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Immunogenicity of a recombinant malaria vaccine based on receptor binding domain of *Plasmodium falciparum* EBA-175

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Abstract

Malaria parasites require specific receptor–ligand interactions to invade host erythrocytes. The 175 kDa *Plasmodium falciparum* erythrocyte binding antigen (EBA-175) binds sialic acid residues on glycophorin A to mediate erythrocyte invasion. The amino-terminal, conserved, cysteine-rich region of EBA-175, referred to as F2, contains receptor-binding sites. We propose to develop a recombinant malaria vaccine based on region F2. Recombinant *P. falciparum* region F2 (PfF2) was expressed in *Escherichia coli*, purified from inclusion bodies under denaturing conditions by metal affinity chromatography, renatured by oxidative refolding and purified further by ion-exchange and gel filtration chromatography. Recombinant PfF2 was characterized and shown to be pure, homogeneous and functionally active in that it binds human erythrocytes with specificity. The immunogenicity of recombinant PfF2 formulated with three human compatible adjuvants, namely, Montanide ISA720, AS02A and alum was tested in mice. All the formulations tested elicited high titer antibodies that block erythrocyte invasion in vitro. The AS02 formulation yielded sera with the highest end-point ELISA titers followed by Montanide ISA720 and alum. Analysis of cellular immune responses indicated that all formulations resulted in significant splenocyte proliferation. Analysis of cytokines secreted by proliferating splenocytes indicated that all the adjuvant formulations tested induced Th1 type responses. These results suggest that recombinant PfF2 formulated with human compatible adjuvants is immunogenic and can elicit high titer invasion inhibitory antibodies providing support for further clinical development of this promising vaccine candidate.

Keywords: Malaria vaccine; Erythrocyte invasion; Erythrocyte binding proteins; EBA-175

1. Introduction

Plasmodium falciparum is responsible for majority of the mortality associated with malaria. All the clinical symptoms of malaria including severe complications like anemia, cerebral malaria, placental malaria and renal failure are associated with the blood-stage of *P. falciparum* infection [1]. Each cycle of blood-stage development involves red cell invasion, parasite multiplication, red cell rupture, and egress of next generation merozoites and re-invasion of red cells. Erythrocyte invasion requires specific receptor–ligand interactions. *P. falciparum* merozoites commonly use sialic acid residues on glycophorin A as receptors for invasion [2]. Binding to sialic acid-glycophorin A is mediated by a 175 kDa erythrocyte binding antigen (EBA-175) [3]. *P. falciparum* is also known to invade erythrocytes by alternate pathways that are independent of the interaction of EBA-175 with sialic acid-glycophorin A [4]. EBA-175 belongs to a family of erythrocyte binding proteins (EBPs) that includes *Plasmodium vivax* and *Plasmodium knowlesi* Duffy-binding proteins (PvDBP and PkDBP), which bind the Duffy-blood group antigen during invasion, and *P. falciparum* paralogs such as EBA-140, EBA-181 and EBL-1, which bind receptors other than sialic acid-glycophorin A [4,5]. The receptor-binding domains of EBPs lie in conserved N-terminal cysteine-rich regions that

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are referred to as Duffy-binding-like (DBL) domains after the binding domains that were first identified from PvDBP and PkDBP [4–8]. The N-terminal cysteine-rich region of EBA-175 contains a tandem duplication of DBL domains, F1 and F2, at the N-terminal end, which contain the receptor-binding sites for sialic acid residues [7,8].

Immunization with recombinant F2 region of *P. falciparum* EBA-175 (PfF2) formulated with Freund's adjuvant yields high titer antibodies that efficiently inhibit erythrocyte invasion by *P. falciparum* in vitro. Importantly, antibodies raised against recombinant PfF2 block invasion by *P. falciparum* field isolates that invade erythrocytes using multiple pathways [9]. These results provide support for the development of an asexual blood stage malaria vaccine based on PfF2.

