Evaluation of Three *Pichia pastoris*-Expressed *Plasmodium falciparum* Merozoite Proteins as a Combination Vaccine against Infection with Blood-Stage Parasites

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Because invasion of erythrocytes by *Plasmodium falciparum* merozoites involves multiple receptor-ligand interactions, it may be necessary to develop a multivalent malaria vaccine that is comprised of distinct parasite ligands. PfAMA-1, PfMSP1, and PfEBA-175 are merozoite proteins that play important roles in invasion. We have constructed a PfCP-2.9 chimeric protein consisting of PfAMA-1 and PfMSP1 and tested it for immunogenicity in animal models and humans. The F2 subdomain of PfEBA-175 (PfEBA-175II F2) was identified as the binding domain for glycophorin A on erythrocytes. In this study, we used the codon frequencies of the yeast *Pichia pastoris* to redesign and synthesize a gene encoding the F2 domain. We found that the codon-optimized gene was expressed at a high level in *P. pastoris* as a soluble protein with a yield of about 300 mg/liter. The expressed protein was able to bind normal erythrocytes but not those treated with neuraminidase or trypsin. Moreover, the protein was recognized by the sera of malaria patients and was highly immunogenic in mice, rabbits, and rhesus monkeys. Immunoglobulin G isolated from both immunized rabbits and monkeys inhibited *in vitro* parasite growth. Immunization of animals with a combination of PfEBA-175II F2 and PfCP-2.9 did not result in antigen (Ag) competition in animals. Moreover, antibodies to both PfEBA-175II F2 and PfCP-2.9, isolated from rabbits immunized with both constructs, inhibited parasite growth *in vitro*. The combination of high yield, functional folding, antibody inhibition, and lack of Ag competition provides support for inclusion of these merozoite proteins in a combination vaccine against infection with blood-stage parasites.

Plasmodium falciparum and *Plasmodium vivax* are the causative agents of the majority of malaria cases in the world today. Of the two, *P. falciparum* is responsible for the most virulent form of the disease, causing over 2 million deaths per year, usually in children under 5 years of age. Malaria infections have traditionally been treated by chemotherapy. Another approach has been to use insecticides against the *Anopheles* sp. mosquito vectors that transfer the parasites between hosts. Because of the emergence and rapid spread of drug-resistant parasites and insecticide-resistant mosquitoes, there is an urgent need for the development of new tools to control malaria. Vaccination is one such tool that may control and even eradicate the disease from the world. Based on the life cycle of the parasite, merozoite invasion of host erythrocytes is an optimal target for vaccines against infection with blood-stage parasites. However, merozoite invasion is a complex process involving several steps. The initial step requires species-specific interactions between erythrocyte receptors and parasite ligands. Disruption of these interactions would, in principle, prevent invasion and all of the clinical manifestations of infection. The invasion of human erythrocytes by *P. vivax* requires recognition of the Duffy blood group antigen (Ag) (11, 22), while invasion by *P. falciparum* involves multiple alternative ligand-receptor interactions (5, 9, 14). Glycophorin A and band 3 on human erythrocytes are thought to be receptors for invasion of *P. falciparum*. Proposed ligands include merozoite surface pro-

teins or proteins situated in micronemes and rhoptries of merozoite.

These *P. falciparum* ligands and receptors interact via either a sialic acid-dependent or an independent invasion pathway. One *P. falciparum* ligand is the 175-kDa erythrocyte binding Ag (EBA-175), which is released as a soluble protein from micronemes at the time of schizont rupture (3). A number of investigations indicate that the protein specifically binds to normal human erythrocytes but does not bind to erythrocytes that are deficient in glycophorin A or that have been treated with neuraminidase (21). An N-terminal cysteine-rich region comprised of 616 amino acids, known as region II (RII), has been identified as the receptor-binding domain of EBA-175 (25). The sequence of RII is conserved among *P. falciparum* isolates (13) and is also homologous to the cysteine-rich erythrocyte binding domains of the *P. vivax* Duffy binding proteins (1). RII is composed of two subdomains, designated F1 and F2. Investigation of the subdomains has revealed that the binding function resides within the F2 subdomain. Antibodies against this subdomain blocked the binding of the molecule to glycophorin A on erythrocytes as well as parasite invasion *in vitro* (6, 17, 20). The dependence on the binding of glycophorin A for invasion places this interaction in the sialic acid-dependent invasion pathway.

