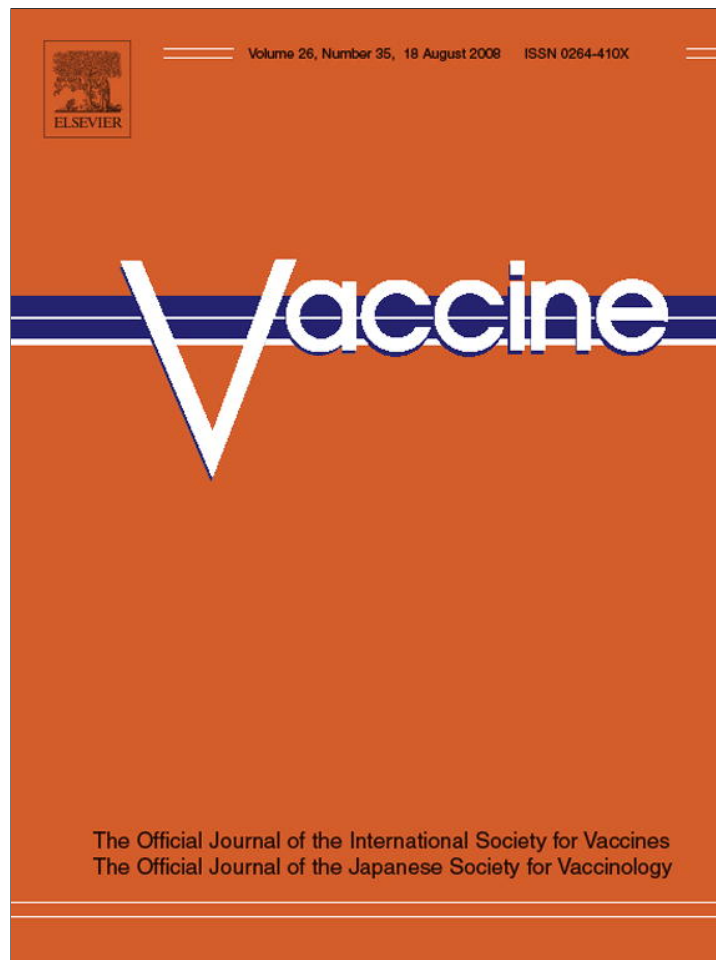


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Immunogenicity of a recombinant malaria vaccine candidate, domain I + II of AMA-1 ectodomain, from Indian *P. falciparum* alleles[☆]

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ARTICLE INFO

Article history:

Received 25 January 2008

Received in revised form 21 May 2008

Accepted 11 June 2008

Available online 30 June 2008

Keywords:

Malaria

Vaccine

Apical membrane antigen

Recombinant protein

Domain I + II

Growth inhibition assay

ABSTRACT

Among the few vaccine candidates under development, apical membrane antigen (AMA-1) of *Plasmodium falciparum* is one of the most promising erythrocyte stage malaria vaccine candidates under consideration. The overall structure of AMA-1 appears to be conserved as compared to other surface proteins, but there are numerous amino acid substitutions identified among different *P. falciparum* isolates. Antisera raised against recombinant AMA-1 or naturally acquired human antibodies were strongly inhibitory only towards homologous parasites. In an attempt to examine the strain specificity of antibodies elicited to AMA-1, we have cloned, expressed and purified two allelic variants of domain I + II of AMA-1 ectodomain from Indian *P. falciparum* isolates in bacteria. One of these is a new haplotype not reported so far and varies in 18 aa positions from the geographically diverse forms 3D7 and 15 from FVO. Refolded proteins were recognized by a conformation specific monoclonal antibody 4G2.dc1 and hyper immune sera. Immunization of mice and rabbits with the purified proteins using CFA/IFA adjuvant generated high titer polyclonal antibodies. Both the alleles induced high levels of IgG1, IgG2a and IgG2b and a low level of IgG3 in mice. Lymphocyte proliferation assays using splenocytes from immunized mice showed significant proliferative responses and cytokines interleukin-2 (IL-2), IL-4, IL-10 and IFN- γ presence in the culture supernatants. The anti-AMA-1 rabbit antibodies obtained with both the proteins were active in an in vitro parasite growth invasion/inhibition assay. These results suggest that recombinant AMA-1 domain I + II formulated with CFA/IFA adjuvant elicited cellular and humoral responses and is capable of inducing high titer invasion inhibitory antibodies supporting further development of this vaccine candidate.

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1. Introduction

Among the four species of human malaria parasites, *Plasmodium falciparum* causes most malaria related deaths, causing nearly 25% of child deaths in Africa [1]. The morbidity and mortality associated with malaria infection result from repeated cycles of asexual replication in a host's red blood cells. During malaria infection, the merozoite invades new red blood cell during a short duration of the infection cycle. Hence any intervention that could block this step can lead to the control of malaria parasite replication [2]. It has been shown that antibodies are effective in controlling parasite growth in vivo and in vitro through passive transfer experiments

[3,4]. Hence in order to accelerate the development of natural protective immunity, the induction of appropriate antibody responses should be an important component of any vaccine strategy [2]. The pathology associated with malaria is related largely to the asexual blood stages of the parasite and the antigens expressed during the blood stage of *P. falciparum* infection are targets for malaria vaccine development efforts [5]. As there is an increased incidence of drug resistance in parasites and insecticide resistance in malarial vectors, vaccine development efforts have become a high priority area. Among the few vaccine candidates under development, Apical membrane antigen (AMA-1) of *P. falciparum* is one of the most promising erythrocyte stage malaria vaccine candidates, because of its role in merozoite invasion of erythrocytes [6–9].

The 83 kDa AMA-1 antigen comprises ecto-, transmembrane and cytoplasmic domains and is shown to be expressed in sporozoite, hepatic as well as erythrocytic stages of the parasite life cycle [10–13]. AMA-1 undergoes proteolytic processing into smaller fragments, and at the time of merozoite release, a 66 kDa processed form of the antigen is distributed around the merozoite surface

[☆] Sequences reported in this manuscript have been deposited in GenBank with accession numbers DQ455557, DQ455558.

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[12,14–16]. The ectodomain has 16 interspecies conserved cysteine residues that form eight intra-molecular disulphide bonds [17]. The fact that targeted AMA-1 gene disruption has been unsuccessful in *Plasmodium* suggests this molecule has an essential role in parasite survival [18].

