obtained from various geographical locations [11,12]. While MSP-1_{33} portion is dimorphic with a low 47% sequence similarity between the dimorphs, the MSP-1_{19} region is highly conserved among field isolates with substitutions mainly at four places with several additional polymorphic sites in field isolates [3,11]. MSP-1_{19} has 12 cysteine residues those are conserved across the species [13,14] making this region a prime focus for developing a MSP-1 based vaccine [15]. Recombinant MSP-1_{42} and MSP-1_{19} have shown to be protective in animals against lethal parasite challenge [16–18]. MSP-1_{19}, though a target of protective antibodies, there is evidence that it may lack sufficient number of T-cell epitopes in order to elicit a broader response in genetically diverse populations [12,19]. The inclusion of additional epitopes from the N-terminal MSP-1_{42} has shown to enhance the protective efficacy of MSP-1_{19} vaccine [20]. Recent reports on human trials using MSP-1_{42} recombinant proteins suggest that immunizations resulted in generation of biologically active antibodies and reactive T-cells [21]. Although the biological function of MSP-1 is not known, there are reports that MSP-1 has erythrocyte binding activity and thus may participate in erythrocyte invasion [22,23].

In the Indian subcontinent, *P. falciparum* accounts for almost 47% of total malaria cases countrywide and its prevalence is 80–90% in hilly, forested areas. We have initiated this investigation in order to assess the immunogenicity of the allelic variant proteins using the standard conventional Freund's adjuvants. For this, we have cloned, expressed and purified three allelic variants of MSP-1_{42} using a simple two-step chromatographic method and examined

☆ Sequences reported in this manuscript have been deposited in GenBank with accession numbers FJ919372, FJ919373 and FJ919374.

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the strain specificity of antibodies elicited to these MSP-1 alleles. One of the allele is extremely rare with an arginine in place of a cysteine residue at position 1679 and with two more aa variations. Purified and refolded proteins were recognized by conformation specific monoclonal antibodies and hyper-immune sera. The purified proteins formulated with CFA/IFA adjuvant have generated high titer polyclonal antibodies in mice and rabbits and these antibodies inhibited *P. falciparum* parasites' growth in an in vitro assay.

2. Materials and methods

2.1. Cloning and expression of the recombinant MSP-1_{42}

Genomic DNA was isolated from *P. falciparum* isolates collected from the parasite bank at National Institute of Malaria Research, Delhi, by proteinase K digestion. 42 kDa fragments were amplified using *Taq* DNA polymerase (Bangalore Genei) and appropriate primers for the gene encoding msp-1_{42} (aa 1351–1726) with BamH1 and Sph1 sites (forward: ggt gct gaa ccc gca cca; reverse: tga aca tgg aaa att). The PCR-amplified products were cloned into pGEM-T cloning Vector (Promega) and analyzed by DNA restriction endonuclease and nucleotide sequence analyses. The expression plasmid, PQE-30 XA (QIAGEN) was restriction digested with BamH1 and Sph1, and gel-purified gene inserts from the pGEM-T cloning vector were ligated before transformation into *E. coli* strain M15 and analyzed by DNA restriction digestion and nucleotide sequencing. For expression in shake flasks, cells were grown to an optical density at 600 nm of 0.5 and induced with 0.1 mM IPTG. Cells were harvested after 2 h and cell pastes were stored at −80 °C.

2.2. Purification of MSP-1

The *E. coli* cell pastes were solubilized in 3× PBS (phosphate buffered saline), 2% sarkosyl and 1 mM PMSF (pH 7.4). The cells after sonication were centrifuged and the supernatants containing the recombinant proteins were incubated with Ni^{2+}–NTA chelating resin (Qiagen Inc.) according to manufacturer's specifications. Bound proteins were eluted with 250 mM imidazole/20 mM phosphate buffer/0.1% sarkosyl (pH 8.0) and refolded by rapid dilution in the presence of GSH–GSSG at 4 °C for ∼40 h. The protein solution was pH adjusted (7.2 for PfRK9; 6.6 for PfS76 and PfS2) and passed through pre-equilibrated Fractogel EMD SO3-650 (M) (Merck, Germany). The resin-bound protein was washed with phosphate buffer–1 mM EDTA (50 cv), followed by phosphate buffer/100 mM NaCl and eluted with 1× PBS containing 1 mM EDTA. Purity was checked using coomassie blue stained SDS-PAGE.

