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Plasmodium falciparum Merozoite Surface Protein 1 (MSP-1)–MSP-3 Chimeric Protein: Immunogenicity Determined with Human-Compatible Adjuvants and Induction of Protective Immune Response▽†

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A chimeric gene, *MSP-Fu$_{24}$*, was constructed by genetically coupling immunodominant, conserved regions of the two leading malaria vaccine candidates, *Plasmodium falciparum* merozoite surface protein 1 (C-terminal 19-kDa region [PfMSP-1$_{19}$]) and merozoite surface protein 3 (11-kDa conserved region [PfMSP-3$_{11}$]). The recombinant MSP-Fu$_{24}$ protein was produced in *Escherichia coli* cells and purified to homogeneity by a two-step purification process with a yield of ~30 mg/liter. Analyses of conformational properties of MSP-Fu$_{24}$ using PfMSP-1$_{19}$-specific monoclonal antibody showed that the conformational epitopes of PfMSP-1$_{19}$ that may be critical for the generation of the antiparasitic immune response remained intact in the fusion protein. Recombinant MSP-Fu$_{24}$ was highly immunogenic in mice and in rabbits when formulated with two different human-compatible adjuvants and induced an immune response against both PfMSP-1$_{19}$ and PfMSP-3$_{11}$. Purified anti-MSP-Fu$_{24}$ antibodies showed invasion inhibition of *P. falciparum* 3D7 and FCR parasites, and this effect was found to be dependent on antibodies specific for the PfMSP-1$_{19}$ component. The protective potential of MSP-Fu$_{24}$ was demonstrated by *in vitro* parasite growth inhibition using an antibody-dependent cell inhibition (ADCI) assay with anti-MSP-Fu$_{24}$ antibodies. Overall, the antiparasitic activity was mediated by a combination of growth-inhibitory antibodies generated by both the PfMSP-1$_{19}$ and PfMSP-3$_{11}$ components of the MSP-Fu$_{24}$ protein. The antiparasitic activities elicited by anti-MSP-Fu$_{24}$ antibodies were comparable to those elicited by antibodies generated with immunization with a physical mixture of two component antigens, PfMSP-1$_{19}$ and PfMSP-3$_{11}$. The fusion protein induces a protective immune response with human-compatible adjuvants and may form a part of a multicomponent malaria vaccine.

Malaria is among the major parasitic diseases in tropical and subtropical countries. With as many as 300 to 500 million new cases each year, malaria accounts for the death of over 2 million people globally each year, and most are children (41). Among the four species of *Plasmodium* that infect humans, the most threatening is *Plasmodium falciparum*. The extensive spread of drug-resistant *P. falciparum* strains as well as the insecticide-resistant mosquito necessitates the development of a malaria vaccine on an urgent basis. Collectively, the major objective of the ongoing vaccine effort in this field is to develop a multistage, multivalent vaccine against *P. falciparum* (34).

The blood-stage cycle of the parasite is responsible for malaria pathogenesis. Intervention at this stage of the parasite's development through vaccination is likely to reduce malaria-related clinical symptoms. As a major interface between host and pathogen, the merozoite surface is an obvious target for the development of a malaria vaccine. A number of potential vaccine candidate antigens identified so far are located on or associated with the surface of the merozoite or in apical organelles. These include merozoite surface protein 1 (MSP-1), MSP-2, MSP-3, MSP-4, MSP-5, MSP-8, RAP1/2, AMA-1, and EBA-175, which are implicated in the process of merozoite invasion of the erythrocyte (23).

MSP-1 is one of the most extensively studied proteins of *P. falciparum* (18). It is synthesized as a ~200-kDa precursor and then processed in two steps: the primary processing step produces a complex of four fragments that are present on the merozoite surface, and the secondary processing step at invasion results in the shedding of the complex from the surface, except for the C-terminal 19-kDa domain (MSP-1$_{19}$), which remains anchored to the parasite surface by a glycosylphosphatidylinositol (GPI) moiety (2). The C-terminal 19-kDa fragment of MSP-1 is well conserved among *P. falciparum* isolates and contains two epidermal growth factor (EGF)-like domains that play a role in merozoite invasion. Substantial data from studies with *P. falciparum* MSP-1 and *in vivo* immunization studies of mice with *Plasmodium yoelii* and *Plasmodium chabaudi* indicate that the protective immune responses are directed against the C-terminal 19-kDa domain (10, 12, 15, 20, 27, 35). The inhibition of MSP-1 processing by conformation-specific antibodies (Abs) was previously proposed to be one of the possible mechanism for the inhibition of merozoite invasion (1).

Another merozoite surface protein, MSP-3, was also shown to be the target of the protective immune responses in humans (29). The PfMSP-3 protein contains three blocks of four tandem heptad repeats based on the AXXAXX motif at the N

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terminus, a glutamic acid-rich domain, and a putative leucine zipper sequence at the C terminus (25). Although a clear surface localization of PfMSP-3 is known, it lacks any transmembrane domain or glycosylphosphatidylinositol (GPI) anchor site (24, 25) and is therefore considered to be loosely associated with the merozoite surface by interactions with other merozoite surface proteins. PfMSP-3 was identified as a candidate vaccine antigen by an antibody-dependent cellular inhibition (ADCI) assay using human immune sera (28). The potential of PfMSP-3 as a vaccine candidate was further illustrated by ADCI using mice antibodies and was further confirmed by the suppression of *P. falciparum* growth in an immunocompromised mouse after the passive transfer of human antibodies purified on MSP-3 peptides together with human monocytes (28, 40, 42). The immunization of *Aotus* and *Saimiri* monkeys with recombinant PfMSP-3 or its fragments provided protection against parasite challenge (6, 16). A 70-amino-acid-long conserved domain of PfMSP-3, referred to here as the PfMSP-3$_{11}$ region, was identified as the target of protective antibodies in human immune responses (40). The presence of high titers of cytophilic antibodies, IgG3, against this conserved region of MSP-3 has been correlated with protection against the parasite. In addition, immunization of humans with a synthetic peptide corresponding to this region was previously shown to induce antiparasitic antibodies that suppress parasite growth in an ADCI assay (11).

It is generally believed that a combination vaccine for malaria is likely to be more effective than vaccines based on a single antigen, and attempts are being made to develop a malaria vaccine by using a mixture of more than one antigen or by combining immunologically relevant proteins of the target antigens as fusion proteins (31, 43, 45). In the present study, we have constructed a fusion chimera (MSP-Fu$_{24}$) consisting of PfMSP-1$_{19}$ and PfMSP-3$_{11}$ and produced the corresponding recombinant MSP-Fu$_{24}$ protein in *Escherichia coli* cells. The two individual components, PfMSP-1$_{19}$ and PfMSP-3$_{11}$, were also expressed and purified separately; the immunological properties of MSP-Fu$_{24}$ were compared with a physical mixture of the two individual components. MSP-Fu$_{24}$ retained the native conformation of the PfMSP-1$_{19}$ component and was highly immunogenic in small animals. The anti-MSP-Fu$_{24}$ antibodies inhibited parasite invasion into host red blood cells (RBCs) and also inhibited parasite growth in a monocyte-dependent manner, suggesting the potential of the fusion protein as a malaria vaccine candidate.

MATERIALS AND METHODS

Construction of plasmids expressing PfMSP-3$_{11}$, PfMSP-1$_{19}$, and MSP-Fu$_{24}$. To design a synthetic gene corresponding to the C-terminal 19-kDa fragment of PfMSP-1, the amino acid sequence of PfMSP-1$_{19}$ corresponding to residues 1526 to 1619 of the *P. falciparum* Welcome strain (GenBank accession no. P04933) was back-translated to the nucleotide sequence based on the *E. coli* codon frequency table (available at http://www.kazusa.or.jp/codon). This synthetic gene was used as a template to amplify PfMSP-1$_{19}$ with the NcoI-XhoI site with the following primer set: forward primer 5′-GTG ACA CCA TGG GTA ACA TTT CTC AGC ATC AGT G-3′ and reverse primer 5′-GCC CTC GAG TTA GTG GTG GTG GTG GTG GTG GGA ACT GCA GAA AAT ACC ATC-3′. The amplified product was cloned into the NcoI and XhoI sites of pET28a(+), a kanamycin-based vector (Novagen), in frame with the coding sequence of the 6×His tag at its C terminus, to obtain the pET28a-*PfMSP-1$_{19}$* construct.

The conserved 11-kDa fragment of PfMSP-3 corresponding to 70 amino acids (163 to 230 amino acids) was amplified from a PfMSP-3 synthetic gene with the following set of primers: forward primer 5′-GGC GGC CAT GGC AAA GAA TGC TTA CGA AAA GGC C-3′ and reverse primer 5′-GGC CTC GAG TTA GTG GTG GTG GTG GTG GTG GTC GTT TTC CTT AGA GAT GTT TTC-3′. The amplified product was cloned into the NcoI and XhoI sites of pET28a$^+$, in frame with the coding sequence of the 6×His tag at its C terminus, to obtain the pET28a-*PfMSP-3$_{11}$* construct.