Monoclonal and polyclonal antibodies against AMA-1 and their respective Fab fragments were shown to block merozoite invasion of erythrocytes [6,8,9,19–21]. It has also been shown that anti-AMA-1 antibodies of infected humans can inhibit invasion of host erythrocytes interrupting the parasite multiplication in the host [7,9,22,23]. Recombinant AMA-1 vaccines have provided protection in a number of rodent and primate models against homologous parasite challenges [8,24–26]. The structure elucidation of AMA-1 suggested a three domain structure, namely domains I, II and III, for the ectodomain [17]. It has been shown that the ability of this molecule to induce a protective immune response is dependent on its structure stabilized by the presence of disulphide bonds [24,25,27–29]. Phase I clinical trial results with recombinant Pf AMA-1 are encouraging as it could elicit antibodies with *P. falciparum* parasite growth inhibitory activity [30,31].

Our previous study has shown that the construct encompassing domains I and II accounts for generating most of the inhibitory antibodies [32]. Other immunizations have also confirmed that domains I and II are important targets of polyclonal inhibitory antibodies [33]. Though the overall structure of AMA-1 appears to be conserved as compared to other surface proteins, numerous amino acid substitutions have been identified among different *P. falciparum* isolates [34–36]. In spite of a conserved tertiary structure, there are more than 60 residue positions showing sequence polymorphism in the ectodomain [37,38]. Among *P. falciparum* strains, almost 10% of amino acids in a total 622 are shown to be polymorphic spreading in all the three domains of the ectodomain. There is enough experimental evidence supporting the population genetic studies to show that the sequence polymorphisms in AMA-1 are to allow parasites to overcome the inhibitory activity of anti-AMA-1 antibodies [34,35]. Antisera raised against recombinant AMA-1 or naturally acquired human antibodies were strongly inhibitory towards homologous parasites but were less inhibitory for other strains of the parasite [22,39]. Recent reports suggest that a combination vaccine containing several forms of AMA-1 could induce protection against several alleles of challenging parasites [22,40]. Though the protective immune responses are shown to be directed against the domain I+II region of the ectodomain, a recent report showed that monoclonal antibodies specific for AMA-1 domain III were also inhibited parasite invasion of erythrocytes [21]. Epidemiological observations and results from experimental models validated this antigen as a promising molecule to be explored as a candidate vaccine against *plasmodium*.

In India, *P. falciparum* accounts for 47% of total malaria cases countrywide and in hilly, forested area the prevalence of this species is 80–90%. We undertook this investigation with an aim to assess the immunogenicity of the allelic variant proteins by using the standard conventional Freund's adjuvant. We planned to isolate and characterize domain I+II allelic variants and examined the strain specificity of antibodies elicited to AMA-1 from Indian *P. falciparum* isolates of diverse origin. For this, we have cloned, expressed and purified two allelic variants of domain I+II of AMA-1 ectodomain. The two allelic variants differ in 18 aa positions in total. The purified proteins, when formulated with CFA/IFA adjuvant, were able to generate high titer polyclonal antibodies in mice and rabbits with *P. falciparum* growth inhibitory activity in vitro and these antibodies showed growth inhibition at very low concentrations.

2. Materials and methods

2.1. Cloning and expression of the recombinant AMA-1 domain I+II

P. falciparum parasite isolates were obtained from the parasite bank of National Institute of Malaria Research, Delhi. Genomic DNA was prepared from these isolates by proteinase K digestion. PCR primers were designed (Bioserve Biotechnologies) for the gene fragments encoding subdomains I and II (aa 83–442) (domains as defined by Hodder et al., ref. [17]). Gene sequences corresponding to domain I+II fragments were amplified from genomic DNA by using *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and appropriate primers with BamH1 and Kpn1 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 positive clones were selected by DNA restriction endonuclease analyzes and further confirmed by nucleotide sequence analyzes. The expression plasmid, PQE-30 XA (QIAGEN) was restriction digested with BamH1 and Kpn1, and gel-purified gene inserts from the pGEM-T cloning vectors were ligated before transformation into *Escherichia coli* strain M15. Bacterial colonies containing expected fragments were picked and analyzed by restriction digestion following plasmid DNA preparation (Qiagen Inc., Valencia, California). Selected clones were further confirmed by nucleotide sequencing of the plasmid DNA samples. Glycerol (8%) stocks were made for each bacterial clone from an overnight culture and stored at -80°C . For expression, cells were grown in shake flasks to an optical density at 600 nm of 0.5 and then induced with a final concentration of 0.1 mM isopropyl-dithiogalactopyranoside (IPTG). Cells were harvested after 2 h of induction by centrifugation at 5000 rpm and 4°C for 30 min. The cell pellets were stored in aliquots at -80°C and taken for purification as and when required.

2.2. Purification of the domain I+II

The *E. coli* cell pastes were solubilized in a buffer containing 3X phosphate buffered saline (PBS), 2% sodium *N*-lauroylsarcosine (Sarkosyl) and 1 mM PMSF (pH 7.4). The cells were disrupted by sonication (Sonifier 250, Branson Ultrasonics Corporation, CT, USA) followed by centrifugation ($12,000 \times g$, for 45 min at 4°C). Supernatants containing the recombinant proteins were incubated by a batch method with nickel nitrilotriacetic acid (Ni-NTA)-agarose chelating resin (1 ml of resin/l culture's cell paste) (Qiagen Inc.) at room temperature (RT) ($\sim 22^{\circ}\text{C}$) for 1 h in the presence of 10 mM imidazole. The resin was then loaded into a fritted column, and the unbound proteins were allowed to flow through. The resin was washed with a minimum of 40 column volumes (cv) of 3X PBS, 10 mM imidazole containing 0.1% Sarkosyl (pH 7.4) followed by 20 cv of 20 mM phosphate buffer, 10 mM imidazole (pH 8.0). Bound proteins were eluted with 400 mM imidazole in 20 mM phosphate buffer containing 0.1% Sarkosyl (pH 8.0). Proteins eluted from the Ni-NTA-agarose were rapidly diluted to 30–40 $\mu\text{g}/\text{ml}$ in a redox-coupled GSH-GSSG solution and allowed to fold at 4°C for a minimum of 40 h. After refolding and adjustment of pH, the protein solution was passed through pre-equilibrated Fractogel EMD SO3-650 (M) (MERCK, Germany) (0.5 ml of resin/l culture's cell paste). The resin-bound protein was washed with a minimum of 50 cv of 20 mM NaH_2PO_4 –1 mM EDTA, followed by a 20 cv wash with 20 mM phosphate buffer containing 100 mM NaCl. The bound protein was eluted with 1X PBS containing 1 mM EDTA. Purity was analyzed on 10% SDS-PAGE and stained with Coomassie blue.