2.3. SDS-PAGE and immunoblot analyses

Proteins were run on 10% SDS-PAGE after denaturing with sample buffer in the presence and/or absence of a reducing agent (DTT) and stained with coomassie blue followed by destaining. For Western blot analyses, proteins were electrophoretically transferred to nitrocellulose membranes, blocked with 0.5% casein and 0.1% Tween 20 in PBS for ∼2 h at RT. Primary antibodies appropriately diluted in PBST (PBS with 0.05% Tween 20) were incubated for 1 h at RT. The blots were washed with PBST and incubated with 1:5000 dilution of HRP conjugated species-specific IgG (Bangalore Genei) for 1 h. The blots were washed with PBST and developed with DAB/H_2O_2 substrate as per the manufacturer's recommendation.

2.4. Reduction and alkylation, and free thiol analyses

For the reduction and alkylation analysis, protein was reduced by incubation with a 100-fold molar excess of dithiothreitol over cysteines in the presence of 4 M urea, followed by alkylation with a 1000-fold molar excess of iodoacetamide over cysteines for 1 h at room temperature in the dark. For free thiol analysis, free sulfhydryl groups were estimated in the presence and absence of 4 M guanidine–HCl using Ellman's reagent (5,5′-dithiobis-3-nitrobenzoic acid). L-Cystine was used to plot the standard curve.

2.5. Immunization of mice and rabbits

Swiss mice (6–8 weeks, $n = 6$) and New Zealand White rabbits ($n = 2$) were immunized subcutaneously with 30/100 μg each protein, respectively, emulsified in Freund's adjuvants (Sigma). Immunizations were done three times giving 3 weeks interval. Sera were collected 15 days after last immunization. Mice were bled out 15 days after last immunization, and spleen samples were collected in RPMI 1640 medium. As controls, two mice/rabbits were immunized with the same volume of adjuvant in 1× PBS. The experimental protocols were approved by JNU Institutional Animal Ethics Committee and performed accordingly.

2.6. T-cell proliferation assay

Spleens of immunized mice were dissected out aseptically and individual cell suspension was prepared in RPMI 1640 medium. The cells were washed and resuspended in medium containing 5% fetal bovine serum to get a 10×10^6 cells/ml. Splenocytes were cultured in a round bottom 96-well tissue culture plate (Costar, MA) containing 100 μl aliquots of the cell suspension in the presence of purified proteins at 4 and 6 μg/ml in triplicate. 2 μg/ml of PHA was added as a positive control in each plate in triplicates. The cultures were grown for 4 days at 37 °C in a 5% CO_2 incubator with a humidified atmosphere. These cells were pulsed with 1 μCi ^{3}H-thymidine per well for 18 h. Cells were harvested (PHD Cell Harvester, Cambridge Technology, MA) on to glass fiber filters to measure thymidine incorporation by liquid scintillation (Beckman beta scintillation counter, Beckman Coulter, CA). Results were expressed as stimulation indexes (SI) calculated as mean of counts per minute (cpm) of test triplicates divided by mean of cpm of control triplicates.

2.7. ELISA

Immunized mice and rabbit sera were analyzed by ELISA as described [24]. Polystyrene 96-well micro-titer plates were coated with 100 ng/well of proteins and incubated at 4 °C overnight. The plates were blocked at RT for 1 h with PBST in 5% casein and washed with PBST. Individual sera in consecutive two-fold dilutions were incubated for 2 h at RT. The plates were washed and incubated with 1:5000-diluted species-specific anti-IgG conjugated with horseradish peroxidase (HRP) for 2 h. They were washed and developed with OPD/H_2O_2 substrate. The absorbance at 490 nm was recorded (Microscan, ECIL, India) and comparative ELISA titers were calculated using regression analysis on the titration curve. The ELISA was repeated three times for each individual serum sample, in triplicate wells on separate days. For anti-MSP-1 IgG subclass analysis, individual sera from mice were tested at a starting dilution of 1:100 in duplicate followed by four-fold serial dilutions followed by incubation with secondary antibodies goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Sigma) at 1:1000 dilutions. After 2 h incubation and washing, HRP conjugated anti-goat IgG at 1:2000 dilutions was added and reacted for 2 h at RT. Rest of the procedure remained same. As controls, pre-immune sera or sera from mice immunized with another irrelevant protein (PfAMA-1) were used in the assay.

