Production and Preclinical Evaluation of *Plasmodium falciparum* MSP-119 and MSP-311 Chimeric Protein, PfMSP-Fu24

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A *Plasmodium falciparum* chimeric protein, PfMSP-Fu24, was constructed by genetically coupling immunodominant, conserved regions of two merozoite surface proteins, the 19-kDa region C-terminal region of merozoite surface protein 1 (PfMSP-19) and an 11-kDa conserved region of merozoite surface protein 3 (PfMSP-311), to augment the immunogenicity potential of these blood-stage malaria vaccine candidates. Here we describe an improved, efficient, and scalable process to produce high-quality PfMSP-Fu24. The chimeric protein was produced in *Escherichia coli* SHuffle T7 Express lysY cells that express disulfide isomerase DsbC. A two-step purification process comprising metal affinity followed by cation exchange chromatography was developed, and we were able to obtain PfMSP-Fu24 with purity above 99% and with a considerable yield of 23 mg/liter. Immunogenicity of PfMSP-Fu24 formulated with several adjuvants, including Adjuplex, Alhydrogel, Adjuphos, Alhydrogel plus glucopyranosyl lipid adjuvant, aqueous (GLA-AF), Adjuphos + GLA-AF, glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE), and Freund's adjuvant, was evaluated. PfMSP-Fu24 formulated with GLA-SE and Freund's adjuvant in mice and with Alhydrogel and Freund's adjuvant in rabbits produced high titers of PfMSP-119 and PfMSP-311-specific functional antibodies. Some of the adjuvant formulations induced inhibitory antibody responses and inhibited in vitro growth of *P. falciparum* parasites in the presence as well as in the absence of human monocytes. These results suggest that PfMSP-Fu24 can form a constituent of a multistage malaria vaccine.

*Plasmodium falciparum* is responsible for causing over 2 million deaths annually, and 90% of these deaths are reported to occur in children under the age of 5 years. An effective vaccine represents an urgent need for more efficacious, second-generation blood-stage vaccines might be achieved with the use of a suitable adjuvant which can improve the quality and magnitude of the immune response and is safe for use in humans. Aluminum-containing adjuvants that are licensed for use in human vaccines are aluminum hydroxide (Alhydrogel) and aluminum phosphate (Adjuphos). Both these adjuvants are composed of loose aggregates of noncrystalline gel-like forms of aluminum salts and absorb antigens via several mechanisms, among which electrostatic adsorption and ligand exchange appear to be important. Aluminum-containing adjuvants are associated with strong humoral and Th2 responses. Alhydrogel is a mainstay in current malaria vaccine formulations. Toll-like receptor (TLR) ligands are known as immune-potentiating adjuvants and exhibit high potential for prophylactic and therapeutic vaccines.
Production of and Immune Response to PfMSP-Fu24

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with HIV have indicated that it has potent immunostimulatory effects without reactogenicity and enhances protective immune responses via both cell-mediated and antibody-mediated mechanisms (21).

The approach using construction of fusion protein chimeras has been advocated for blood-stage vaccine development. Several studies reported that chimeric proteins based on merozoite surface antigens show significantly increased immune responses compared to single antigens (22, 23). We had constructed a fusion chimera protein by fusing two merozoite antigens, the 19-kDa conserved carboxy-terminal region of merozoite surface protein 1 (MSP-119) and an 11-kDa conserved region rich in B and Th epitopes of merozoite surface protein 3 (PfMSP-311) (24). PfMSP-1 and PfMSP-3 are two of the leading blood-stage vaccine candidates. Merozoite surface protein 1 (MSP-1) is synthesized as an ∼195-kDa precursor protein that is proteolytically processed to form a multisubunit complex, expressed on the surface of merozoites (25). A 42-kDa glycosylphosphatidylinositol-anchored component, PfMSP-142, is further cleaved at the time of invasion, leaving only a 19-kDa C-terminal domain (PfMSP-119) attached to the merozoite surface (25). The PfMSP-119 fragment has 12 cysteine residues which through disulfide linkage form two highly conserved epidermal growth factor (EGF)-like domains (26). PfMSP-119 antibodies have also been shown to correlate with naturally acquired immunity in several epidemiological studies (27). Several studies have shown that immunization with recombinant MSP-119 fails to produce high-titer antibodies (28–31). However, inclusions of exogenous T cell epitopes have been shown to improve the immunogenicity of the PfMSP-119 fragment (32).

PfMSP-3 is another major merozoite surface protein that is being developed as a vaccine candidate on the basis of its involvement in antibody-mediated, monocyte-dependent protection acquired by humans upon natural exposure (33, 34). Unlike PfMSP-1, PfMSP-3 lacks the characteristic transmembrane domain and is thought to associate loosely with the merozoite surface. PfMSP-3 protein contains three blocks of four tandem heptad repeats based on the AXXAXX motif at the N terminus, a glutamic acid-rich domain, and a putative leucine zipper sequence at the C terminus (35). It has been demonstrated that high levels of cytotoxic antibodies to PfMSP-3 are directed toward the conserved N-terminal region of PfMSP-3, which also contains T-helper epitopes (36, 37).