2.3. SDS-PAGE and immunoblot analyzes

Proteins were mixed with sample buffer in the presence and/or absence of DTT as reducing agent, and run on 10% SDS-PAGE stained with Coomassie blue followed by destaining (50% methanol + 10% acetic acid). For Western blots, proteins were transferred onto nitrocellulose membranes electrophoretically, then blocked with 0.5% casein and 0.1% Tween-20 in phosphate buffered saline (PBS) for ~2 h at RT. Appropriate dilution of primary antibody in PBST (PBS with 0.05% Tween-20) was incubated for 1 h at RT. The blots were washed with PBST and incubated with 1:5000 dilution of HRP conjugated secondary antibody (Bangalore Genei, Bangalore, India) for 1 h. The blots were washed with PBST and developed with DAB/H₂O₂ substrate as per the manufacturer's recommendation. *P. falciparum* lysate prepared from in vitro culture was also run on SDS-PAGE following same conditions. After electrophoresis, proteins were transferred onto nitrocellulose membranes followed by blocking. The blots were incubated with 1:2000 dilution of the rabbit sera containing antibodies induced against the proteins as described above.

2.4. Molecular mass, reduction and alkylation, and free thiol analyzes

Purified protein samples were analyzed for mass by matrix-assisted laser desorption ionization–time of-flight (MALDI–TOF) by mass spectrometry with a sinapinic acid matrix. Protein reduction was achieved by incubation with a 100-fold molar excess of dithiothreitol over cysteines in the presence of 4 M urea, and alkylation was carried out with a 1000-fold molar excess of iodoacetamide over cysteines for 1 h at room temperature in the dark. Free sulfhydryl groups were estimated in the presence and absence of 4 M guanidine–HCl by using Ellman's reagent (5,5-dithio-bis-3-nitrobenzoic acid). L-Cysteine was used to plot the standard curve.

2.5. Immunization of mice

Groups of six Swiss mice (6–8 weeks old) were immunized subcutaneously with 30 µg of each protein emulsified in Freund's complete adjuvant. Immunizations were done three times giving two weeks interval between immunizations. Fifteen days after last immunization, mice were bled; spleen was dissected out and collected in RPMI 1640 medium. Two more mice were immunized with the same volume of adjuvant in 1X PBS and were taken as control. All the experimental protocols were approved by JNU Institutional Animal Ethics Committee and performed accordingly.

2.6. Lymphocyte proliferation assay

Splenic cell suspension from each mouse was prepared by grinding tissues in RPMI 1640 medium. The cells were washed in medium and then 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, Cambridge, MA) containing 100 µl aliquots of the cell suspension in presence of purified proteins at 1, 2, 4 and 6 µg/ml in triplicates. As a positive control, 2 µg/ml of PHA was added in each plate in triplicates. The cells were cultured for 4 days at 37 °C in a 5% CO₂ incubator at humidified atmosphere. Splenocytes were pulsed with 1 µCi ³H-thymidine per well for 18 h. Cells were harvested (PHD Cell Harvester, Cambridge Technology, MA) on to glass fibre filters to measure thymidine incorporation in a liquid scintillation counter (Beckman beta scintillation counter, Beckman Coulter, Fullerton, CA). Results were expressed as stimulation indices (SI) calculated as mean of counts

per minute (cpm) of test triplicates divided by mean of cpm of control triplicates.

2.7. Cytokine analysis

Cytokine levels in supernatants of antigen stimulated splenocytes of immunized mice were analyzed using Mouse Th1/Th2 ELISA kit (eBiosciences, San Diego, CA). For this, supernatants from splenocyte cultures stimulated with antigens (6 µg/ml) were collected at 48 and 72 h and stored at –80 °C until use. For the cytokine analysis, frozen samples were taken out and analyzed for the quantitative estimation of interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4 and IL-10 using a standard curve obtained with known concentrations of recombinant cytokines. Splenocyte culture supernatants from mice immunized with adjuvant alone were also used in the analysis.

2.8. Preparation of immunized rabbit sera

New Zealand White rabbits (*n*=3) were immunized subcutaneously with 100 µg of each protein emulsified in Freund's complete adjuvant (Sigma). Immunizations were done three times with a three weeks interval between immunizations. Fifteen days after last immunization, sera were collected. Control rabbits were immunized with the same volume of adjuvant in 1X PBS.

2.9. ELISA

Immunized sera were evaluated by enzyme-linked immunosorbent assay (ELISA). Polystyrene 96-well microtiter plates were coated with 100 ng/well of proteins incubating overnight at 4 °C. The plates were blocked at RT for 1 h with PBST containing 5% casein and then washed with PBST. Consecutive 2-fold dilutions of individual rabbit sera were incubated for 2 h at RT. The plates were washed and incubated with 1:5000 diluted horseradish peroxidase conjugated secondary antibody for 2 h. After washing enzyme-specific substrate, *o*-phenylenediamine/H₂O₂ was added in each well. The reaction was terminated with 5N sulphuric acid and absorbance was read at 490 nm in an ELISA reader (Microscan, ECIL, India). The absorbance was recorded, 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. Anti-AMA-1 IgG subclass antibodies were also detected by ELISA in the similar way using secondary antibodies anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (Sigma) at 1:1000 dilutions. Individual sera were tested at a starting dilution of 1:100 in duplicate followed by 4-fold serial dilutions. Pre-immune sera and sera from mice immunized with another irrelevant protein (PfMSP-1₄₂) were also used in the experiment as controls.

2.10. Indirect-immunofluorescence assay (IFA)

Localization of AMA-1 was determined by IFA in thin blood smears of *P. falciparum* schizonts on multitest slides. Cultures of three different Indian *P. falciparum* isolates were used for IFA. Blood smears were fixed with chilled acetone + methanol mixture (1:1) and then blocked with PBS containing 1% BSA (PBS-BSA). Smears were treated with sera dilutions at 1:100 in PBS-BSA and incubated for 2 h at room temperature. Slides were then washed three times with PBST and incubated with a 1:100 dilution of mouse anti-rabbit IgG–FITC-labeled antibody (Jackson ImmunoResearch, PA, 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.