2.8. Indirect-immunofluorescence assay (IFA)

Localization of MSP-1 was determined by IFA in thin blood smears of *P. falciparum* schizonts on multi-test slides. Cultures of four different Indian *P. falciparum* isolates were used for IFA. Blood smears were fixed with chilled acetone + methanol mixture (1:1) and blocked with PBS–1% BSA. Smears were treated with sera dilutions at 1:100 in PBS–BSA and incubated for 2 h at RT. Slides were then washed with PBST and incubated with mouse anti-rabbit IgG–FITC-labeled antibody at 1:100 dilution (Jackson ImmunoResearch, USA) for 1 h. Slides were washed, anti-fade solution was applied, mounted in 60% glycerol and read under a UV fluorescence microscope. IFA titers were determined as the last serum dilution with a positive recognition of the parasite compared to the negative adjuvant control rabbit serum diluted 1:20.

2.9. Parasite growth inhibition assay

Immunized and pre-immunized rabbit serum samples were subjected for immunoglobulin-G fractionation by ammonium sulphate at a saturation of 40%. Precipitated IgG was dialysed against 1 × PBS, pH 7.2, extensively and the purity was checked by immunoelectrophoresis. Parasite growth inhibition assay was done for each dilution of individual IgG preparation in triplicate. Parasite in culture media without negative or pre-immune or immune sera was the control. The culture with negative or pre-immune sera (IgG) is taken as test control for parasite growth compared to immune IgG of various constructs. The effect of immunoglobulin-G fractions were evaluated against two local *P. falciparum* culture lines (3d7 type), one was chloroquine-sensitive (FDL-B) and other resistant (FDL-NG). FDL-B and FDL-NG isolates were collected from patients

Table 1
(A) Alignment of MSP-1_{42} DNA sequences (nt 4030–5157) from PfS76, PfS2 and PfRK9 isolates. Identical nucleotides are indicated by dashes. (B) Nucleotide and aa variations in MSP-1_{42}. Positions of nucleotides are given in vertical notation (Miller et al. 1993). Deduced aa are shown in boldface.

A

```
PfS76  GCAATATCTGTCACAATGGATAATATCCTCTCAGGATTTGAAAATGAATATGATGTTATATATTTAAAACCTTTAGCTGGAGTATATAGAAGCTTAAAAA
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  AACAAATTGAAAAAAACATTTTTACATTTAATTTAAATTTGAACGATATCTTAAATTCACGTCTTAAGAAACGAAAATATTTCTTAGATGTATTAGAATC
PfS2   ---------G----------------------------------------------------------------------------------------
PfRK9  --------------------A----------------------------------------------------------------------------

PfS76  TGATTTAATGCAATTTAAACATATATCCTCAAATGAATACATTATTGAAGATTCATTTAAATTATTGAATTCAGAACAAAAAAACACACTTTTAAAAAGT
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  TACAAATATATAAAAGAATCAGTAGAAAATGATATTAAATTTGCACAGGAAGGTATAAGTTATTATGAAAAGGTTTTAGCGAAATATAAGGATGATTTAG
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  AATCAATTAAAAAAGTTATCAAAGAAGAAAAGGAGAAGTTCCCATCATCACCACCAACAACACCTCCGTCACCAGCAAAAACAGACGAACAAAAGAAGGA
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ------------------------------------***-----------------------------------------------------------

PfS76  AAGTAAGTTCCTTCCATTTTTAACAAACATTGAGACCTTATACAATAACTTAGTTAATAAAATTGACGATTACTTAATTAACTTAAAGGCAAAGATTAAC
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  GATTGTAATGTTGAAAAAGATGAAGCACATGTTAAAATAACTAAACTTAGTGATTTAAAAGCAATTGATGACAAAATAGATCTTTTTAAAAACCCTTACG
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------A--A---

PfS76  ACTTCGAAGCAATTAAAAAATTGATAAATGATGATACGAAAAAAGATATGCTTGGCAAATTACTTAGTACAGGATTAGTTCAAAATTTTCCTAATACAAT
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  AATATCAAAATTAATTGAAGGAAAATTCCAAGATATGTTAAACATTTCACAACACCAATGCGTAAAAAAACAATGTCCAGAAAATTCTGGATGTTTCAGA
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  CATTTAGATGAAAGAGAAGAATGTAAATGTTTATTAAATTACAAACAAGAAGGTGATAAATGTGTTGAAAATCCAAATCCTACTTGTAACGAAAATAATG
PfS2   ----------------------------------------------------------------------------C-----------G---
PfRK9  ----------------------------------------------------------------------------------------------------

PfS76  GTGGATGTGATGCAGATGCCACATGTACCGAAGAAGATTCAGGTAGCAGCAGAAAGAAAATCACATGTGAATGTACTAAACCTGATTCTTATCCACTTTT
PfS2   ----------------------------------------------------------------------------------------------------
PfRK9  --------------------A-------------------------A-G-----------------------------------------------

PfS76  CGATGGTATTTTCTGCAGTTCCTCTAAC
PfS2   ----------------------------
PfRK9  ----------------------------
```