Merozoite surface protein 1 (MSP1) is spread evenly over the entire surface of the merozoite and may be anchored via epidermal growth factor-like regions in the C terminus of the protein (7). Upon invasion, the proteolytic cleavage just N terminal of these epidermal growth factor-like domains leaves only a 19-kDa fragment attached to the cell surface (2). Recently, this 19-kDa C-terminal fragment, MSP1-19, was iden-

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tified as the parasite ligand that binds to human erythrocyte band 3 (8). This finding suggested that the MSP1-19/band 3 interaction plays a role in the sialic acid-independent pathway. Apical membrane antigen 1 (AMA-1) is a type I integral membrane protein that is expressed in micronemes and transported to the cell surface when merozoites are released. Its C-terminal disulfide-bonded domain [AMA-1 (III)] is the target of inhibitory antibodies isolated from regions where malaria is endemic, although its role in invasion of the parasite is unclear (15).

Due to multiple alternative pathways for invasion by *P. falciparum*, it may be necessary to develop a multicomponent malaria vaccine incorporating distinct parasite ligands. We have constructed a *P. falciparum* AMA-1 (III)/MSP1-19 chimeric protein (designated PfCP-2.9) as a potential vaccine candidate that has entered clinical trials (18). Antibodies against this protein strongly inhibited merozoite invasion in vitro, mediated by a combination of inhibitory antibodies generated by the individual components of PfCP-2.9. In this study, we expressed a codon-optimized gene encoding PfEBA-175II F2 in the yeast *Pichia pastoris*. The recombinant protein was characterized and tested for its immunogenicity in animal models, including nonhuman primates. Immunization with PfEBA-175II F2 plus PfCP-2.9 was carried out to evaluate Ag competition of the combination as well as the function of antibodies specific for each individual component. Incorporation of these three merozoite proteins into a vaccine combination would be of great interest, since such a vaccine could potentially achieve protection via inhibition of distinct invasion pathways used by merozoites.

MATERIALS AND METHODS

Parasite. The *P. falciparum* FCC1/HN line was isolated from Hainan Island, China, and its continuous cultivation in vitro was established in 1979. This line has been adapted to rabbit serum for cultivation. The parasite was maintained in RPMI 1640 medium containing 15% rabbit serum.

Synthesis of PfEBA-175II F2 gene. The DNA sequence encoding PfEBA-175II F2 of the Pf3D7 line was redesigned using *Pichia* codon usage (GenBank U32207). The 942-bp sequence of the resulting synthetic gene was divided into 12 oligonucleotides with a length of about 100 nucleotides (nt). The overlapping region between two oligonucleotides varied from 16 to 20 nt. The 12 oligonucleotide fragments were assembled by an asymmetric PCR-based method as described before (19) to generate the 942-bp fragment. The product was isolated from the PCR by 0.8% agarose electrophoresis and inserted into plasmid pBKS via XhoI and EcoRI restriction sites. The recombinant plasmid was transformed into *Escherichia coli* DH5 α , and transformants containing the insert were sequenced using an ABI PRISM 377 DNA sequencer to identify a clone with an error-free sequence of the gene.

Expression of PfEBA-175II F2 gene in *Pichia pastoris*. The synthetic PfEBA-175II f2 gene was first inserted into *Pichia* expression vector pPIC9 (Invitrogen Co., CA) via XhoI and EcoRI such that the *Saccharomyces cerevisiae* pre-pro-alpha-mating factor signal sequence was introduced in frame with the PfEBA-175II f2 coding sequences, and then a BamHI/Sall fragment was cut out from the plasmid and ligated into pPIC9k vector, which is identical to pPIC9 except for the Kan^r gene that confers resistance to G418. The resulting recombinant plasmids were linearized by Sall before they were transferred by electroporation into *Pichia pastoris* GS115. Selection of His⁺ transformants and G418-resistant clones was carried out according to the instruction manual (Invitrogen Co., CA). The selected clones were cultured first in MGY medium at 30°C overnight till the optical density at 600 nm (OD₆₀₀) reached 2 to 6 and then in BMMY medium containing 0.5% methanol for inducing expression. Methanol was added to the culture at a final concentration of 0.5% at 24-h intervals. To express the protein in a 15-liter fermentor, the 250-ml culture of the expressing clone was first grown at 30°C for 22 h and then inoculated into the 15-liter fermentor containing 6,000 ml of minimal salts fermentation medium (per liter of the medium containing

