INFECTION AND IMMUNITY, Jan. 2005, p. 287–297 Vol. 73, No. 1
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.1.287–297.2005

The Clinical-Grade 42-Kilodalton Fragment of Merozoite Surface Protein 1 of *Plasmodium falciparum* Strain FVO Expressed in *Escherichia coli* Protects *Aotus nancymai* against Challenge with Homologous Erythrocytic-Stage Parasites

Christian A. Darko,[1]†‡ Evelina Angov,[1]*† William E. Collins,[2] Elke S. Bergmann-Leitner,[1] Autumn S. Girouard,[1] Stacy L. Hitt,[1] Jana S. McBride,[3] Carter L. Diggs,[4] Anthony A. Holder,[5] Carole A. Long,[6] John W. Barnwell,[2] and Jeffrey A. Lyon[1]

Department of Immunology, Walter Reed Army Institute of Research, Silver Spring,[1] and Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville,[6] Maryland; Division of Parasitic Diseases, Centers for Disease Control and Prevention, Chamblee, Georgia[2]; USAID Malaria Vaccine Development Program, U.S. Agency for International Development, Washington, D.C.[4]; School of Biological Sciences, University of Edinburgh, Edinburgh, Scotland[3]; and Division of Parasitology, National Institute for Medical Research, London, United Kingdom[5]

Received 2 July 2004/Returned for modification 9 August 2004/Accepted 20 September 2004

A 42-kDa fragment from the C terminus of major merozoite surface protein 1 (MSP1) is among the leading malaria vaccine candidates that target infection by asexual erythrocytic-stage malaria parasites. The $MSP1_{42}$ gene fragment from the Vietnam-Oak Knoll (FVO) strain of *Plasmodium falciparum* was expressed as a soluble protein in *Escherichia coli* and purified according to good manufacturing practices. This clinical-grade recombinant protein retained some important elements of correct structure, as it was reactive with several functional, conformation-dependent monoclonal antibodies raised against *P. falciparum* malaria parasites, it induced antibodies (Abs) that were reactive to parasites in immunofluorescent Ab tests, and it induced strong growth and invasion inhibitory antisera in New Zealand White rabbits. The antigen quality was further evaluated by vaccinating *Aotus nancymai* monkeys and challenging them with homologous *P. falciparum* FVO erythrocytic-stage malaria parasites. The trial included two control groups, one vaccinated with the sexual-stage-specific antigen of *Plasmodium vivax*, Pvs25, as a negative control, and the other vaccinated with baculovirus-expressed $MSP1_{42}$ (FVO) as a positive control. Enzyme-linked immunosorbent assay (ELISA) Ab titers induced by *E. coli* $MSP1_{42}$ were significantly higher than those induced by the baculovirus-expressed antigen. None of the six monkeys that were vaccinated with the *E. coli* $MSP1_{42}$ antigen required treatment for uncontrolled parasitemia, but two required treatment for anemia. Protective immunity in these monkeys correlated with the ELISA Ab titer against the p19 fragment and the epidermal growth factor (EGF)-like domain 2 fragment of $MSP1_{42}$, but not the $MSP1_{42}$ protein itself or the EGF-like domain 1 fragment. Soluble $MSP1_{42}$ (FVO) expressed in *E. coli* offers excellent promise as a component of a vaccine against erythrocytic-stage falciparum malaria.

Plasmodium falciparum malaria afflicts more than 200 million people per year (41) and is estimated to kill one African child every 30 s (2). The development of vaccines against this disease is increasingly important due to the spread of multidrug-resistant malaria parasites.

The major surface protein from merozoites (merozoite surface protein 1 [MSP1]) is among the leading erythrocytic-stage candidates for inclusion in a multistage malaria vaccine. In *P. falciparum*, this protein ranges in size from 185 to 200 kDa and is attached at its C terminus to the parasite plasma membrane via a glycosylphosphatidylinositol anchor (15). MSP1 is present on the merozoite's surface as a complex of fragments (25, 27) derived by proteolytic processing of the precursor (21, 22, 25). During erythrocytic invasion by merozoites, $MSP1_{42}$, which is the major fragment from the C terminus, is processed secondarily, producing a 33-kDa fragment ($MSP1_{33}$) from the N terminus and a 19-kDa fragment from the C terminus ($MSP1_{19}$). $MSP1_{19}$ is highly structured, folding into two epidermal growth factor (EGF)-like domains known as EGF-like domain 1 and EGF-like domain 2 (29). $MSP1_{19}$ remains attached to the merozoite surface and is present on ring forms in newly invaded erythrocytes (4, 5). Sequence variation among $MSP1_{42}$ molecules from different *P. falciparum* strains is primarily dimorphic (28).

Both $MSP1_{42}$ and $MSP1_{19}$ are established targets of protective immunity in animal models, and in the case of the rodent malaria model, *Plasmodium yoelii*, the protection observed is strain specific (33). Additional evidence for the importance of this region of MSP1 in immunity includes the observation that

* Corresponding author. Mailing address: Department of Immunology, Walter Reed Army Institute of Research, 503 Robert Grant Ave., Silver Spring, MD 20910. Phone (301) 319-9614. Fax: (301) 319-7358. E-mail: Evelina.angov@na.amedd.army.mil.

† C.D. and E.A. contributed equally to this work.

‡ Present address: Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD 20910.

$MSP1_{19}$-specific monoclonal antibodies (MAb) inhibit *P. falciparum* growth in vitro (3, 10) and passively protect mice against *P. yoelii* malaria infection (24, 26). It has also been shown that MAbs that inhibit *P. falciparum* growth in vitro also inhibit the secondary processing of $MSP1_{42}$ (6) and bind to conformational epitopes that are lost when cystines are reduced. $MSP1_{19}$-specific Abs have proven to be the major contributors to the invasion-inhibitory response in humans who are immune to malaria (30), and double domain-specific p19 as well as EGF domain 2-specific antibodies affinity purified from human immune sera prevent parasite invasion in vitro (13). Several studies have shown that rabbit antisera raised against recombinant $MSP1_{42}$ inhibit *P. falciparum* growth in vitro (1, 8, 35).

The active vaccination of *Aotus nancymai* monkeys with *Escherichia coli* (35)- and baculovirus (7, 36, 37)-expressed recombinant *P. falciparum* $MSP1_{42}$ induces a significant homologous protective effect against infection by erythrocytic-stage *P. falciparum* strain FVO. $MSP1_{42}$ purified from transgenic mouse milk also induces protection, but this result depends on removing two N-glycosylation sites from the protein (36). Since *P. falciparum* does not N-glycosylate proteins, expression strategies that avoid glycosylation should be most suited for malaria vaccines based on $MSP1_{42}$. *E. coli* offers this possibility, and two approaches can be taken. One is to allow *E. coli* to express and fold the protein such that it is either soluble or can be solubilized by the use of mild detergents (1), and the other is to purify the protein from inclusion bodies and refold it in vitro (35). The present work follows the first approach.

Our objective was to manufacture a clinical-grade *E. coli*-expressed recombinant $MSP1_{42}$ (FVO) antigen (Ag) that preserved various elements of the correct antigen structure, as evidenced by its binding of functional MAbs specific for conformation-dependent epitopes. Furthermore, we wanted to show that this Ag was capable of inducing functional immunity by using growth inhibition assays to evaluate vaccinated rabbit sera or by challenging vaccinated *A. nancymai* monkeys with the highly virulent FVO strain of *P. falciparum*, which is homologous to the vaccine. For the vaccination experiments, we chose to use Freund's adjuvant because, to date, it is the only adjuvant that has shown the capacity to induce protective immunity in the *Aotus* monkey model. Taking this approach allowed us firstly to determine if we met our objective of manufacturing efficacious clinical-grade FVO strain $MSP1_{42}$, which has not been accomplished previously, and secondly to compare the performance of our antigen with those of several non-clinical-grade Ags that have already been evaluated. This comparison was facilitated by the use of baculovirus-expressed FVO strain $MSP1_{42}$ (37), which has been compared with other $MSP1_{42}$ preparations (35–37), as a positive control. Our clinical-grade *E. coli*-derived $MSP1_{42}$ induced a strong protective effect in *Aotus* monkeys, protecting all animals against uncontrolled parasitemia, while all animals in the negative control group, who were immunized with the 25-kDa sexual-stage antigen from *Plasmodium vivax* (Pvs25) (20), were treated for uncontrolled parasitemia, as were two of five animals in the positive control group, who were vaccinated with baculovirus-expressed $MSP1_{42}$. This soluble *E. coli*-expressed $MSP1_{42}$ (FVO) Ag is a strong prospect as a component of an erythrocytic-stage malaria vaccine.

MATERIALS AND METHODS

Construction of expression vector pET(AT)PfMSP1$_{42}$ (FVO). Initial experiments showed that we could not express the wild-type $MSP1_{42}$ gene fragment (GenBank accession no. L20092) in *E. coli* in a quantity that was sufficient for purification. This problem was solved by introducing a synonymous mutation in codon 141 (Ile) at bp 423 (C→A) (numbered from the start of the native $MSP1_{42}$ sequence from L20092), permitting expression at the level of 0.05 mg/g of cell paste. The rationale for this mutation will be reported elsewhere (unpublished data). The mutation was introduced by PCR to generate two overlapping fragments of the $MSP1_{42}$ gene that were annealed and extended prior to PCR amplification to prepare the final product. The primers used to prepare the overlapping fragments were FVO-PCR1 (5′-GGGTCGGTACCATGGCAGTA ACTCCTTCCGTAATTGAT-3′), which was paired with EA5 (5′-AAAAGGG AAGGTATTTCTCATT-3′), and FVO-PCR2, (5′GGATCAGATGCGGCCGC TTAACTGCAGAAAATACCATCGAAAAGTGGA-3′), which was paired with EA3 (5′-TAAAAAATATATAAACGACAAAC-3′). The base change relative to the native sequence in primer EA3 is underlined.