B

	414	415	444	447	472	501	502	505	507	508
	139	150	666	723	636	143	266	516	788	800
PfS 76	GAA	TTT	AAG	CCT	TAC	TGT	AAT	ACA	AGC	AGA
	E	**F**	**K**	**P**	**Y**	**C**	**N**	**T**	**S**	**R**
PfS 2	-G-	---	---	---	---	C--	G--	---	---	---
PfRK 9	---	A--	***	A--	A--	---	---	-A-	-A-	G--
	G	**I**	**-**	**T**	**N**	**R**	**D**	**K**	**N**	**G**
	1387	**1391**	**1496**	**1582**	**1583**	**1679**	**1683**	**1691**	**1700**	**1701**

reported with symptomatic malaria in a local clinic of Delhi in 1994 and 1995, respectively. They were adapted and maintained in vitro by candle-jar technique. Parasites were cultured in human O+ RBCs in RPMI 1640 media enriched with 10% (v/v) AB+ serum and supplemented with 25 mM HEPES buffer and 25 mM sodium bicarbonate. Assay was done in synchronous culture with ring form at 5% haematocrit containing 1% parasitaemia in 96-well flat bottom tissue culture plate. Purified IgG was dosed in wells in triplicate at 100 and 50 μg/ml concentrations. To determine the effect of antibodies in total parasite growth, assay was done for 48 h. Growth of the parasite from each well was monitored microscopically in JSB stained smears by counting total number of parasites per 10,000 RBCs. Percent parasite growth inhibition (PGI) was calculated by the formula $(1 - Nt/Nc) \times 100$, where Nt and Nc represent the number of parasites in the test and control wells, respectively.

2.10. Statistical analysis

Data were analyzed using either Microsoft Excel 2002 or Sigma plot 8.02 software tools. Student's *t*-test was used to compare the data groups. In ELISA, serum dilution required to get the specific OD values were calculated using linear regression analyzes.

3. Results

3.1. Cloning, expression and purification

Fifteen *P. falciparum* isolates were screened and DNA sequence analysis of the clones from these gave three different alleles for further analysis (Table 1A). The sequence of one of the alleles PfS76 was exactly same as that of 3D7 (E-TSR) isolate and the other PfRK9 was E-KNG type isolate with six amino acids different from 3D7 sequence at 1391 (I/F), 1582 (T/P), 1583 (N/Y), 1691 (K/T), 1700 (N/S), 1701 (G/R) and with a deletion of K at position 1496 (Table 1B). The third one PfS2 was new allele with an arginine in place of a cysteine at position 1679 and with variations at 1387 (G/E) and 1683 (D/N). All of the nucleotide changes seen in these isolates were nonsynonymous. These three allelic variants of MSP-1_{42} were cloned and expressed in *E. coli*. Expressions were standardized by varying parameters such as concentration of IPTG, duration of induction and induction temperatures. The protein induction level was considerably high at a very low IPTG concentration of 0.1 mM. Most of the proteins were expressed as inclusion bodies in the temperature range 25–37 °C and a buffer containing 2% sarkosyl was used for the solubilization. All the constructs had a 6xHis-tag at N-terminal, and hence Ni–NTA agarose affinity chromatography was used as a first step in purification. The non-specifically bound impurities to bound proteins were removed by extensive washing. Elution of the proteins with 250 mM imidazole yielded nearly 75% purified proteins. For all the allelic variants, same binding, washing and elution conditions were used. A refolding step was standardized to get the recombinant proteins with close to native structure after testing several experimental conditions. Eluted proteins were refolded using rapid dilution method at 30–40 μg/ml in the presence of a redox couple GSH:GSSG solution and at 4 °C for a minimum of 40 h. Following concentration and purification using Fractogel EMD SO3-650 (M), a cation exchange resin, yielded purified protein for both the alleles. It involved several conditions such as standardizing the pH and salt concentrations for binding, washing and elution. This simple two-step purification yielded nearly 75–80% pure recombinant proteins (Fig. 1, panel I). The final yield of purified protein was 2–3 mg/l on shake flask level.