To generate a fusion construct consisting of PfMSP-3$_{11}$ and PfMSP-1$_{19}$, the 11-kDa fragment of PfMSP-3 was amplified again with different sets of primers: forward primer 5′-GGC GGC CAT GGC AAA GAA TGC TTA CGA AAA GGC C-3′ and reverse primer 5′-GCC GCC CAT GGC GTC GTT TTC CTT AGA GAT GTT TTC-3′. The amplified PCR product was purified and digested with NcoI. The excised fragment was cloned into the NcoI site of pET28a-*PfMSP-1$_{19}$* to generate the pET28a-*MSP-Fu$_{24}$* construct.

All constructs were sequenced from both ends to confirm the orientation and sequence of the inserts and transformed into *E. coli* BLR(DE3) cells (Novagen) for the expression of recombinant proteins with 6×His tags.

Expression and purification of recombinant proteins. *E. coli* BLR(DE3) cells containing recombinant plasmids pET28a-*PfMSP-1$_{19}$*, pET28a-*PfMSP-3$_{11}$*, and pET28a-*MSP-Fu$_{24}$* were grown in Luria broth containing kanamycin (30 μg/ml) at 37°C until an optical density at 600 nm (OD$_{600}$) of 0.6 to 0.7 was reached. The expressions of the respective recombinant proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C, and the expressed proteins were analyzed and localized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting of the soluble and insoluble fractions of the *E. coli* cells after disruption.

For the purification of recombinant MSP-Fu$_{24}$ and PfMSP-1$_{19}$, the *E. coli* cell pellets from the respective 6-liter shake flask cultures were washed with phosphate-buffed saline (PBS) and resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM imidazole, 1% Triton X-100, 25 mg liter^{-1} lysozyme, 5 mM benzamidine HCl). The bacterial cells were lysed on ice by sonication, and the lysate was centrifuged at 12,000 rpm for 45 min at 4°C. The clarified supernatant was loaded onto a column containing precharged streamline chelating matrix (GE Healthcare). The column was subsequently washed with 10 column volumes of equilibration buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM imidazole), followed by 10 column volumes each of wash buffer 1 (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 40 mM imidazole) and wash buffer 2 (20 mM Tris-HCl [pH 8.0], 10 mM NaCl, 40 mM imidazole). Bound protein was eluted with a linear gradient of imidazole (40 mM to 1 M) in 20 mM Tris–10 mM NaCl (pH 8.0) buffer. The eluted fractions were analyzed by SDS-PAGE, and fractions containing the recombinant protein were pooled. The pooled protein was further purified by anion-exchange chromatography on a column of Q-Sepharose resin (GE Healthcare) equilibrated with equilibration buffer (20 mM Tris-HCl [pH 8.0], 10 mM NaCl). The bound proteins were eluted with a linear gradient of NaCl (10 mM to 1 M) in Tris-HCl buffer (pH 8.0). Eluates were analyzed by SDS-PAGE, fractions containing a single protein band of MSP-Fu$_{24}$ or PfMSP-1$_{19}$ were pooled, and the protein concentration was determined by a bicinchoninic acid assay (BCA).

For the purification of PfMSP-3$_{11}$, the cell pellet from the 6-liter shake flask culture was lysed by sonication, and the inclusion bodies (IBs) were collected by centrifugation at 12,000 rpm. The IB pellet was resuspended in solubilization buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM imidazole, 8 M urea) and kept for 12 to 15 h under stirring at room temperature (RT). The solubilized IBs were centrifuged at 12,000 rpm for 45 min at room temperature, and clarified supernatant was loaded onto a column containing precharged streamline chelating matrix (GE Healthcare). The column was sequentially washed with 10 column volumes of equilibration buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM imidazole, 8 M urea), wash buffer 1 containing 8 M urea, and wash buffer 2 containing 8 M urea. The bound protein was eluted by a linear gradient of imidazole (40 mM to 500 mM) in elution buffer (20 mM Tris-HCl [pH 8.0], 10 mM NaCl, 8 M urea). The fractions were analyzed by SDS-PAGE, and those fractions containing the PfMSP-3$_{11}$ protein were pooled and dialyzed sequentially against 6 M, 4 M, and 2 M urea in dialysis buffer (20 mM Tris-HCl [pH 8.0]), followed by a final change in 20 mM Tris–10 mM NaCl (pH 8.0). Dialyzed PfMSP-3$_{11}$ was further purified by anion-exchange chromatography by using the Q Sepharose matrix, as mentioned above.

The homogeneity of purified MSP-Fu$_{24}$, PfMSP-3$_{11}$, and PfMSP-1$_{19}$ was assessed by SDS-PAGE under reducing and nonreducing conditions, on an analytical gel permeation chromatography column, and by reverse-phase chromatography on an analytical C$_8$ column (Supelcosil; 5 by 4.9 cm, 5 μm). Endotoxin contents in the protein samples were estimated by using a *Limulus* amebocyte lysate (LAL) gel clot assay (Charles River Endosafe), and host cell protein contamination was estimated by immunoblotting as well as by enzyme-linked immunosorbent assay (ELISA) using anti-*E. coli* antibodies (Cygnus Technolo-

gies). The reactivity of the recombinant proteins with monoclonal and polyclonal antibodies was analyzed by immunoblotting according to standard protocols. Briefly, the recombinant proteins were separated by SDS-PAGE under reducing or nonreducing conditions and blotted onto a nitrocellulose membrane, followed by the blocking of the membrane using 5% nonfat milk in PBS (pH 7.4). The blots were sequentially incubated with the respective monoclonal or polyclonal antibodies in PBS (pH 7.4) containing 0.5% milk and 0.05% Tween 20, followed by the respective horseradish peroxidase-conjugated secondary antibody, after prior washing with PBS containing 0.05% Tween 20 (PBS-T). The protein bands were detected after developing the reaction mixture with 3,3′-diaminobenzidine tetrahydrochloride (DAB) in PBS and hydrogen peroxide (H_2O_2).

Immunization of mice and rabbits with recombinant antigens formulated with Freund's adjuvant (IFA), alum, and Montanide ISA720. Three different adjuvants used in the immunization study were Freund's complete adjuvant (CFA) and Freund's incomplete adjuvant (Sigma), alum (Alhydrogel; Superfos, Denmark), and Montanide ISA 720 (Seppic Inc., France). Vaccine formulations were prepared with the individual antigen or their mixture according to the manufacturers' instructions. Groups of five BALB/c mice 4 to 6 weeks of age were immunized intramuscularly with 25 μg of the individual antigens (MSP-Fu_{24}, PfMSP-3_{11}, or PfMSP-1_{19}) or a mixture of PfMSP-3_{11} and PfMSP-1_{19} (25 μg each) in each of the adjuvant formulations. Control groups received only PBS mixed with adjuvants. Immunized animals were given booster doses with the respective formulations on days 28 and 56 and were bled on days 0, 14, 42, 55, and 70 after the first immunization. Four groups of two New Zealand White rabbits were immunized intradermally with 200 μg of MSP-Fu_{24} or with a mixture of PfMSP-3_{11} and PfMSP-1_{19} (200 μg each) emulsified with either Montanide ISA720 or alum. Two booster immunizations were carried out intramuscularly on day 28 and day 56 by using the respective adjuvant formulations, and mice were bled on day 70 after the first immunization. The sera obtained were used for immunoassay and IgG purification. For the duration of this study, mice and rabbits were housed and used strictly in accordance with the guidelines set by the National Institutes of Health in 1985, and the study was approved by the institutional animal ethics committee of the International Centre for Genetic Engineering and Biotechnology (ICGEB).

IgG purification from rabbit and mouse sera. Total IgG was purified from sera obtained from immunized groups of mice and rabbits as well as from the control groups by using a protein G-Sepharose column (Pharmacia) according to the manufacturer's recommendations. Briefly, a serum sample, after equal dilution with binding buffer (20 mM sodium phosphate buffer [pH 7.0]), was loaded onto a protein G-Sepharose column preequilibrated with binding buffer. The column was washed with 10 column volumes of the binding buffer. The bound IgG was eluted with 0.2 M glycine-HCl (pH 3.0), and eluted fractions were analyzed by SDS-PAGE. The fractions containing purified IgGs were pooled and dialyzed against PBS.

Human immune sera from a region where malaria is endemic. Forty-two human sera were collected from healthy individuals residing in an area where *P. falciparum* is endemic (Orissa, India); these individuals were without any blood-stage infection at the time of collection and had no recent history of any malaria infection. Consent from these individuals and approval from the Human Volunteers Research Ethical Committee of the International Centre for Genetic Engineering and Biotechnology were obtained prior to the study. Serum samples were also collected from individuals who had no known history of malaria and had never visited an area where malaria is endemic.