MgSO₄, 14.0 g; CaSO₄ · 2H₂O, 0.9 g; K₂SO₄, 18 g; KOH, 4 g; 26 ml of 85% H₃PO₄ and 4 ml of PTM1 trace metals solution). The cells were grown at 30°C and harvested at 72 h after methanol induction. Time point samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for detection of expressed product.

Protein purification. To facilitate purification, a DNA sequence encoding a six-His tag was added to the C terminus of the gene. Purification of PfEBA-175II F2 protein from the fermentation supernatant was performed by using two steps including Ni-nitrilotriacetic acid (NTA) and gel filtration (Superdex 75) chromatography. For the first step of purification, the supernatant was dialyzed at 4°C extensively against phosphate buffer. The dialyzed material was applied to an Ni-NTA column (QIAGEN, Germany). The column was washed three times with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The second step of purification was carried out with gel filtration chromatography (Superdex 75; Amersham Biosciences, United Kingdom) using 0.05 M sodium phosphate, pH 7.0, plus 0.15 M NaCl as elution buffer. The fractions containing the target protein were pooled, and the purity of the protein was analyzed by densitometry of a Coomassie blue-stained SDS-polyacrylamide gel. The protein concentration was measured using the Bradford method. Preparation of purified PfCP-2.9 recombinant protein was performed as previously described (18).

Erythrocyte binding assays. Fermentation supernatant containing the expressed PfEBA-175II F2 protein was extensively dialyzed against serum-free RPMI 1640 medium before used for binding. The supernatant was incubated with normal or neuraminidase- as well as trypsin-treated erythrocytes at room temperature on a rocker for 60 min. The bound protein was eluted from the erythrocytes with 1 M NaCl. The eluted samples were analyzed by SDS-PAGE and further confirmed by Western blotting using pooled sera obtained from malaria patients.

Immunization studies. The purified PfEBA-175II F2 protein or PfCP-2.9 or a combination of the two Ags was formulated with the Montanide ISA720 adjuvant (SEPPIC, France) by mixing a 70% volume of the adjuvant with a 30% volume of the Ag solution using a homogenizer at 4,000 rpm for 4 min. New Zealand White rabbits as well as BALB/c mice used in this study were purchased from Songjiang Animal Facility of the Chinese Academy of Sciences, Shanghai, China. BALB/c female mice 6 to 8 weeks old and 18 to 20 g in weight were used in this study. The mice were maintained in the animal facility of the Second Military Medical University under specific-pathogen-free conditions, in compliance with the Second Military Medical University animal care policies. The mice (five per group) were immunized subcutaneously three times at 3-week intervals with 20 μ g of each protein or a combination of two Ags (20 μ g each) emulsified in the adjuvant. Rabbits (2 to 2.5 kg, 4 to 6 months, male, three rabbits per group) housed at the same animal facility were immunized subcutaneously four times at 3-week intervals with 100 μ g of each protein or a combination of two Ags (100 μ g each) emulsified in the adjuvant. The monkey experiment was performed at the Kunming Institute of Zoology, Chinese Academy of Sciences. The rhesus monkeys (*Macaca mulatta*, 3 to 5 kg, 3 to 4 years of age, four monkeys per group) with a history of no malaria exposure were screened for lack of antibodies to *P. falciparum* by immunofluorescence assay (IFA) and immunized intramuscularly four times at days 0, 30, 90, and 146 with 100 μ g PfEBA-175II F2 protein emulsified in the adjuvant. The monkeys were maintained in filter-topped, temperature-controlled cages after immunization in compliance with the animal care policies of the Kunming Institute of Zoology, Chinese Academy of Sciences. Temperatures of the immunized monkeys were measured, and local reactions at injection sites were observed daily for 3 days after each immunization. Sera obtained from each animal before immunization and about 1 week after each immunization were analyzed for specific antibodies by enzyme-linked immunosorbent assay (ELISA) as well as IFA. The purified recombinant PfCSP protein derived from *Pichia pastoris* was used as a control antigen for immunization (18).