3.2. Reduction and alkylation, and free thiol analyses

As the MSP-1 proteins from PfS76 and PfRK9 contain 13 cysteine residues, there may be at least one free cysteine present in these molecules. Ellman's test with both PfS36 and PfRK9 MSP-1 proteins resulted in 0.93 and 0.96 μmol of free cysteines per μmol of proteins suggesting the presence of a free cysteine. These values are slightly lesser than the expected 1 μmol, may be because these proteins are only nearly 75–80% pure. Ellman's test results were not consistent for PfS2 MSP-1 presumably due to the unstable nature of this product. Further, on SDS-PAGE the reduced and alky-

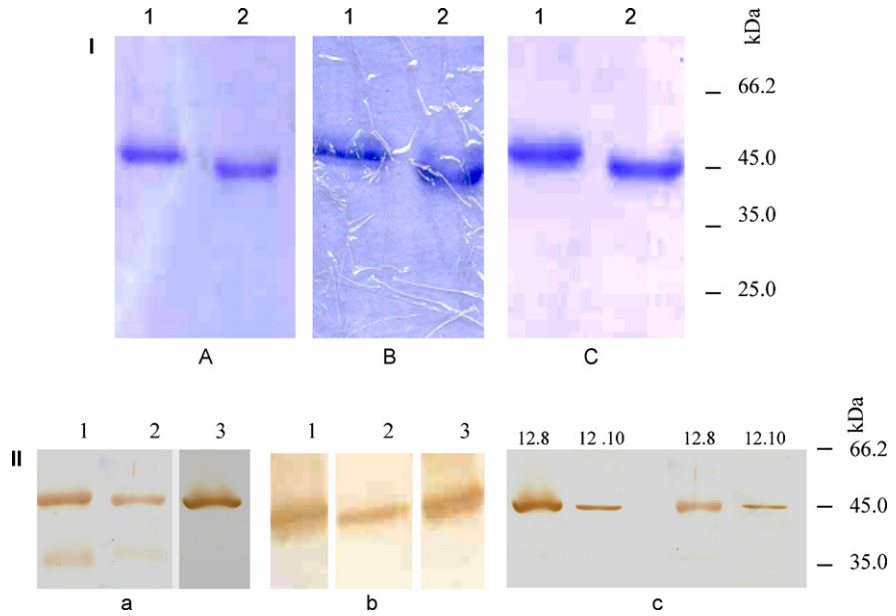

Fig. 1. SDS-PAGE analysis of MSP-1 allelic variants at different purification steps. Proteins detected by coomassie blue staining (Panel I) and immunobloting (Panel II). Panel I: MSP-1_{42} from (A) PfS76; (B) PfS2; (C) PfRK9. Lane 1: Fractogel EMD SO3-650(M) purified sample under non-reducing conditions and Lane 2: Fractogel EMD SO3-650(M) purified sample under reducing conditions. Panel II: Western blot analysis of purified allelic proteins of PfS76, PfS2 and PfRK9. 200 ng of purified and refolded proteins were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and reacted with (a) polyclonal mice sera (1:5000) followed by anti mice IgG (1:5000), (b) a pool of patients' sera (1:1000) followed by anti-human IgG (1:5000) and (c) monoclonal antibodies 12.8 and 12.10 (1 μg/ml) followed by 1:5000 anti-mice IgG. There was no recognition of bands for these proteins with normal mice/human serum or with an irrelevant antibody (raised against Pf AMA-1 Domain I+II [24]).

Table 2
ELISA and lymphocyte proliferation responses in mice immunized with Pf. MSP-1 variant proteins. Stimulation index shown are the average from three experiments carried out in different days in triplicate wells with antigen concentration at 4 and 6 μg/ml. Values of cpm for negative controls were 1535 ± 97 (immunized with antigen/adjuvant) or 1542 ± 118 (with adjuvant only); cpm for positive controls with PHA were 28,119 ± 676 (with antigen/adjuvant) or 28,121 ± 728 (with adjuvant only). These values were 14,806 ± 1165 and 14,324 ± 1443 for PfRK9/MSP-1 and PfS76/MSP-1, respectively.