ELISA and competitive ELISA. Antibody responses in mice as well as the reactivity of human sera and monoclonal antibodies (MAbs) (12.10, 2F10, and 5.2) with the respective antigens were evaluated by ELISA. All ELISAs were carried out using a 100-μl reaction mixture volume. Briefly, 96-well microplates (Dynatech) were coated with 100 ng of the antigens (MSP-Fu_{24}, PfMSP-3_{11}, or PfMSP-1_{19}; antigen concentration, 1 μg/ml) diluted in 0.06 M carbonate-bicarbonate buffer (pH 9.6) per well. Plates were washed with 1× PBS containing 0.05% Tween 20 (PBS-T), and the wells were blocked with 5% low-fat milk in PBS (pH 7.2) for 1 h at room temperature. Antigen-coated wells were sequentially incubated with serial dilutions of immune sera from mouse or rabbit and then with enzyme-labeled secondary antibody (horseradish peroxidase-labeled anti-mouse or anti-human immunoglobulin [IgG]). In between these incubations, the plates were washed with PBS-T. The enzyme reaction mixture was developed with *o*-phenylenediamine dihydrochloride–H_2O_2 in a citrate phosphate buffer (pH 5.0), the reaction was stopped with 8 M H_2SO_4, and the OD was recorded at 490 nm by use of a microplate reader (Molecular Devices). An OD cutoff of 0.1 (mean plus 2 standard deviations [SDs]) was selected for antibody titer determinations. To coat the reduced/denatured protein, SDS and β-mercaptoethanol were added to the protein solution to final concentrations of 1% (vol/vol) and 5% (vol/vol), respectively, kept in boiling water for 5 min, and coated into 96-well microplates at a concentration of 100 ng/well.

To detect the reactivities of MSP-Fu_{24}, PfMSp-1_{19}, and PfMSP-3_{11} with anti-PfMSP-1 conformation-specific monoclonal antibodies, ELISA was carried out by using MAb 12.10 and MAb 5.2 (62.5 ng/ml) under nonreducing and reducing conditions as described above. The assay for the reactivity of each of the recombinant proteins with anti-penta-His antibody (dilution, 1:1,000; Qiagen) was carried out in parallel as a positive control to ascertain equal amounts of coating of the antigens under reduced and nonreduced conditions. To detect subclasses of IgG in sera of mice immunized with MSP-Fu_{24} or with a mixture of PfMSP-1_{19} and PfMSP-3_{11}, ELISA was performed as described above by using immunized mouse sera (1:10,000 dilutions); MSP-Fu_{24} as a capture antigen; secondary goat antibodies specific for mouse IgG1, IgG2a, IgG2b, and IgG3 (Sigma) at dilutions of 1:1,000; and horseradish peroxidase (HRP)-linked tertiary anti-goat antibodies (Sigma). The statistical significance of the results was determined by use of an unpaired Student's *t* test.

A competitive ELISA was carried out to assess the efficacy of purified anti-MSP-Fu_{24} antibodies to competitively block the binding of anti-PfMSP-1_{19} MAbs. For this, wells of a microtiter plate were coated with 100 ng of the PfMSP-1_{19} protein (100 μl of antigen at a concentration of 1 μg/ml) as described above. These wells were incubated with serial dilutions of the purified anti-MSP-Fu_{24} rabbit antibodies (2.5 μg/ml to 1.2 ng/ml) for 2 h at 37°C. After washing with PBS-T, the wells were incubated with 6.25 ng of mouse MAb 5.2 or 12.10 (100-μl solution in 1× PBS at a concentration of 62.5 ng/ml) for 2 h at 37°C. The plates were washed, and the binding of the MAbs was detected with anti-mouse IgG conjugated to horseradish peroxidase. The reactivity of PfMSP-1_{19} with MAbs after a primary incubation with anti-MSP-Fu_{24} antibodies was compared with the reactivity without any incubation.

Immunofluorescence assay. Antibodies to the recombinant proteins were tested for their reactivity with native parasite proteins by an immunofluorescence assay (IFA). The assay was performed essentially as described previously (36, 46). Briefly, multispot parasite slides were made from *P. falciparum* (3D7) cultures, air dried, fixed with a mixture of acetone-methanol (9:1), and then blocked in blocking buffer (10% fetal calf serum [FCS] in PBS) for 1 h at 37°C. Slides were washed and incubated for 1 h with purified IgGs against each of the antigens, appropriately diluted in blocking buffer, and kept in a sealed humid chamber for 2 h at 37°C. Slides were washed with PBS-T and subsequently incubated with anti-mouse and anti-rabbit secondary antibodies conjugated to fluorescence dye (fluorescein isothiocyanate [FITC] or Cy-3; dilution, 1:250) for 1 h at room temperature in the dark. Later, the washed slides were stained with DAPI (4′,6′-diamidino-2-phenylindole) at a concentration of 2 μg/ml, followed by two washes with PBS-T and one wash with PBS. The washed slides were then mounted with a coverslip in the presence of Antifade mounting reagent (Bio-Rad) and were viewed by using a Nikon fluorescence microscope (SE300) with a 100× oil immersion objective.

Lymphoproliferative cellular responses and cytokine analysis. Groups of BALB/c mice were immunized with the recombinant proteins formulated in Montanide ISA720 or alum as described above. Fourteen days later, spleens of two mice from each immunized group were isolated, and single-cell suspensions were prepared from them. Cells were plated at 5×10^6 cells/ml in a final volume of 200 μl per well in 96-well flat-bottom plates containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 100 U of penicillin-streptomycin/ml. Cells were cultured in the presence or absence of a graded concentration of homologous antigen (4 days) and concanavalin A (2 days) in a humidified atmosphere with 5% CO_2 at 37°C. Splenocytes were pulse-labeled with 1 μCi per well of [^{3}H]thymidine (Amersham Pharmacia Biotech) per well and harvested onto glass fiber filters by using a PHD cell harvester (Cambridge Technology, Cambridge, MA) 16 h later. The [^{3}H]thymidine incorporation was determined by β-emission liquid scintillation spectroscopy (Betaplate; Pharmacia, Sweden). The geometric mean of the counts per minute for each set of triplicate wells was calculated, and the stimulation indices were calculated as the counts per minute for the test antigen divided by the counts per minute for the control. The statistical significance of the results was determined by use of a Student's *t* test.

For determinations of cytokine production, 10^6 splenocytes were cultured in a volume of 200 μl in 96-well flat-bottom plates in the presence or absence of an antigen. Culture supernatants were collected after 60 h for gamma interferon (IFN-γ) analysis. IFN-γ was measured by using a murine cytokine immunoassay kit (Duo Set ELISA developmental system; R&D Systems, Minneapolis, MN) according to the procedure recommended by the manufacturer.

Parasite culture and invasion inhibition assay. *P. falciparum* parasite strains 3D7 and FCR were cultured by using methods described previously by Trager

and Jensen (44). Parasite cultures were maintained under mixed gas (5% CO_2, 5% O_2, and 90% N_2) at 37°C in O^+ erythrocytes and RPMI 1640 medium supplemented with 10% O^+ human sera, 0.2% sodium bicarbonate, 0.2% glucose, and gentamicin (10 μg/ml). *P. falciparum* cultures were synchronized by sorbitol treatment (22).

Invasion inhibition assays were performed as described previously (36, 46). Briefly, ring-stage malaria parasites were synchronized with sorbitol lysis and allowed to mature through to the schizont stage. Hematocrit and parasitemia were adjusted to 2% and 0.5%, respectively. Purified IgG from rabbit sera (preimmune and immune) was added to the parasite culture in 96-well plates at final concentrations of 0.5, 0.25, and 0.125 mg/ml. Cultures were incubated at 37°C in a mixed-gas (5% CO_2, 5% O_2, and 90% N_2) cabinet for 26 h to allow for schizont rupture and merozoite invasion. These assays were performed in triplicate. For microscopic analysis, smears were made from triplicate wells and stained with Giemsa stain, and the numbers of ring-stage parasites per 5,000 RBCs were determined for each well. The percent inhibition of the parasite invasion was calculated using the following formula: percent inhibition = 1 − [(percent invasion of purified IgG from immune sera)/(percent invasion of purified IgG from preimmune sera)] × 100.

ADCI assay. The antibody-dependent cell inhibition (ADCI) assay was carried out by using purified IgG from mouse sera according to a procedure described previously by Oeuvray et al. (28). Briefly, human monocytes were purified from blood samples of healthy donors by using CD14 (monocyte/macrophage) microbeads (Miltenyi Biotech) according to the manufacturer's protocol. The study was approved by the Human Volunteers Research Ethical Committee of the International Centre for Genetic Engineering and Biotechnology prior to the study. Tightly synchronized *P. falciparum* 3D7 schizont-stage parasites (0.5% parasitemia and 1% hematocrit) were cocultured in a 96-well flat-bottom microculture plate with adherent human monocytes (2×10^6 monocytes) in complete medium (RPMI medium containing 10% human sera). Purified antibodies at a concentration of 50 μg/ml were added to these cultures and incubated at 37°C for 96 h in a mixed-gas environment (5% O_2, 5% CO_2, and 90% N_2). After 48 and 72 h of growth, 50 μl of complete medium was added to each well. After 96 h of growth, parasitemia was estimated for a thin Giemsa-stained smear of RBCs from each well by counting parasitized erythrocytes in more than 10,000 erythrocytes. Control wells consisted of (i) parasite alone, (ii) parasite and control IgG (purified from naïve mouse sera), (iii) parasites and monocytes, (iv) parasites and purified IgG without monocytes, and (v) parasites, control IgG, and monocytes. The specific growth inhibition index (SGI) was calculated as follows: 1 − [(percent parasitemia with monocytes and test antibodies/percent parasitemia with test antibodies)/(percent parasitemia with monocytes and control IgG/percent parasitemia with control IgG)] × 100.