2.11. Parasite growth inhibition assay

We did our initial experiments with three rabbits for each construct. Serum samples, both pre-immune and immune sera from rabbits were subjected for immunoglobulin-G fractionation by ammonium sulphate at a saturation of 40%. IgG precipitate was dialysed against 1XPBS, pH 7.2 till the removal of excess ammonium sulphate and the purity of the product was checked by immunoelectrophoresis. Parasite growth inhibition assay was done in triplicate for each dilution of individual IgG preparation. Parasite in culture media without negative or preimmune or immune sera was the control, because it determines the growth of parasites in normal media. The culture with negative or pre-immune sera (IgG) is taken as test control for parasite growth compared to immune IgG of various constructs. Parasite growth inhibition assay was done in two lines (3D7 type) with their difference at chloroquine susceptibility. The culture was set up with ring form at 1% parasitemia and at 5% hematocrit; culture was done for 48 h with a volume of 200 μ l media in each well. The effects of immunoglobulin-G fractions were evaluated in *P. falciparum* culture lines, both chloroquine sensitive (CQS) and chloroquine resistant (CQR) in vitro following the published method [41]. The CQS isolate, FDL-B and CQR isolate, FDL-NG were collected from patients 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 [42]. 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% hematocrit containing 1% parasitemia in 96-well flat bottom tissue culture plate. Individual IgG fractions were dosed in wells in triplicate at different protein concentrations starting from 100, 50, 25, 10, 5 and 2 μ g/well, either in the absence or presence of the immunogen. The volume of culture per well was kept 200 μ l including media, IgG fractions and parasite inoculums and culture only in enriched media was taken as control. The parasites were allowed to grow at 37 °C in a candle-jar to determine the effect of test substances in total parasite growth after 48 h. Growth of the parasite from each well was monitored microscopically in Giemsa stained smears by counting total number of parasites per 10,000 RBCs. Percent parasite growth inhibition (PGI) was calculated by the formula; $(1 - \text{NPT}/\text{NPC}) \times 100$, where NPT and NPC represent the number of parasites in the test and control wells, respectively.

2.12. 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. Linear regression analyzes were used in ELISA to calculate the serum dilution required to get the specific OD values.

3. Results

3.1. Expression and purification

As many as 10 *P. falciparum* isolates were screened and DNA sequence analysis of the clones from these gave two different alleles for further expression analysis. The sequence of one of the alleles PfS76 was exactly same as that of 3D7 isolate and the other PfrK9 had 18 aa difference from 3D7 sequence. These two allelic variants of domain I + II of AMA-1 ectodomain were cloned and expressed in

E. coli. Expression was standardized using various conditions such as concentration of IPTG, induction temperatures and duration of induction. The level of protein induction was very high even 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 were solubilized with a buffer containing 2% Sarkosyl. As the constructs had a 6xHis tag at N-terminal, Ni-NTA agarose affinity chromatography was used as a first step in purification. The bound proteins were washed extensively to remove the non-specifically bound impurities. Elution of the proteins with 400 mM imidazole yielded nearly 75% purified proteins. For both the allelic variants, same binding, washing and elution conditions were used. In order to get the recombinant proteins with close to native structure, a refolding step was standardized after testing several experimental conditions. Eluted proteins were refolded using rapid dilution method at 30–40 μ g/ml in a redox coupled GSH: GSSG solution and at 4 °C for a minimum of 40 h. Concentration and further purification using Fractogel EMD SO3-650 (M), a cation exchange resin, yielded purified protein for both the alleles. This was achieved after standardizing the pH conditions and salt concentrations for binding, washing and elution. This simple two-step purification yielded nearly 80–85% pure recombinant proteins (Fig. 1, panel I). The final yield of purified protein was 4–5 mg/l on shake flask level.

3.2. Molecular mass and disulphide bonds

The molecular masses of both the constructs were within the experimental error limits of measurements of the MALDI-TOF mass spectrometer, as calculated from the deduced amino acid sequence. The observed average masses were 45,034 for PfS76 (45,039) and 45,129 for PfrK9 (45,094) respectively (the values in the brackets correspond to the predicted masses). There are 10 cysteine residues, and hence expected to have five disulphide bonds in domain I + II. Ellman's test with both the recombinant products for the analysis of free cysteines resulted a very low value of free cysteines (data not shown). The absence of measurable amounts of free cysteines in the recombinant products have indicated correct folding resulting in complete disulphide bond formation. Under non-reducing conditions, both the recombinant proteins moved as tight single bands. All constructs showed significant mobility shifts when subjected to reduction and alkylation followed by SDS-PAGE analysis (Fig. 1, panel I). This further suggested the formation of disulphide bond stabilized folding in these recombinant products.

3.3. Recognition by immune sera and monoclonal antibody

A conformation specific monoclonal antibody, 4G2dc1 (kind gift from Dr. Alan Thomas), known to bind to a conformational dependent epitope within the region encompassing domain I + II [15] recognized the recombinant domain I + II on Western blots (Fig. 1, panel II a) and ELISA (data not shown) run under non-reduced conditions. Both the 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 b) and a pool of *P. falciparum* infected patients' sera (Fig. 1, panel II c). 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 (PfMSP-1₄₂) did not show any recognition with the polyclonal mice/rabbit sera from animals immunized with the recombinant constructs (fig not shown).

The two allelic proteins vary in 18 aa positions between each other. Among these, 13 are in domain I and 5 in domain II region.