Immunogen	Mice#	ELISA titer ($\times 10^4$)	SI
RK9/MSP-1	A1	7.6004	
	A2	6.9924	
	A3	8.0320	9.87(1.18)
	A4	6.0420	
	A5	5.8383	
	A6	6.1398	
S76/MSP-1	M1	8.0154	
	M2	13.5600	
	M3	7.1888	9.41(1.13)
	M4	7.6763	
	M5	7.0535	
	M6	8.1785	

lated products of all the three recombinant proteins showed shift in mobility as compared to refolded proteins. These results further confirm the formation of disulfide bond stabilised structures within the recombinant products.

3.3. Recognition by polyclonal and monoclonal antibodies

Two conformation specific monoclonal antibodies, 12.8 and 12.10 (kind gift from Dr. Jana McBride, UK), known to bind to a conformational dependent epitope within the region encompassing MSP-1_{19} recognized the two of the recombinant proteins on Western blots (Fig. 1, panel II(c)) and ELISA (data not shown) run under non-reduced conditions. PfS2 did not show any recognition due to the absence of a cysteine and related change in conformational structure. All the three recombinant constructs under non-reducing conditions were recognized on Western blots at the appropriate molecular masses by polyclonal rabbit/mouse sera (Fig. 1, panel II(a)) and a pool of *P. falciparum* infected patients' sera (Fig. 1, panel II(b)). There was no recognition of these bands when analyzed with normal human or rabbit/mice sera, respectively, at the same concentrations (data not shown). Also an irrelevant his-tagged protein (Pf AMA-1 Domain I + II) did not show any recognition with the polyclonal mice/rabbit sera from animals immunized with the recombinant constructs [24] (figure not shown).

3.4. Immunogenicity of recombinant MSP-1 proteins

Immunization with each of the two MSP-1_{42} fragments in rabbits ($n = 2$) and mice ($n = 6$) induced high titer antibodies in both animals. PfS2 derived MSP-1 was not used for immunization experiments as this recombinant product was found to be highly unstable. Splenocytes from mice immunized with both the proteins formulated in CFA/IFA adjuvants showed significant proliferation at 4 and 6 μg/ml of antigen concentration (Table 2). Splenocytes of mice immunized with adjuvant alone showed no proliferation suggesting antigen specificity of the proliferation observed. Splenocytes on PHA stimulation gave very high proliferation for both antigen/adjuvant and adjuvant alone immunized groups (Table 2). Individual sera from a group of six mice were analyzed for anti-MSP-1 antibodies of IgG1, IgG2a, IgG2b and IgG3 isotypes (Fig. 2). All the mice immunized with both the allelic proteins showed high titers for IgG; IgG1 or IgG2a was the predominant isotype for all the mice and IgG3 the least dominant (Table 2, Fig. 2). The mice immunized with PfS76/MSP-1 protein showed higher titers of IgG1, IgG2a and IgG2b as compared to those immunized with PfRK9. The differences between groups were significant in case of IgG1 ($p = 0.006$), IgG2a and IgG2b ($p = 0.032$). However for both the allelic proteins the ELISA titers for IgG2b were considerably higher than IgG2a. The stimulation indices for the mice immunized by both the antigens were high (Table 2) and comparable ($p = 0.27$). Both the antigens induced high proliferation as compared to the adjuvant control

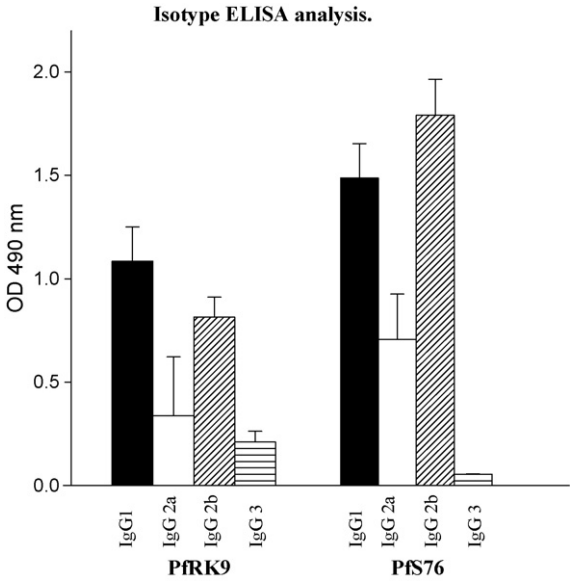

Fig. 2. IgG subclass antibody responses in mice immunized with MSP-1 allelic proteins from PfRK9 and PfS76. 1:2000 dilutions of mice sera and 1:1000 dilutions of secondary antibodies were used for all the isotype IgGs. Error bars represent SDs. Negative control used was the sera from the adjuvant alone immunized mice.

