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Biochemical and Immunological Characterization of Bacterially Expressed and Refolded *Plasmodium falciparum* 42-Kilodalton C-Terminal Merozoite Surface Protein 1

Sanjay Singh,* Michael C. Kennedy, Carole A. Long, Allan J. Saul, Louis H. Miller, and Anthony W. Stowers*

Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville Maryland 20852

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Protection against *Plasmodium falciparum* can be induced by vaccination in animal models with merozoite surface protein 1 (MSP1), which makes this protein an attractive vaccine candidate for malaria. In an attempt to produce a product that is easily scaleable and inexpensive, we expressed the C-terminal 42 kDa of MSP1 ($MSP1_{42}$) in *Escherichia coli*, refolded the protein to its native form from insoluble inclusion bodies, and tested its ability to elicit antibodies with in vitro and in vivo activities. Biochemical, biophysical, and immunological characterization confirmed that refolded *E. coli* $MSP1_{42}$ was homogeneous and highly immunogenic. In a formulation suitable for human use, rabbit antibodies were raised against refolded *E. coli* $MSP1_{42}$ and tested in vitro in a *P. falciparum* growth invasion assay. The antibodies inhibited the growth of parasites expressing either homologous or heterologous forms of *P. falciparum* $MSP1_{42}$. However, the inhibitory activity was primarily a consequence of antibodies directed against the C- terminal 19 kDa of MSP1 ($MSP1_{19}$). Vaccination of nonhuman primates with *E. coli* $MSP1_{42}$ in Freund's adjuvant protected six of seven *Aotus* monkeys from virulent infection with *P. falciparum*. The protection correlated with antibody-dependent mechanisms. Thus, this new construct, *E. coli* $MSP1_{42}$, is a viable candidate for human vaccine trials.

The malarial parasite remains a scourge on human civilization, and in recent years the incidence of malaria has been increasing (5, 25). Vaccination against *Plasmodium falciparum* has the potential to reduce severe malaria-associated morbidity and mortality in areas with the most intense transmission, and it may do so without necessarily preventing blood stage infection (20, 24). Most blood stage vaccine research has been focused on antigens that are expressed on the surface of merozoites (11). Merozoites are released from rupturing red blood cells (RBCs) and quickly invade other RBCs. Merozoite surface protein-specific antibodies, therefore, have only a brief period of time in which they can be active (26). The most widely studied merozoite surface protein is merozoite surface protein 1 (MSP1) (15). This molecule is polymorphic and has a complex folding pattern (8, 21).

P. falciparum MSP1 is a large (~200-kDa) protein. MSP1 is processed into a complex of polypeptides on the merozoite surface, including an 82-kDa N-terminal polypeptide and 30- and 38-kDa central regions, as well as the 42-kDa C-terminal region ($MSP1_{42}$) (1). At the time of RBC invasion, $MSP1_{42}$ is further processed by proteolytic cleavage into a 33-kDa fragment ($MSP1_{33}$), which is shed from the parasite with the rest of the MSP1 complex, and a C-terminal 19-kDa fragment ($MSP1_{19}$). Only the C-terminal $MSP1_{19}$ fragment remains on the merozoite surface and is carried into parasitized RBCs (2). This so-called secondary processing of MSP1 is completed during the successful invasion of a RBC, suggesting that it is a necessary step (3, 7).

The $MSP1_{19}$ and $MSP1_{42}$ regions of *P. falciparum* MSP1 are leading malaria vaccine candidates (15). Studies with rodent malaria and challenge studies with *P. falciparum* in primates have indicated that vaccines based on $MSP1_{19}$ and $MSP1_{42}$ confer protection against malaria (6, 9, 12, 13, 29, 30). Recently, O'Donnell et al. (22) convincingly demonstrated not only that most sera from two high-transmission areas in Papua New Guinea were able to inhibit parasite invasion in vitro but also that the inhibitory activity was primarily directed against MSP1. By constructing a chimeric transfected *P. falciparum* line, D-10 (D10-PcMEGF), which expressed an antigenically distinct $MSP1_{19}$ domain from the distantly related rodent species *Plasmodium chabaudi*, these authors showed that $MSP1_{19}$-specific antibodies comprised a large component of the total invasion-inhibiting response of sera from many *P. falciparum*-immune adults in Papua New Guinea (22). There are two implications of these results that can be used for malaria vaccine development. First, antibodies specific to $MSP1_{19}$ may play a major role in reducing parasite multiplication rates during natural immunity. And second, although in animal models protection elicited with vaccines based on MSP1 requires high antibody titers, the lower levels of antibody obtainable in natural infections have an effect on in vitro parasite growth.

As a part of a strategy for malaria vaccine development based on recombinant MSP1, the following different expression systems for MSP1 production have been evaluated: *Saccharomyces cerevisiae* (18, 19), *Pichia pastoris* (4), baculovirus-

* Corresponding author. Mailing address for Sanjay Singh: Malaria Vaccine Development Unit, NIAID/NIH, Bldg. TW1, Rm. 1210A, 5640 Fisher Lane, Rockville, MD 20852. Phone: (301) 435-2917. Fax: (301) 480-1962. E-mail: ssingh@niaid.nih.gov. Present address for Anthony W. Stowers: CSL Ltd., 45 Poplar Rd., Parkville, Victoria 3052, Australia. Phone: 61-3-9389-1911. Fax: 61-3-9389-1434. E-mail: anthony.stowers@csl.com.au.