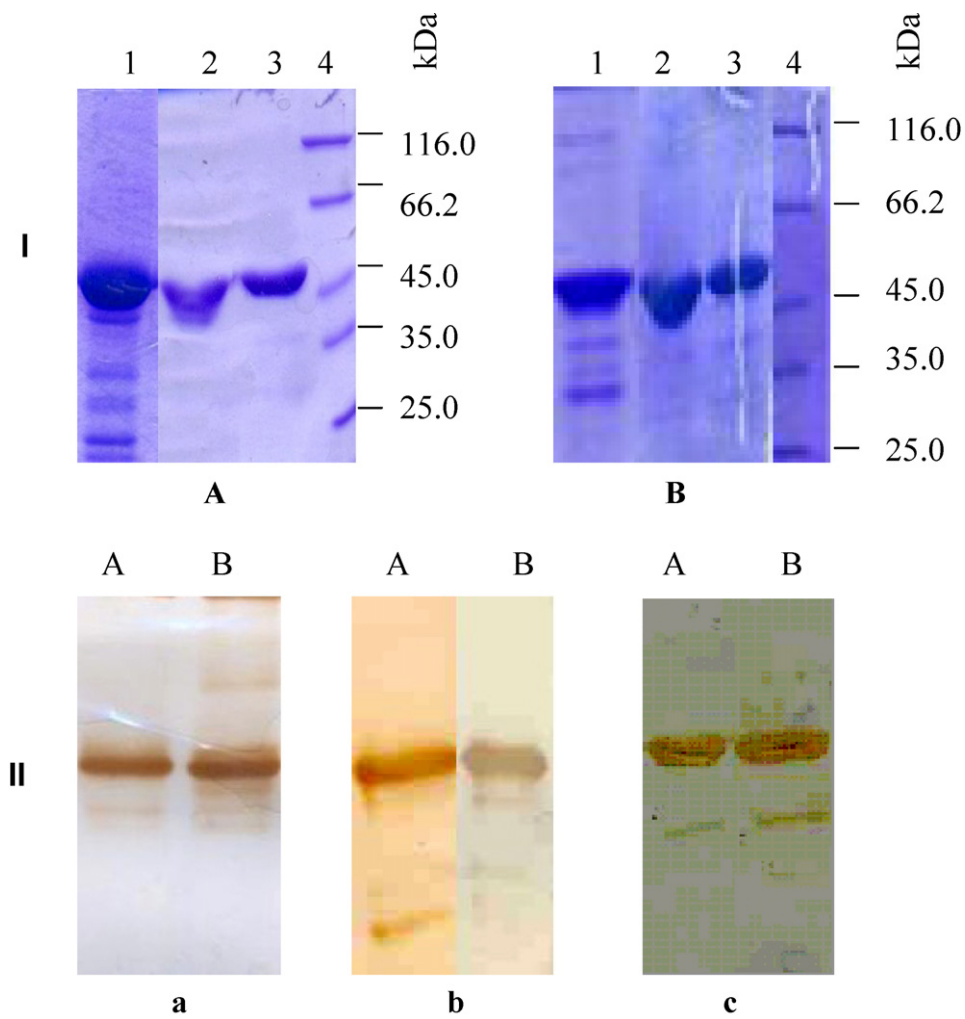


Fig. 1. SDS-PAGE analysis of AMA-1 domain I+II allelic variants during purification steps. Proteins detected by coomassie blue staining (panel I) and immunoblotting (panel II). Panel I (A) protein PfrK9; (B) protein Pfs76. Lane (1) Ni-NTA eluted sample under reducing conditions, (2) fractogel EMD SO3-650(M) purified sample under non-reducing conditions, (3) fractogel EMD SO3-650(M) purified sample under reducing conditions, (4) molecular weight marker. Panel II, Western blot analysis of purified allelic proteins PfrK9 and Pfs76. 200 ng of purified and refolded proteins were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and reacted with (a): 4G2.dc1 monoclonal antibody (1 µg/ml) followed by 1:5000 anti-rat IgG; (b): polyclonal mice sera (1:5000) followed by anti mice IgG (1:5000), and (c): a pool of patients's sera (1:1000) followed by anti-human IgG (1:5000). There was no recognition of bands with normal mice/human serum.

For the PfrK9 allele, there were 26 polymorphic amino acid (aa) positions; among these, aa at 8 positions are identical to 3D7 (all in domain I), 11 identical to FVO (6 in domain I and 5 in domain II) whereas 7 does not match with either of the two (6 in domain I and one in domain II), 3D7 and FVO isolates. Fig. 2 shows the aa variations between both the allelic type of domain I+II as compared to the widely diverse FVO and 3D7 types.

3.4. Immunogenicity of recombinant AMA-1 proteins

Immunization of rabbits ($n=3$) and mice ($n=6$) with each of the two double domain fragments induced high titer antibodies in both animals. Splenocytes from mice vaccinated with both the proteins formulated in CFA/IFA adjuvants showed significant proliferation at 4 and 6 µg/ml of antigen concentration. In ELISA and prolifer-

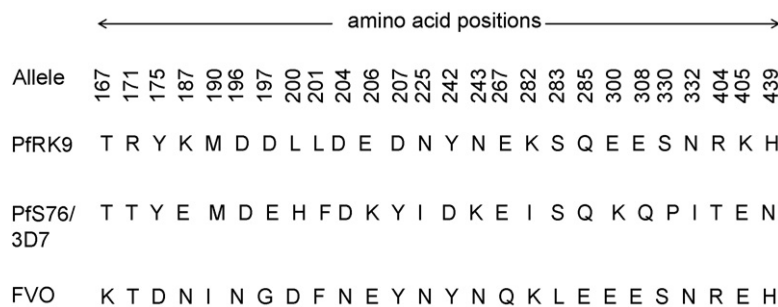


Fig. 2. Amino acid variations and their positions in the region encompassing domain I+II of AMA-1 among PfrK9, Pfs76 (3D7) and FVO isolates.

Table 1
Lymphocyte proliferation and cytokine responses

Protein	Stimulation index	Cytokine concn. (pg/ml of supernatant)			
		IL-2	IL-4	IL-10	IFN- γ
PfRK9	5.9 \pm 1.5	170 \pm 18	22 \pm 10	266 \pm 15	446 \pm 148
PfS76	6.1 \pm 2.6	299 \pm 169	9 \pm 3	250 \pm 53	416 \pm 98

Lymphocyte proliferation and cytokine responses in mice immunized with Pf. AMA-1 domain I+II variant proteins. The cytokine data were taken as an average from triplicate experiments on each of 48 and 72 h secretion. Stimulation index shown are the average from three experiments carried out in different days in triplicate wells with antigen concentration at 4 and 6 μ g/ml.