Depletion of antigen-specific antibodies and reversal of invasion/growth inhibition. Antibodies specific to MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$ were depleted from total antibodies purified from rabbit/mouse sera by using the respective recombinant antigens. Briefly, purified IgGs from rabbit or mouse sera (0.25 mg/ml and 0.05 mg/ml, respectively) of the MSP-Fu$_{24}$–Montanide ISA720 groups were mixed with the recombinant antigen (MSP-Fu$_{24}$, PfMSP1$_{19}$, or PfMSP-3$_{11}$) at different final concentrations (0.025 mg/ml, 0.05 mg/ml, or 0.1 mg/ml for rabbit antibodies and 0.05 mg/ml for mouse antibodies) and incubated at RT for 2 h. This antigen-antibody mix was passed three times through a 1-ml Hi-Trap Ni-nitrilotriacetic acid (NTA) column (GE Healthcare) preequilibrated with 20 mM Tris-HCl (pH 8.0) to remove the protein-antibody complexes, and the flowthrough was collected, concentrated, and dialyzed against incomplete RPMI (iRPMI) medium. The respective antigen-depleted IgGs were used subsequently for invasion inhibition or ADCI assays as described above. The growth inhibition (invasion inhibition or ADCI) by depleted antibodies was compared with that by antibodies without any depletion. Recombinant *P. falciparum* histidine-rich protein 2 (PfHRP-2) was used for antibody depletion as a negative control for these experiments.

RESULTS

Expression of MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$ in *E. coli* and purification of recombinant proteins. Cloned *PfMSP-1$_{19}$* and *PfMSP-3$_{11}$* gene sequences were genetically coupled to generate *MSP-Fu$_{24}$*, a chimeric gene construct, in the pET28a$^+$ vector (Fig. 1A and B), and the corresponding recombinant protein was expressed in *E. coli* BLR(DE3) cells. For comparison, the individual *PfMSP-1$_{19}$* and *PfMSP-3$_{11}$* fragments were also cloned into the pET28a$^+$ vector and expressed in *E. coli* BLR(DE3) cells. The MSP-Fu$_{24}$ protein was expressed as a soluble protein, and its expression level of MSP-Fu$_{24}$ was two- to three-fold higher than the expression of individual PfMSP-1$_{19}$ and PfMSP-3$_{11}$ proteins under similar conditions. PfMSP-1$_{19}$ was also expressed as a soluble protein in the cytosolic fraction, whereas PfMSP-3$_{11}$ was expressed as an insoluble protein and aggregated in the inclusion bodies (IBs) of *E. coli* cells.

MSP-Fu$_{24}$ and PfMSP-1$_{19}$ were purified to homogeneity from soluble fractions by a combination of metal affinity and ion-exchange chromatography. These purified recombinant proteins showed apparent mobilities of ~24 kDa and ~19 kDa, respectively, on SDS-PAGE gels (Fig. 1C and F). Reverse-phase high-performance liquid chromatography (RP-HPLC) and gel permeation chromatography analyses suggested that the purified MSP-Fu$_{24}$ protein was >98.0% pure and was in a monomeric form (Fig. 1E). Recombinant PfMSP-3$_{11}$ was purified to homogeneity from the inclusion body fraction and refolded; it moved as a single band at ~11.0 kDa under both reducing and nonreducing conditions on SDS-PAGE gels (Fig. 1G). The three purified proteins (MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$) eluted as monomers by gel permeation chromatography (see Fig. S1 in the supplemental material). The final yields for MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$ were 30.0 mg/liter, 15.0 mg/liter, and 10.0 mg/liter, respectively. The final preparations of MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$ contained less than 12.5, 0.25, and 5.0 endotoxin units (EU) per 25 μg of protein, respectively. Host cell proteins were not observed in any of the protein samples, as determined by an ELISA and Western blot analysis.

Immunological characterization of the fusion chimera. The conformational integrity of the PfMSP-1$_{19}$ component in MSP-Fu$_{24}$ was assessed by its reactivity with conformation-specific PfMSP-1$_{19}$ monoclonal antibodies (MAbs 5.2 and 12.10) by immunoblotting. MSP-Fu$_{24}$ also showed a strong reactivity with MAb 5.2 by immunoblotting (Fig. 1D), as in the case of PfMSP-1$_{19}$. ELISA was also carried out to determine the reactivities of the recombinant proteins (MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP3$_{11}$) with MAbs. ELISA showed that purified MSP-Fu$_{24}$ and PfMSP-1$_{19}$ reacted strongly with monoclonal antibodies 5.2 and 12.10, which are specific for disulfide-dependent conformational epitopes of native PfMSP-1$_{19}$ (Fig. 2). The reactivities of MSP-Fu$_{24}$ and PfMSP-1$_{19}$ with these MAbs declined considerably under denatured conditions, whereas the reactivities of MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$ with anti-penta-His antibody under native and denatured conditions were found to be similar in a parallel experiment that ascertained the equal coating of the antigens in both cases (Fig. 2). However, there was no reactivity of PfMSP-3$_{11}$ with any of the anti-MSP-1 MAbs under both native and reduced/denatured conditions. MSP-Fu$_{24}$ was also recognized by polyclonal antibody raised against PfMSP-1$_{19}$ and PfMSP-3$_{11}$ (data not shown).

The recognition of MSP-Fu$_{24}$ by human immune sera from a region where malaria is endemic was assessed by ELISA and compared with the reactivities of PfMSP-1$_{19}$ and PfMSP-3$_{11}$. Thirty-two out of 44 sera (~73%) showed reactivity with MSP-Fu$_{24}$. Sera that showed reactivity with both PfMSP-3$_{11}$ and PfMSP-1$_{19}$ or with either of the two antigens also showed reactivity with MSP-Fu$_{24}$. Sera from control individuals showed no reactivity with any of the antigens (see Fig. S2 in the supplemental material).