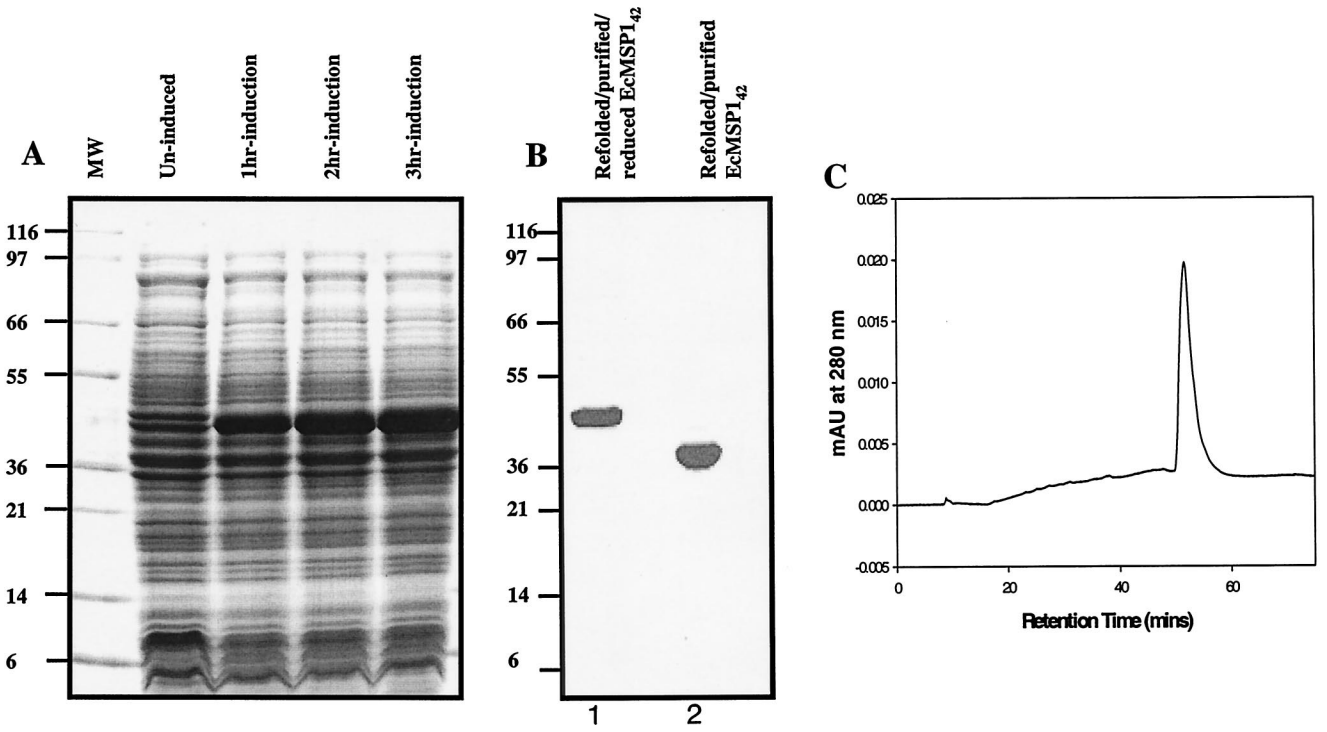

FIG. 1. Expression and analysis of refolded and purified recombinant *E. coli* MSP1$_{42}$. (A) Expression of recombinant *E. coli* MSP1$_{42}$ in *E. coli*. Cell pellets were solubilized in 50 mM Tris (pH 8.0) containing 8 M urea and 10 mM dithiothreitol. Each solubilized cell pellet was diluted in 2× SDS-PAGE sample buffer. Solubilized samples were electrophoresed under reducing conditions on an SDS–4 to 20% PAGE gel. There was time-dependent accumulation of a band at 42 kDa after induction. Lane MW contained molecular weight standards. (B) Coomassie blue stain analysis on an SDS–4 to 20% PAGE gel under nonreducing conditions (lane 2) and reducing conditions (dithiothreitol treatment followed by alkylation with iodoacetamide) (lane 1). The shift in mobility upon reduction indicates the presence of disulfide linkages. EcMSP1$_{42}$, *E. coli* MSP1$_{42}$. (C) Reverse-phase high-performance liquid chromatography profile of refolded and purified *E. coli* MSP1$_{42}$. Refolded *E. coli* MSP1$_{42}$ eluted as a single sharp peak, indicating that it contained a single, homogeneous conformer population. mAU, milliabsorbance unit.

infected insect cells (29), and milk from transgenic mice (30). Recombinant MSP1$_{42}$ produced in baculovirus-infected insect cells (6, 29) and transgenic milk (30) elicits protective responses in an in vivo model system but has yet to be scaled up for human clinical trials. The purpose of the present study was to examine *Escherichia coli* expression for the production of MSP1$_{42}$. The *E. coli* protein expression system, which was the first commercialized system for recombinant protein production, is cost-effective and very efficient for nonglycosylated proteins, such as MSP1$_{42}$. MSP1 is a nonglycosylated protein in its native form, and glycosylation blocks the efficacy of MSP1$_{42}$ produced in transgenic milk (30). Here, we describe methods to produce recombinant MSP1$_{42}$ in its correctly folded conformation, to examine the ability of antibodies raised against recombinant MSP1$_{42}$ to block erythrocyte invasion by *P. falciparum* in vitro, and to examine the in vivo efficacy of MSP1$_{42}$ in *Aotus nancymai* monkeys against a lethal challenge with *P. falciparum*.