We had earlier produced PfMSP-Fu24 in E. coli BLR(DE3) cells at the shake flask level and showed highly enhanced immunogenicity, including high levels of functional PfMSP-119 and PfMSP-3-specific antibody responses in small animals (24). These findings warranted new studies to further develop PfMSP-Fu24 as a vaccine candidate and to search for an adjuvant system that is appropriate for human use. In order to take this vaccine candidate to phase 1 clinical trials, we optimized the process for robust, scalable production of PfMSP-Fu24. In the present study, we report the production of PfMSP-Fu24 in E. coli ShuffLe 30 and optimization of a batch fermentation process on a 10-liter scale. We tested the preclinical immunogenicity of this clinical-grade recombinant PfMSP-Fu24 in combination with the following adjuvants: (i) Freund’s adjuvant, because of its known efficacy due to its high immunogenicity and widespread use in vaccine development in animals; (ii) Adjuplex, an experimental adjuvant that has been reported to increase vaccine-induced serum IgG antibody titers (21); (iii) Alhydrogel and Adjuphos, which stimulate a Th2-type immune response characterized by increased antibody titers (38); and (iv) GLA-SE, a Th1-biased adjuvant that has recently been tested with few malaria antigens in mice (39–41). We found that both the magnitude and the type of the immune response were affected by the choice of adjuvant. PfMSP-Fu24 formulated with Freund’s adjuvant as well as with Adjuplex and GLA-SE induced high-titer antibodies against PfMSP-119 as well as the PfMSP-3 component, while PfMSP-Fu24 formulated with Alhydrogel and Adjuphos induced low antibody titers in mice. However, in rabbits, Freund’s adjuvant and Alhydrogel induced high antibody titers compared to GLA-SE adjuvant. Finally, PfMSP-Fu24-specific IgG from some the adjuvant formulations induced significant inhibitory antibody responses (i.e., >50% inhibition of parasite growth) by blocking erythrocyte invasion as well as by antibody-dependent cellular inhibition mechanisms.

**MATERIALS AND METHODS**

**Plasmid, bacterial strains, antibodies, and matrices.** Plasmid pET28a (Novagen) was used for cloning of PfMSP-Fu24 (24). E. coli strain SHuffle 3030H (NEB, Ipswich, MA) was used for expression of PfMSP-Fu24. Horseradish peroxidase (HRP)-conjugated secondary antibodies and an antibody isotyping kit were purchased from Sigma. Alhydrogel and Adjuphos (Brenntag, Denmark), Adjuplex (Advance Adjuvants, USA), GLA-SE and GLA-AF (IDRI, Seattle, USA), and complete Freund’s adjuvant and incomplete Freund’s adjuvant (Sigma-Aldrich) were used for immunizations. Streamline chelating agent, SP-Sepharose FF, and Superdex S-75 were obtained from GE Healthcare, Sweden.

**Large-scale expression and purification of recombinant PfMSP-Fu24.** Batch cultivations were performed to optimize the expression of PfMSP-Fu24. The construction of the codon-optimized PfMSP-Fu24 chimera gene was described earlier (24). The gene was cloned in pET28a(+) and the resultant construct, pET28a(+)synPFMSP-Fu24, was transformed into either chemically competent E. coli BLR(DE3) cells to yield E. coli BLR(DE3)-pET28a(+)synPFMSP-Fu24 or E. coli ShuffLe T7 Express lysY to yield E. coli ShuffLe T7 Express lysY-pET28a(+)synPFMSP-Fu24.