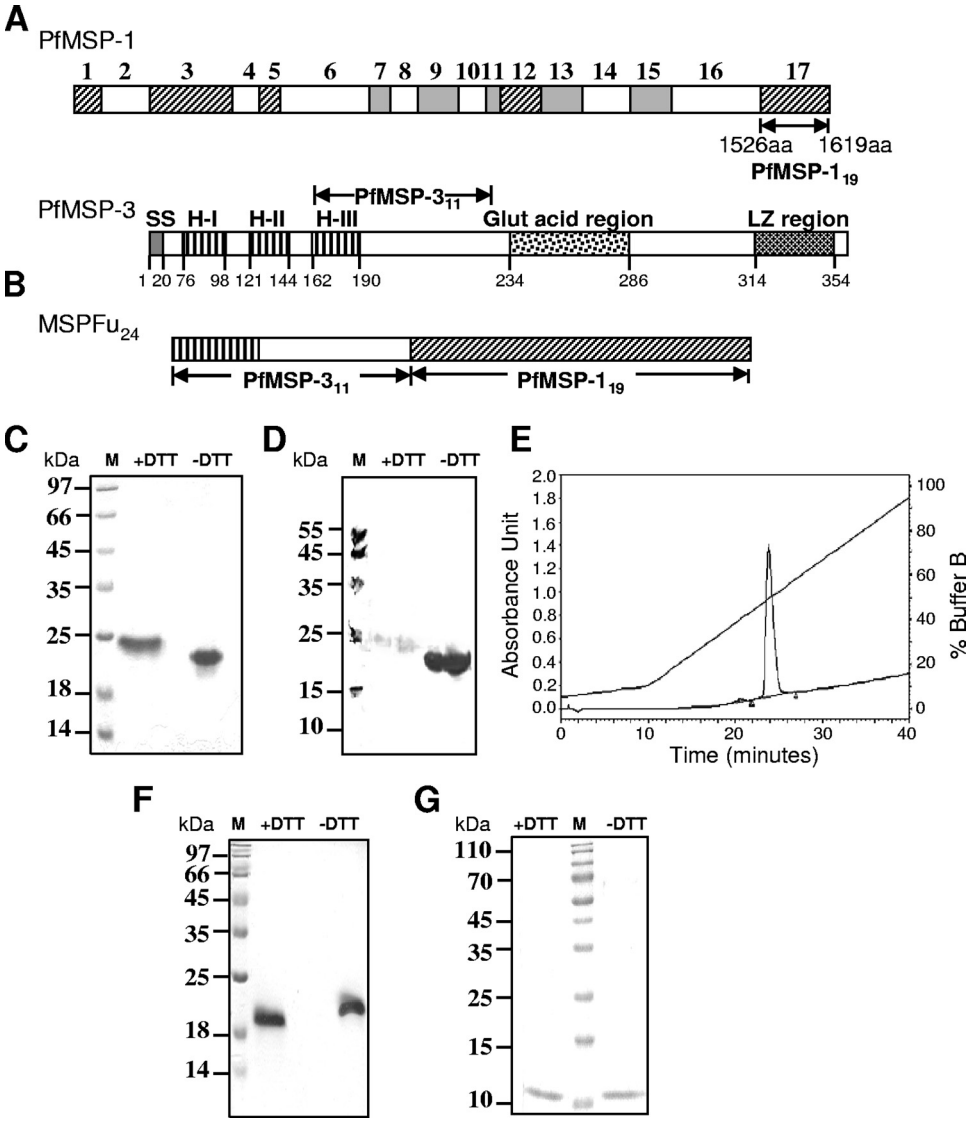

FIG. 1. Design and expression of the chimeric protein MSP-Fu_{24}. (A) Schematic diagram of *P. falciparum* MSP-1 (PfMSP-1) and PfMSP-3 showing the locations of the MSP-1_{19} and MSP-3_{11} regions. The conserved (hatched), semiconserved (filled), and variable (open) sequence blocks of PfMSP-1 are marked. The signal sequences (SS), heptad repeat regions (HI to HIII), glutamic acid-rich region, and leucine zipper region (LZ region) of PfMSP-3 are also marked. (B) Schematic diagram of MSP-Fu_{24} consisting of MSP-1_{19} and MSP-3_{11}. (C to E) Expression and purification of recombinant MSP-Fu_{24}. (C) Coomassie blue-stained SDS-PAGE gel showing purified recombinant MSP-Fu_{24} under reducing (with dithiothreitol [+DTT]) and nonreducing (−DTT) conditions. (D) Western blot analysis of MSP-Fu_{24} under reducing (+DTT) and nonreducing (−DTT) conditions using MAb 5.2. (E) RP-HPLC profile of purified MSP-Fu_{24} that eluted as a single sharp peak. (F) Coomassie blue-stained SDS-PAGE gel showing purified recombinant MSP-1_{19} under reducing (+DTT) and nonreducing (−DTT) conditions. (G) Coomassie blue-stained SDS-PAGE gel showing purified recombinant MSP-3_{11} under reducing (+DTT) and nonreducing (−DTT) conditions. M, molecular mass marker.

Immunogenicity of MSP-Fu_{24} and mixture of its individual components. The immunogenicity of MSP-Fu_{24}, formulated with three different adjuvants (CFA/IFA, Montanide ISA 720, and alum) was evaluated by using BALB/c mice. In parallel sets of experiments, groups of BALB/c mice were immunized separately with each of the individual components (PfMSP-1_{19} and PfMSP-3_{11}) as well as with their mixture for comparisons of immune responses. Antigen-specific antibodies were detected for each group after primary immunization, and antibody levels increased with each subsequent immunization. In all groups, antibody titers reached a peak after the second boost of immunization (data not shown).

As shown in Fig. 3, immunization with MSP-Fu_{24} induced antibodies recognizing both PfMSP-1_{19} and PfMSP3_{11}. Immunization with MSP-Fu_{24} or with the physical mixture of the two component antigens elicited antibodies recognizing both of the components in all the adjuvant groups. The antibody titers elicited for each of the component antigens in MSP-Fu_{24}- and in mixture-immunized groups were comparable to the titer value elicited by the respective single-antigen-formulation group. MSP-Fu_{24} induced high antibody titers in rabbits when formulated with two human-compatible adjuvants, Montanide ISA720 or alum (end point titers of $\sim 3.5 \times 10^5$ and $\sim 16.0 \times 10^5$, respectively). The mixture of the two antigens also induced

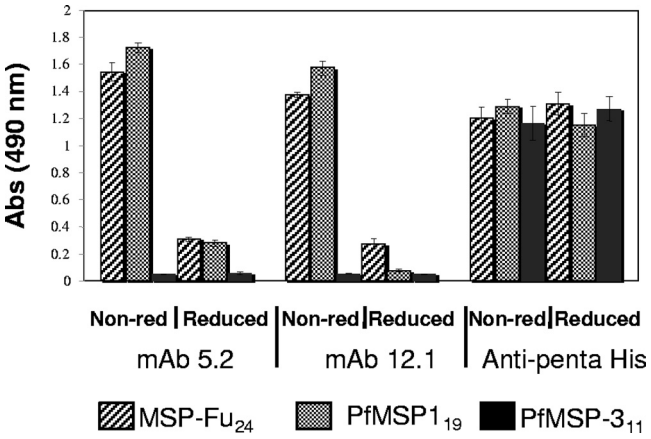

FIG. 2. Reactivity of MSP-Fu$_{24}$, PfMSp-1$_{19}$, and PfMSP-3$_{11}$ with anti-PfMSP-1 conformation-specific MAb 12.1 and MAb 5.2 by ELISA under nonreducing (Non-red) and reducing conditions. The assay for the reactivity of each recombinant protein with anti-penta-His antibody was carried out in parallel as a positive control to ascertain an equal amount of coating of the antigens under reducing and nonreducing conditions.

nearly similar levels of antibody titers when immunized in formulations with Montanide ISA720 or alum (end point titers of $\sim 19.3 \times 10^5$ and 9.4×10^5, respectively).

The IgG profiles between immunization groups for different adjuvant formulations were compared. The MSP-Fu$_{24}$-Montanide and mixture-Montanide groups showed similar levels ($P > 0.05$) of IgG subtypes. However, the mixture-alum immunization groups showed low levels of all the isotypes compared to the MSP-Fu$_{24}$-alum group (see Fig. S3 in the supplemental material).

MSP-Fu$_{24}$, PfMSP-3$_{11}$, and PfMSP-1$_{19}$ induce T-cell responses in immunized mice. T-cell responses of mice immunized with MSP-Fu$_{24}$, PfMSP-1$_{19}$, PfMSP3$_{11}$, and a mixture of the individual antigens were determined. Table S1 in the supplemental material summarizes the cellular responses induced upon immunization with different formulations of MSP-Fu$_{24}$, PfMSP-1$_{19}$, PfMSP3$_{11}$, and the mixture in BALB/c mice. Compared to the adjuvant control, MSP-Fu$_{24}$, PfMSP-1$_{19}$, PfMSP3$_{11}$, and mixture formulations induced significant proliferation ($P > 0.05$). However, there was variation in the proliferative responses depending upon the adjuvant (see Table S1 in the supplemental material). Splenocytes from alum groups showed significantly lower ($P > 0.05$) proliferation than the corresponding Montanide groups. After stimulation with any of these antigens, MSP-Fu$_{24}$, PfMSP-3$_{11}$, and PfMSP-1$_{19}$, the splenocytes of the MSP-Fu$_{24}$ immunization group showed significantly higher ($P > 0.05$) proliferation than splenocytes from the corresponding mixture groups. Similarly, for a given adjuvant, splenocytes from the MSP-Fu$_{24}$-immunized group showed significantly higher levels of proliferation when stimulated with the respective component antigens (PfMSP-1$_{19}$ and PfMSP-3$_{11}$) than the groups immunized with the individual antigen (see Table S1 in the supplemental material).

As shown in Table S2 in the supplemental material, MSP-Fu$_{24}$, PfMSP-1$_{19}$, PfMSP3$_{11}$, and the mixture formulated in Montanide ISA720 elicited higher IFN-γ responses than the respective alum formulation groups ($P > 0.05$). For a given adjuvant, splenocytes from the MSP-Fu$_{24}$ group stimulated with MSP-1$_{19}$ or MSP-3$_{11}$ showed IFN-γ secretion comparable to the secretion by splenocytes from the MSP-1$_{19}$ or MSP-3$_{11}$ group, respectively, after stimulation with the respective antigens. IFN-γ secretion was variable with physical mixture immunization groups; splenocytes from the mixture-Montanide group showed IFN-γ secretion comparable to that of the PfMSP-1$_{19}$–Montanide and PfMSP-3$_{11}$–Montanide groups. However, the mixture-alum group showed lower levels of IFN-γ compared to the PfMSP-1$_{19}$–alum and PfMSP-3$_{11}$–alum groups (see Table S2 in the supplemental material).