The two products, FVO-PCR1/EA5 and EA3/FVO-PCR2, were purified by gel extraction with a QIAEX II kit. An equimolar mixture of the two oligonucleotides was then used in combination with the flanking primers FVO-PCR1 and FVO-PCR2 to produce a full $MSP1_{42}$ (FVO) gene fragment possessing NcoI and NotI restriction sites at the 5′ and 3′ ends, respectively.

Molecular cloning and bacterial transformations were performed as follows. The $MSP1_{42}$ (FVO) gene fragment and the expression vector pET(AT) $PfMSP1_{42}$ (3D7) (1) were digested with NcoI and NotI and purified by extraction from agarose gels (QIAEX II; Qiagen, Chatsworth, Calif.). The DNAs were ligated with T4 DNA ligase (Roche Diagnostics Corporation, Indianapolis, Ind.), producing the plasmid pET(AT)$PfMSP1_{42}$ (FVO), which was used to transform (34) *E. coli* BL21 DE3 [F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)] (Invitrogen, Carlsbad, Calif.) to tetracycline resistance (Boehringer Mannheim, Indianapolis, Ind.). The $MSP1_{42}$ insert was fused N-terminally to 17 non-MSP1 amino acids that included an N-terminal hexahistidine tag (Fig. 1).

GMP fermentation and expression. Based on conditions obtained from laboratory scale 10-liter fermentations, a 300-liter good manufacturing practices (GMP) fermentor containing Super Broth medium supplemented with 15 μg of tetracycline/ml was inoculated with 3 liters of fresh stationary-phase culture in accordance with batch production record (BPR) 444-00. Fermentation continued at 37°C to an optical density at 600 nm (OD_{600}) of 4.5. The culture temperature was reduced from 37 to 25°C prior to the induction of protein expression with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2.5 h. At the end of the induction, the cells were harvested by use of a Sharples AS-26 centrifuge at 27,666 × *g* at a rate of 3 liters/min, and the *E. coli* cell paste was stored at −80°C at the Pilot Bioproduction Facility, Department of Biologics Research, Walter Reed Army Institute of Research (WRAIR), Silver Spring, Md.

GMP purification of BPR 444-00, lot no. 0792. The cell paste (2.8 kg) was lysed in buffer (8.4 liters) containing 10 mM sodium phosphate (pH 6.2), 50 mM sodium chloride, 80 mM imidazole, 2 mM magnesium chloride, and 50 U of benzonase/ml. After cell lysis by microfluidization, Tween 80 (NOF Corporation, Hyogo, Japan) and sodium chloride were added to final concentrations of 1.0% (vol/vol) and 1.0 M, respectively, and the lysate was incubated on ice for 30 min with stirring prior to centrifugation at 27,666 × *g* for 1 h at 4°C. The clarified lysate was collected and filtered through 0.45-μm-pore-size filters (Millipore Corp., Bedford, Mass.). All subsequent steps were carried out at 4°C. Two chromatographic steps were used to purify the $MSP1_{42}$ (FVO) Ag to homogeneity.

For Ni^{2+}-nitrilotriacetic acid Superflow (Qiagen) chromatography, a 230-ml column (12:1 [wt/vol] cell paste-to-resin ratio) was equilibrated with a solution containing 10 mM sodium chloride (pH 6.2), 1.0 M sodium chloride, 80 mM imidazole (Ni buffer), and 1.0% (vol/vol) Tween 80 (NOF Corporation). The column was loaded at a flow rate of 32 ml/min and washed with 20 column volumes of Ni buffer containing 1.0% Tween 80. The column was washed with 20 volumes of buffer containing 10 mM sodium phosphate (pH 6.2), 0.5 M sodium chloride, 80 mM imidazole, and 1.0% (vol/vol) Tween 80 followed by 20 volumes of buffer containing 10 mM sodium phosphate (pH 8.5), 75 mM sodium chloride, and 20 mM imidazole. $MSP1_{42}$ (FVO) was eluted by applying 5 column volumes of buffer containing 10 mM sodium phosphate (pH 8.5), 75 mM sodium chloride, 1 M imidazole, and 0.2% Tween 80 to the column.

For Q Sepharose chromatography, a Q Sepharose Fast Flow column (Amersham-Pharmacia Biotech, Piscataway, N.J.) (cell paste-to-resin ratio of 5:1 [wt/vol]) was equilibrated with buffer containing 10 mM glycine (pH 9.6), 35 mM sodium chloride, 2 mM EDTA, and 0.2% Tween 80 (Q buffer). The eluate from the Ni chromatography step was diluted with an equal volume of buffer contain-

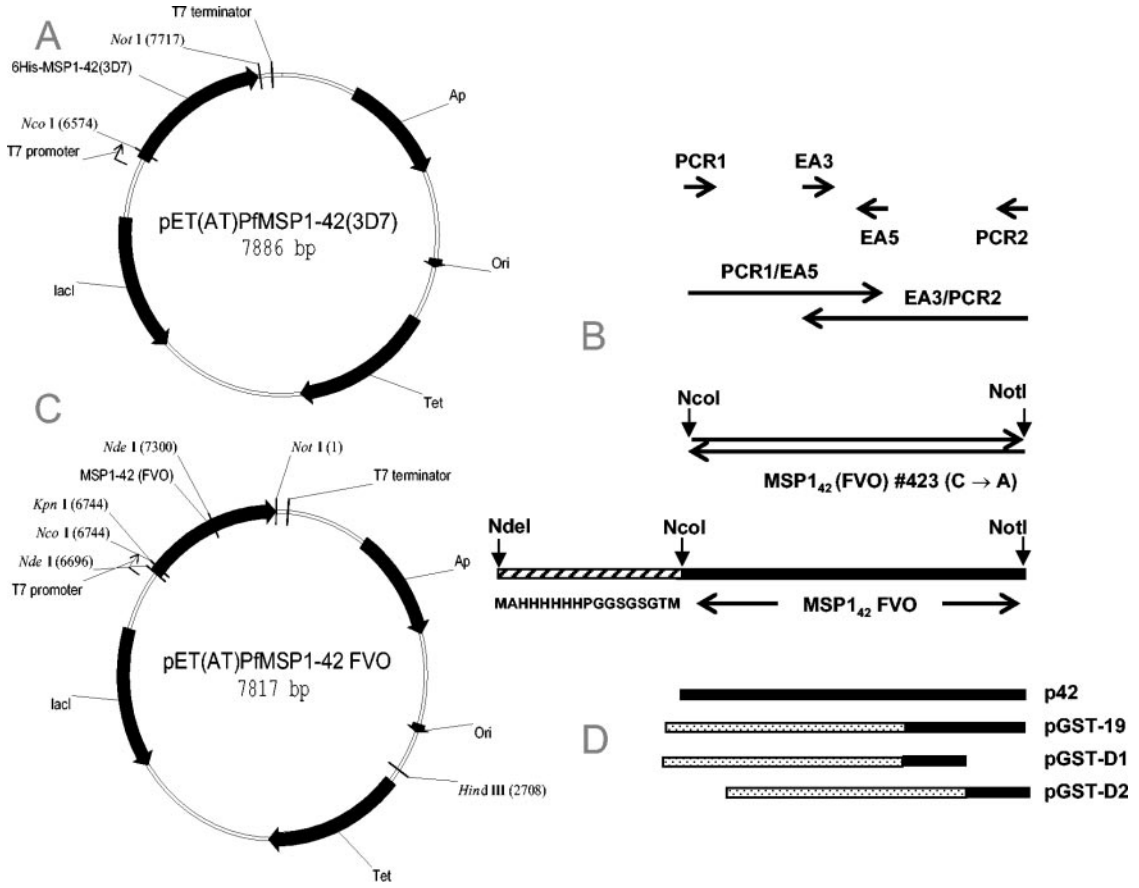

FIG. 1. Construction of *E. coli* MSP1$_{42}$ (FVO) expression plasmid. (A) pET(AT)PfMSP1$_{42}$(3D7) plasmid map. (B) Strategy for PCR-generated mutagenesis of PfMSP1$_{42}$(FVO) strain insert. (C) pET(AT)PfMSP1$_{42}$(FVO) plasmid map. (D) Diagram designating the subunit fragments of MSP1$_{42}$ (FVO) used in ELISAs. The N-terminal black bars designate GST in the fusions.

ing 10 mM glycine (pH 9.6), 4 mM EDTA, and 0.2% Tween 80, and the pH was adjusted to 9.6 by the addition of 1 N sodium hydroxide. The column was loaded at a flow rate of 65 ml/min and then washed with 5 column volumes of Q buffer. The column was washed with 30 column volumes of wash buffer containing 10 mM glycine (pH 9.6), 0.1 M sodium chloride, 2 mM EDTA, and 0.2% Tween 80 at a flow rate of 82 ml/min, and the protein was eluted with 3 column volumes of buffer containing 10 mM sodium phosphate (pH 7.2), 0.2 M sodium chloride, 2 mM EDTA, and 0.2% Tween 80. The buffer was exchanged with a solution containing 1.7 mM potassium phosphate (monobasic), 5 mM sodium phosphate (dibasic), 0.15 M sodium chloride, and 0.1% Tween 80 (pH 7.1) by ultrafiltration with a UFP-3C-6A filter (molecular weight cutoff, 3,000; A/G Technology, Needham, Mass.), and the protein was concentrated to 0.5 mg/ml. The retentate was filter sterilized by use of a Millipak 60 0.22-μm-pore-size filtration unit, and the final purified bulk was stored at −80°C.