Batch cultivations of E. coli BLR(DE3)-pET28a(+)synPFMSP-Fu24 as well as E. coli ShuffLe T7 Express lysY-pET28a(+)synPFMSP-Fu24 were performed with the following setup: airflow rate, 0.5 vvm (volume of gas per volume of liquid per min); stirrer speed, 500 rpm; pH 6.8; temperature, 37°C for E. coli BLR(DE3)-pET28a(+)synPFMSP-Fu24 and 30°C for E. coli ShuffLe T7 Express lysY-pET28a(+)synPFMSP-Fu24; initial dissolved oxygen (DO2) level, 100%. DO2 level was maintained at 40% saturation during the cultivation by controlling agitation rate and airflow rate through proportional-integral-derivative (PID) controller. If required, the inlet air was enriched with pure oxygen. pH was controlled at 6.8 by the addition of 2 N NaOH. Sampling was performed at regular intervals to measure cell density by taking absorbance at 600 nm and to monitor PfMSP-Fu24 expression by SDS-PAGE. For batch cultivation, cells were induced at an optical density at 600 nm (OD600) of ∼5.0 with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown further at 37°C for E. coli BLR(DE3)-pET28a(+)synPFMSPFu24 and 30°C for E. coli ShuffLe T7 Express lysY-pET28a(+)synPFMSP-Fu24 for 4 h. E. coli cell biomass obtained from the fermentation culture was suspended in lysis buffer (10 mM Tris HCl [pH 8], 500 mM NaCl, 10 mM imidazole, 100 mM NaH2PO4 1% Tween 20 [25 mg liter−1], 5 mM benzamidine HCl) and lysed by passing through a Dyno-Mill (KDL, Germany) at a flow rate of 100 ml min−1. More than 95% of the cells were lysed after three passages through a Dyno-Mill. Lysed culture was clarified through online centrifugation (Carr Pilot centrifuge) at 15,000 × g, and supernatant containing PfMSP-Fu24 protein was loaded onto packed XK50/20 columns (GE Healthcare) for immobilized metal affinity chromatography (IMAC), containing Streamline chelating agent (GE Health-
Characterization of recombinant PfMSP-Fu24. Recombinant PfMSP-Fu24 was analyzed for its molecular mass by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) at the National Centre of Genetic Engineering and Biotechnology, India, and N-terminal sequencing by Edman degradation followed by high-pressure liquid chromatography (HPLC) at the National Institute of Immunology, India. Recombinant PfMSP-Fu24 was characterized for purity and homogeneity by the following methods. PfMSP-Fu24 was analyzed for its purity on 15% SDS-PAGE gels and stained with Coomassie to observe shifts in mobility due to reduction of disulfide bonds by separating the antigen on SDS-PAGE gel before and after reduction with β-mercaptoethanol (β-ME). The antigenicity of expressed proteins was confirmed by Western blotting using polyclonal antibodies against purified PfMSP-Fu24 raised in mice to confirm the identity of full-length PfMSP-Fu24. HRP-conjugated goat anti-mouse immunoglobulin (Sigma) was used to detect the primary antibodies using diaminobenzidine (DAB). PfMSP-Fu24 was analyzed for dimers and purity by analytical gel permeation chromatography with Superdex-75 (GE Healthcare). The conformation integrity of the PfMSP-1α fragment in recombinant PfMSP-Fu24 was analyzed by enzyme-linked immunosorbent assay (ELISA) using conformation sensitive monoclonal antibodies (Mabs) 2E10 and 1H4. Mab 2E10 and 1H4 were made commercially against bacterially expressed, highly pure PfMSP-1α (A&G Pharmaceuticals, Columbia, MD) (42). For the assay, the ELISA plate was coated with PfMSP-Fu24 and PfMSP-1α, in coating buffer (50 mM carbonate buffer, pH 9.6) at a concentration of 0.5 ng/well in both the folded and denaturing forms. PfMSP-Fu24 and PfMSP-1α were denatured by boiling the sample with 20% (vol/vol) β-ME for 5 min followed by incubation with 50 ml of 20% (wt/vol) iodoacetamide for 30 min at 37°C. The proteins used for coating were probed with Mabs 2E10 and 1H4 at a dilution of 1:2,000 in triplicate wells followed by HRP-conjugated goat anti-mouse immunoglobulin (Sigma) at a dilution of 1:1,000. In addition, recombinant PfMSP-Fu24 was also probed with anti-PfMSP-1α-anti-MSP3 antibody (43) (dilution, 1:1,000), followed by HRP-conjugated goat anti-mouse immunoglobulin (Sigma) at a dilution of 1:1,000 in parallel as another control. The ELISA was developed by the addition of 100 ml/well of O-phenylenediamine dihydrochloride (1 mg/ml; Sigma) and hydrogen peroxide (Merck) as the substrate. The reaction was stopped by adding 2 N sulfuric acid (Merck), and the absorbance was measured at 490 nm using an ELISA reader (Molecular Devices). Mean absorbance values and standard deviations for the triplicate wells of each antibody group were calculated and used for plotting graphs.

PfMSP-Fu24 was tested for homogeneity by reverse-phase chromatography using a C8 column (Supelco; Sigma-Aldrich). The gradient used for elution was developed using buffer A (0.05% trifluoroacetic acid in water) and buffer B (0.05% trifluoroacetic acid in acetonitrile). The column was initially washed in 10% buffer B, and the gradient reached 95% buffer B in 40 min. Residual host cell protein (HCP) content was determined by enzyme-linked immunosorbent assay (ELISA) and Western blotting with commercially available kits (Cygnus Technologies, USA). The HCP standard recommended by the manufacturer was used. Endotoxin content in PfMSP-Fu24 was estimated using a Limulus amoebocyte lysate (LAL) gel clot assay (Endosafe; Charles River, USA). The presence of free thiols groups, if any, was detected by using Ellman’s reagent (5,5′-dithio-bis-2-nitrobenzoic acid). Different concentrations of 1-cysteine were used to plot the standard curve for estimation of free thiol groups in the protein.

Expression, purification, and characterization of recombinant proteins. PfMSP-1α and PfMSP-31a were expressed, purified, and characterized as reported in our earlier paper (24).

Immunization of mice with PfMSP-Fu24. Six- to eight-week-old female BALB/c mice were obtained from the Jackson Laboratory and bred in the small-animal facilities of the International Centre for Genetic Engineering and Biotechnology (ICGEB) under pathogen-free conditions. New Zealand White female rabbits were obtained from National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. All animals were fed, housed, and looked after according to recommendations established by the Guide for the Care and Use of Laboratory Animals (ICGEB, India). ICGEB is licensed to conduct animal studies for research purposes under the registration number 18/1999/ CPCSEA (dated 1 October 1999). All the experimental protocols were approved by the ICGEB Institutional Animal Ethics Committee (IAEC).