MATERIALS AND METHODS

Expression and fermentation conditions for *E. coli* MSP1$_{42}$. The amino acid sequence of MSP1$_{42}$ FVO (MSP1$_{42}$ of the Vietnam-Oak Knoll or FVO strain; GenBank accession no. L20092) was used to construct a synthetic gene. The coding sequence of the gene was optimized for expression in *E. coli* by normalizing its AT content on the basis of previously published values for *E. coli* codon bias. This construct, corresponding to amino acids A-1 to S-355, was generated by PCR techniques and was subcloned into a pCR-blunt vector from Invitrogen. The MSP1$_{42}$ FVO gene was then inserted downstream of the T7 promoter by using an *Nde*I and *Xho*I site in the *E. coli* expression vector pET24d+ (Novagen Inc., Madison, Wis.) to obtain plasmid pPfMSP1$_{42}$FVOPET. The transcribed sequence of pPfMSP1$_{42}$FVOPET contains an additional LEHHHHHH at the C terminus. *E. coli* BL21(DE3) cells (Novagen) were transformed with pPfMSP1$_{42}$ FVOPET and used for expression of recombinant *E. coli* MSP1$_{42}$ FVO protein. Fermentation was performed in a 1.9-liter culture by using defined medium containing (per liter) 13.3 g of KH_2PO_4, 4.0 g of NH_4HPO_4, 1.7 g of citric acid monohydrate, 1.2 g of $MgSO_4 \cdot 7H_2O$, 4.5 mg of thiamine-HCl, 25 g of dextrose, 35 mg of kanamycin, and 1 ml of PTM4 trace salts. NH_4OH was the nitrogen source, and glucose was the carbon source. Fermentation was carried out at 37°C, and once the optical density at 550 nm reached 35, the culture was induced by adding isopropyl-1-thio-β-galactopyranoside (IPTG) to a final concentration of 1 mM. Induction was continued for 3 h before harvesting by centrifugation and cell pellet storage at −80°C.

Refolding and purification of *E. coli* MSP1$_{42}$. A portion of the frozen cell pellet was resuspended in 10 volumes of lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 mM NaCl, 5 mM dithiothreitol) and lysed at 19,000 lb/in^2 by using a microfluidizer (Microfluidics Corporation, Newton, Mass.). The resulting lysate was mixed with an equal volume of freshly prepared urea wash buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 2 M urea, 1% Triton X-100) and stirred for 1 h at 4°C. The lysate was centrifuged for 30 min at 10,000 × *g*, and recombinant *E. coli* MSP1$_{42}$ was detected in the pellet formed by inclusion bodies. The inclusion body pellet was resuspended in solubilization buffer (10 mM Tris-HCl [pH 8.0], 8 M guanidine-HCl, 100 mM NaCl, 10 mM β-mercaptoethanol) and stirred with a magnetic stirrer for 2 h at room temperature. The guanidine-solubilized material was clarified by centrifugation at 20,000 × *g* for 30 min at 4°C. The denatured supernatant was then refolded by 33-fold rapid dilution in a redox refolding buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 50 mM NaCl, 0.5 M arginine, 1 M urea, 25 mM cysteine, 1 mM cystamine). The refolding solution was incubated for 24 h at 4°C with continuous stirring and then

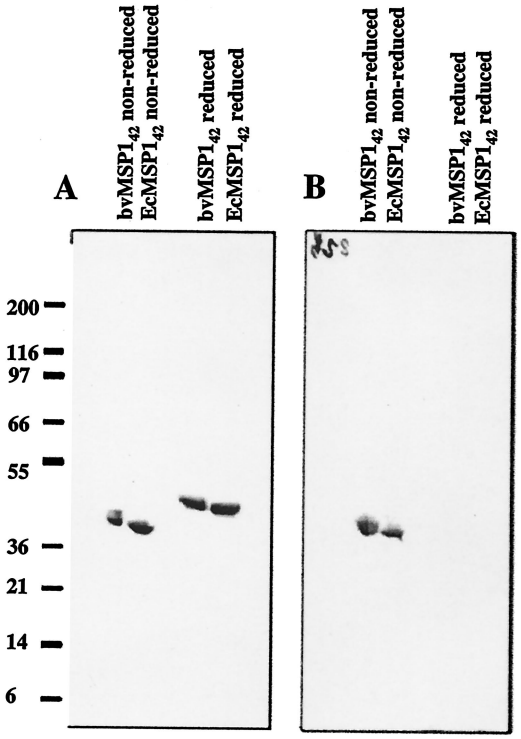

FIG. 2. Comparative analysis of refolded and purified recombinant *E. coli* MSP1$_{42}$ (EcMSP1$_{42}$) with naturally refolded and purified baculovirus MSP1$_{42}$ (bvMSP1$_{42}$). The positions of molecular mass markers (in kilodaltons) are indicated on the left. (A) Coomassie blue stain analysis of SDS–4 to 20% PAGE gel. (B) Western blot developed by using the anti-baculovirus MSP1$_{42}$ FVO 223 monoclonal antibody.

dialyzed for 36 h against 50 mM Tris-HCl (pH 8.0)–750 mM urea. The dialyzed solution was clarified by centrifugation and applied to a Q-Sepharose Hi Trap column (Amersham Pharmacia) equilibrated with binding buffer containing 50 mM Tris-HCl (pH 8.0), 250 mM urea, and 12 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). After sample application, the column was washed with 15 column volumes of binding buffer, and then the *E. coli* MSP1$_{42}$ was eluted with a linear gradient to 100% elution buffer (50 mM Tris-HCl [pH 8.0], 1 M NaCl, 12 mM CHAPS). Fractions containing *E. coli* MSP1$_{42}$ were pooled and loaded onto an Ni-nitrilotriacetic acid (NTA) Superflow (Qiagen) column preequilibrated in 2× phosphate-buffered saline (PBS). The Ni-NTA column was washed with 5 column volumes of 2× PBS, and protein was eluted from the column by using 1× PBS containing 250 mM imidazole. Final purification of the refolded *E. coli* MSP1$_{42}$ eluted from the Ni-NTA was carried out by using a Superdex 75 column (Amersham Pharmacia) with PBS.