SDS-PAGE and immunoblotting. The protein was analyzed by Tris-glycine SDS-PAGE under nonreducing or reducing (10% 2-mercaptoethanol) conditions followed by Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, Calif.) staining or immunoblotting as previously described (1). Nitrocellulose membranes were probed with either polyclonal mouse anti-FVO MSP1$_{42}$ antibodies (kindly provided by Sanjai Kumar of the Food and Drug Administration [FDA], Bethesda, Md) or mouse MAbs diluted in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Tween 20. The MAbs used for evaluations of proper epitope structure included 2.2 (16, 27), 12.8 (3, 27), 7.5 (16, 27), 12.10 (3, 27), and 5.2 (9).

Protein purity. Protein purity was measured by the following methods: Coomassie blue R250 total protein staining of a 10-μg sample, Western blotting to reveal host cell protein contamination by probing with polyclonal rabbit anti-*E. coli* antibodies (kindly provided by Joe Cohen of GlaxoSmithKline Biologicals, Rixensart, Belgium), and quantification of the *E. coli* host cell protein by use of an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (Cygnus Technologies, Plainville, Mass.). Host cell endotoxin levels were quantified by use of the Pyrotell *Limulus* amebocyte lysate gel clot formulation according to the manufacturer's protocol (Cape Cod Associates, Mass.). The residual Ni^{2+} content was quantified by inductively coupled plasma optical emission spectrometry (ICP-OES) at Research Triangle Institute (Research Triangle Park, N.C.).

GST fusion protein expression plasmids and protein expression. Glutathione *S*-transferase (GST)-EGF domain 1 (FVO), GST-EGF domain 2 (FVO), and GST-MSP1$_{19}$ (FVO) expression plasmids were constructed previously (10) and were used to transform *E. coli* BL21 DE3 to ampicillin resistance. Terrific Broth (Difco/Becton-Dickinson, Sparks, Md.) was inoculated with a 1/100 volume of fresh stationary-phase culture. The culture was grown at 37°C to an OD_{600} of 0.6, cooled to 25°C, induced by the addition of 0.1 mM IPTG (final concentration), and cultured for an additional 2 h. The cells were collected by centrifugation at 6,000 × *g* for 15 min, and the cell paste was stored frozen at −70°C.

GST fusion protein purification. The cell paste was suspended in lysis buffer (ice cold PBS–0.01 M dithiothreitol) at a paste-to-buffer ratio of 1:3 (wt/vol). Next, the cells were lysed by microfluidization in one pass; Triton X-100 (final concentration, 1% [vol/vol]), $MgCl_2$ (final concentration, 0.01 M), and DNase I (final concentration, 100 μg/ml) were added; and the sample was incubated on ice for 30 min. The lysate was clarified by centrifugation at 27,666 × *g* for 1 h at 4°C. The supernatant was collected and chromatographed on a 1-ml column of glutathione-Sepharose beads (Pharmacia) equilibrated with PBS–10 mM dithiothreitol–1% Triton X-100 (equilibration buffer). The loaded column was washed with 20 column volumes of equilibration buffer, and the fusion protein was eluted with 0.005 M reduced glutathione–0.05 M Tris-HCl, pH 8.0. The sample was stored at 0 to 4°C for 2 days to allow cystine formation and was then stored at −70°C. The protein concentration was quantified by the use of Folin Ciocalteau reagent (Lowry), and its purity was evaluated by SDS-PAGE under reducing and nonreducing conditions. The purified protein structure was evaluated by reac-

tions with MAbs specific for conformational epitopes in immunoblotting assays and ELISAs.

ELISAs. Microtiter plate (Immulon-2 HB; Thermo Labsystems, Franklin, Mass.) wells were coated with 0.4 pmol of recombinant antigen in 50 μl of PBS and then incubated overnight at 4°C. The plates were blocked for 1 h at room temperature with 150 μl of blocking buffer (1% fraction V bovine serum albumin in PBS). Sera (primary antibodies) were diluted serially with blocking buffer, and the primary antibodies (50 μl) were incubated for 1 h at room temperature. The plates were washed four times with wash buffer (0.05% Tween 20 and 0.0025% chlorohexidine in 1× PBS) by use of a microplate washer (SkanWasher 300 version B; Skatron Instruments, Sterling, Va.). For measurements of *Aotus* monkey Ab responses, a goat anti-*A. nancymai* IgG heavy-plus-light chain peroxidase-labeled conjugate was diluted 1:8,000 in blocking buffer and added to plates at 50 μl/well. The plates were incubated for 1 h at room temperature, washed, and developed by the addition of 100 μl of the ABTS peroxidase substrate system (KPL, Gaithersburg, Md.) to each well. The absorbance at 405 nm of each well was measured after 30 min by use of a microplate reader (EL 312e automated microplate reader; Bio-tek Instruments, Inc., Winooski, Vt.). The midpoint antibody titer was calculated as the serum dilution that produced an absorbance of 1.0 optical density unit in the ELISA assay. Rabbit antibodies were measured in the same manner, except that the conjugate was goat anti-rabbit IgG Fc–alkaline phosphatase diluted to 1:1,000 (Promega, Madison, Wis.) and the substrate was *p*-nitrophenyl phosphate (Sigma), the product of which was measured at 405 nm.

For analyses comparing $MSP1_{42}$ fragment-specific Ab titers, the plate antigens were normalized to one another. Equimolar coatings (0.4 pmol/well) of each ELISA plate antigen were confirmed by detection with an antibody against the GST moiety of the fusion protein (in the case of p19, EGF1, and EGF2) or against MAb 5.2 (in the case of $MSP1_{42}$ and p19).

Rabbit immunization and inhibition of parasite growth and invasion. New Zealand White rabbits were immunized four times at 3-week intervals. They were primed with 200 μg of *E. coli*-expressed $MSP1_{42}$ (FVO) in Freund's complete adjuvant (FCA) and boosted with 50 μg of Ag in incomplete Freund's adjuvant (IFA). Sera were collected prior to immunization and 3 weeks after the last immunization and were adsorbed with type A human erythrocytes (18).

Sera were evaluated for their inhibition of malaria parasite invasion and growth by measurements of lactate dehydrogenase levels in late trophozoite-stage *P. falciparum* parasites by a protocol (C. A. Long, personal communication) adapted from a previously developed method (31). *P. falciparum* strains 3D7 and FVO were synchronized by thermal cycling as described previously (17) and were collected for use at the beginning of schizogony. Cultures were adjusted to 0.3% parasitemia with type A human erythrocytes, and assays were conducted at a final hematocrit of 1%, with test serum concentrations ranging from 5 to 20%. Samples with *P. falciparum* 3D7 and FVO were collected 40 and 46 h, respectively, after initiation. Inhibition was calculated as follows: % inhibition = $[(OD_{\text{immune serum}} - OD_{RBC}) \div (OD_{\text{preimmune serum}} - OD_{RBC})] \times 100$.

Immune sera from rabbits that were vaccinated with an irrelevant Ag in Freund's adjuvant (with the same regimen as that for $MSP1_{42}$) and preimmune or normal rabbit sera were used interchangeably as negative control samples in this assay.

***A. nancymai* vaccination.** The monkeys used for this study were *A. nancymai* monkeys of either sex with intact spleens and with no history of *Plasmodium* species infections, as determined by parasitological and serological examinations; they were in good health and free of tuberculosis. The monkeys weighed no less than 700 g at the start of the study and were stratified into groups of six animals according to weight and sex. Groups were then assigned randomly to the vaccine and control groups. Six monkeys were assigned per group.

Animals were vaccinated twice with 50 μg of *E. coli*-expressed $MSP1_{42}$, baculovirus-expressed $MSP1_{42}$ (37), or recombinant *Saccharomyces cerevisiae* Pvs25 (20) emulsified in 0.5 ml of FCA or IFA (Difco Laboratories, Irvine, Calif.) for the first and second immunizations, respectively. The hair was closely shaved from the back region of the monkeys to allow visualization of any reactions, and four 125-μl subcutaneous inoculations were given. Immunizations were given 7 weeks apart, and the animals were challenged 7 weeks after the last immunization.

All *Aotus* monkeys enrolled at the start of this study were healthy, but two animals, one of which was assigned to the Pvs25 control group and one of which was assigned to the baculovirus $MSP1_{42}$ group, died prior to challenge from complications unrelated to immunization.

***A. nancymai* challenge.** The *P. falciparum* Vietnam Oak Knoll (FVO) strain is adapted to *A. nancymai* and produces a rapid, reproducible, lethal, high-density parasitemia in naïve monkeys with intact spleens. For challenge experiments, parasitized erythrocytes from a donor monkey were diluted to 20,000 parasitized red blood cells (PRBC)/ml in sterile RPMI 1640 tissue culture medium, and 0.5 ml was administered intravenously via the femoral vein.