Groups of six BALB/c mice were immunized subcutaneously with 25 μg of PfMSP-Fu24 formulated with Adjuplex (Advance Adjuvants, USA), Alhydrogel and Adjuphos (Brenntag, Denmark), GLA-SE and GLA-AF (IDRI, Seattle, WA, USA), and Alhydrogel+GLA-AF, Adjuphos+GLA-AF, complete Freund’s adjuvant, and incomplete Freund’s adjuvant (Sigma-Aldrich). Vaccine formulations were made according to the manufacturer’s instructions. Control groups received only phosphate-buffered saline (PBS). In parallel, mice (n = 5) were immunized with 25 μg of recombinant PfMSP-1α or PfMSP-31a as described in references 33, 34, and 36, formulated with Freund’s adjuvant or Alhydrogel. Booster doses were given on days 28 and 56 postimmunization, and bleeding was done on days 0, 14, 42, 70, and 84.

Rabbits (n = 3) were immunized subcutaneously with 100 μg of PfMSP-Fu24 formulated with complete Freund’s adjuvant (CFA), Alhydrogel, and GLA-SE followed by 2 booster immunizations (100 μg each) on days 28 and 56 and were bled 14 days after the last immunization.

Enzyme-linked immunosorbent assay (ELISA). The titers of PfMSP-Fu24, PfMSP-1α, and PfMSP-31a-specific antibodies in the serum samples were determined by ELISA. Ninety-six-well microplates (Nunc) were coated with 500 ng per well of PfMSP-1α, or 200 ng per well of PfMSP-31a, or PfMSP-Fu24, in 0.06 M carbonate-hexameric (pH 9.6) and incubated overnight at 4°C. Antibody-coated plates were washed and blocked with 2% low-fat milk in PBS (pH 7.2) for 2 h at 37°C. Two-fold serial dilutions of mouse or rabbit sera (100 μl) were added to antigen-coated wells in duplicate and incubated for 1 h at room temperature. Bound antibodies were detected using HRP-conjugated secondary antibodies specific for mouse or rabbit antibody immunoglobulin G (IgG), and IgG subclass-specific antibodies for mouse IgG (IgG1, IgG2a, IgG2b, and IgG3) were used for detection. Between incubations, the plates were washed with a 0.05% solution of Tween 20 in PBS by the ELISA plate washer AquaMax 2000 (Molecular Devices). Bound secondary Abs were detected by adding 100 μl of O-phenylenediamine dihydrochloride and H2O2 substrate solution at room temperature for 20 min, and the reaction was stopped with 50 μl of 2 N H2SO4. The plates were read at an absorbance of 492 nm using a VersaMax ELISA reader (Molecular Devices). Cutoff values were determined as the mean plus three standard deviations (SDs) for the preimmunization sera.

Immunofluorescence assay (IFA) for PfMSP-Fu24. Anti-PfMSP-Fu24 antibodies raised in mice immunized with different adjuvant formulations were tested for their abilities to recognize the native protein within...
the parasite by IFA as described earlier (44). Synchronized P. falciparum 3D7 parasites were air dried, fixed on 12-well immunofluorescence slides as described in references 24 and 43, as per the manufacturer’s instructions. Antiserum was eluted with 0.2 M glycine-HCl (pH 3.0). Eluted fractions were analyzed by densitometry.

Purified IgG from mouse (pooled) and rabbit (individual) immune sera was tested for its ability to inhibit growth of the parasite by IFA as described earlier (44). Synchronized P. falciparum 3D7 parasites were air dried, fixed on 12-well immunofluorescence slides as described in references 24 and 43, as per the manufacturer’s instructions. Antiserum was eluted with 0.2 M glycine-HCl (pH 3.0). Eluted fractions were analyzed by densitometry.

Table 1: Comparative analysis of PfMSP-Fu$a$4 expression in E. coli BLR(DE3) and E. coli SHuffle at 10-liter fermentation

| Strain (DE3) | Expression | Cell biomass (g/liter) | Amt (mg/liter) of PfMSP-Fu$a$4 after IMAC | Purity (%) by HPLC after IMAC | Amt (mg/liter) of PfMSP-Fu$a$4 after ion exchange | Final purity (%) by RP-HPLC | Native protein mobility shift |
|--------------|------------|------------------------|------------------------------------------|--------------------------------|----------------------------------|-----------------------------|-----------------------------|
| BLR(DE3)     | 1-fold     | 25                     | −30                                      | 50                             | −2                               | 84                          | Double bands seen at ~19 and 24 kDa |
| SHuffle      | 4-fold     | 24                     | −80                                      | 80                             | −23                              | 99                          | Single band seen at ~19 kDa     |

$^a$ Analyzed by densitometry.
$^b$ RP, reverse phase.

Results

Expression, purification, and characterization of PfMSP-Fu$a$4

Our first goal was to work out fermentation conditions for scalable production of PfMSP-Fu$a$4 with high biomass and therefore high protein yield. We transformed E. coli BLR(DE3) cells using the plasmid (pET28a-MSP-Fu$a$4) construct used to produce the protein on a shake flask scale (24) (the PfMSP-Fu$a$4 construct is schematically represented in Fig. 1). From three 10-liter batch fermentations using semidefinite media, we obtained a wet biomass of 25 g/liter. Plasmid stability tests showed that there was no
detectable loss of plasmid during fermentation. PfMSP-Fu24 was purified from soluble fractions by a combination of metal affinity and cation-exchange chromatography to a purity less than or equal to 84%. The quantitative yields of purified PfMSP-Fu24 produced were ~20 mg (~2 mg/liter of E. coli BLR(DE3) fermentation culture) (Table 1 and Fig. 2).