Collection of human serum samples. Blood samples used in this study were collected from patients infected with *P. falciparum* in Hainan and Yunnan provinces of China, where malaria is endemic. Infection of the *P. falciparum* parasite was determined by examination of a finger prick thick and thin blood smear for malaria parasites and measurement of temperature. Sixty-five enrolled subjects had a positive blood smear for asexual forms of *P. falciparum* and symptoms consistent with the presence of the malaria parasite. Participants were excluded from enrollment if they were infected with a mixture of *P. falciparum* and *P. vivax*. Blood sampling was performed after oral consent for participation was obtained from all subjects, who were volunteers aware of the objective of the study. Venous blood was collected into sterile tubes, and serum samples were stored at -20°C until used.

FIG. 1. Production of rPfEBA-175II F2 protein. A: Expression of PfEBA-175II f2 gene in *P. pastoris*. Fifteen microliters of *Pichia* fermentation culture supernatant collected immediately before and 12, 24, and 48 h after induction was loaded on a 12.5% SDS-polyacrylamide gel followed by Coomassie blue staining. Lane 1, prestained molecular mass marker; lane 2, before induction (0 h); lane 3, 12 h after induction; lane 4, 24 h after induction; lane 5, 48 h after induction. B: The homogeneity of the recombinant protein was determined by the analysis of a Coomassie blue-stained SDS-polyacrylamide gel. Lane 1, 7.5 µg of the purified protein; lane 2, 15 µg of the purified protein. Numbers at left are molecular masses in kilodaltons.

ELISA. ELISA was performed in triplicate on diluted serum samples in 96-well flat-bottomed microtiter plates. Microtiter plates (Thermo Lab Systems) were coated for 1 h at 37°C with 100 µl of recombinant protein at the concentration of 1.0 µg/ml diluted in carbonate buffer (0.159% Na₂CO₃, 0.293% NaHCO₃, pH 9.6). Plates were blocked with phosphate-buffered saline (PBS) containing 3% skim milk at 37°C for 1 h. Serum samples at various dilutions were added to the plates (100 µl) and incubated at 37°C for 1 h. Horseradish peroxidase-conjugated goat anti-rhesus monkey (Southern Biotechnology Associates), goat anti-rabbit, or goat anti-mouse immunoglobulin G (IgG) (SAB Company, China) with 1:1,000 dilutions in PBS containing 3% skim milk was added to the plates for a further 1 h of incubation at 37°C. For every step, plates were washed three times with PBST (PBS containing 0.05% Tween 20) and once with PBS using a plate washer. Bound secondary antibodies were detected by adding 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) substrate solution at room temperature for 10 min, and the reaction was stopped by adding a solution of 50 µl of 2 M H₂SO₄. The plates were read at an absorbance of 450 nm (ELx800 ELISA reader; BIO-TEC Instrument Inc.). Cutoff values were determined as the mean plus 3 standard deviations for the preimmunization sera (five preimmunization serum samples were used to calculate each of the cutoff values).

IFA. IFA was performed as previously described (24). Cultured parasites of *P. falciparum* FCC1/HN isolates were used as Ags. IFA slides were prepared using culture material with a 5 to 10% parasitemia. Serum samples were incubated at various dilutions with the slides containing parasite-infected erythrocytes for 1 h at room temperature. After being extensively washed with PBS, the slides were incubated with fluorescein isothiocyanate-labeled secondary antibody—goat anti-rhesus monkey (Southern Biotechnology Associates) or goat anti-rabbit or goat anti-mouse (SAB Company, China) IgG—with 1:1,000 dilutions in PBS for 1 h at room temperature. The bound secondary antibodies were examined with a fluorescence microscope. Endpoint titers were determined as the last dilution above the background at which fluorescent parasites were observed in preimmunized serum samples.