Analysis of refolded *E. coli* MSP1$_{42}$. Reverse-phase high-performance liquid chromatography analysis of refolded *E. coli* MSP1$_{42}$ was carried out with a Dynamax 300Å C_4 column (Varian Inc., Walnut Creek, Calif.). The gradient used for elution was developed from buffer A (0.1% trifluoroacetic acid in water) and buffer B (0.1% trifluoroacetic acid in 90% acetonitrile–10% water). The column was initially equilibrated with 90% buffer A and 10% buffer B and reached a composition of 10% buffer A and 90% buffer B in 75 min. N-terminal amino acid sequencing was performed by the Biological Resources Branch, National Institute of Allergy and Infectious Diseases. Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Ill.). Electrophoresis and immunoblotting of sodium dodecyl sulfate (SDS) gels were performed by using standard methods, except that 2.5% bovine serum albumin was used to block the binding sites on nitrocellulose after protein transfer. All washes were performed in 1× PBS containing 0.02% Tween 20 and 0.2% Triton X-100. For development of the blot, the nitrocellulose was treated with a 1:1,000 dilution of five individual conformation-specific monoclonal antibodies raised against baculovirus MSP1$_{42}$ FVO (13). The primary antibody was detected with goat anti-mouse alkaline phosphatase-conjugated secondary antibody (Kirkegard and Perry, Gaithersburg, Md.). Detection was performed by using a 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium colorimetric kit (Kirkegaard and Perry).

Preparation of rabbit antisera against *E. coli* MSP1$_{42}$. Rabbit antisera against recombinant refolded *E. coli* MSP1$_{42}$ were raised by immunization with 50 μg of the antigen emulsified in 100 μl of Montanide ISA 720 (SEPPIC, Fairfield, N.J.). The primary immunization was intramuscular (zero time), and subsequent immunizations were subcutaneous (days 21 and 42). Sera were collected on days 42 and 63. Each rabbit serum was heat inactivated at 56°C for 20 min and then sterilized by filtration through a 0.22-μm-pore-size filter.

Parasite culture and GIA. The following *P. falciparum* culture-adapted clones were used for growth inhibition assays (GIAs): *P. falciparum* FVO (27), D10-PfM3′ (Pf-D10), and chimeric parasite D10-PcMEGF (Pf-D10Pc) (22). The chimeric parasite Pf-D10Pc is identical to the parental Pf-D10 clone except that the Pf-D10 MSP1$_{19}$ domain is replaced by the domain from *P. chabaudi*. Prior to use, each clone was cultured by using standard techniques but was preadapted to grow in 25% normal rabbit serum without a loss of viability. The GIA employed is a reproducible method developed in our laboratory (17). Percentages of inhibition were determined by using the following formula: $100 - \{[(A_{650}$ of immune sample $- A_{650}$ of RBC alone)$/(A_{650}$ of preimmune control $- A_{650}$ of RBC alone)$] \times 100\}$.

Vaccination and challenge infection of malaria-naive *Aotus* monkeys. Fourteen monkeys were randomly assigned to two groups containing seven monkeys each. One vaccine group received *E. coli* MSP1$_{42}$, and the control group received an unrelated recombinant *Plasmodium vivax* antigen, Pvs25H, as a negative control.

The monkeys received three vaccinations consisting of 100 μg of *E. coli* MSP1$_{42}$ or Pvs25H emulsified in 400 μl of the adjuvant 3 weeks apart, as described previously (29). The initial vaccination preparations were emulsified with complete Freund's adjuvant (Sigma, St. Louis, Mo.), and the subsequent two vaccination preparations were emulsified with Montanide ISA 51. Sera were collected from the vaccinated monkeys on day 15 after the third vaccination, and then the monkeys were challenged by intravenous injection of 5×10^4 infected RBCs from a donor monkey infected with the highly virulent *P. falciparum* FVO strain. Parasitemia was monitored daily by examining Giemsa-stained thin films until treatment. The monkeys were treated when the parasitemia reached >4.5% or the hematocrit fell below 25%. All monkeys not treated previously were treated on day 28 after challenge. The treatment consisted of mefloquine administered in a single dose of 25 mg/kg of body weight by intubation.

Measurement of antibody responses. Enzyme-linked immunosorbent assays (ELISAs) and indirect immunofluorescence assays were performed as previously described (21). Serum dilutions that gave an absorbance that was 0.5 U above the background value were designated the endpoint of the ELISA titer.

Statistical methods. *Aotus* monkeys that control their parasitemia (i.e., the parasitemia remains less than 4.5%) either self-cure or suffer from anemia and require treatment. At this point it is impossible to say what would have occurred to a monkey's parasite burden; the monkey may have self-cured, continued to control the parasitemia, or lost control and suffered from a virulent infection. Thus, the primary endpoint data included only data collected until the first monkey was treated for anemia rather than parasitemia. At that time, all monkeys were ranked in the following order. Monkeys that were treated for parasitemia prior to the day of data collection were ranked first, in order of the first day of treatment and then cumulative parasitemia (the sum of a monkey's daily parasite burden). Then the monkeys that required treatment for low hematocrit (thus triggering the endpoint) were ranked in the same fashion. Finally, monkeys that did not require treatment until that point were ranked in order of their cumulative parasitemias (29). A nonparametric, unpaired Mann-Whitney U test was then performed to compare the test group to the control group. Secondary statistical comparisons were also made. Nonlinear Spearman's regression analysis was performed to correlate antibody responses to protection from challenge. Unpaired Mann-Whitney U tests were also used to compare data between the vaccine groups (e.g., days until patent).