To resolve the problem of low yield, we expressed PfMSP-Fu24 in the E. coli SHuffle strain, which has disulfide isomerase (Dsbc) and allows improved cytoplasmic disulfide bond formation (45). We transformed E. coli SHuffle cells using the same plasmid (pET28a-MSP-Fu24) construct. Each of the three different batches of 10-liter fermentation using semidefined media typically yielded a wet biomass of 24 g/liter. PfMSP-Fu24 protein was then purified from the supernatant to >99% purity by a combination of metal affinity and cation-exchange chromatography (Table 1). Three different 10-liter batch fermentations gave a consistent yield of ~23 mg/liter of the protein from E. coli SHuffle fermentation. Analysis of PfMSP-Fu24 by reverse-phase chromatography revealed the presence of a single symmetric peak, indicating that the protein was highly pure and homogeneous (Fig. 2B). The protein migrated as a single homogeneous band on SDS-PAGE with a molecular mass of ~19.2 kDa (Fig. 2C). Polyclonal antibodies against PfMSP-Fu24 recognized both the native and denatured forms of recombinant PfMSP-Fu24 (Fig. 3A). The endotoxin level in PfMSP-Fu24 as determined by the Limulus amebocyte lysate assay was less than 0.25 endotoxin units (EU) per 25 μg of protein.

FIG 2 Characterization of purified PfMSP-Fu24 produced on a 10-liter cultivation scale. (A and B) Purity of PfMSP-Fu24 produced in E. coli BLR(DE3) (A) and E. coli SHuffle (B) on SDS-PAGE gels after Coomassie staining. PfMSP-Fu24 was loaded on SDS-PAGE gels under reduced and nonreduced conditions. M, molecular mass marker. (C) Reverse-phase chromatography profile of purified recombinant PfMSP-Fu24 produced in E. coli SHuffle that eluted as a single peak, indicating 99% purity.

FIG 3 Identity and conformational integrity of PfMSP-Fu24. (A) Immunoblot analysis of purified recombinant PfMSP-Fu24. Immunoblots of 15% SDS-polyacrylamide gels of purified recombinant PfMSP-Fu24 were probed with polyclonal anti-PfMSP-Fu24 mouse serum at a 1:10,000 dilution under reducing and nonreducing conditions. M, molecular mass markers. (B) The reactivity of native and denatured PfMSP-Fu24 was tested with the PfMSP-119 conformation-specific MAbs 2E10 and 1H4 by ELISA at a dilution of 1:10,000.
The HCP content was determined by ELISA using an anti-
E. coli
antibody kit capable of quantifying the lowest concentration of
HCP, and the purity of PfMSP-Fu24 was tested with a Western blot
HCP determination kit (Cygnus). No host cell protein contami-
nation was observed in purified PfMSP-Fu24, as determined by an
ELISA (0.3 ng/ml [0.03%]) and by Western blotting (data not
shown). The protective immune responses induced by PfMSP-119
have been shown to be dependent on protein conformation (31).
We
tested the conformational integrity of the PfMSP-119 compo-
nent in PfMSP-Fu 24 by two conformation-sensitive MAbs, 1H4
and 2E10 (42). ELISA showed that purified PfMSP-Fu24 reacted
strongly with both 1H4 and 2E10, confirming that PfMSP-Fu24
retained conformational epitopes of native PfMSP-119 (Fig. 3B).
Additionally, the reactivity of PfMSP-Fu24 with these MAbs de-
clined considerably under denatured conditions, revealing that
the critical epitopes were present in the same conformation as the
native protein. Ellman’s test indicated that there were no free cy-
ssteines present in PfMSP-Fu24. Polyclonal antibodies against full-
length PfMSP-3 (43) and PfMSP-119 (42) recognized the native
and denatured forms of PfMSP-Fu24 (Fig. 3B).