Table 3
The ELISA titers presented are the reciprocal of the dilution that gave an OD (490) of 0.5 for each plate antigen. The percentage parasite growth inhibition values presented are the mean and SDs of the results in triplicate from rabbit IgGs for all two rabbits per group for each of the antigen at an IgG concentration of 100 μg/ml.

Protein	Rabbit code	ELISA titer ($\times 10^5$)	% growth inhibition	
			FDL-B	FDL-NG
PfS76	R2	5.78	56.3 ± 2.7	64.3 ± 6.1
	R6	6.29	48.7 ± 8.1	44.35 ± 1.9
PfRK9	R3	1.61	29.7 ± 2.7	31.4 ± 8.1
	R5	6.72	54.4 ± 10.7	44.3 ± 6.1
PfRK9 + PfS76	R8	5.30[a]	67.7 ± 2.7	68.55 ± 4
	R9	5.81[a]	60.1 ± 2.7	60.05 ± 4.2
PfRK9 + +PfS76	R8	2.62[b]		
	R9	6.18[b]		

[a] Plate antigen PfS76.
[b] Plate antigen PfRK9.

($p < 0.001$) with CFA/IFA formulation suggesting antigen specificity of the proliferation.

3.5. Antigenicity of recombinant MSP-1

The reactivity of rabbit anti-MSP-1 antibodies with *P. falciparum* MSP-1 was confirmed by IFA. On IFA all of these antibodies (rabbits) reacted with schizont stage of *P. falciparum* (3D7 and FDL lines) at a dilution of 1:100 (figure not shown). Almost 90–95% of schizonts showed reactivity at 1:100 dilutions of sera and at 5 μg purified IgG concentration. The reactivity of rabbit anti-MSP-1 antibodies with *P. falciparum* MSP-1 was confirmed by immunoblot analyzes.

We wanted to see whether the difference in chloroquine sensitivity may cause any difference in the growth of the two 3D7 type parasite lines in the presence of immune IgG. The ELISA and the total parasite growth inhibition (PGI) results obtained from both strains FDL-B and FDL-NG (chloroquine resistant and chloroquine-sensitive, respectively) are summarized in Table 3. Antisera from all the rabbits immunized showed substantial inhibitory activity in both the strains at 100 μg/ml of IgG concentration (Table 3). The results were not significant ($p = 0.9074$) between FDL-B and FDL-NG isolates.

4. Discussion

In the present study we have isolated three allelic variant forms of MSP-1_{42} from Indian isolates of *P. falciparum* of which two are analyzed for their immunogenicity in mice and rabbits. The third one was a new allele with one cysteine residue less that made it very unstable to store. The sequence of PfS76 was same as that of 3D7 and PfRK9 differs in six amino acids from 3D7 with a deletion of K at 1496. Three variations are in the MSP-1_{33} kDa region and three in the EGF-2 domain of MSP-1_{19}, the EGF-1 being completely conserved. In the PQ 30 expression vector, the level of expression was very high that resulted in the formation of inclusion body proteins for all the alleles. Very similar purification conditions could be used for all the three. Using simple two-step chromatography, his-tag affinity followed by a cation exchange chromatography; nearly 70–75% pure proteins could be generated. Under non-reducing conditions, the proteins moved as tight bands suggesting them to be monomeric single population. Ellman's test suggested the presence of a single free cysteine in the preparation in the case of MSP-1 proteins from PfS76 and PfRK9. Reduction and alkylation analysis suggested the formation of disulfide bonds in the refolded recombinant products.

Both the Mabs 12.8 and 12.10 known to bind to an epitope in the region encompassing MSP-1_{19} recognized both the proteins under non-reduced conditions suggesting that the recombinant products are refolded correctly. Recognition of these two proteins by a pool of *P. falciparum* infected human sera at expected molecular masses when subjected to SDS-PAGE analysis under non-reducing conditions further confirms that the critical conformational epitopes were maintained within the recombinant products.