TABLE 1. *E. coli* $MSP1_{42}$ (FVO) induces a strong protective antiparasite immune response

Ag	Treatment status[a]	No. of parasites/μl of blood		Day of patency rank
		Cumulative day 11 parasitemia	Day 11 rank	
Pvs25	P	125,361	1	6
	P	99,231	2	7
	P	39,461	3	7
	P	23,521	4	7
	P	18,761	5	6
Median		39,461	3	7
Baculovirus $MSP1_{42}$	P	5,920	6	6
	P	1,690	7	7
	A	790	8	6
	A	160	10	7
	none	141	11	7
Median		790	8	7
E. coli $MSP1_{42}$	A	612	9	9
	A	31	13	11
	none	34	12	13
	none	25	14	26
	none	13	15.5	12
	none	13	15.5	12
Median		28	13.5	12

[a] P, treatment for uncontrolled parasitemia; A, treatment for anemia; none, no treatment.

Beginning 3 days after the challenge, Giemsa-stained blood smears were made to quantify the numbers of parasites per microliter of blood (12). When parasite counts exceeded 80,000/μl of blood, parasite densities were quantified from thin blood smears. Daily blood smears were prepared and evaluated for 56 days after the challenge. Monkeys that developed high-density parasitemia (>200,000 parasites/μl of blood) were treated with mefloquine (Roche Laboratories, Nutley, N.J.) and quinine (Marion Merrel Dow, Inc., Kansas City, Kans.). Animals that developed anemia were cured with drugs and treated by iron supplementation and transfusion of whole blood if the hematocrit fell below 20%. Iron supplementation is a standard practice by veterinary staff for the treatment of malaria-induced anemia; it helps to speed recovery and blood replacement in severely anemic animals.

Statistical analysis. (i) Rabbits. Data groups for vaccinated rabbits satisfied the criteria of equal variance and normality after natural logarithm transformation to stabilize variance (Box Cox; Minitab) and were further evaluated by analysis of variance to discern if differences existed among the various groupings of data; differences were then isolated by Tukey's posttest. Correlations between ELISA Ab titers and parasite inhibition were established by Pearson's regression analysis.

(ii) *Aotus* monkeys. The data from the *Aotus* trial did not satisfy the criteria of equal variance after transformation, and thus the results were evaluated by nonparametric methods. For the *Aotus* vaccine trial, the primary end point was the cumulative day 11 parasitemia, which was the summation of an individual animal's daily levels of parasitemia from the day of challenge until the 11th day after challenge. The 11th day after challenge was the day prior to treatment of the first animal for uncontrolled parasitemia. Day 11 parasitemia levels were ranked as shown in Table 1, and end points for experimental groups were compared first by using Kruskal-Wallis tests to determine if any of the treatment groups had different primary end points. If a difference was discovered, it was isolated by secondary pairwise comparisons by use of the Mann-Whitney test. In addition to cumulative day 11 parasitemia as the primary end point, the day of patency was evaluated as a secondary end point. The relationships between the primary day 11 end point and Ab titers were evaluated by Spearman's regression analysis.

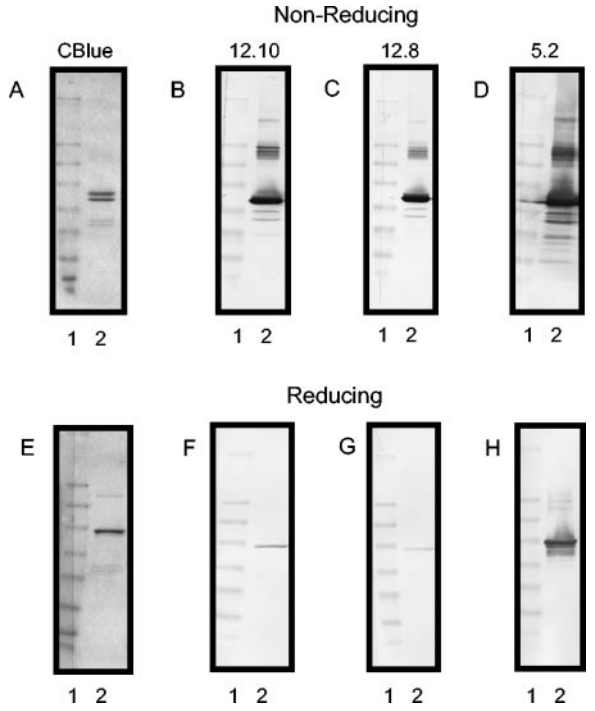

FIG. 2. GMP-generated MSP1$_{42}$ (FVO) electrophoresed in SDS under nonreducing conditions reacts in Western blots with functional conformation-dependent MAbs. Electrophoresis was performed under nonreducing (top) and reducing (bottom) conditions. (A and E) Coomassie blue-stained gel; (B and F) MAb 12.10; (C and G) MAb 12.8; (D and H) MAb 5.2. Lanes: 1, marker; 2, purified bulk MSP1$_{42}$ (FVO). Protein molecular size markers (SeeBlue; Invitrogen), 250, 98, 64, 50, 36, 30, 16, and 6 kDa.

RESULTS

Cloning. The MSP1$_{42}$ (FVO) gene fragment was prepared from the *P. falciparum* FVO genomic DNA by PCR in several steps (Fig. 1B) to introduce a synonymous codon by mutating nucleotide 423 (C to A) within codon 141. The PCR fragment was subcloned into the expression vector pET(AT)PfMSP1$_{42}$ (3D7) (Fig. 1A). The final construct (Fig. 1C) encodes 17 non-MSP1$_{42}$ amino acids that include the N-terminal hexahistidine tag for Ni^{2+}-chelating chromatography, a spacer sequence, and the entire MSP1$_{42}$ (FVO) protein sequence from Ala_1 to Ser_{355}.

Expression and purification. The expression of soluble MSP1$_{42}$ was induced with IPTG, and the protein was purified under GMP conditions by a two-step chromatographic method that included a Ni^{2+}-nitrilotriacetic acid-Sepharose affinity resin followed by a Q-Fast Flow Sepharose ion exchanger. The final protein purity was >95% according to SDS-PAGE and Coomassie blue staining (Fig. 2A and E). This result was supported by the observations that no contaminating *E. coli* proteins were observed in Western blots probed with polyclonal rabbit anti-*E. coli* antibodies (data not shown) and that a 50-μg dose of vaccine contained <2 ng of contaminating *E. coli* host protein, as measured by an ELISA for host protein quantification (data not shown). In addition, a 50-μg dose of vaccine contained 60 endotoxin units (EU), well below the FDA's acceptable limits (350 EU/70 kg of human body weight). The residual Ni^{2+} content remaining after purification was <2 μg/g of MSP1$_{42}$ (FVO) according to ICP-OES.

Antigen characterization. Coomassie blue staining of SDS-PAGE gels revealed a doublet at 42 kDa, a 30-kDa proteolytic fragment, and some high-molecular-weight aggregates under nonreducing conditions (Fig. 2A, lane 2). A single major monomer that migrated at approximately 50 kDa and minor components at 90 and 30 kDa (Fig. 2E, lane 2) were present after electrophoresis under reducing conditions.

Western blots probed with either polyclonal mouse anti-MSP1$_{42}$ (FVO) or rabbit anti-*E. coli* antibodies revealed that all Coomassie blue-detectable bands originated from MSP1$_{42}$ (data not shown). The N-terminal sequence of the 30-kDa fragment corresponded to the N terminus of MSP1$_{42}$, indicating that this fragment is truncated at the C terminus of MSP1$_{42}$.

The final bulk product (Fig. 2, lanes 2) was characterized structurally by immunoblotting and probing with various MSP1$_{19}$-specific MAbs that react with conformational epitopes. MAbs 12.10 (Fig. 2B and F) and 12.8 (Fig. 2C and G) reacted strongly with MSP1$_{42}$ under nonreducing conditions, but nearly all of their reactivity was eliminated by reduction. The same results were obtained with MAbs 2.2 and 7.2 (data not shown). Monomeric MSP1$_{42}$ as well as the high-molecular-weight aggregates reacted with MAb 5.2 (Fig. 2D and E); the epitope recognized by MAb 5.2 reforms more readily under these conditions than the epitopes recognized by the other MAbs. The reactivity with MAb 5.2 observed under reducing conditions could be completely eliminated by reducing and alkylating the Ag (data not shown).

Rabbit immunization. The induction of antibodies was measured by ELISAs with the following capture antigens, which were homologous to the FVO vaccine strain: full-length *E. coli* MSP1$_{42}$, C-terminal MSP1$_{19}$ (p19), EGF-like domain 1 (EGF1), and EGF-like domain 2 (EGF2). Fragment-specific Ab titers induced by the *E. coli* MSP1$_{42}$ (FVO) vaccine were ranked as follows: MSP1$_{42}$ > p19 = EGF1 > EGF2 (Fig. 3A). The *E. coli* MSP1$_{42}$ (FVO) vaccine induced two times more MSP1$_{42}$-specific Ab than p19-specific Ab and about five times more MSP1$_{42}$-specific Ab than EGF1-specific Ab, suggesting that most of the Ab induced was specific to conformational epitopes associated with the whole MSP1$_{42}$ antigen but not its fragments. The Ab titer against EGF2 was about 50-fold lower than the titer against MSP1$_{42}$.

The immune rabbit sera had potent in vitro growth and invasion inhibitory activities against both the homologous FVO parasite and the heterologous 3D7 parasite (Fig. 3B), although the activity against the FVO parasite was significantly stronger at all three serum dilutions tested (Student's *t* test; $P \leq 0.024$). Inhibition with 20% serum was not significantly higher than inhibition with 10% serum when tested against either the FVO or 3D7 parasite. The strongest correlation between ELISA Ab titers and growth and invasion inhibition was observed for the EGF2-specific Ab and inhibition of the FVO parasite with 20% serum (Pearson's $R = -0.784$; $P = 0.021$).