FIG 4 Antibody responses against PfMSP-Fu24. (A) BALB/c mice (n = 6) were immunized and given two boosters of 25 μg of PfMSP-Fu24 formulated with
Adjuplex, Alhydrogel, Adjuphos, GLA-SE, Alhydrogel + GLA-AF, Adjuphos + GLA-AF, and Freund’s adjuvants. (B) New Zealand White rabbits (n = 3) were
immunized and given two boosters of 100 μg of PfMSP-Fu24 formulated, GLA-SE, Alhydrogel, and Freund’s adjuvants. ELISA was used to assess the titers of
antibody against PfMSP-Fu24, PfMSP-3,11, and PfMSP-119. (C) Comparison of the antibody response to PfMSP-119 and PfMSP-3,11, in BALB/c mice immunized
with PfMSP-Fu24, PfMSP-119, or PfMSP-3,11, in Freund’s adjuvant. Sera from different immunized groups were tested at a dilution of 1:10,000 for recognition of
PfMSP-119 and PfMSP-3,11, by ELISA. Values are averages and SDs.
The PfMSP-Fu24 chimera protein induced specific antibody responses with different adjuvant formulations. We compared the immunogenicity of PfMSP-Fu24 formulated with adjuvants, including Adjuplex, GLA-SE, Alhydrogel, Adjuphos, Alhydrogel + GLA-AF, and Adjuphos + GLA-AF, in mice. Sera were tested for antibody responses specific to PfMSP-Fu24 and to the PfMSP-119 and PfMSP-311 components. Mice immunized with PfMSP-Fu24 formulated with GLA-SE, Freund’s adjuvant, and Adjuplex exhibited the highest antibody responses against PfMSP-Fu24, PfMSP-119, and PfMSP-311, followed by Alhydrogel/H11001 GLA-AF and Adjuphos/H11001 GLA-AF (Fig. 4A). PfMSP-Fu24 formulated with Alhydrogel was generally less immunogenic and elicited lower antibody responses against PfMSP-Fu24, PfMSP-119, and PfMSP-311, followed by Alhydrogel + GLA-AF and Adjuphos + GLA-AF (Fig. 4A). PfMSP-Fu24 formulated with Alhydrogel was generally less immunogenic and elicited lower antibody responses against PfMSP-Fu24, PfMSP-119, and PfMSP-311 (Fig. 4A). PfMSP-Fu24 formulated with Adjuphos was not immunogenic and did not elicit any antibody responses against any antigen. Next, the antibody response induced by immunization with PfMSP-Fu24 was compared with that induced by immunization with a single antigen, PfMSP-119 or PfMSP-311 (Fig. 4A). PfMSP-Fu24 formulated with Adjuphos was not immunogenic and did not elicit any antibody responses against any antigen. Next, the antibody response induced by immunization with PfMSP-Fu24 was compared with that induced by immunization with a single antigen, PfMSP-119 or PfMSP-311. Mice immunized with PfMSP-Fu24 exhibited ~5-fold-higher PfMSP-119-specific and ~4-fold-higher PfMSP-311-specific antibody titers than were seen in either PfMSP-119 or PfMSP-311-immunized mice (Fig. 4C). These data indicate that the antibody response to both PfMSP-119 and PfMSP-311 was significantly increased when the two antigens were fused in PfMSP-Fu24.

As adjuvants are known to skew the immune response toward either a Th1-type antibody response (dominated by IgG2a and IgG2b) or a Th2-type antibody response (dominated by IgG1) (40), IgG subclass distribution was assessed in mice immunized with PfMSP-Fu24 formulated with different adjuvants. Mice immunized with PfMSP-Fu24 exhibited high titers of PfMSP-Fu24-specific IgG1 antibodies (Fig. 5A). The presence of IgG1 isotypes was suggestive of a predominant Th2-type profile. In addition, we observed that PfMSP-Fu24-immunized mice showed significantly higher serum IgG1 responses to PfMSP-119, whereas these mice showed a mixed IgG1 and IgG2a response against PfMSP-311 (Fig. 5B and C).

The immunogenicity of PfMSP-Fu24 formulated with Freund’s adjuvant, Alhydrogel, and GLA-SE was also tested in rabbits with the same immunization regimen. Higher antibody titers for both PfMSP-119 and PfMSP-311 were induced with Freund’s adjuvant and Alhydrogel formulation than with the GLA-SE formulation (Fig. 4B). The data indicate that the antibody response in rabbits was different from that in mice.

Anti-PfMSP-Fu24 antibody recognizes the native parasite protein. Sera raised against PfMSP-Fu24 formulated with different adjuvants were analyzed for their ability to recognize the native parasite protein in blood-stage P. falciparum 3D7 parasites by immunofluorescence. Antibodies to PfMSP-Fu24 from all the adjuvant groups showed strong reactivity with the P. falciparum merozoite surface proteins MSP-1 and MSP-3 (Fig. 6).

In vitro parasite growth inhibition by anti-PfMSP-Fu24 antibodies. Next, we investigated whether total IgGs purified from mice and rabbits immunized with PfMSP-Fu24 formulated with
different adjuvants were able to neutralize *P. falciparum* 3D7, 7G8, and Dd2 in a GIA assay *in vitro*. IgGs purified from mice immunized with PfMSP-Fu24 formulated with different adjuvants were capable of *in vitro* parasite neutralization with various levels of efficacy (Fig. 7). IgGs from the Freund’s adjuvant group were highly inhibitory against all three parasite strains, 3D7, 7G8, and Dd2 (50.8%, 64.38%, and 67.08% inhibition, respectively). Purified IgGs from the Adjuplex, GLA-SE, and Adjuphos/GLA-AF formulations showed more inhibition against Dd2 parasites (52.96%, 63.7%, and 70.3%, respectively) than 7G8 parasites (58.7%, 57.3%, and 53.74%, respectively). In contrast, the antibodies from Alhydrogel+GLA-AF and GLA-SE formulations showed no growth inhibition against any of the three parasite strains, 3D7 (~4.3% inhibition), Dd2 (~8.2% inhibition), and 7G8 (~0.99% inhibition) (Fig. 7B).