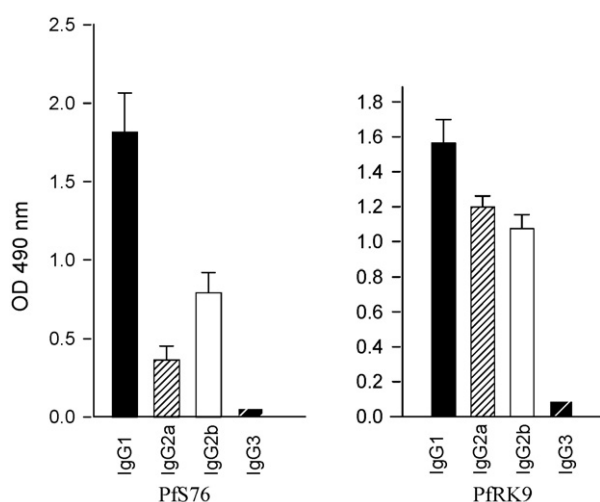


Fig. 3. Isotype ELISA analysis. IgG subclass antibody responses in mice immunized with allelic proteins Pfrk9 and Pfs76 were analyzed on ELISA. Sera dilutions were 1:2000 and the secondary antibody dilutions were 1:1000 for all the isotype IgGs. Error bars represents S.D.s. The sera from mice immunized with adjuvant alone were used as negative control.

eration assays, heterologous proteins were also used, the results (data not given) were comparable as that of homologous proteins. 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 (data not shown). Individual sera from a group of six mice were analyzed for anti-AMA-1 antibodies of IgG1, IgG2a, IgG2b and IgG3 isotypes (Fig. 3). All the mice immunized with both the allelic proteins showed high titers for IgG; IgG1 was the predominant isotype for all the mice and IgG3 the least dominant. The mice immunized with Pfs76 protein showed higher titers of IgG1 and lower titers of IgG2a and IgG2b as compared to those immunized with Pfrk9. The differences between groups were not very significant in case of IgG1, whereas those were significant for IgG2a

and IgG2b (p values were 0.0539 for IgG1, 0.000027 for IgG2a and 0.0065 for IgG2b). However for Pfrk9 allelic protein the ELISA titers for IgG2a and IgG2b were comparable. Table 1 represents the cellular responses seen for the AMA-1 immunized mice. The stimulation indices for the mice immunized by both the antigens were high and comparable. Both the antigens induced high proliferation as compared to the adjuvant control with CFA/IFA formulation suggesting antigen specificity of the proliferation. In order to analyze the contribution of cytokines associated with Th1 or Th2 immune responses, the levels of IFN- γ , IL-2, IL-4 and IL-10 were measured. Culture supernatants from mice splenocytes immunized with the allelic proteins were collected after 48 and 72 h of stimulation and their cytokine levels were determined by an ELISA. Both the antigens have stimulated higher levels of IL-2, IFN- γ (Th1) and IL-10 (Th2) and comparatively lower levels of IL-4. The cytokine induction levels in the case of splenocyte culture supernatants of adjuvant alone immunized mice were less than 10 pg/ml for all cytokines except for IL-2 where the values were 46 ± 7 .

3.5. Antigenicity of recombinant AMA-1

On IFA all of these antibodies (rabbits) reacted with schizont stage of *P. falciparum* (3D7 and FDL lines) at a dilution of 1:100 (Fig. 4). Nearly 90–95% of schizonts showed reactivity at 1:100 dilutions of sera and at 5 μ g purified IgG concentration. We found similar intensity of antibody reactions in schizont stage of 3D7 line by IFA as observed in two parasites used in this study. The reactivity of rabbit anti-double domain antibodies with *P. falciparum* AMA-1 was confirmed by immunoblot analyzes. Rabbit antibodies obtained on immunization with domain constructs (domain I+II) predominantly recognized two bands (fig not shown) corresponding to the previously identified 83-kDa and 66-kDa full-length and processed forms of AMA1 in *P. falciparum* [15,16].

We were interested to see any difference may affect the growth of the two 3D7 type parasite lines with difference in chloroquine sensitivity in presence of immune IgG. Results obtained from the ELISA and the total PGI of both strains CQR and CQS (CQR – chloroquine resistant and CQS – chloroquine sensitive) are summarized

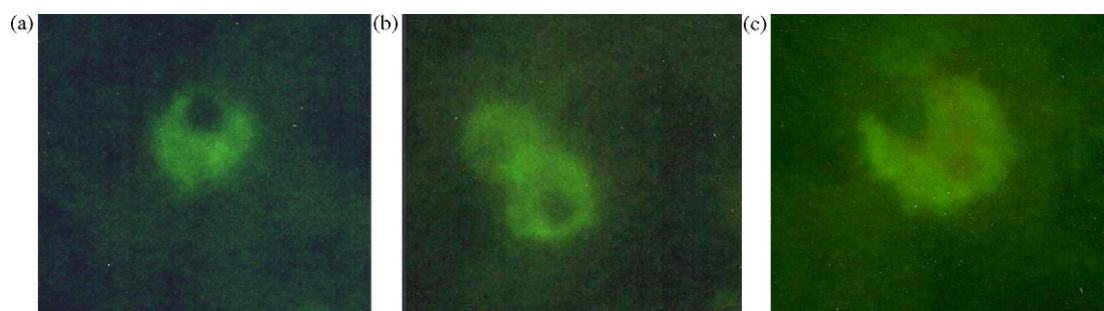


Fig. 4. (a–c) Rabbit antibodies against anti-AMA-1 domain I+II recognizes schizont (segmenter) stage parasites on IFA. *P. falciparum* culture enriched with schizonts was smeared on multitest slides. Parasites were fixed with chilled acetone+methanol mixture and were tested with antisera. Reactivity of antibodies with merozoites in the segmenter is shown with fluorescence.

Table 2
Summary of ELISA and GIA results for rabbits immunized with AMA-1 domain I + II proteins PfrK9 and Pfs76

Protein	Rabbit code	ELISA titer $\times 10^5$	% Growth inhibition	
			CQS	CQR
PfrK9	R1	1.15	70	74
	R3	1.85	± 9	± 8
	R4	1.25		
Pfs76	R2	5.65	57	53
	R5	7.05	± 15	± 7
	R6	17.63		
PfrK9	R7	10.06 ^a	41	48
+Pfs76	R8	13.59 ^b	± 16	± 7

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 S.D.s of the results from rabbit IgGs for all three rabbits per group for each of the antigen at an IgG concentration of 100 $\mu\text{g/ml}$.

^a Coat antigen PfrK9.