***Aotus* vaccination and challenge.** Seven weeks after the second immunization, the monkeys were challenged with 10,000 *P. falciparum* strain FVO PRBC. By the 15th day after challenge, all monkeys in the control group had been treated for

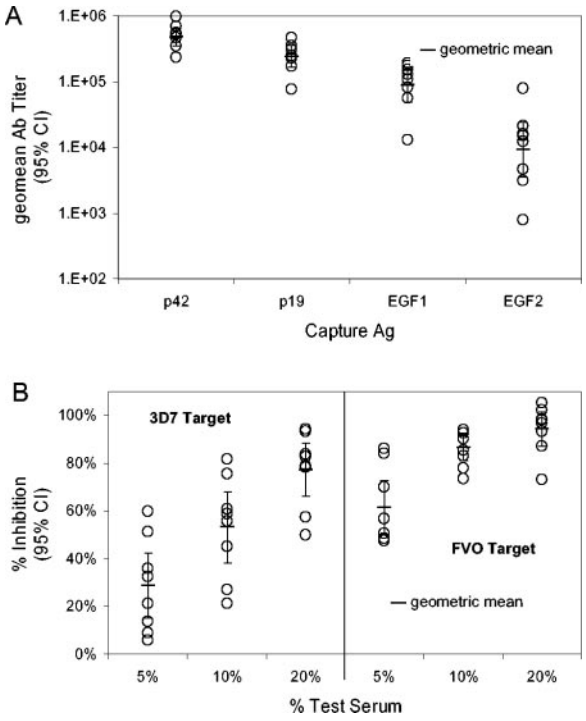

FIG. 3. Abs from rabbits vaccinated with *E. coli* $MSP1_{42}$ (FVO) are strongly inhibitory in growth inhibition assay (GIA) against both homologous and heterologous *P. falciparum* in vitro and react differentially with homologous strain Ag fragments. Ab titers against $MSP1_{42}$, p19, EGF1, and EGF2 capture antigens were compared by analysis of variance followed by Tukey's posttest to identify which groups differed. The same procedures were used to analyze results from GIA. Correlations between Ab titers (A) and GIA activities (B) were measured by Pearson's linear regression method.

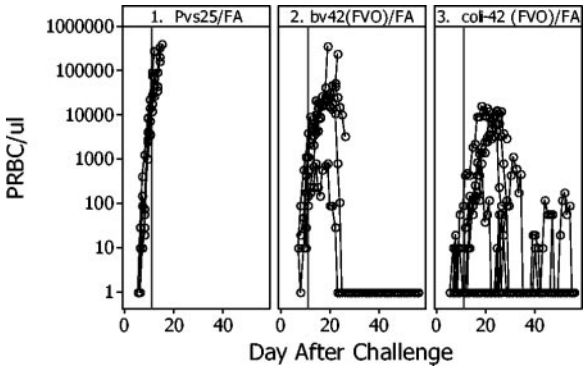

FIG. 4. *A. nancymai* monkeys vaccinated with *E. coli* $MSP1_{42}$ (FVO) controlled parasitemia better than did either the negative control group vaccinated with Pvs25 or the positive control group vaccinated with baculovirus $MSP1_{42}$ (FVO). Shown are daily parasitemia levels for individual monkeys following a challenge 98 days after the first immunization. The monkeys were challenged with 10,000 parasitized erythrocytes of *P. falciparum* strain FVO. Parasitemia was determined by counting erythrocytes on Giemsa-stained smears. Vertical lines indicate day 11 postchallenge. Left panel, control group immunized with Pvs25 plus Freund's adjuvant; middle and right panels, experimental groups that were immunized with baculovirus-expressed $MSP1_{42}$ and *E. coli*-expressed $MSP1_{42}$, respectively.

uncontrolled acutely rising parasitemia. In contrast, no animals in either of the $MSP1_{42}$-vaccinated groups required treatment by this time (Fig. 4). All six animals that were vaccinated with *E. coli* $MSP1_{42}$ began resolving their infections spontaneously at relatively low levels of parasitemia; two of these animals were subsequently treated for anemia (days 26 and 28), and none were treated for uncontrolled infection. Between the 19th and 28th day after challenge, four of the five monkeys that were vaccinated with baculovirus $MSP1_{42}$ were treated for either anemia (days 26 and 28) or uncontrolled parasitemia (days 19 and 23). One animal from this group self-cured its infection (Table 1).

An evaluation of the primary end point, cumulative day 11 parasitemia, showed that at least one group among the three in the trial differed significantly from the other two ($P = 0.002$ by Kruskal-Wallis test, both unadjusted and adjusted for ties). Pairwise isolation of the differences by the use of Mann-Whitney tests showed that both of the $MSP1_{42}$-vaccinated groups had lower cumulative day 11 parasitemia levels than the Pvs25 control group (for *E. coli* $MSP1_{42}$, $P = 0.0081$; for baculovirus $MSP1_{42}$, $P = 0.0122$). The two $MSP1_{42}$-vaccinated groups also differed from one another ($P = 0.0225$), with the *E. coli* $MSP1_{42}$-vaccinated group being the better protected of the two. An evaluation of the secondary end point, the day of patency, by the same process produced slightly different results ($P = 0.005$ by Kruskal-Wallis test; $P = 0.003$ when adjusted for ties). The isolation of these differences by use of a Mann-Whitney test showed that the *E. coli* $MSP1_{42}$-vaccinated group differed from the Pvs25 control group ($P = 0.0081$) but that the baculovirus $MSP1_{42}$-vaccinated group did not ($P = 1$). The days of patency for the *E. coli* $MSP1_{42}$ and baculovirus $MSP1_{42}$ groups also differed from one another ($P = 0.0081$). Thus, *E. coli* $MSP1_{42}$ was significantly more efficacious than baculovirus $MSP1_{42}$, as measured by differences in both the day 11 primary end points and the day of patency secondary end points.

***Aotus* serology and immune correlates.** The induction of antibodies was measured by ELISAs with the same Ag set as that described for rabbits. The kinetics of Ab induction by these fragments were measured with pooled sera (Fig. 5). A linear regression analysis of four contiguous points from the central parts of the kinetic plots ($R^2 > 0.91$ in all cases) showed that the rate of induction of $MSP1_{42}$-specific Ab by *E. coli* $MSP1_{42}$ exceeded that by baculovirus $MSP1_{42}$ 1.4-fold for EGF1, 2.5-fold for $MSP1_{42}$ and p19, and 6.5-fold for EGF2. For both groups of vaccines, the $MSP1_{42}$- and EGF1-specific Abs began to plateau 3 weeks after the second immunization, but the p19- and EGF2-specific Ab titers did not reach a plateau even by the day of challenge, which was 7 weeks after the second immunization.

On the day of parasite challenge, fragment-specific Ab titers induced by both $MSP1_{42}$ vaccines ranked as follows: $MSP1_{42} >$ p19 = EGF1 > EGF2 (Fig. 6). Both $MSP1_{42}$ vaccines induced four times more $MSP1_{42}$-specific Ab than p19-specific Ab and about five times more $MSP1_{42}$-specific Ab than EGF1-specific Ab, suggesting that, like the case for the rabbits, most of the Ab induced was specific to conformational epitopes associated with the whole $MSP1_{42}$ antigen. The Ab titer against EGF2 was about 50-fold lower than the titer against $MSP1_{42}$, and the titer specific for the p33 product from the N terminus of $MSP1_{42}$ was the same as that observed for EGF2 (data not shown). When compared with baculovirus $MSP1_{42}$, *E. coli*

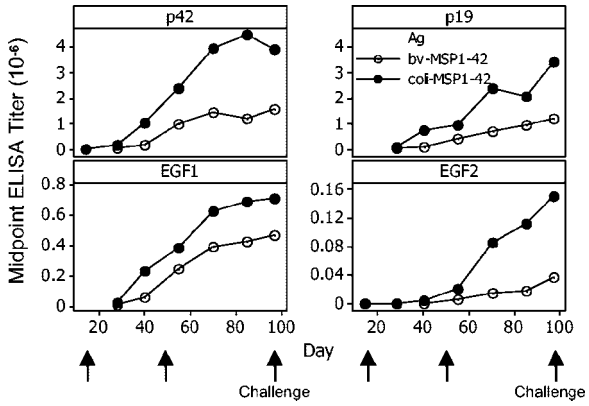

FIG. 5. Ab induction kinetics for *E. coli* MSP1$_{42}$ exceed Ab induction kinetics for baculovirus MSP1$_{42}$. Midpoint Ab titers were measured at each time point by ELISAs against the following capture Ags: MSP1$_{42}$, p19, EGF1, and EGF2. Results are shown for *Aotus* monkeys vaccinated with baculovirus-expressed MSP1$_{42}$ (open circles) and *E. coli*-expressed MSP1$_{42}$ (filled circles). Arrows at the abscissa denote vaccinations; the arrows labeled "challenge" denote the parasite challenge date.

MSP1$_{42}$ induced significantly more MSP1$_{42}$-specific (2.6-fold), p19-specific (2.7-fold), EGF1-specific (3.7-fold), and EGF2-specific (8.8-fold) Ab ($P = 0.006$ by Kruskal-Wallis test for all responses). Antisera taken on the day of parasite challenge from the two groups of vaccines were also tested by a fluorescent antibody test (FAT) with *P. falciparum* FVO (data not shown). The results of the FAT provide additional evidence supporting the difference in the immunogenicity of the two Ags, as the FAT Ab titers for the *E. coli* Ag vaccinees were eightfold higher than those for the baculovirus Ag vaccinees. The day-of-challenge antisera were also tested by ELISAs, with both *E. coli* MSP1$_{42}$ and baculovirus MSP1$_{42}$ as capture Ags (data not shown). When the capture Ag was the *E. coli* MSP1$_{42}$ Ag, the ratio of mean Ab titer for the *E. coli* Ag vaccinees to that for the baculovirus Ag vaccinees was 2.6, while this ratio was 2.4 when the baculovirus MSP1$_{42}$ Ag was used. Thus, the choice of capture Ag for the ELISA analysis did not affect the interpretation of the results.