We also tested whether total IgGs purified from rabbit immune sera could inhibit *in vitro* parasite growth. IgGs from Freund’s adjuvant and Alhydrogel formulations showed strain-transcending parasite neutralization activity and potently inhibited erythrocyte invasion of *P. falciparum* strains 3D7 (81.2% inhibition by Freund’s adjuvant versus 52.9% inhibition by Alhydrogel), Dd2 (91.2% inhibition by Freund’s adjuvant versus 51.5% inhibition by Alhydrogel), and 7G8 (86.7% inhibition by Freund’s adjuvant versus 50.9% inhibition by Alhydrogel) (Fig. 7B). In contrast, IgGs purified from rabbits that received PfMSP-Fu24 in GLA-SE showed no growth inhibition against any of the three parasite strains, 3D7 (~4.3% inhibition), Dd2 (~8.2% inhibition), and 7G8 (~0.99% inhibition) (Fig. 7B).

**Anti-PfMSP-Fu24 antibodies inhibit parasite growth in an ADCI assay.** Next, we tested whether anti-PfMSP-Fu24 antibodies mediate the monocye-dependent antibody-mediated cellular inhibition (ADCI) of *P. falciparum*. PfMSP-3-specific antibodies have been reported to inhibit parasite growth *in vitro* in a monocye-dependent manner (15). The specific growth inhibitory index (SGI) was calculated for all these ADCI assays. IgGs purified from sera from rabbits immunized with PfMSP-Fu24 formulated with Freund’s and Alhydrogel showed potent effects against parasite strain 3D7 (SGI with Freund’s adjuvant formulation = 56%; SGI with Alhydrogel formulation = 46%), and this was comparable to the ADCI activity of the IgGs purified from PfMSP-3,11 immune sera (SGI = 52%). In contrast, no ADCI activity was seen with IgGs from the GLA-SE adjuvant group (Fig. 8).
DISCUSSION

It is necessary to be able to produce substantial quantities of stable and pure recombinant protein with a process that can be scaled up and is reproducible (46). The main aims of the present study were to (i) optimize the large-scale production of PfMSP-Fu24, (ii) assess the immunogenicity of PfMSP-Fu24 in different adjuvant formulations, and (iii) investigate PfMSP-Fu24-specific antibodies for inhibition of erythrocyte invasion by three different strains of P. falciparum in vitro.

In our earlier work, we had expressed recombinant PfMSP-Fu24 as a soluble protein at the shake flask level in E. coli BLR(DE3) and had gotten a yield of ~30 mg/liter (24). However, when the same PfMSP-Fu24 protein was expressed in E. coli BLR(DE3) on a 10-liter fermentation scale, the yield of protein dropped significantly, to approximately ~20 mg of 10-liter fermentation culture. In order to improve the yield and purity of the chimera protein, an alternative E. coli expression system was used to express PfMSP-Fu24 in a 10-liter fermentation. We were able to significantly scale up the production of PfMSP-Fu24 using E. coli SHuffle cells that express a signal-truncated disulfide bond isomerase (DsbC) in the cytoplasm and rearrange incorrect disulfide bonds (45). A 10-liter fermentation batch yielded ~230 mg of PfMSP-Fu24 which was highly pure and homogeneous, as indicated by SDS-PAGE, reverse-phase chromatography, and gel permeation chromatography. The purified PfMSP-Fu24 had a low endotoxin content and was free of thiol and host cell proteins.

PfMSP-1,9 is highly structured and folds into two epidermal growth factor-like domains due to the presence of six disulfide
Immunization of mice with PfMSP-Fu24 resulted in an increased antibody response to PfMSP-1 float compared to immunization with either PfMSP-1 float alone or a mixture of PfMSP-1 float and PfMSP-3 float, as we reported previously (24). MSP-1 float is known to be poorly immunogenic, eliciting low levels of antibodies and poor T-cell activation and functional T helper cells in humans (29, 31). A number of studies have shown that the addition or presence of exogenous helper T-cell epitopes provides help to B cells, thereby enhancing the immunogenicity of PfMSP-1 float (32). The induction of high levels of antibodies and the evidence of IgG class switching in PfMSP-Fu24-immunized mice support the critical role of T helper cells in the PfMSP-Fu24-induced immune response. Furthermore, the T cell response was restricted to the PfMSP-3 float domain but clearly provided adequate help for the activation and differentiation of both PfMSP-1 float- and MSP-3 float-specific B cells (24). Thus, a significant increase in PfMSP-1 float-specific antibody response may be due to T cell epitopes contributed by the PfMSP-3 float portion of this chimeric protein (32, 49, 50).

One of the major concerns for any fusion chimera vaccine construct in general is the possibility of interference in eliciting desired immune responses against individual components. Our results showed that there was no evidence for antigenic competition between PfMSP-1 float and PfMSP-3 float. This is consistent with other chimeric malaria vaccine constructs. For example, no antigenic competition was observed with PCP2.9, a fusion chimera of PfMSP-1 float and PFAMA-1 (51), or when PCP2.9 was administered in combination with another recombinant antigen, PIHEA-175 (F2) (52). In another study, a fusion construct of Plasmodium yoelii MSP-1 and MSP-8 induced an antibody response comparable to that of the two components (22). Similarly, immunizations with the GLURP-MSP3 hybrid molecule generated a stronger antibody response against the individual GLURP and MSP3 domains, thereby validating the value of the approach (53).