Anti-MSP-Fu$_{24}$ antibody recognizes the native parasite protein and conformation-specific epitopes in PfMSP-1$_{19}$. Affinity-purified anti-MSP-Fu$_{24}$ antibodies from all immunization groups were assessed for their ability to recognize the native parasite protein in the blood stage of *P. falciparum* 3D7 para-

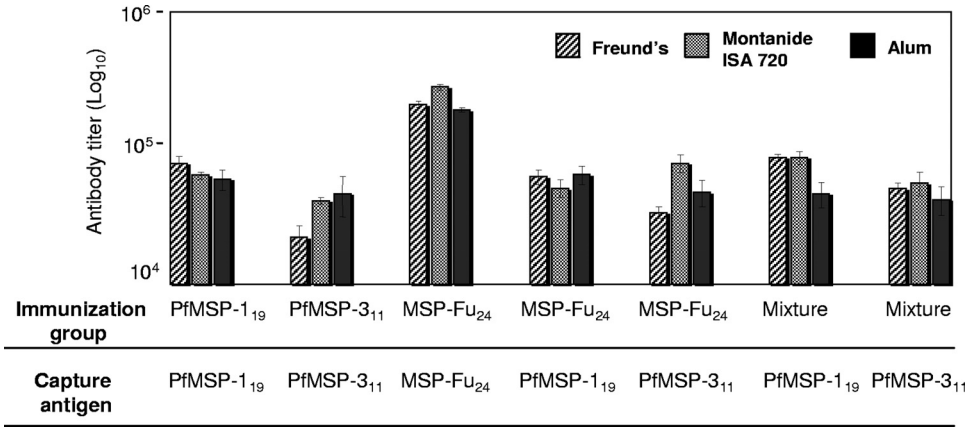

FIG. 3. Immune response in groups of BALB/c mice immunized with MSP-Fu$_{24}$, PfMSP1$_{19}$, PfMSP-3$_{11}$, or a mixture of PfMSP1$_{19}$ and PfMSP-3$_{11}$ formulated in different adjuvants. End point titers for anti-PfMSP1$_{19}$, anti-PfMSP-3$_{11}$, and anti-MSP-Fu$_{24}$ were determined by ELISA using the respective capture antigens and mouse sera collected on day 70. The highest dilution of sera showing an OD greater than or equal to the reactivity of the preimmune sera plus 2 SDs was considered to be the end point titer. Recombinant PfMSP-1$_{19}$ and PfMSP-3$_{11}$ were used as the capture antigens to determine the immune response to the component antigens (PfMSP-1$_{19}$ and PfMSP-3$_{11}$) in the sera from group of mice immunized with MSP-Fu$_{24}$ or with a physical mixture of the two components.

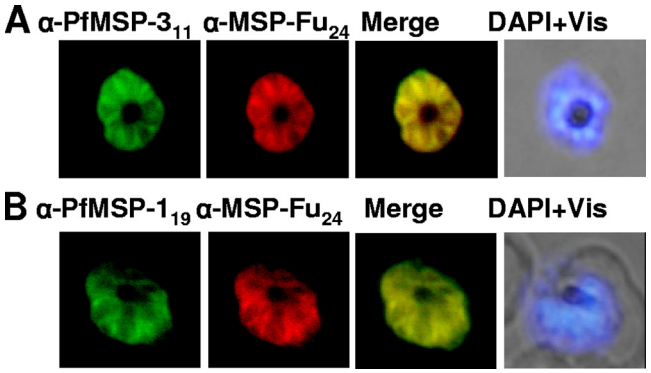

FIG. 4. Immunofluorescence assay with *P. falciparum* 3D7 parasites using anti-MSP-Fu$_{24}$ antibodies. Shown are fluorescence and bright-field images of acetone-methanol-fixed *P. falciparum* 3D7 parasites at the schizont stage immunostained with purified anti-MSP-Fu$_{24}$ (red) and anti-PfMSP-1$_{19}$ (green) or anti-MSP-3$_{11}$ (green) antibody. Parasite nuclei were stained with DAPI (blue).

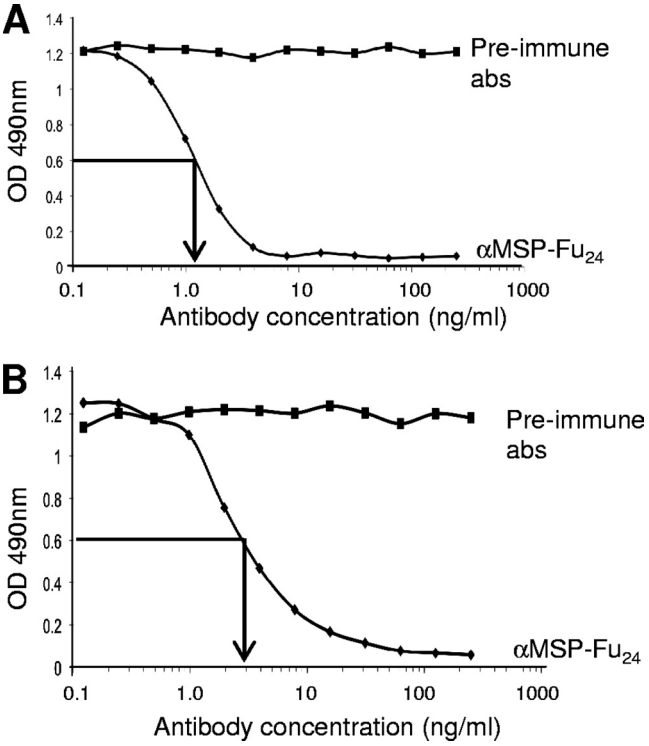

FIG. 5. Inhibition of binding of conformation-dependent anti-PfMSP1$_{19}$ MAb 12.10 (A) and MAb 5.2 (B) with recombinant PfMSP-1$_{19}$ by purified anti-MSP-Fu$_{24}$ antibodies. The reactivity of monoclonal antibodies with PfMSP1$_{19}$ preincubated with different concentrations of purified anti-MSP-Fu$_{24}$ antibodies was assessed by use of a competitive ELISA. The arrow indicates the concentration of anti-MSP-Fu$_{24}$ antibodies that brings about a 50% decrease in reactivity. Antibodies purified from preimmune sera were used as negative controls.

sites by immunofluorescence assay (IFA). Antibodies to MSP-Fu$_{24}$ showed strong reactivity on the surface of *P. falciparum* merozoites. IFA end point titers varied from 1:40,000 to 1:8,0000; the highest antibody titers were observed for rabbit and mouse sera immunized with MSP-Fu$_{24}$ formulated in Montanide ISA720. Fluorescence staining with anti-MSP-Fu$_{24}$ antibody colocalized with the staining by anti-PfMSP-1$_{19}$ as well as with anti-PfMSP-3$_{11}$ antibodies (Fig. 4). Similar results were also obtained with purified IgG from mice and rabbits immunized with a mixture of PfMSP-1$_{19}$ and PfMSP-3$_{11}$ (data not shown).

Competitive ELISA was carried out to investigate if the purified antibody from rabbit sera immunized with MSP-Fu$_{24}$ contained conformation-specific and/or invasion- and processing-inhibiting antibodies directed against PfMSP-1$_{19}$. As shown in Fig. 5, purified anti-MSP-Fu$_{24}$ antibodies inhibited the binding of conformation-specific MAb 5.2 as well as invasion- and processing-inhibitory MAb 12.10 with PfMSP-1$_{19}$ in a concentration-dependent manner. An approximately 50% reduction in the reactivity of MAb 5.2 was observed by competitive ELISA with anti-MSP-Fu$_{24}$ antibodies at a concentration of 0.3 μg/ml; similarly, anti-MSP-Fu$_{24}$ at a concentration 0.15 μg/ml caused a ~50% reduction in the reactivity of MAb 12.10 in this assay. Thus, immunization with MSP-Fu$_{24}$ induced conformation-specific as well as invasion/processing-inhibitory antibodies.

***In vitro* parasite growth inhibition by anti-MSP-Fu$_{24}$ antibodies.** Purified rabbit IgGs were assessed for their efficacy in inhibiting *in vitro* parasite growth of *P. falciparum* strains 3D7 and FCR. Table 1 shows the percent invasion inhibition by purified antibody from rabbits immunized with different antigen-adjuvant formulations. Purified IgGs from sera of rabbits immunized with MSP-Fu$_{24}$ formulations showed up to 75% and 66% parasite growth inhibitions of the 3D7 and FCR parasite strains, respectively, in a dose-dependent manner. Purified antibodies from rabbits immunized with the mixture of the two antigens also showed comparable invasion inhibition levels for both the strains. To ascertain that specific antibodies mediated this growth inhibition, antibodies specific to MSP-Fu$_{24}$, PfMSP-1$_{19}$, or PfMSP-3$_{11}$ were depleted from purified anti-MSP-Fu$_{24}$ rabbit antibodies by using different concentrations of recombinant MSP-Fu$_{24}$, PfMSP-1$_{19}$, and PfMSP-3$_{11}$, respectively. These antibody samples depleted of specific IgGs were then assessed for their efficacies in inhibiting *P. falciparum* 3D7 parasite invasion. The depletion of anti-MSP-Fu$_{24}$-specific antibodies showed up to an 80% reversal in the growth inhibition rate in a concentration-dependent manner, whereas PfMSP-1$_{19}$-depleted antibody showed a ~70% reversal (Fig. 6). However, PfMSP-3$_{11}$ showed only a ~23% reversal in growth inhibition at the same concentration. Recombinant PfHRP-2, used as a negative control in a depletion assay, did not show any reversal of invasion inhibition.

