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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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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 form 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 × 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) (∼22 °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 μg/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 $NaHPO_4$–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.

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