Growth inhibition assay. Function of specific antibodies was determined by measuring their ability to inhibit growth of the parasite in vitro. Parasites of the FCC1/HN isolate were maintained in RPMI 1640 medium containing 15% rabbit sera. Protein A beads (Pharmacia Biotech, Piscataway, NJ) were applied to isolate total IgG from immune sera for the inhibition assay while the PfEBA-175II F2 affinity column was used to isolate specific antibodies against the antigen. The antibodies eluted from the columns were extensively dialyzed before use. The inhibition assay was performed with the starting culture containing 2% hematocrit and approximately 0.5% parasitemia with the majority being late trophozoites and schizonts. One hundred seventy microliters of the culture

FIG. 2. Erythrocyte binding assay of rPfEBA-175II F2. The dialyzed expression supernatant was added to normal erythrocytes and to enzymatically treated erythrocytes. The eluent was separated by SDS-PAGE and detected by Western blotting using pooled sera from malaria patients. Lane 1, expression supernatant; lane 2, rat erythrocytes; lane 3, human erythrocytes; lane 4, neuraminidase-treated human erythrocytes; lane 5, trypsin-treated human erythrocytes; lane 6, rabbit erythrocytes; lane 7, mouse erythrocytes; lane 8, human erythrocytes with the supernatant from a *Pichia* culture transformed with control plasmid pPIC9k.

suspension and 30 µl of various concentrations of antibodies were added in triplicate wells to 96-well flat-bottomed plates and incubated at 37°C for 24 h. Thin blood smears were prepared to determine parasitemia. The inhibition rate was determined according to the following equation: % inhibition = $(P_c - P_t)/P_c \times 100\%$, where P_c is parasitemia of IgG isolated from preimmune sera and P_t is parasitemia of IgG from immune sera.

RESULTS

Construction and expression of recombinant PfEBA-175II F2 protein. Since codon optimization can improve the stability and expression of malaria genes (16, 19, 26), we redesigned the DNA sequence of the PfEBA-175II f2 gene (3D7 strain) using the codon frequencies of the yeast *Pichia pastoris*. It is thought that N glycosylation of *Plasmodium* proteins is a rare event (4) and that overglycosylation of proteins occurs in the yeast expression system. Therefore, a potential glycosylation site in PfEBA-175II F2 was eliminated by changing the amino acid Asn to Gln. The redesigned DNA sequence of the PfEBA-175II f2 gene was synthesized and expressed in *P. pastoris*. As shown in Fig. 1A, upon induction by methanol, the recombinant PfEBA-175II F2 (rPfEBA-175II F2) protein was produced and secreted from the cells with the predicted size of the full-length protein (molecular mass, 36 kDa). The calculated yield of about 300 mg/liter of protein in the supernatant was measured by scanning densitometry of Coomassie brilliant blue staining gels once the conditions for fermentation were optimized. To facilitate the purification of rPfEBA-175II F2, a six-His tag was incorporated at the C terminus of the molecule that allowed protein isolation by Ni-NTA columns. As shown in Fig. 1B, the recombinant protein was purified to near homogeneity from the supernatant with >95% purity by a combined purification process of Ni-NTA with gel filtration chromatography.

Characterization of rPfEBA-175II F2 protein. To characterize the protein, Western blot analysis was performed using pooled sera obtained from malaria patients in southern China. As shown in Fig. 2 (lane 1), in addition to a major band of the product, a degraded protein with a lower molecular mass was detected in the expression supernatant. However, purification of the product by Ni-NTA eliminated this degraded protein, indicating that the deletion occurred at the C terminus of the molecule. Further analysis of rPfEBA-175II F2 by Edman degradation was carried out to determine the N-terminal sequences of the purified protein. The results showed that the sequence at amino acid positions 1 to 10 was identical to the

designed sequence. Thus, based on observations of the entire N-terminal sequence and recognition of the six-His tag by Ni-NTA resin, isolation of full-length purified rPfEBA-175II F2 was achieved.

The F2 subdomain of PfEBA-175 has been shown to be involved in the binding of the molecule to glycophorin A on human erythrocytes. The binding appeared to be dependent on both sialic acid residues and the peptide sequence of glycophorin A (25). To determine if rPfEBA-175II F2 protein binds to glycoprotein A on erythrocytes, the ability of the protein from the expression supernatant to bind the erythrocytes of mice, rats, rabbits, and humans was tested. As shown in Fig. 2, a protein eluted from normal human erythrocytes incubated with the expression supernatant had a molecular mass similar to that of the expressed product and interacted with pooled sera obtained from malaria patients (Fig. 2, lane 3). However, no specific protein was detected in eluent from rat (Fig. 2, lane 2) or rabbit erythrocytes (Fig. 2, lane 6). In addition, the protein was also observed in the eluent from mouse erythrocytes, although it was likely degraded (Fig. 2, lane 7). To determine if the binding to human erythrocytes is sialic acid dependent, the erythrocytes were treated with neuraminidase or trypsin prior to the binding assay. The results showed that the protein failed to bind the enzymatically treated erythrocytes (Fig. 2, lanes 4 and 5).