RESULTS

Expression, in vitro refolding, and purification of *E. coli* MSP1$_{42}$. Fermentation of *E. coli* BL21(DE3) cells containing the pPfMSP1$_{42}$FVOPET plasmid in defined medium showed that there was time-dependent accumulation of a ~42-kDa protein when the preparation was induced with IPTG (Fig. 1A). Based on an SDS-polyacrylamide gel electrophoresis (PAGE) analysis and light microscopy, expression of recombi-

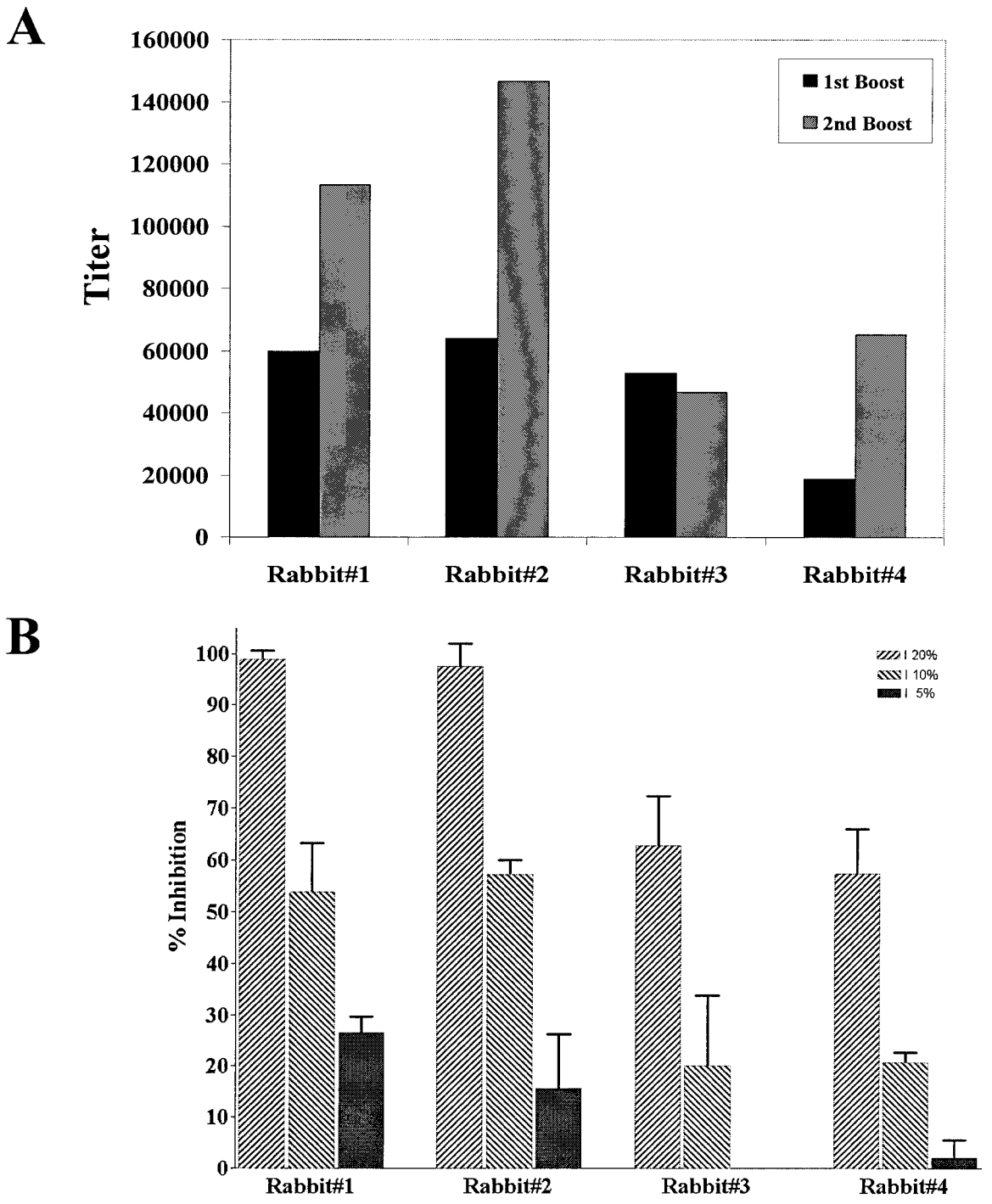

FIG. 3. Antibody responses of rabbits to immunization with *E. coli* $MSP1_{42}$. (A) Magnitude of the antibody response to *E. coli* $MSP1_{42}$ immunization as determined by ELISA. (B) Evaluation of the concentration-dependent growth-inhibitory response of *E. coli* $MSP1_{42}$-immunized rabbit sera to the homologous parasite line (*P. falciparum* FVO). The error bars indicate the standard deviations observed for nine samples from three independent experiments.

nant *E. coli* $MSP1_{42}$ was associated with the insoluble fraction of the cell lysate in the form of inclusion bodies. The recombinant *E. coli* $MSP1_{42}$ was isolated from the inclusion bodies by solubilization in denaturant and reductant buffer, followed by rapid dilution in refolding buffer. Refolded *E. coli* $MSP1_{42}$ was further purified by using three stringent chromatography purification processes involving three different chemistries. This purification yielded a homogeneous product. As determined by SDS-PAGE, the refolded *E. coli* $MSP1_{42}$ had an observed molecular mass under nonreducing conditions of ~39 kDa,