Anti-MSP-Fu$_{24}$ antibodies inhibit parasite growth in an ADCI assay. We investigated the efficacy of purified anti-MSP-Fu$_{24}$ mouse antibodies in inhibiting parasite growth in cooperation with monocytes in an ADCI assay. As shown in Fig. 7A, IgGs purified from the MSP-Fu$_{24}$–alum and MSP-Fu$_{24}$–Montanide groups of mice significantly ($P > 0.05$) inhibited parasite growth (SGI of up to 63%) in cooperation with monocytes in a concentration-dependent manner. It was also observed that the ADCI effect was dependent on the IgG concentration. Purified IgG from mice immunized with a physical mixture of the two antigens also inhibited parasite growth (SGI of up to 53%) in a similar manner. However, no significant differences were observed between growth inhibition by IgGs from MSP-Fu$_{24}$ and mixture immunization groups for a given adjuvant

TABLE 1. Inhibition of *P. falciparum* invasion of erythrocytes by purified antibodies from sera of rabbits immunized with MSP-Fu_{24} or with a mixture of PfMSP-3_{11} and PfMSP1_{19} formulated with Montanide ISA720 or alum[a]

Immunization group	Parasite strain	% Parasitemia[b] (SD) at concn of IgG Ab (mg/ml) of[c]:			% Invasion inhibition[d] at concn of IgG Abs (mg/ml) of:		
		0.125	0.25	0.5	0.125	0.25	0.5
Preimmune	3D7	3.68 (±0.75)	3.62 (±0.34)	4.36 (±0.33)			
	FCR	3.78 (±0.07)	3.95 (±0.14)	4.19 (±0.05)			
$PfMSP1_{42}$	3D7	2.01 (±0.29)	2.18 (±0.104)	1.61 (±0.28)	45.4	39.78	63.0
	FCR	2.36 (±0.09)	2.25 (±0.07)	1.74 (±0.08)	37.57	43.04	58.47
MSP-Fu_{24}-Montanide	3D7	1.9 (±0.08)	1.77 (±0.44)	1.37 (±0.05)	48.36	51.7	68.6
	FCR	2.07 (±0.07)	1.98 (±0.15)	1.52 (±0.03)	45.24	49.87	63.80
MSP-Fu_{24}-alum	3D7	1.94 (±0.43)	1.45 (±0.12)	1.11 (±0.14)	47.28	59.94	74.5
	FCR	2.53 (±0.07)	2.3 (±0.08)	1.39 (±0.08)	32.98	41.77	66.83
Mixture-Montanide	3D7	2.38 (±0.07)	2.37 (±0.35)	1.97 (±0.33)	35.32	34.5	54.81
	FCR	3.22 (±0.09)	2.7 (±0.09)	2.01 (±0.14)	14.81	31.65	51.95
Mixture-alum	3D7	2.37 (±0.31)	2.16 (±0.45)	2.31 (±0.35)	35.6	40.1	47.0
	FCR	3.42 (±0.16)	2.38 (±0.09)	2.05 (±0.13)	9.44	39.58	51.07

[a] Anti-$PfMSP\text{-}1_{42}$ rabbit antibodies were used as a control.
[b] Parasitemia values are the averages of triplicate determinations.
[c] Final concentration of IgG in a well.
[d] Calculated against the respective preimmune Ab well. The proportion of parasitized red blood cells in test wells was statistically significantly high compared to those in the respective control wells ($P < 0.005$ by chi-square test).

(Fig. 7A). Similar levels of inhibition were observed with anti-PfMSP-3 and anti-MSP-Fu_{24} antibodies; however, no significant inhibition was observed using anti-$PfMSP\text{-}1_{19}$ antibodies. To ascertain the specificity of growth inhibition, the anti-MSP-Fu_{24} antibodies were depleted by using recombinant $PfMSP\text{-}1_{19}$ or PfMSP-3_{11}. The depleted anti-MSP-Fu_{24} antibodies were then used for ADCI assays. A reversal of growth inhibition was observed after the depletion of PfMSP-3_{11}-specific antibodies (Fig. 7B), indicating that the observed ADCI effect was mediated largely by the presence of antibodies specific to PfMSP-3_{11} in a fusion chimera.

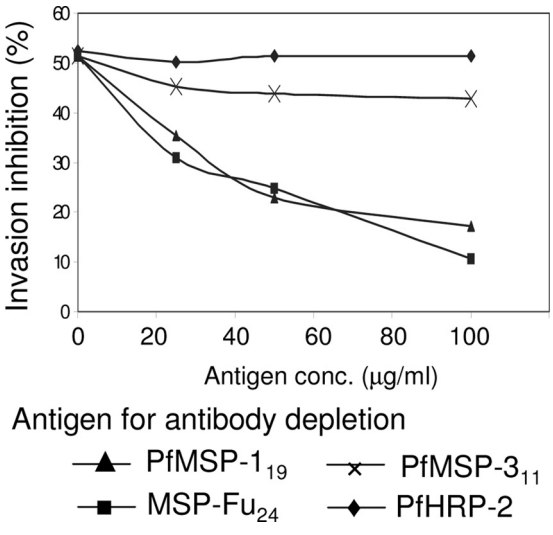

FIG. 6. Reversal of anti-MSP-Fu_{24} antibody-mediated inhibition of *P. falciparum* invasion after depletion of antigen-specific antibodies. The invasion inhibition by anti-MSP-Fu_{24} antibodies (250 μg/ml) was assessed after the preincubation of antibodies with different amount of recombinant MSP-Fu_{24}, $PfMSP1_{19}$, or PfMSP-3_{11} to deplete antibodies specific to the respective antigens. PfHRP-2 was also used as one of the antigens for antibody depletion in the assay as a negative control. The percent reversal of invasion inhibition was calculated based upon invasion inhibition without any antibody depletion.

DISCUSSION

It is generally believed that subunit vaccines containing more than one malaria antigen will have more of a chance of success than those based on a single antigen. The advantages of a combination vaccine is the possibility of a simultaneous attack on more than one parasite target and the countering of parasite growth by different mechanisms. A combination vaccine may also provide a synergetic response. However, such an approach would necessarily require the development and analysis of individual components of the combination vaccine. Proteins that appear on the surface of merozoites of *P. falciparum* are considered possible candidates for blood-stage malaria vaccines, and out of the many merozoite surface proteins identified so far, PfMSP-1 and PfMSP-3 are among the most promising vaccine candidate antigens. Interestingly, these two antigens seem to generate protective immune responses by entirely different mechanisms: while the antibody response to the C-terminal fragments of PfMSP-1 is supposed to be invasion inhibitory, the protective response of MSP-3 is believed to be in a monocyte-dependent manner through ADCI (23, 28). In the present study, we have produced a chimeric malaria protein containing the C-terminal fragment of $PfMSP\text{-}1_{19}$ fused downstream with a conserved, immunologically relevant region of PfMSP-3; in addition, we also produced the two individual component polypeptides separately. The main aim of the study was to compare immune responses of the fusion protein, MSP-Fu_{24}, with those of a mixture of its individual components ($PfMSP\text{-}1_{19}$ and PfMSP-3_{11}) and also to assess the antiparasitic

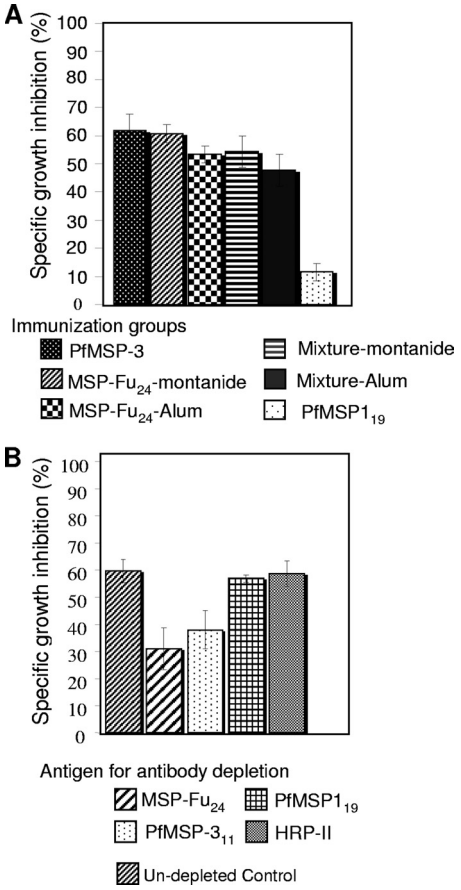

FIG. 7. (A) Specific growth inhibition (SGI) index of antibody-dependent cellular inhibition (ADCI) of parasite growth by total IgG purified from sera of mice immunized with MSP-Fu_{24}, $PfMSP1_{19}$, $PfMSP\text{-}3_{11}$, or a mixture of $PfMSP1_{19}$ and $PfMSP\text{-}3_{11}$ formulated in Montanide ISA720 or alum. Parasites at the schizont stage were cocultured with human monocytes in the presence of purified antibodies (50 μg/ml of culture in the assay), and parasite growth was monitored after 96 h. (B) Reversal of antibody-dependent cellular inhibition of parasite growth by depletion of antigen-specific antibodies. The SGI index of ADCI of parasite growth purified from sera of mice immunized with MSP-Fu_{24}–Montanide after incubation of antibodies with recombinant MSP-Fu_{24}, $PfMSP1_{19}$, or $PfMSP\text{-}3_{11}$ to deplete antibodies specific to the respective antigen was determined.

efficacies induced by the immune responses generated. The three recombinant proteins were expressed in *E. coli* cells, purified to homogeneity, and characterized by different standard procedures.