Here, we have tested the immunogenicity of recombinant PfF2 formulated with adjuvants compatible for use in humans. Recombinant PfF2 was expressed in *Escherichia coli*, purified from inclusion bodies under denaturing conditions, refolded by rapid dilution and purified to homogeneity by ion-exchange and gel-filtration chromatography using methods described earlier [9]. Biochemical and biophysical characterization confirmed that recombinant PfF2 was pure, homogenous and functionally active in that it bound erythrocytes with specificity. In an effort to identify an optimal adjuvant formulation for use in human clinical trials, we tested the immunogenicity of recombinant PfF2 formulated in three human-compatible adjuvants, namely, Montanide ISA720, AS02A and alum. Each formulation yielded high titer invasion inhibitory antibodies in BALB/c mice with the AS02A formulation yielding sera with highest ELISA titers. Results reported here indicate that recombinant PfF2 formulated in human compatible adjuvants is highly immunogenic and can elicit high titer antibodies that block invasion providing support for further development of a recombinant malaria vaccine based on PfF2.

2. Materials and methods

2.1. Expression of recombinant receptor binding domain of P. falciparum EBA-175 (PfF2)

E. coli BL21(DE3) cells (Novagen) were transformed with plasmid pET28a+ PfF2, which contains DNA encoding PfF2 fused to a 6-His tag at the carboxyl terminus cloned downstream of the T7 promoter in the expression plasmid pET28a+ (Novagen) as previously described [9]. *E. coli* BL21(DE3) contains the gene for T7 RNA polymerase under the control of the IPTG inducible *lac* promoter. Two milliliters of an overnight culture of *E. coli* BL21(DE3) pET28a+ PfF2 grown in Luria broth (LB) containing kanamycin at 30 $\mu g\,mL^{-1}$ was used to inoculate 200 mL of semi-defined medium (Na_2HPO_4, 6.0 $g\,L^{-1}$; KH_2PO_4, 3 $g\,L^{-1}$; NaCl, 0.5 $g\,L^{-1}$; NH_4Cl, 0.2 $g\,L^{-1}$; yeast extract, 1.0 $g\,L^{-1}$; glucose, 1.0 $g\,L^{-1}$; 1.0 mM $MgSO_4$; 0.1 mM $CaCl_2$; kanamycin, 50 $mg\,L^{-1}$, pH 7.2) and grown to log phase. The log phase culture (200 mL) was used to inoculate a 20 L fermentor (Applikon AG, Netherlands) containing 10 L of semi-defined medium at 37 °C. Dissolved oxygen of the culture was maintained at 40% by controlling the agitation rate and air inlet flow rate. The pH was maintained at 6.8 by addition of 2.0 N NaOH. Cell density was monitored by measuring the optical density of the culture at 600 nm (OD_{600}). Expression of PfF2 was induced with 1.0 mM IPTG when cell density of the culture reached an OD_{600} of five. *E. coli* cells were harvested by centrifugation 4 h after induction. The cell pellet was stored at −80 °C until use.

2.2. Refolding and purification of recombinant PfF2

Recombinant PfF2 accumulates in inclusion bodies. It was purified from inclusion bodies under denaturing conditions by metal affinity chromatography, refolded by rapid dilution and purified to homogeneity by ion-exchange and gel-filtration chromatography as previously described [9]. The protein concentration was measured spectrophotometrically, adjusted to 0.5 $mg\,mL^{-1}$ and stored at −80 °C until further use.

2.3. Reverse phase chromatography with recombinant PfF2

Refolded and purified PfF2 was tested for homogeneity by reverse phase high-pressure liquid chromatography (RP-HPLC) using a C-8 column (Supelco) following previously described method [9].

2.4. Erythrocyte binding assay with recombinant PfF2

The functional activity of PfF2 was tested using an erythrocyte-binding assay as described earlier [9].

2.5. Testing for endotoxin levels

Endotoxin levels were measured with Limulus amebocyte lysate (LAL) kit using gel clot assay as recommended by the manufacturer (Salesworth).

2.6. Formulation of PfF2 with adjuvants

PfF2 was formulated with the following adjuvants, Montanide ISA720, AS02A and alum according to manufacturer's instructions. Briefly, formulations with different adjuvants were prepared as follows. In case of Montanide ISA720, 2.3 parts Montanide ISA720 (Seppic) were mixed with one part of antigen (v/v). For AS02A, 1.3 parts of AS02A (GlaxoSmithKline, Belgium) were mixed with one part of antigen (v/v). For formulation with alum hydroxide, the pH of alum (18 $mg\,mL^{-1}$) was first adjusted to 7.0 with NaOH and then mixed with PfF2 in the ratio of one part adjuvant:one part