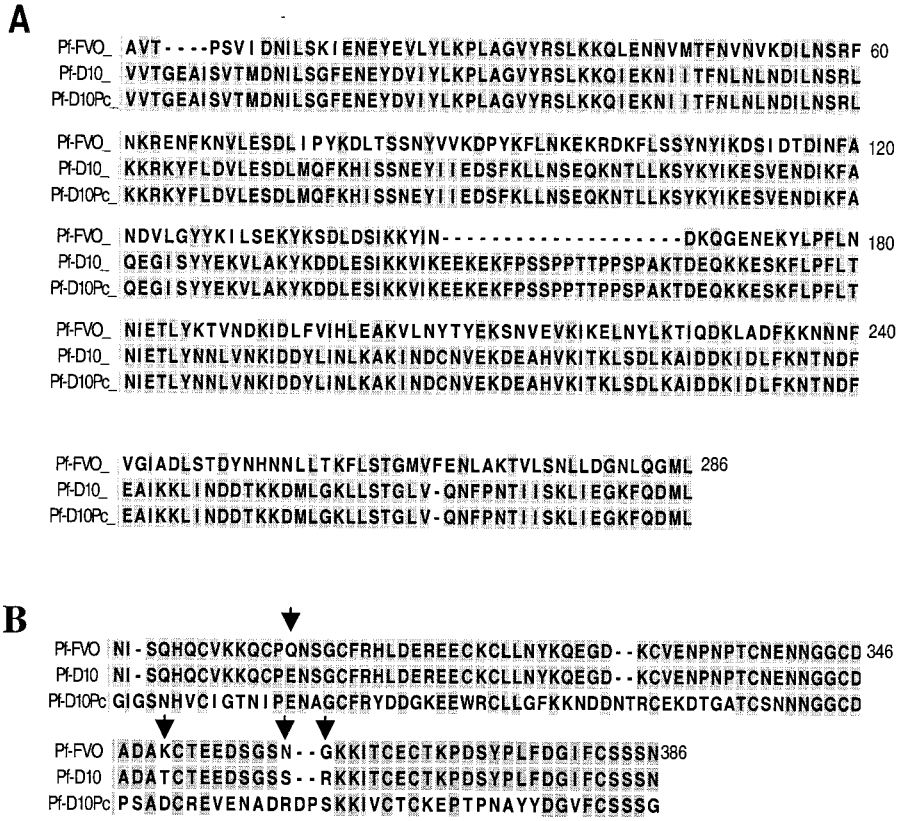

FIG. 4. Alignment of the sequences of MSP-1_{42} from the three *Plasmodium* parasites used in the GIA, including *P. falciparum* FVO (Pf-FVO) (GenBank/EMBL/DDBJ accession no. L20092), the Pf-D10 cloned line (GenBank/EMBL/DDBJ accession no. AAA29653), and the *P. chabaudi adami* (GenBank/EMBL/DDBJ accession no. AF149303)-*P. falciparum* $MSP1_{19}$ chimera created by O'Donnell et al. (Pf-D10Pc) (22). (A) $MSP1_{33}$ alignment. *P. falciparum* FVO has the Wellecome type sequence, and Pf-D10 and Pf-D10Pc have the MAD 20 type of sequence. (B) $MSP1_{19}$ alignment. The arrows indicate the four amino acid differences between *P. falciparum* FVO and Pf-D10.

and the molecular mass under reducing conditions was ~42 kDa (Fig. 1B). Densitometry scanning of the Coomassie blue-stained gel indicated that the purity of the refolded and purified *E. coli* $MSP1_{42}$ was more than 94%.

The homogeneity of the refolded and purified *E. coli* $MSP1_{42}$ was analyzed by reverse-phase high-performance liquid chromatography, which can separate different conformers of a protein based on the change in surface hydrophobicity. Refolded *E. coli* $MSP1_{42}$ eluted in a single peak during reverse-phase chromatography on a C_4 column (Fig. 1C), suggesting that purified *E. coli* $MSP1_{42}$ is homogeneous. N-terminal sequencing of the refolded *E. coli* $MSP1_{42}$ yielded the expected sequence (AVTPSVIDNILSKIE) with successful cleavage of the bacterial *N*-formyl methionine. The mobilities of reduced and nonreduced samples of naturally refolded and purified recombinant baculovirus $MSP1_{42}$ and refolded *E. coli* $MSP1_{42}$ on SDS-PAGE gels were compared (Fig. 2A). The shifts in mobility after reduction appeared to be similar, although baculovirus $MSP1_{42}$ migrated more slowly than refolded *E. coli* $MSP1_{42}$under both nonreduced and reduced conditions. This slower migration resulted from the glycosylation of the baculovirus $MSP1_{42}$ (29). Immunological characterization was performed by using conformation-specific monoclonal antibodies and a positive control (baculovirus $MSP1_{42}$). All five conformation-specific monoclonal antibodies reacted with nonreduced *E. coli* $MSP1_{42}$ but not with reduced *E. coli* $MSP1_{42}$. An example of the reactivity pattern is shown in Fig. 2B. Monoclonal antibody bv223 reacted with nonreduced refolded *E. coli* $MSP1_{42}$ and baculovirus $MSP1_{42}$ identically and did not react with the reduced samples.

Elicitation of an immune response known to occur in natural infections. A vaccine formulation suitable for human use (10) was prepared by using the *E. coli* $MSP1_{42}$ protein (50 μg of protein emulsified in 100 μl of Montanide ISA 720). This formulation was used to immunize rabbits, and the immune sera were tested for inhibition of the growth of *P. falciparum* parasites in vitro. Immune sera from all four rabbits had high titers (Fig. 3A) and effectively blocked parasite invasion by the *P. falciparum* FVO laboratory clone (homologous to the immunogen) in a concentration-dependent manner (Fig. 3B). These data suggest that at whole-blood concentrations (effectively 100% serum), parasite growth inhibition would have been total.