For a number of merozoite surface proteins that are rich in cysteine residues, the generation of parasite-inhibitory antibodies is dependent on specific protein conformations stabilized by several disulfide linkages, which makes it critical to produce such vaccine target recombinant proteins that conformationally resemble their native counterparts (5, 17, 26, 45). $PfMSP\text{-}1_{19}$ is highly structured, contains six disulfide linkages, and folds into two epidermal growth factor-like domains, which provide it with conformational stability (8, 26). A number of MAbs that show reactivity with $PfMSP\text{-}1_{19}$ only when it attains an appropriate conformation have been developed (45). Our results of immunoblotting and ELISA using different conformation-dependent MAbs showed that MSP-Fu_{24} contained immunologically relevant conformational epitopes of $PfMSP\text{-}1_{19}$, suggesting that the $MSP\text{-}1_{19}$ component in MSP-Fu_{24} has acquired a conformation similar to that of the native protein. The complete loss of reactivity of the conformational MAbs with the fusion protein under reducing conditions indicated that the tertiary structure of the protein is stabilized by disulfide linkages. Furthermore, MSP-Fu_{24} was recognized by the majority of sera from individuals residing in an area where malaria is endemic, indicating that it contained an epitope(s) present in its component antigens that may be the targets of the antibody response generated during natural exposure to *P. falciparum*. In addition, anti-MSP-Fu_{24} antibodies recognized both native PfMSP-1 and PfMSP-3 on the parasite surface.

We tested the immunogenicities of MSP-Fu_{24} and a physical mixture of its two-component antigens using three different adjuvants, alum, Montanide ISA720, and CFA/IFA, in small animals. Two of these adjuvants, alum and Montanide ISA720, were used previously in malaria vaccine trials for monkeys as well as humans (11, 14, 21, 37). We found that immunization with MSP-Fu_{24} induced a significant antibody response with all three adjuvant formulations. Considering that most vaccine target malaria recombinant antigens are poorly immunogenic, the high antibody response to the fusion protein in alum and Montanide ISA720 is encouraging. Antigenic competition or immunodominance of individual component antigens could be a potential hindrance in the development of combination-antigen-based vaccines. Immunization with MSP-Fu_{24} in different adjuvant formulations elicited an immune response to each of its components that was comparable to that induced by immunization with individual components. These results suggested a lack of antigenic competition when the two antigens were presented together as a fusion protein or as a physical mixture of the two. Such a lack of antigenic competition was observed previously by workers using other combination malaria vaccine constructs. For example, no antigenic competition was observed for studies with PfCP 2.9, a fusion chimera of $PfMSP\text{-}1_{19}$ and PfAMA-1 (31), or when this fusion protein was administered in combination with another recombinant antigen, PfEBA-175 (F2), as a single formulation (47). Similar observations were made when the C-terminal region of the PfCSP, including the repeats, was administered in combination with PfCP 2.9 (48). In another study, a fusion construct of *P. yoelii* MSP-1 and MSP-8 also induced an antibody response comparable to that of the two components (38). It appears that the immunodominant regions of the malaria proteins selected in these studies and in the present one have inherently strong structural preferences, which are maintained when they are presented in fusion chimeras.

The recombinant fusion chimera stimulated T lymphocytes in an *in vitro* assay with various stimulation indices depending on the adjuvant used. In general, the level of splenocyte proliferation was higher in MSP-Fu_{24}-immunized groups than in the physical mixture groups. Cytokines play an important role in determining the IgG subclass. IFN-γ is a product of Th1 cells and is generally associated with the production of IgG2a, also an indicator of cell-mediated immunity. The production of IFN-γ and IgG subtype analysis of the immune response indicated that both Th1 and Th2 subsets of T-helper cells are elicited by the fusion protein.

Although many adjuvant formulations have been shown to

induce high-titer antibodies against malarial antigens, it was also shown that the antibody titers alone are not sufficient to ensure protection, even when the antigen contained protective epitopes (19); the kind of antibody response generated by synthetic immunogens is equally important. Particularly for PfMSP-1_{19}, several studies have shown that it was not the total antibody titers but the levels of specific invasion-inhibitory and processing-inhibitory antibodies that correlated with protection (9, 12, 20, 30, 45). Therefore, it was important to determine the fine specificity of anti-PfMSP-1_{19} antibody induced by the fusion chimera that may be indicative of its antiparasitic efficacy. Of several MAbs (5.2, IE1, 12.10, 2F10, 111.4, and 12.8) raised against PfMSP-1, MAbs 5.2, 2F10, 12.10, and 12.8 have been shown to be directed against conformation-specific epitopes of PfMSP-1_{19}. In addition, MAbs 12.10 and 12.8 also inhibit the proteolytic processing of PfMSP-1 during merozoite invasion (1, 7, 13). We carried out a competition ELISA-based analysis of mouse immune sera to assess the presence of anti-MSP-Fu_{24} antibodies that share a conformation-specific epitope(s) with these invasion/processing-inhibitory MAbs. The degree of competition in this assay is influenced mainly by the relative affinities of serum antibodies and the MAbs, the degree of overlap of the epitopes, and the concentration of serum antibodies. Similar assays were used previously to assess the induction of PfMSP-1_{19}-specific invasion-inhibitory antibodies against immunization of mice/rabbits with PfMSP-1_{42} formulations (36). Our results show that immunization with MSP-Fu_{24} elicited antibodies against the PfMSP-1_{19} component that share epitopes with conformation-specific and invasion/processing-inhibiting MAbs; these results also suggest that MSP-Fu_{24} can induce invasion-inhibitory/processing antibodies when used as an immunogen with human-compatible adjuvants.

In the absence of any direct correlates of protection against malaria, *in vitro* assays such as invasion inhibition and ADCI assays are used to assess the antiparasitic efficacy of the immune response generated to an immunogen (11, 36, 39, 40, 46). We found that purified Abs from sera of rabbits immunized with MSP-Fu_{24} strongly inhibited the parasite invasion of not only homologous *P. falciparum* strain 3D7 but also strain FCR. Furthermore, the depletion of MSP-Fu_{24}- and PfMSP-1_{19}-specific antibodies from total anti-MSP-Fu_{24} antibodies decreased this inhibition, indicating the specificity of the invasion inhibition assay. On the other hand, the depletion of PfMSP-3-specific antibodies did not cause any significant reduction in invasion inhibition, suggesting that the antibody response directed against the MSP-1_{19} component in MSP-Fu_{24} was mainly responsible for the invasion inhibition.

It was previously shown that some merozoite surface proteins, such as PfMSP-3, PfMSP-6, and PfGLURP, provide ADCI-mediated protection against the parasite compared to the direct invasion inhibition by PfMSP-1 (28, 29, 40, 42). For ADCI of malaria parasites, the antibodies against these proteins act in cooperation with host monocytes. These antibodies form a complex with the respective merozoite proteins and then interact with FcγRIIA on the monocytes, which induces the release of killing factors such as tumor necrosis factor alpha (TNF-α) (3). The human FcγIIa receptor is the primary trigger molecule for ADCI and exists in two alloforms, FcγRIIA-Arg/Arg_{131} and FcγRIIA-His/His_{131} (4, 32). Mouse IgG1 and IgG2b isotype antibodies bind well with the more prevalent FcγIIa receptor FcγRIIA-Arg/Arg_{131} (33). Our results showed that anti-MSP-Fu_{24} antibodies from the alum and Montanide ISA720 immunization groups significantly inhibited parasite growth in a dose-dependent manner in the ADCI assay. The ADCI-mediated parasite growth inhibition by anti-MSP-Fu_{24} antibodies was comparable to that by the antibodies induced by a mixture of the two component antigens. Furthermore, the depletion of anti-PfMSP-3_{11} antibodies decreased the level of parasite growth inhibition, suggesting that anti-MSP-Fu_{24} antibodies directed against PfMSP-3_{11} contribute to the protective immune response against the parasite through ADCI-related mechanisms. Although the heterogeneous combination of mouse antibodies with human monocytes in these assays may not be entirely satisfactory or close to the natural conditions, it is not possible to generate anti-MSP-Fu_{24} antibodies in humans for these assays in the present study. Therefore, to assess the efficacy of antibodies induced by the recombinant antigen MSP-Fu_{24}, to elicit ADCI, we have used mouse antibodies with human monocytes as the next-best approximation for these *in vitro* assays. It may be important that such a heterogeneous combination was used previously for a similar set of ADCI experiments (42).

The multivalent vaccine concept is an attractive strategy for the development of a malaria vaccine, even though each of the component antigens of such a vaccine would need to be developed separately. The use of a fusion protein consisting of the immunodominant region from different antigens may provide a feasible alternative strategy. In this study, we generated a fusion protein, MSP-Fu_{24}, consisting of immunodominant regions of PfMSP-3 and PfMSP-1. A simple protocol was established to express MSP-Fu_{24} as a soluble protein in relatively high yields, which can be easily scaled up for large-scale production under cyclic GMP (cGMP) conditions. Given that it has not been easy to produce PfMSP-1_{19} as a soluble protein with satisfactory yields, it is quite remarkable that its fusion with a specific fragment of PfMSP-3 has resulted in an easy-to-purify fusion protein with the appropriate folding of the PfMSP-1_{19} component. The fusion protein formulated with human-compatible adjuvants is highly immunogenic in small animals, and the two-component polypeptides have maintained their functional identity in the context of the immune response to the fusion protein. Our data suggest that the fusion protein has the potential to be developed as a malaria vaccine candidate.

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