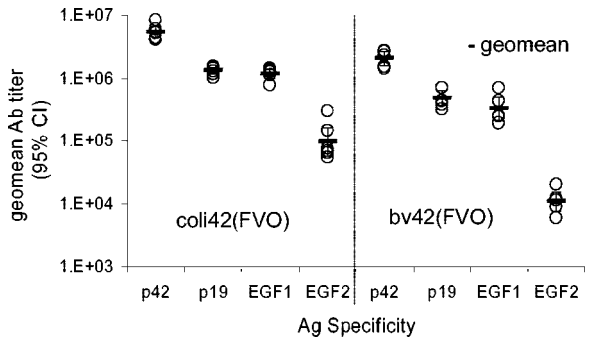

FIG. 6. MSP1$_{42}$ domain-specific Ab titers induced by *E. coli* MSP1$_{42}$ (FVO) on the day of challenge were significantly higher than those induced by baculovirus MSP1$_{42}$ (FVO). Midpoint Ab titers were measured at each time point by ELISAs against the following capture Ags: MSP1$_{42}$, p19, EGF1, and EGF2. Results are shown for *Aotus* monkeys vaccinated with baculovirus-expressed MSP1$_{42}$ (right) and *E. coli*-expressed MSP1$_{42}$ (left). Sera were collected on the day of challenge prior to the challenge.

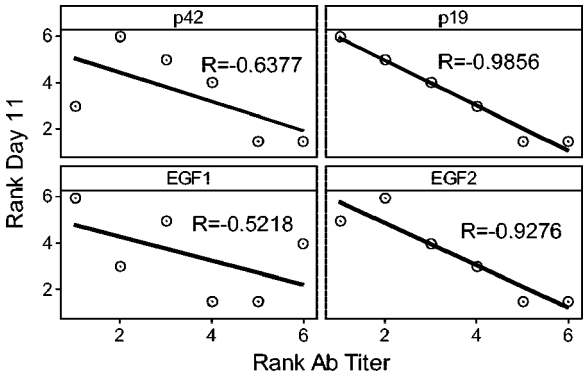

FIG. 7. p19- and EGF2-specific Abs induced by vaccination with *E. coli* MSP1$_{42}$ correlate with protection, but Abs induced by baculovirus MSP1$_{42}$ do not (not shown). Ranked cumulative day 11 parasitemia levels were compared with ranked MSP1$_{42}$-, p19-, EGF1-, and EGF2-specific antibody titers by Spearman's linear regression method. The numbers above the regression lines are correlation coefficients.

Figure 7 shows Spearman's correlations between the various serologic responses measured for Fig. 6 and the primary end point. The p19-specific and EGF2-specific Ab titers induced by *E. coli* MSP1$_{42}$-FCA correlated strongly with protection ($R = -0.9856$ and -0.9276, respectively). None of the Ab responses induced by baculovirus MSP1$_{42}$ correlated with the primary end point that was used to define protection.

DISCUSSION

Several approaches to developing recombinant protein vaccines based on the MSP1$_{42}$ gene fragment from *P. falciparum* have been reported. Three constructs have been expressed in *E. coli* (1, 23, 35), two have been expressed in baculovirus (8, 37), and one has been expressed in transgenic mice (36).

Results from various preclinical studies in which *A. nancymai* monkeys vaccinated with the FVO allele of MSP1$_{42}$ were challenged with the homologous strain of *P. falciparum* (23, 35–37) led Singh et al. to conclude that the protein expressed in *E. coli* performed at least as well as that expressed in baculovirus and that it offers the best prospect as a vaccine candidate owing to more favorable manufacturing issues (35). There are two leading approaches for purifying the MSP1$_{42}$ protein expressed in *E. coli*, either cytoplasmic expression as a soluble protein (1) or extraction from inclusion bodies and refolding in vitro (35). The present work took the former approach, and we described here the manufacture and characterization of clinical-grade *P. falciparum* MSP1$_{42}$ (FVO) expressed in a soluble form in *E. coli* and the testing of this Ag for efficacy in *Aotus* monkeys challenged with a homologous strain of erythrocytic-stage malaria parasites.

Our soluble *E. coli* MSP1$_{42}$ product met all FDA standards for purity and safety which are required for clinical testing. The measured residual endotoxin levels were significantly below the FDA's acceptable limits (FDA limit, 350 EU/dose/70 kg of human body weight; this product, 60 EU/dose/70 kg), and *E. coli* host proteins were below the limits of detection by ELISA. The product is comprised predominantly of full-length MSP1$_{42}$ and a minor portion of truncated MSP1$_{30}$ derived from the N terminus (confirmed by N-terminal amino acid sequencing) as

well as some high-molecular-mass multimeric forms (~90 kDa). Immunoblotting showed that various immunologically relevant epitopes of the $MSP1_{42}$ protein are folded correctly owing to their reactivities with conformation-dependent MAbs (including MAbs 12.10 and 12.8) raised against *P. falciparum* blood-stage parasites. The structure is stabilized by disulfide bonds, as evidenced by the loss of functional MAb binding in immunoblots with reduced antigen (Fig. 2). Additional evidence of the correct structure includes the induction of IFA-reactive Abs, the induction of rabbit Abs that inhibit parasite growth and development in vitro, and the induction of protective immunity in *A. nancymai*.

Both the *E. coli* $MSP1_{42}$ vaccine and the baculovirus $MSP1_{42}$ control vaccine elicited protective host responses against homologous challenge. By day 15, all monkeys in the Pvs25-vaccinated negative control group, but none in either $MSP1_{42}$ group, had been treated for uncontrolled parasitemia. A comparison of differences in the end points of the treatment groups by Kruskal-Wallis analysis, followed by Mann-Whitney tests to isolate the differences, showed that the *E. coli* $MSP1_{42}$ vaccine was significantly more protective than the baculovirus $MSP1_{42}$ vaccine for cumulative day 11 parasitemia ($P = 0.0225$) and the day of patency end point ($P = 0.0081$).

The difference in the protective effects of the *E. coli* $MSP1_{42}$ and baculovirus $MSP1_{42}$ vaccines might be due to differences in the quantity and fine specificities of Ab that they induced. On the day of challenge with live parasites, Ab titers induced by the *E. coli* $MSP1_{42}$ vaccine were substantially higher than those induced by the baculovirus $MSP1_{42}$ vaccine, varying from 2.6 to 8.8 times higher according to the domain specificity of the Abs (Fig. 6). *E. coli* $MSP1_{42}$ induced C-terminal p19 fragment- and EGF-like domain 2-specific Ab responses that correlated strongly with protection in vivo (Spearman's $R = -0.9856$ and -0.9267, respectively), but the responses induced against EGF-like domain 1 and full-length $MSP1_{42}$ did not correlate with protection (Spearman's $R = 0.5218$ and 0.6377, respectively). These results are consistent with those of Guevara-Patino et al. (14), who showed that an Ab to EGF-like domain 1 can block the activity of functional growth inhibitory Abs, and of Egan et al. (13), who showed that human IgG affinity purified from protective immunoglobulin by the use of EGF-like domain 2 inhibits parasite growth in vitro, as does double-domain p19-specific IgG depleted of its EGF1 and EGF2 specificities. None of the Abs induced by the baculovirus $MSP1_{42}$ vaccine correlated with protection in vivo. It may also be relevant that a reasonably strong negative correlation was observed between EGF2-specific Ab titers induced by vaccinating rabbits with $MSP1_{42}$ (FVO) and the growth and invasion inhibition of *P. falciparum* FVO by those sera in vitro (Pearson's $R = -0.784$; $P = 0.024$). The strong growth inhibitory effect of the rabbit sera against the heterologous strain 3D7 parasite suggests that this vaccine may be capable of inducing a protective effect against multiple parasite strains.

There are several possible explanations for the differences observed between the immunogenicity and the efficacy of the soluble *E. coli* $MSP1_{42}$ and baculovirus $MSP1_{42}$ vaccine candidates. One is that *E. coli* $MSP1_{42}$ may be comprised of noncovalently associated multimers rather than monomers. In support of this, we found that the elution of this antigen from Ni-chelate columns required 1 M imidazole, which has been observed previously for multimeric proteins, while most monomeric proteins bearing hexahistidine tags can be eluted with 100 to 250 mM imidazole (32). If the induction of protective immunity is dependent on a multimeric Ag, the required effector Ab responses may be directed to quaternary epitopes that are present in the assembled Ag but not the monomeric Ag. A second explanation for the differences is that simple mannosylation at the two N-glycosylation sites on baculovirus $MSP1_{42}$ may have altered the antigenicity and reduced the immunogenicity at either the B-cell or T-cell level. Other explanations include the possibilities that subpyrogenic levels of endotoxin present in the *E. coli* preparation provided for an additional adjuvant effect and that the location of the histidine tag affected the relative antigenicities and immunogenicities of the two vaccines. The His tag which is fused to the N terminus of *E. coli* $MSP1_{42}$ and to the C terminus of baculovirus-$MSP1_{42}$ may have altered the T-cell or B-cell responses to vaccination. B-cell epitope mapping by mutagenesis showed that the functionally relevant MAb 12.10 epitope contains critical contact residues located near the C terminus of the protein (40). Introducing an additional sequence at the C terminus of $MSP1_{42}$ of our baculovirus product may have interfered with the induction of immunity to this important epitope. This hypothesis is supported by our observation that *E. coli* $MSP1_{42}$ induced 8.8-fold more Ab specific to EGF-like domain 2 than did baculovirus $MSP1_{42}$ and that this Ab response correlated strongly with protection, as measured by the cumulative day 11 parasitemia. Along this line, it should be noted that a strong protective effect was observed in *Aotus lemurinus griseimembra* vaccinated with baculovirus-expressed $MSP1_{42}$ from the FUP strain of *P. falciparum* and that this Ag was not modified at its C terminus (7). While these results may suggest that mannosylation did not effect the protection induced by this baculovirus-expressed Ag and may support the idea that it is important to avoid modifying the C-terminal end of $MSP1_{42}$, it is important to consider that, aside from the differences in virulence of the FUP strain in *A. nancymai* (11) and *A. lemurinus griseimembra*, Chang et al. gave four immunizations, with each containing a diminishing amount of FCA (7).