Reactivity of naturally acquired human antibodies with rPfEBA-175II F2. To determine if the rPfEBA-175II F2 protein is recognized by individual serum obtained from regions where malaria is endemic, 65 serum samples were collected from malaria patients in the Yunnan (32 samples, 4 to 55 years of age) and Hainan (33 samples, 6 to 41 years of age) provinces of China and tested at a 1/200 dilution for interaction with the recombinant protein by ELISA. Fifty-four samples (83%) had an absorbance above the cutoff value ($OD_{450} = 0.082$) that was determined by normal human sera (20 serum samples), 37 (56.9%) had absorbance between the cutoff value and $OD_{450} = 1.0$, 8 (12.3%) were between $OD_{450} = 1.0$ and 1.5, and 9 (13.8%) were above $OD_{450} = 1.5$. These data are indicative of a high prevalence of antibodies against the F2 domain of the molecule in naturally infected populations and of the highly Ag-reactive nature of the cysteine-rich protein produced in the yeast expression system.

Immunogenicity of the rPfEBA-175II F2 protein in animals. To evaluate the immunogenicity of rPfEBA-175II F2, purified protein was prepared for immunization of mice, rabbits, and rhesus monkeys. The animals were immunized with the protein formulated with the adjuvant Montanide ISA720. Serum samples were obtained immediately before immunization and about 1 week after each of the immunizations. Antibody titers of the serum samples against the recombinant protein were measured with ELISA. High levels of specific antibody titers were elicited, and subsequent boosting of the protein dramatically increased the ELISA titers in all immunized animals. The highest level of the antibodies achieved in rabbits was 1/1,389,224 after a third immunization, compared with 1/61,435 in BALB/c mice and 1/41,026 in rhesus monkeys. Moreover, the antibodies in the sera obtained from all of the immunized animals after a third immunization recognized the cultured blood-stage parasites in an IFA. The immune sera showed a pattern of punctate fluorescence on the apical end of

FIG. 3. Recognition of native PfEBA-175 by antibodies against normal or denatured rPfEBA-175II F2. ELISA analysis of serum samples of rabbits immunized with normal rPfEBA-175II F2 (group 1), denatured and alkylated rPfEBA-175II F2 (group 2), or adjuvant ISA720 (group 3), diluted 1/500 and incubated with the concentrated serum-free supernatant of parasite culture.

merozoites as previously described (data not shown) (26). Interestingly, the interaction with cultured parasites was completely blocked by adding purified rPfEBA-175II F2 protein to the sera, indicating that the majority of the antibodies induced by the recombinant protein that recognized the native PfEBA-175 exist on the parasite.

To further examine whether the high level of antibodies and the recognition of cultured parasites by the antibodies are dependent on the conformation of the recombinant protein, rabbits were immunized with denatured and alkylated protein formulated with ISA720 adjuvant. Analysis of the sera by ELISA measured a much lower antibody titer (1/3,228) in the sera of rabbits immunized with the denatured and alkylated protein than in animals immunized with the normal recombinant protein (1/1,389,224). Moreover, sera from the rabbits immunized with the denatured and alkylated proteins did not recognize the cultured blood-stage parasites in an IFA (data not shown). We prepared a serum-free culture supernatant of a *P. falciparum* FCC1/HN isolate that contains soluble PfEBA-175 protein released by the parasites during schizogony. An ELISA was performed to test the interaction of the native protein with rabbit immune sera. As shown in Fig. 3, sera of two rabbits immunized with normal rPfEBA-175II F2 protein recognized the protein in the supernatant when diluted 1:500 ($OD_{450} = 0.7547$ and 0.6964 , respectively) while sera of two rabbits immunized with the denatured and alkylated protein showed OD_{450} values (0.2675 and 0.2566 , respectively) similar to those of preimmune sera (0.3155 and 0.2903 , respectively), indicating that proper folding of the protein is essential for its immunogenicity as well as for recognition of the native protein by antibodies.