^b Coat antigen Pfs76.

in Table 2. Of all the antisera, R3 showed substantial inhibitory activity in both the strains even at 25 $\mu\text{g/ml}$ of IgG concentration (data not shown). Fig. 5 shows the parasite growth inhibition at varying IgG concentrations. The inhibition was reversed or considerably reduced on the addition of the respective proteins at 0.25 μM concentration along with the IgGs in the culture (Fig. 6). The results were significantly different ($p = 0.04$) between CQR/PfrK9 and CQS/Pfs76. When PfrK9 or Pfs76 were used individually at 100 μg per dose for immunization, it was enough to produce sufficient inhibitory antibodies. But with a combination of both the proteins at 50 μg each per dose may not be sufficient to produce inhibitory antibodies as high as single preparation at 100 μg per dose. The combination has the effect of halving the response to either of the alleles, but still showed specific inhibitory activity.

4. Discussion

It is seen from various studies that antibodies against AMA-1 inhibit merozoite invasion of host RBC with greater extent on

homologous parasites as compared to a heterologous parasite challenge [7,28,39,40]. Passive and active immunization experiments with murine and monkey malaria have shown that antibody mediated protection is strain specific [25,26]. The ectodomain of AMA-1 has been characterized for several plasmodium species in various systems and shown to be important in protection [18]. Similar to many other *Plasmodium* antigens, the conformational structure of AMA-1 has been shown to be critical for inducing inhibitory anti-AMA-1 antibodies [6,28]. The full-length ectodomain has eight disulphide bonds as compared to five in domain I + II. In an earlier study, we had shown that the region encompassing domain I + II is capable of generating antibodies that were inhibitory in an in vitro invasion/inhibition assay [32]. We have also observed that the level of expression of the smaller fragment was very high as compared to the full-length molecule under similar conditions. While most of the studies utilized the full-length ectodomain molecule, there are very few studies with the inter-species domains such as domain I + II [22,32,33].

In the present study we have isolated two allelic variant forms of domain I + II of AMA-1 ectodomain from Indian isolates of *P. falciparum* of which one is new and analyzed their immunogenicity in mice and rabbits. The two allelic variants differed in 18 aa positions from a total of ~ 360 aa. Among these, 12 aa difference occurs in domain I and the rest in domain II. PfrK9 allele varies in 15 aa positions with the sequence of FVO isolate, where 14 aa difference occurs in domain I and only one in domain II. In the PQ 30 expression vector, the level of expression was very high that resulted in the formation of inclusion body proteins for both the alleles. As the theoretical pIs were 6.4 (Pfs76) and 6.52 (PfrK9), the same purification conditions could be used. Using simple two-step chromatography, his-tag affinity followed by a cation exchange chromatography, nearly 80–85% pure proteins could be generated. Under non-reducing conditions, the proteins moved as tight bands suggesting them to be monomeric single population. The Ellmann's test also suggested the absence of any detectable amount of free cysteines in the preparation. Reduction and alkylation analysis suggested the formation of disulphide bonds in the refolded recombinant products. Molecular weight determination on mass spectrometric data was very close to the theoretical masses and well within the experimental limits of errors.

The authenticity of these recombinant products was analyzed using a monoclonal antibody 4G2.dc1 that is known to bind to an epitope in the region encompassing domain I + II [15]. The 4G2 Mab recognized both the proteins under non-reduced conditions suggesting that the recombinant products are refolded correctly. Furthermore, these two proteins were recognized by a pool of *P. falciparum* infected human sera at expected molecular masses when

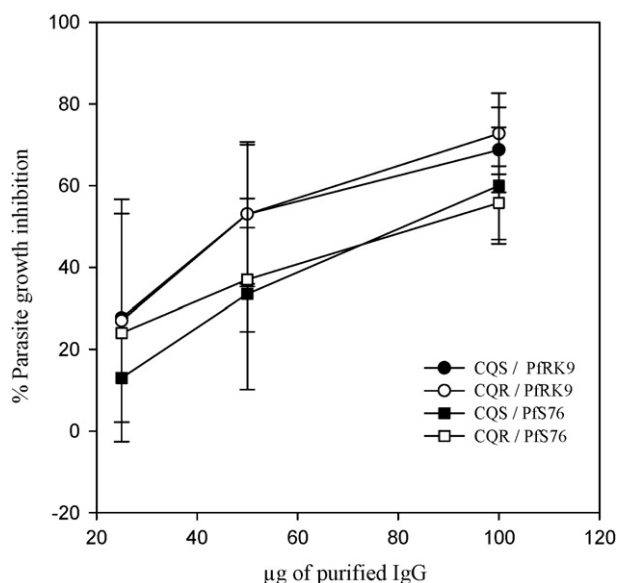


Fig. 5. Dose dependant growth inhibition when varying amounts of anti-AMA-1 domain I + II rabbit antibodies ($n = 3$) were added to the culture medium. Two parasite cell lines of 3D7 type, one chloroquine sensitive (CQS) and the other chloroquine resistant (CQR) were used for each of the anti-AMA-1 rabbit IgG immunized with PfrK9 and Pfs76. Error bars represent S.D.s. The p value was 0.04 for CQR/PfrK9 and CQR/Pfs76. In all other cases $p > 0.05$.

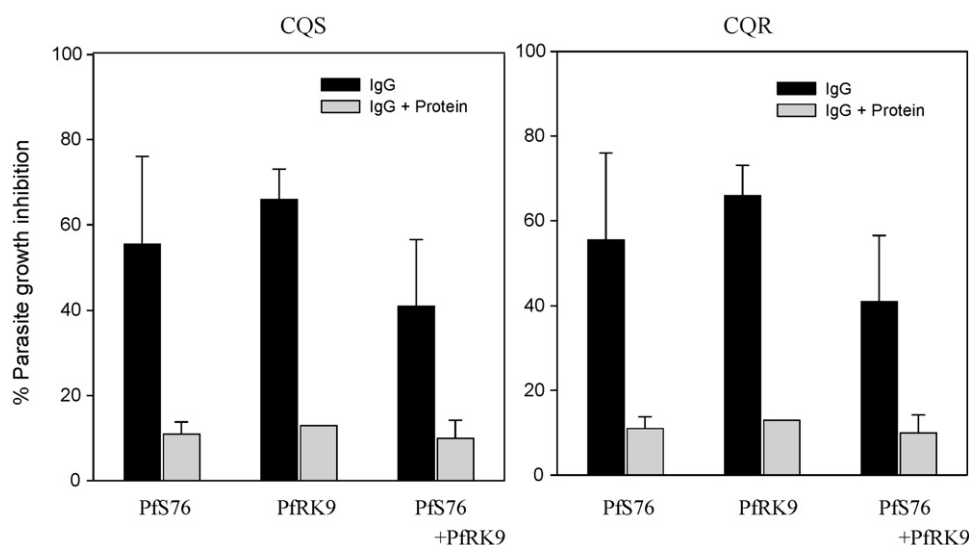


Fig. 6. Reversal of inhibition of *P. falciparum* parasites in presence of antigen ($0.25 \mu\text{M}$). The values plotted are the average of GIA done with IgG from three rabbits each ($100 \mu\text{g/ml}$) analyzed in triplicate wells. Error bars represent S.D.s.