In order to better understand the relationship of the immunity induced by this soluble *E. coli*-expressed $MSP1_{42}$ vaccine and other $MSP1_{42}$ vaccine candidates, we analyzed the various trials that were reported previously and were conducted similarly to this trial, i.e., *A. nancymai* monkeys vaccinated with $MSP1_{42}$ (FVO) given with Freund's adjuvant and challenged with erythrocytes infected with *P. falciparum* strain FVO. Results from previous trials suggested that an inverse relationship exists between occurrences of anemia and self-curing in vaccinated animals, with anemia appearing when there is an unfavorable balance between the immune status and challenge virulence. Given the same challenge, a vaccine that induces the most self-cure outcomes compared to anemia outcomes should be the most potent. The data related to these conclusions are discussed below.

Retrospectively, it appears that the protective effect of our *E. coli* $MSP1_{42}$ vaccine is also stronger than the immunity induced by the baculovirus $MSP1_{42}$ vaccine in other comparable trials (36, 37) as well as the immunity induced by secreted transgenic mouse-expressed $MSP1_{42}$ (36) or *E. coli*-expressed, refolded $MSP1_{42}$ (35). All of these trials are summarized in

TABLE 2. Summary of MSP1$_{42}$ (FVO) vaccine trials in *A. nancymai* monkeys

Parameter	Value for study with indicated antigen (Reference)[a]						
	Bv (37)	Bv (36)	Tg (36)	Tg (36)	*E. coli* refold (36)	Bv (this study)	*E. coli* (this study)
Ag dose (μg)	250 × 3	100 × 3	100 × 3	100 × 3	100 × 3	50 × 2	50 × 2
Adjuvant	FCA/IFA	FCA/IFA	FCA/IFA	FCA/IFA	FCA/ISA 51	FCA/IFA	FCA/IFA
Interval (wks)	3 × 3	3 × 3	3 × 3	3 × 3	3 × 3	2 × 7	2 × 7
Days to challenge	12	15	15	15	15	49	49
No. of PRBC	1×10^4	1×10^4	1×10^4	5×10^4	5×10^4	1×10^4	1×10^4
Treatment days (controls)	12–14	16–18	16–18	10–13	12–13	12–14	12–14
Total *n*	7	7	5	7	7	5	6
No. of animals treated for uncontrolled parasitemia	1	2	1	2	1	2	0
No. of animals treated for anemia	4	1	2	4	5	2	2
No. of animals with no treatment	2	4	2	1	1	1	4

[a] Bv, baculovirus-expressed MSP1$_{42}$ (FVO); Tg, transgenic mouse-expressed MSP1$_{42}$ (FVO); *E. coli* refold, *E. coli*-expressed MSP1$_{42}$ that was refolded during purification.

Table 2. Our conclusion is supported by several observations. First, we compared soluble *E. coli*-expressed MSP1$_{42}$ and baculovirus-expressed MSP1$_{42}$ in this present trial after recalculating our primary end points as described previously (37). A Kruskal-Wallis analysis followed by a Mann-Whitney analysis of these recalculated data showed that the difference in the end points of our soluble *E. coli*-expressed MSP1$_{42}$ vaccine group and the Pvs25 control group was significant at the 95% confidence limit ($P = 0.008$) but that the difference in the end points of the baculovirus-expressed MSP1$_{42}$ vaccine group and the Pvs25 control group was not ($P = 0.0947$). The weak protective effect of baculovirus-expressed MSP1$_{42}$ in the present trial compared with the protective effect observed previously may be due to the administration of two rather than three immunizations and to the difference in the immunization schedules between the two trials. Second, Stowers et al. observed that baculovirus MSP1$_{42}$ and NG-transgenic mouse-expressed MSP1$_{42}$ were equally potent for inducing protection against a challenge (38). An important observation from that study is that the self-cure rates after vaccinations with baculovirus MSP1$_{42}$ and NG-transgenic mouse-expressed MSP1$_{42}$ were higher when control animals were treated for parasitemia between 16 and 18 days after challenge (57%) than when they were treated between 12 and 14 days after challenge (14 to 28%). The converse was true for anemia rates; the anemia rates for both the baculovirus MSP1$_{42}$ and the NG-transgenic mouse-expressed MSP1$_{42}$ were lower when control animals were treated for parasitemia between 16 and 18 days after challenge (14 to 40%) and were higher when controls were treated between 12 and 14 days after challenge (57%). Thus, trial-to-trial variation in vaccine potency depends on the challenge, and comparisons of trials conducted at different times are best achieved if control animals from those trials are treated for uncontrolled parasitemia within the same time frame after challenge. Third, although a direct comparison was not performed, Singh et al. concluded that refolded *E. coli*-expressed MSP1$_{42}$ (FVO) and baculovirus MSP1$_{42}$ are equally potent vaccine candidates (35). In that experiment, control animals were treated between 11 and 14 days after challenge. The self-cure rate for the refolded *E. coli*-expressed MSP1$_{42}$ (FVO) Ag was 14%, and the anemia rate was 71%, whereas in other studies with the baculovirus Ag tested in equally challenged animals, self-cure and anemia rates were 28 and 56%, respectively (37). Given that ISA-51 and incomplete Freund's adjuvant are virtually identical formulations, the choice of adjuvant for boosting with refolded *E. coli*-expressed MSP1$_{42}$ (FVO) probably had little effect on the efficacy of this Ag. The soluble *E. coli* MSP1$_{42}$ (FVO) Ag described in the present study seems to be more potent than any of the previous candidates because its associated self-cure (67%) and anemia (33%) rates were the inverse of those observed for other vaccine candidates (14 to 28% and 57 to 72%, respectively) in challenge experiments in which control animals were treated between 11 and 14 days after challenge. The differences between this study and those conducted previously should be reiterated. For this study, animals received two immunizations at 7-week intervals and were challenged 7 weeks after the last immunization with 10,000 PRBC. In previous studies, animals received three immunizations at 3-week intervals and were challenged 2 weeks after the last immunization with 50,000 PRBC. Despite the difference in inoculum sizes used in these studies, all control animals were first treated from 11 to 14 days after challenge (Table 2), indicating that they were challenged equally.

Our results show that MSP1$_{42}$ expressed in a soluble form by *E. coli* and purified according to GMP specifications can induce a very potent protective immune response in *A. nancymai* when it is coadministered with Freund's adjuvant. Future studies must be directed toward the identification, development, and characterization of adjuvants that will be suitable for human use and that can induce protective immunity, but whether the *Aotus* monkey represents an appropriate venue for the discovery of such adjuvants remains a point of open debate (19, 39).

ACKNOWLEDGMENTS

This work was supported by the U.S. Agency for International Development under project number 936–6001, award number AAG-P-00-98-00006, and award number AAG-P-00-98-00005, by the National Institutes of Health and Centers for Disease Control and Prevention Interagency Agreement under grant number NIAID YIAI-0438-03/CDC C100-042, and by the United States Army Medical Research and Materiel Command.

The authors' views are private and are not to be construed as official policy of the Department of Defense or the U.S. Army.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*. All procedures were reviewed and approved by the Institute's Animal Care and Use Committee and were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

We thank Brian A. Bell and Jay F. Wood, WRAIR, Pilot Bioproduction Facility. We also thank Sally Robinson and Scott Bowden from the Department of Immunology at WRAIR for technical support for ELISAs and Elizabeth Duncan, also from the Department of Immunology, for parasite invasion and inhibition assay technical support.