In vitro growth inhibition assay. To analyze the function of antibodies against the PfEBA-175II F2 protein, protein A-Sepharose beads were used to isolate total IgG from sera collected after the fourth immunization. Isolated IgG was detected by its ability to inhibit the invasion of parasites in vitro. As shown in Fig. 4, IgG isolated from the sera of both rabbits

FIG. 4. Inhibition of parasite growth in vitro by antibodies to rPfEBA-175II F2. Total IgG was isolated with protein A beads from the pooled sera of rhesus monkeys (A) or rabbits (B) immunized with either rPfEBA-175II F2 or PfCSP recombinant protein. The IgG was detected based on its ability to inhibit the growth of *P. falciparum* strain FCC1/HN in vitro as described in Materials and Methods.

and monkeys immunized with the rPfEBA-175II F2 protein inhibited parasite growth in vitro in a dose-dependent manner, whereas the IgG from preimmune sera or the sera from rabbits immunized with PfCSP protein had no effect on parasite growth. At concentrations of 1.0 mg/ml, the antibodies isolated from monkey sera inhibited parasite growth by 85%, while IgG from the rabbit sera inhibited growth by 82% at a concentration of 0.7 mg/ml.

Immunization of animals with combined Ags. The rabbits and mice were immunized with either the combined rPfEBA-175II F2 and PfCP-2.9 Ags or the individual proteins formulated with ISA720 adjuvant. Four groups of rabbits were immunized with PfCP-2.9, rPfEBA-175II F2, a combination of PfCP-2.9 and rPfEBA-175II F2, or the adjuvant only. Both anti-PfCP-2.9 and anti-rPfEBA-175II F2 antibodies were measured by ELISA in sera from animals immunized with the combined or individual immunogens. The results showed that a high level of antibody production against both PfCP-2.9 and rPfEBA-175II F2 was induced in all immunized animals (Fig. 5). The antibody titers of sera obtained after the last immunization from the group of animals that received individual Ags were compared with those from the group receiving the combined Ags. No significant differences were found in antibody titers against either PfCP-2.9 or rPfEBA-175II F2 ($P > 0.1$, Wilcoxon). Moreover, in mouse experiments, the combination of the two Ags enhanced antibody response to each protein

FIG. 5. Comparison of specific antibody ELISA titers in rabbits immunized with individual and combined antigens. Time point serum samples were incubated with either rPfEBA-175II F2 (A) or PfCP-2.9 (B) and analyzed by ELISA to determine specific antibody ELISA titers (each value was derived from the geometric means of individuals in a group).

compared with the individual groups (data not shown). To investigate whether antibodies induced by individual components could inhibit in vitro parasite growth, we prepared affinity columns to isolate the specific antibodies from the immune sera. The result showed that both the anti-rPfEBA-175II F2 and anti-PfCP-2.9 antibodies eluted from the columns inhibited parasite growth in vitro in a dose-dependent manner (Fig. 6), indicating that the individual components of the combination induced inhibitory antibodies.

DISCUSSION

Plasmodium merozoites are a major target of blood-stage malaria vaccine development because they are the only form that is exposed to the host immune system during this stage. Intervention in merozoite invasion of erythrocytes would in principle block the erythrocytic life cycle of the parasite and prevent the clinical manifestations of infection. However, it has been shown that invasion of erythrocytes involves multiple alternative pathways, including both sialic acid receptor-dependent and -independent pathways. MSP1-19 was recently revealed to play a role in the sialic acid-independent pathway via an MSP1-19/erythrocyte-band 3 interaction (8), while it has

FIG. 6. Specificity of inhibitory antibodies (Ab) to rPfEBA-175II F2 and PfCP-2.9. Both rPfEBA-175II F2 and PfCP-2.9 affinity columns were prepared to isolate specific antibodies from the sera of rabbits immunized with the combination of PfEBA-175II F2 and PfCP-2.9 recombinant proteins. Eluted antibodies from the columns were assayed for inhibition of parasite growth as described in Materials and Methods. Anti-PfCSP antibodies were used as a negative control for the inhibition assay.

been proposed that PfEBA-175 plays a role in the sialic acid-dependent invasion pathway. Thus, a multivalent malaria vaccine against blood-stage parasites incorporating these parasite ligands may be more efficacious at blocking various invasion pathways (10, 23). Moreover, such a vaccine may also provide an approach to address the issue of parasite variation and host genetic restriction.