PfS2, being highly unstable, was not used for immunization. The stability of the MSP-1 protein is attributed to its conformational structure and the absence of a single cysteine would have contributed to a structural difference that made it unstable. The immunization results show that the two allelic products are highly immunogenic in mice and rabbits in combination with IFA/CFA adjuvants. The antibody titers were around 500,000 for both the antigens in all the animals immunized, when the immunogen was 100 μg (rabbits) and around 75,000 when the immunogen was 30 μg (mice) per dose at three doses. When a combination of the two alleles was used for immunization (50 μg each) in two rabbits, irrespective of the coating antigen (PfRK9/PfS76) similar antibody titers were obtained. Splenocytes from mice vaccinated with the proteins showed stimulatory effects on T-cells with significant proliferation with the recombinant proteins. No proliferation was observed with splenocytes of control mice, suggesting antigen specificity of the proliferation. Immunization with both the proteins induced IgG1, IgG2a and IgG2b antibodies in mice. T-cells may confer protection against erythrocytic stages by helping in antibody production. The T helper 1 (Th1) subset promote cellular responses and are associated with the IgG2a production, while the T helper 2 (Th2) subset produce humoral immunity and are associated with IgG1 production. The results from immunization of mice with both the allelic variants showed activation of mixed Th1/Th2 immune responses. It is known that both Th1 and Th2 responses are involved in protective immune response against malaria [25,26]. These results suggest the suitability of these two recombinant products to be considered for further development. The antibodies elicited by immunization have shown biological activity against *P. falciparum* parasites as seen from the parasite growth inhibition assay. The antisera raised against the two allelic proteins seemed to be inhibitory in two 3D7 type of *P. falciparum* parasite lines that is the predominant isotype in India, one of which is chloroquine resistant and the other chloroquine-sensitive and the results are similar to our previous observation [24]. In India, resistance to chloroquine is alarming and the National Drug Policy recommends the use of artesunate plus sulphadoxine–pyrimethamine (AS + SP) combination therapy as first line of treatment in areas showing CQ treatment failure above 10% of cases. Keeping this in mind, we wanted to study the effect of the antibodies in these two parasite lines and see if CQ sensitivity made any difference to antibody reactivity of the two substrains. Serum purified IgG were used in these studies to make sure that the inhibitory activity was specific to the antibodies alone. These results suggest that the recombinant product is capable of generating inhibitory antibodies. All these results suggest that both PfS76 and PfRK9 have retained T helper and inhibitory epitopes necessary for eliciting protective immunity against malaria. A majority of immunodominant T and B epitopes are localized in the conserved or dimorphic regions that are non polymorphic in the 42-kDa protein of MSP-1 [12,20]. It appears that the antibody response elicited by immunization not to be strain specific as both the antibodies produced similar GIA results in two parasite lines. Sera from rabbits immunized with a combination of the two alleles at half dose each showed an additive effect on parasite growth inhibition suggesting that protective epitopes in MSP-1_{42} are conserved. Among the 6 aa variant positions, five variations at 1582 (T/P), 1583 (N/Y), 1691 (K/T), 1700 (N/S), 1701 (G/R) lie within the critical B-epitopic region and are dimorphic [12]. In the present study, though there are variations at 6 aa positions between two proteins, three are in 19 kDa region and did not cause much difference with respect to allele specificity.

In recent years, many laboratories around the world have been successful in producing and analysing various blood stage vaccine candidate antigens against malaria from various alleles of parasite isolates. It has been shown that MSP-1_{42} induced protection is strain specific and dependent on the antigen and adjuvant used [27,28]. In a recent study two of the mostly diverse alleles of yet another blood stage antigen AMA-1 combined into the vaccine yielded a broader immune response than could be achieved by vaccination with either of the one alone [29]. The field parasite isolates are generally heterogeneous and contain many allelic variant forms. A combination of several variant antigens may have a greater impact as a vaccine as compared to a single one. In conclusion, our results suggest that both the MSP-1 allelic variants either in combination or alone by itself could induce significant immune responses in mice and rabbits with Freund's adjuvant. Furthermore, these antibodies were able to inhibit parasite growth considerably (in vitro) suggesting that they are functionally active. Further studies with large-scale preparations and use of human compatible

adjuvant will prove the vaccine potential of these preparations for human use.

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