REFERENCES

1. **Angov, E., B. M. Aufiero, A. M. Turgeon, M. Van Handenhove, C. F. Ockenhouse, K. E. Kester, D. S. Walsh, J. S. McBride, M. C. Dubois, J. Cohen, J. D. Haynes, K. H. Eckels, D. G. Heppner, W. R. Ballou, C. L. Diggs, and J. A. Lyon.** 2003. Development and pre-clinical analysis of a *Plasmodium falciparum* merozoite surface protein-1(42) malaria vaccine. Mol. Biochem. Parasitol. **128:**195–204.
2. **Anonymous.** 2003. Roll back malaria info sheet. World Health Organization, Geneva, Switzerland.
3. **Blackman, M. J., H. G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder.** 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J. Exp. Med. **172:**379–382.
4. **Blackman, M. J., and A. A. Holder.** 1992. Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of $MSP1_{33}$ as a non-covalently associated complex with other fragments of the MSP1. Mol. Biochem. Parasitol. **50:**307–315.
5. **Blackman, M. J., I. T. Ling, S. C. Nicholls, and A. A. Holder.** 1991. Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. Mol. Biochem. Parasitol. **49:**29–33.
6. **Blackman, M. J., T. J. Scott-Finnigan, S. Shai, and A. A. Holder.** 1994. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J. Exp. Med. **180:**389–393.
7. **Chang, S. P., S. E. Case, W. L. Gosnell, A. Hashimoto, K. J. Kramer, L. Q. Tam, C. Q. Hashiro, C. M. Nikaido, H. L. Gibson, C. T. Lee-Ng, P. J. Barr, B. T. Yokota, and G. S. Hut.** 1996. A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects *Aotus* monkeys against malaria. Infect. Immun. **64:**253–261.
8. **Chang, S. P., H. L. Gibson, C. T. Lee-Ng, P. J. Barr, and G. S. Hui.** 1992. A carboxyl-terminal fragment of *Plasmodium falciparum* gp195 expressed by a recombinant baculovirus induces antibodies that completely inhibit parasite growth. J. Immunol. **149:**548–555.
9. **Chang, S. P., K. J. Kramer, K. M. Yamaga, A. Kato, S. E. Case, and W. A. Siddiqui.** 1988. *Plasmodium falciparum*: gene structure and hydropathy profile of the major merozoite surface antigen (gp195) of the Uganda-Palo Alto isolate. Exp. Parasitol. **67:**1–11.
10. **Chappel, J. A., and A. A. Holder.** 1993. Monoclonal antibodies that inhibit *Plasmodium falciparum* invasion in vitro recognise the first growth factor-like domain of merozoite surface protein-1. Mol. Biochem. Parasitol. **60:**303–311.
11. **Collins, W. E., G. G. Galland, J. S. Sullivan, and C. L. Morris.** 1994. Selection of different strains of *Plasmodium falciparum* for testing blood-stage vaccines in *Aotus nancymai* monkeys. Am. J. Trop. Med. Hyg. **51:**224–232.
12. **Earle, W. C., and M. Perez.** 1931. Enumeration of parasites in the blood of malarial patients. J. Lab. Clin. Med. **19:**1124–1130.
13. **Egan, A. F., P. Burghaus, P. Druilhe, A. A. Holder, and E. M. Riley.** 1999. Human antibodies to the 19kDa C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 inhibit parasite growth *in vitro*. Parasite Immunol. **21:**133–139.
14. **Guevara-Patino, J. A., A. A. Holder, J. S. McBride, and M. J. Blackman.** 1997. Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. J. Exp. Med. **186:**1689–1699.
15. **Haldar, K., M. A. Ferguson, and G. A. Cross.** 1985. Acylation of a *Plasmodium falciparum* merozoite surface antigen via *sn*-1,2-diacyl glycerol. J. Biol. Chem. **260:**4969–4974.
16. **Hall, R., J. McBride, G. Morgan, A. Tait, J. W. Zolg, D. Walliker, and J. Scaife.** 1983. Antigens of the erythrocyte stages of the human malaria parasite *Plasmodium falciparum* detected by monoclonal antibodies. Mol. Biochem. Parasitol. **7:**247–265.
17. **Haynes, J. D., and J. K. Moch.** 2002. Automated synchronization of *Plasmodium falciparum* parasites by culture in a temperature-cycling incubator. Methods Mol. Med. **72:**489–497.
18. **Haynes, J. D., J. K. Moch, and D. S. Smoot.** 2002. Erythrocytic malaria growth or invasion inhibition assays with emphasis on suspension culture GIA. Methods Mol. Med. **72:**535–554.
19. **Heppner, D. G., J. F. Cummings, C. Ockenhouse, K. E. Kester, J. A. Lyon, and D. M. Gordon.** 2001. New World monkey efficacy trials for malaria vaccine development: critical path or detour? Trends Parasitol. **17:**419–425.
20. **Hisaeda, H., A. W. Stowers, T. Tsuboi, W. E. Collins, J. S. Sattabongkot, N. Suwanabun, M. Torii, and D. C. Kaslow.** 2000. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. Infect. Immun. **68:**6618–6623.
21. **Holder, A. A., and R. R. Freeman.** 1984. Protective antigens of rodent and human bloodstage malaria. Philos. Trans. R. Soc. Lond. B Biol. Sci. **307:**171–177.
22. **Holder, A. A., J. S. Sandhu, Y. Hillman, L. S. Davey, S. C. Nicholls, H. Cooper, and M. J. Lockyer.** 1987. Processing of the precursor to the major merozoite surface antigens of *Plasmodium falciparum*. Parasitology **94:**199–208.
23. **Kumar, S., A. Yadava, D. B. Keister, J. H. Tian, M. Ohl, K. A. Perdue-Greenfield, L. H. Miller, and D. C. Kaslow.** 1995. Immunogenicity and *in vivo* efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. Mol. Med. **1:**325–332.
24. **Ling, I. T., S. A. Ogun, and A. A. Holder.** 1994. Immunization against malaria with a recombinant protein. Parasite Immunol. **16:**63–67.
25. **Lyon, J. A., J. D. Haynes, C. L. Diggs, J. D. Chulay, C. G. Haidaris, and J. Pratt-Rossiter.** 1987. Monoclonal antibody characterization of the 195-kilodalton major surface glycoprotein of *Plasmodium falciparum* malaria schizonts and merozoites: identification of additional processed products and a serotype-restricted repetitive epitope. J. Immunol. **138:**895–901.
26. **Majarian, W. R., T. M. Daly, W. P. Weidanz, and C. A. Long.** 1984. Passive immunization against murine malaria with an IgG3 monoclonal antibody. J. Immunol. **132:**3131–3137.
27. **McBride, J. S., and H. G. Heidrich.** 1987. Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex. Mol. Biochem. Parasitol. **23:**71–84.
28. **Miller, L. H., T. Roberts, M. Shahabuddin, and T. F. McCutchan.** 1993. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Mol. Biochem. Parasitol. **59:**1–14.
29. **Morgan, W. D., B. Birdsall, T. A. Frenkiel, M. G. Gradwell, P. A. Burghaus, S. E. Syed, C. Uthaipibull, A. A. Holder, and J. Feeney.** 1999. Solution structure of an EGF module pair from the *Plasmodium falciparum* merozoite surface protein 1. J. Mol. Biol. **289:**113–122.
30. **O'Donnell, R. A., T. F. de Koning-Ward, R. A. Burt, M. Bockarie, J. C. Reeder, A. F. Cowman, and B. S. Crabb.** 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J. Exp. Med. **193:**1403–1412.
31. **Prudhomme, J. G., and I. W. Sherman.** 1999. A high capacity in vitro assay for measuring the cytoadherence of *Plasmodium falciparum*-infected erythrocytes. J. Immunol. Methods **229:**169–176.
32. **Qiagen, Inc.** 2003. The QIAexpressionist: a handbook for high-level expression and purification of 6×His-tagged proteins. Qiagen, Inc., Chatsworth, Calif.
33. **Renia, L., I. T. Ling, M. Marussig, F. Miltgen, A. A. Holder, and D. Mazier.** 1997. Immunization with a recombinant C-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 protects mice against homologous but not heterologous *P. yoelii* sporozoite challenge. Infect. Immun. **65:**4419–4423.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Singh, S., M. C. Kennedy, C. A. Long, A. J. Saul, L. H. Miller, and A. W. Stowers.** 2003. Biochemical and immunological characterization of bacterially expressed and refolded *Plasmodium falciparum* 42-kilodalton C-terminal merozoite surface protein 1. Infect. Immun. **71:**6766–6774.
36. **Stowers, A. W., L. H. Chen, Y. Zhang, M. C. Kennedy, L. Zou, L. Lambert, T. J. Rice, D. C. Kaslow, A. Saul, C. A. Long, H. Meade, and L. H. Miller.** 2002. A recombinant vaccine expressed in the milk of transgenic mice protects *Aotus* monkeys from a lethal challenge with *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **99:**339–344.
37. **Stowers, A. W., V. Cioce, R. L. Shimp, M. Lawson, G. Hui, O. Muratova,**

D. C. Kaslow, R. Robinson, C. A. Long, and L. H. Miller. 2001. Efficacy of two alternate vaccines based on *Plasmodium falciparum* merozoite surface protein 1 in an *Aotus* challenge trial. Infect. Immun. **69:**1536–1546.

38. **Stowers, A. W., M. C. Kennedy, B. P. Keegan, A. Saul, C. A. Long, and L. H. Miller.** 2002. Vaccination of monkeys with recombinant *Plasmodium falciparum* apical membrane antigen 1 confers protection against blood-stage malaria. Infect. Immun. **70:**6961–6967.
39. **Stowers, A. W., and L. H. Miller.** 2001. Are trials in New World monkeys on the critical path for blood-stage malaria vaccine development? Trends Parasitol. **17:**415–419.
40. **Uthaipibull, C., B. Aufiero, S. E. Syed, B. Hansen, J. A. Patino, E. Angov, I. T. Ling, K. Fegeding, W. D. Morgan, C. Ockenhouse, B. Birdsall, J. Feeney, J. A. Lyon, and A. A. Holder.** 2001. Inhibitory and blocking monoclonal antibody epitopes on merozoite surface protein 1 of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. **307:**1381–1394.
41. **World Health Organization.** 1997. World malaria situation in 1994. World Health Organization, Geneva, Switzerland.

Editor: W. A. Petri, Jr.