We have constructed a chimeric protein, PfCP-2.9, comprised of AMA-1 (III) and MSP1-19, two targets of inhibitory antibodies. Our previous data showed that the chimeric protein induced antibodies that inhibited parasite growth in vitro and that the activity was mediated by a combination of growth-inhibitory antibodies generated by the individual components of PfCP-2.9. In this study, we produced another merozoite protein, PfEBA-175II F2, in *Pichia pastoris* and combined the three merozoite surface proteins as one vaccine formulation for animal immunization. Ag competition is a major issue for combination vaccine development. Our data indicated a distinct lack of Ag competition in rabbits as well as mice. Moreover, both the anti-PfCP-2.9 and anti-rPfEBA-175II F2 antibodies isolated from rabbit sera immunized with the combination can inhibit parasite growth in vitro. These data provide strong support for the inclusion of the three Ags in a vaccine formulation against blood-stage parasites.

Since most of the merozoite surface proteins are cysteine-rich proteins and generation of inhibitory antibodies was dependent on protein conformation, it is critical to produce recombinant proteins that closely resemble their native counterparts. The *P. pastoris* system has the potential not only for high-level expression of foreign genes but also for production of correctly folded, fully functioning products. Our previous data showed that the PfCP-2.9 produced in this system correctly resembled its native protein (18). In this study, based on the fact that the recombinant PfEBA-175II F2 produced in *Pichia* can bind to human erythrocytes in a sialic acid-dependent manner and that the reduced and alkylated protein did

not induce antibodies that bound cultured parasites in an IFA nor any that interact with the native protein, rPfEBA-175II F2 appears to correctly resemble the native protein.

A combination of high yield and potential of scale-up for mass production is a major requirement for recombinant vaccine development. In this study, we redesigned the DNA sequence of the PfEBA-175II f2 gene (3D7 line) using normal *Pichia* codon frequencies. We did not use the *Pichia* most frequent codon usage for the synthetic gene based on the consideration of potential depletion of rare tRNAs. Although several attempts have been made to successfully express the protein in various systems (20, 26), we achieved high yield of a properly folded protein that can be scaled up for mass production in a 15-liter fermentor, which provides the opportunity to further evaluate this antigen as a vaccine component. The achievement of a high level of expression was likely due to the codon optimization, because expression in *P. pastoris* of the native sequence of the PfEBA-175II f2 gene was not detectable by Western blotting or ELISA, as previously reported (26).

P. falciparum merozoites also invade mouse erythrocytes, and PfEBA-175 can bind to mouse erythrocytes (12). Therefore, we assayed the binding of the recombinant PfEBA-175II F2 protein to mouse erythrocytes. Interestingly, the protein did bind mouse erythrocytes because the protein eluted from mouse erythrocytes interacted with pooled malaria sera (Fig. 2, lane 7). However, degradation of the protein was observed. Further study may be necessary to determine the characteristics of this binding, such as receptor type (e.g., sialic acid-dependent or independent binding).

In summary, we have expressed recombinant PfEBA-175II F2 protein in *Pichia pastoris* at high levels via optimization of codon usage. The recombinant protein resembled the native protein based on its binding properties as well as the recognition of the native protein by rPfEBA-175II F2 antibodies but not by antibodies against the denatured and alkylated recombinant protein. The protein was highly immunogenic in all animals tested, and the antibodies raised inhibited parasite growth in vitro. No Ag competition was observed when mice and rabbits were immunized with a combination of rPfEBA-175II F2 and PfCP-2.9, and no adverse effects were detected in immunized animals, including monkeys. Antibodies against both rPfEBA-175II F2 and PfCP-2.9 from immune sera inhibited growth of cultured parasites. Further investigation is needed to test whether the antibodies induced by distinct antigens of the combination inhibit different invasion pathways of the merozoite.

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