subjected to SDS-PAGE analysis under non-reducing conditions. These results confirm that the critical conformational epitopes were maintained within the domain I+II recombinant products.

Our immunization results show that the two allelic products are equally highly immunogenic in mice and rabbits with IFA/CFA adjuvants. The antibody titers were around 500,000 for both the antigens in all the animals immunized, when the immunogen was $100 \mu\text{g}$ (rabbits) and $30 \mu\text{g}$ (mice) per dose at three doses. When a combination of the two alleles was used for immunization ($50 \mu\text{g}$ each) in two rabbits, similar antibody titers were obtained irrespective of the coating antigen (PfRK9/PfS76). Lymphocyte proliferation assay has shown stimulatory effects on T-cells of mice immunized with the recombinant proteins. Vaccination of mice with both the proteins induced IgG1, IgG2a and IgG2b antibodies and also produced cytokines IL-2, IL-4, IL-10 and IFN- γ . T-cells may confer protection against erythrocytic stages either by helping in antibody production or by the secretion of effector lymphokines, such as gamma interferon (IFN- γ). The T helper 1 (Th1) subset secrete IL-2 and IFN- γ and promote cellular responses and are associated with the IgG2a production, while the T helper 2 (Th2) subset produce interleukin 4 (IL-4) and IL-10, which are important in promoting humoral immunity and are associated with IgG1 production. The results showed activation of mixed Th1/Th2 immune responses after the immunization of mice with both the allelic variants. As both Th1 and Th2 responses seem to be involved in protective immune response against malaria [43,44], our results suggest the validity of these two recombinant products to be considered for further analysis in vaccine purpose. The antibodies elicited by immunization were biologically active against *P. falciparum* parasites as seen from the growth inhibition/invasion assay. The antisera raised against the two allelic proteins seemed to be inhibitory in two 3D7 type of *P. falciparum* parasite lines (predominant isotype in India), with a difference in their sensitivity towards chloroquine as observed both in vivo and in vitro CQ susceptibility. In India, resistance to commonly used antimalarial, CQ is posing a great threat. Now the national drug policy in India has recommended use of combination therapy (AS+SP – artesunate plus sulphadoxine-pyrimethamine) as first line of treatment in areas showing CQ treatment failure above 10% of cases. We were therefore interested to study the effect of the antibodies in these two parasite lines to see if CQ sensitivity made any difference to antibody reactivity of

the two substrains. In order to make sure that the inhibitory activity was specific to the antibodies alone, serum purified IgG was used in these studies. The effective inhibition of merozoites was sensitive even at a low concentration of purified IgG at $\sim 10 \mu\text{g/ml}$ in one of the rabbits R3 (data not shown). This suggests that the recombinant product even at very lower concentrations is capable of generating inhibitory antibodies. Inhibitory activity was considerably reduced or reversed when the assay was done in presence of $0.25 \mu\text{M}$ of the recombinant antigens. The experimental observations from the present study and the 4G2.dc1 Mab binding ability of the recombinant products suggest that both the allelic proteins have retained T-helper and inhibitory epitopes necessary for eliciting protective immunity against malaria.

From Figs. 5 and 6, it appears that the antibody response elicited by immunization with both the proteins not to be strain specific as both the antibodies produced similar GIA results in two parasite lines (3D7 type). Among the 18 aa variant positions, only three variations at positions at 282 (K/I), 330 (S/P), 332 (N/I) lie within the critical B-epitopic region that are maintained by natural selection [45–47]. Among these, only one variation (at aa 282), which is dimorphic [36], lie within domain I region. AMA-1 polymorphisms are shown to be under balancing selection especially for domains I and III [7,28,39,40]. Earlier reports suggest that conserved cross-reactive epitopes can be targets of invasion inhibitory antibodies, in addition to strain specific epitopes [7,28]. Most of the inhibitory antibodies and naturally acquired protective immunity are targeted to domain I [28,48]. Though the relative contributions of dimorphic and polymorphic residues to the evasion of inhibitory antibodies are unknown, some evidences indicate that highly polymorphic sites have more important role than the dimorphic sites [28,39,40]. In the present study, though there are variations at 18 aa positions in two proteins, the presence of one dimorphic aa variation at 282 did not cause much difference with respect to allele specificity.

Though AMA-1 is widely considered as a promising vaccine candidate, there are not many studies to show the impact of the polymorphism with respect to protective immune responses. A recent study incorporating two of the mostly diverse alleles of AMA-1, viz 3D7 and FVO, into the vaccine has yielded a broader immune response than could be achieved by vaccination with either of the one alone [40]. As there can be many allelic variant forms in the field, a combination of several variant antigens may have a greater

impact as a vaccine as compared to a single one. It is to be noted that one of the isolates PFRK9 is a novel allele and have its closest sequence known from genbank, an Indian isolate, with 7 aa difference.

In conclusion, our results show that both the AMA-1 domain I+II allelic variants either in combination or each one alone by itself could induce significant immune responses in mice and rabbits in combination with Freund's adjuvants. Furthermore, these antibodies are functionally active as they were able to inhibit parasite growth in vitro considerably. Further studies with large-scale preparations, diverse parasite lines and use of other adjuvants, those are human compatible would suggest the validity of these preparations for human vaccine purpose.

Acknowledgments

We thank Dr. Alan Thomas, BPRC, The Netherlands and Drs. David Lanar and Arnaldo Barbosa, WRAIR, Washington DC, for the kind gift of Mab 4G2.dc1. The financial assistance from the Department of Science and Technology, New Delhi, India to PVL is greatly acknowledged.

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