To examine heterologous parasite growth inhibition, Pf-D10 was used in the GIA. Figure 4 shows alignments of the sequences of the *P. falciparum* FVO and Pf-D10 MSP1 proteins for both the $MSP1_{33}$ (Fig. 4A) and $MSP1_{19}$ (Fig. 4B) domains. The proteins have extensive differences in $MSP1_{33}$ and have four amino acid substitutions in $MSP1_{19}$. Growth inhibition results that are representative of the data obtained with the

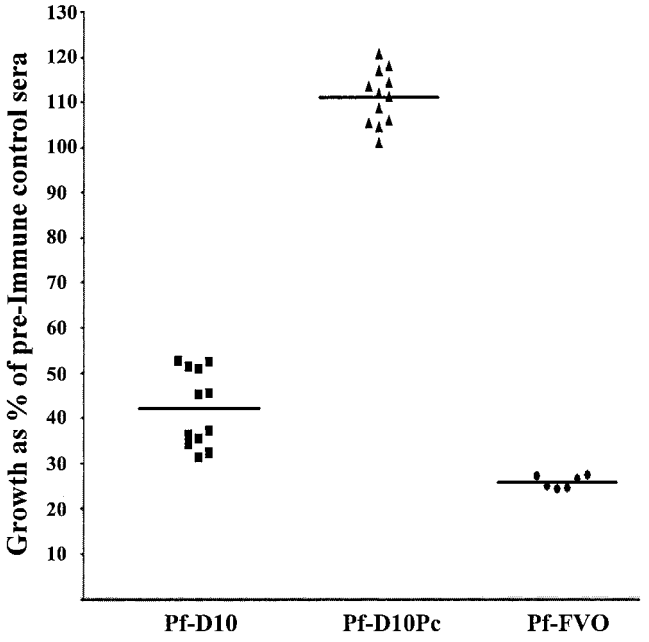

FIG. 5. Inhibition of growth invasion of heterologous *P. falciparum* parasite lines Pf-D10 and Pf-D10Pc determined by using immune sera raised against *E. coli* $MSP1_{42}$ FVO. A GIA in which two different *Plasmodium* strains expressing divergent $MSP1_{19}$ domains were compared revealed the important role of $MSP1_{19}$-specific antibodies. The data show the growth of each parasite strain as a percentage of the growth of the same parasite strain in preimmune control sera (20% immune sera pooled from all four rabbits immunized with refolded *E. coli* $MSP1_{42}$). The data for each group represents 12 different samples from three independent experiments. The horizontal lines indicate the means. Groups were compared by using an unpaired *t* test to determine statistical significance. Pf-FVO, *P. falciparum* FVO.

pooled immune rabbit sera (four rabbits immunized with *E. coli* $MSP1_{42}$) are shown in Fig. 5. Pooled immune rabbit sera at a concentration of 20% inhibited the growth of the heterologous parasite Pf-D10 significantly (58% inhibition). However, this level of inhibition was significantly lower than the level of inhibition of the homologous parasite *P. falciparum* FVO ($P = 0.033$, as determined by a paired Student's *t* test at a serum concentration of 20%).

To investigate whether the significant inhibition of the Pf-D10 parasite was due to growth-inhibitory antibodies directed against the C-terminal $MSP1_{19}$ or the N-terminal $MSP1_{33}$ domain of $MSP1_{42}$, the GIA was repeated with the parasite line Pf-D10Pc. Pf-D10Pc is identical to the parental clone, Pf-D10, in the $MSP1_{33}$ region, but the $MSP1_{19}$ domain is replaced by the domain from *P. chabaudi*. (Fig. 4). No inhibitory effect on Pf-D10Pc parasite growth was observed (Fig. 5).

E. coli $MSP1_{42}$ was used to vaccinate *A. nancymai* monkeys ($n = 7$), which were examined in conjunction with control animals ($n = 7$) that received the irrelevant negative control antigen Pvs25H. Fifteen days after the third vaccination all monkeys were challenged with 5×10^4 *P. falciparum* FVO parasites. The primary technique used to measure efficacy was to rank animals in order of treatment for parasitemia and cumulative parasitemia at the time that the first monkey was treated for anemia, day 18 (see Materials and Methods). Refolded *E. coli* $MSP1_{42}$ was found to be efficacious, and the cumulative parasitemias were significantly less than those of the control animals (Fig. 6) ($P < 0.01$). By day 18, one of the seven animals in the *E. coli* $MSP1_{42}$ group had been treated for parasitemia of >4.5%, compared with six of the seven animals in the control vaccine group. On day 18, four animals were treated for anemia; three of these animals were in the *E. coli* $MSP1_{42}$ group, and the other animal was in the Pvs25H group.

Other secondary markers of protection confirmed these results (Table 1). *E. coli* $MSP1_{42}$-immunized monkeys were significantly different from control animals when the numbers of days to patency, the numbers of days to treatment, the numbers of days to peak parasitemia, and the actual peak parasitemia values were compared ($P = 0.04$, $P = 0.02$, $P = 0.008$, and $P = 0.03$, respectively, as determined by the Mann-Whitney U test). Overall, six of seven control group animals required treatment for parasitemia of >4.5%. One of the seven *E. coli* $MSP1_{42}$-immunized animals required treatment for parasitemia, one animal self-cured, and five animals controlled the parasitemia but required treatment for a decrease in hematocrit to <25%. ELISA titers (against the reference antigen baculovirus $MSP1_{42}$) for the vaccinated monkeys are shown in Table 1. Overall, the antibody titers correlated with protection. There was a significant correlation between the antibody titers to *E. coli* $MSP1_{42}$ and the primary outcome of protection ($P = 0.0004$; Spearman rank $r = -0.79$).

DISCUSSION

We describe a method used to produce from an *E. coli* expression system a recombinant *E. coli* $MSP1_{42}$ that was purified from inclusion bodies and refolded by oxidative rapid dilution. A variety of biochemical, biophysical, and immunological assays were used to demonstrate that the refolded *E. coli* $MSP1_{42}$ is homogeneous and immunogenic. First, refolded *E. coli* $MSP1_{42}$ eluted in a single peak during reverse-phase chromatography on a C_4 column, suggesting that it is composed of a homogeneous population of conformers. Second, *E. coli* $MSP1_{42}$ exhibited a reduction-sensitive shift in mobility during SDS-PAGE, suggesting that disulfide bonds were formed. Third, recombinant $MSP1_{42}$ has been expressed previously in its functional form as a secreted protein in insect cells by using baculovirus vectors (29). The reactivity of *E. coli* $MSP1_{42}$ with conformation-specific monoclonal antibodies is similar to the reactivity of the naturally refolded baculovirus $MSP1_{42}$. Recombinant proteins expressed as secreted proteins in eukaryotic cells are commonly glycosylated. This may be relevant for immunogenicity of vaccine candidate antigens, as previous studies demonstrated that a glycosylated form of $MSP1_{42}$ expressed in transgenic mouse milk did not induce a protective response against malaria (30), while the glycosylated form of $MSP1_{42}$ produced in baculovirus was protective. These differences may be due to the type of glycosylation and the extent of glycosylation on molecules produced in the two different expression systems (30). *E. coli* is thus well suited for the production of nonglycosylated malarial parasite antigens as vaccine candidates (23, 28), and in this report we provide the first description of methods developed for refolding and purification of functional *E. coli* $MSP1_{42}$ produced in a bacterial expression system.