Having established that there was no interference with the immune response to individual components in PfMSP-Fu24, we examined the functional potential of the antibody response. PfMSP-Fu24-elicted potent strain-transcending invasion-inhibitory antibodies in mice from different adjuvant groups, except the Alhydrogel and Adjuphos groups, strongly inhibited parasite invasion, as measured by GIA against P. falciparum. In addition, rabbit sera from the Freund’s adjuvant and Alhydrogel formulation groups were able to directly inhibit the in vitro growth of different strains of P. falciparum. Likewise, these antibodies inhibited the growth of blood-stage parasites in the ADCI assay, which has been shown to correlate with in vivo protection against infection (24). This provided evidence that PfMSP-Fu24-specific antibodies mimicked both the growth inhibition and the ADCI effect of naturally occurring human antibodies against PfMSP-1 float (54) and PfMSP-3 (33, 55), respectively.

In conclusion, we were able to establish a simple protocol to express PfMSP-Fu24 as a soluble protein in a novel expression system with high yields, which can be easily further scaled up for large-scale production under current good manufacturing practice (cGMP) conditions. PfMSP-Fu24 formulated in both preclinical and human-approved adjuvants was more immunogenic than the individual PfMSP-1 float and PfMSP-3 float in small animals. Additionally, PfMSP-Fu24 formulated with some of these adjuvants induced inhibitory antibody responses and inhibited in vitro growth of P. falciparum parasites in the presence and in the ab-

**FIG 8** Antibodies induced by PfMSP-Fu24 also inhibit parasite growth by antibody-dependent cellular inhibition (ADCI). *P. falciparum 3D7* parasites at the schizont stage were cocultured with human monocytes in the presence of 25 μg of purified IgG from rabbits immunized with PfMSP-Fu24 formulated with Freund’s adjuvant, Alhydrogel, and GLA-SE and from rabbits immunized with PfMSP-1 float and PfMSP-3 float formulated with Freund’s adjuvant, and parasite growth was monitored after 96 h. The SGI of ADCI of parasite growth (shown as means and standard deviations) was calculated by using the parasitemia of the culture grown in the presence of immune IgGs plus monocytes compared with parasitemia of the culture grown in the presence of monocytes alone.

linkages, which provide it with conformational stability (47, 48). Maintaining proper conformation of PfMSP-1 float is critical for the generation of protective antibodies. The conformational integrity of the PfMSP-1 float component of PfMSP-Fu24 was confirmed with the conformation-specific monoclonal antibodies 1H4 and 2E10 (42). Several monoclonal antibodies, including 1H4 and 2E10, have been shown to be directed against conformation-specific epitopes of PfMSP-1 float (42). Loss of reactivity of the conformational MABs with PfMSP-Fu24 under reducing conditions indicated that disulfide linkages stabilized the tertiary structure of the protein.

Adjuvants are known to boost the magnitude and duration of antibody responses to antigen. In the comparative assessment of different adjuvants used in this study, mice immunized with the adjuvants most commonly used in human vaccines, Alhydrogel and Adjuphos, were not potent enough to induce high levels of functional PfMSP-1 float-specific and PfMSP-3 float-specific antibodies. Therefore, adjuvants more potent than Alhydrogel were definitively required for the induction of functional antibody responses to blood-stage malaria antigens. The magnitude of the antibody response to both PfMSP-1 float and PfMSP-3 float was improved by the use of GLA-SE and Adjuplex, and these responses were comparable to those obtained with Freund’s adjuvant. While Freund’s adjuvant, GLA-SE, and Adjuplex produced very high antibody titers, other aspects of the antibody response, such as recognition of native antigen on parasite (by IFA) and subclass distribution (predominant IgG1 indicative of Th2-type response), were much less influenced by adjuvants. When aqueous GLA was added to the Alhydrogel or Adjuphos formulation, the combination of both adjuvants led to an increase in antigen-specific antibody response, although the response was still lower than that seen with GLA-SE. In rabbits, Freund’s adjuvant and Alhydrogel formulations elicited higher antibody titers than GLA-SE.

FIG 8 Antibodies induced by PfMSP-Fu24 also inhibit parasite growth by antibody-dependent cellular inhibition (ADCI). *P. falciparum 3D7* parasites at the schizont stage were cocultured with human monocytes in the presence of 25 μg of purified IgG from rabbits immunized with PfMSP-Fu24 formulated with Freund’s adjuvant, Alhydrogel, and GLA-SE and from rabbits immunized with PfMSP-1 float and PfMSP-3 float formulated with Freund’s adjuvant, and parasite growth was monitored after 96 h. The SGI of ADCI of parasite growth (shown as means and standard deviations) was calculated by using the parasitemia of the culture grown in the presence of immune IgGs plus monocytes compared with parasitemia of the culture grown in the presence of monocytes alone.
sence of human monocytes. These results offer the possibility of investigating PiMSP-Fu24 for development as a malaria vaccine candidate.

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