Immunization of rabbits with *E. coli* $MSP1_{42}$ elicits antibod-

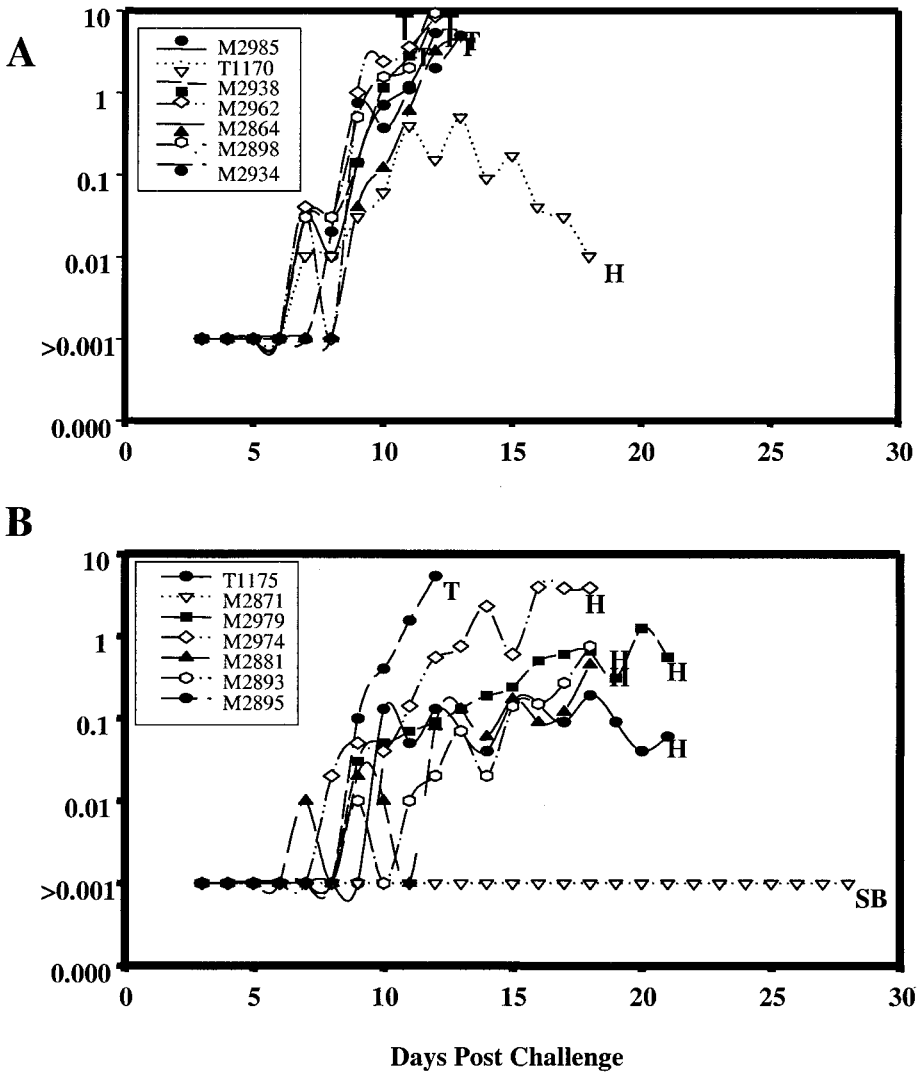

FIG. 6. Course of daily parasitemia in individual monkeys in the *Aotus* vaccine trial. Monkeys were challenged on day 0 with 5×10^4 *P. falciparum* FVO-parasitized erythrocytes 15 days after the third vaccination. (A) Pvs25-vaccinated group. (B) *E. coli* $MSP1_{42}$-vaccinated group. Also indicated are the treatment times for uncontrolled parasitemia of >4.5% (T), hematocrit of <20% (H), and subpatent (SB).

ies with significant concentration-dependent in vitro growth-inhibiting activity for the homologous parasite line *P. falciparum* FVO. The inhibition varies for different rabbit sera and correlates with the ELISA titers in individual rabbit sera against the *E. coli* $MSP1_{42}$.

The data suggest that the target of the antibodies raised against *E. coli* $MSP1_{42}$ that block invasion of human erythrocytes is $MSP1_{19}$. This was shown by comparing the inhibition data for two identical parasites that differ only in the replacement of $MSP1_{19}$ of *P. falciparum* by $MSP1_{19}$ of *P. chabaudi* (Pf-D10 and Pf-D10Pc) (Fig. 4). Invasion by the *P. chabaudi* chimera (Pf-D10Pc) was not inhibited, whereas the level of inhibition for the parasite with the *P. falciparum* sequence (Pf-D10) was around 58% (Fig. 5).

Polymorphisms at four amino acid positions in $MSP1_{19}$ were identified when the FVO and 3D7 sequences were compared (21). These differences may have been selected to minimize cross-protection. We found in the present study that sera raised against the *P. falciparum* FVO sequence (*E. coli* $MSP1_{42}$) inhibited invasion of RBCs by the Pf-D10 parasite, which differs from FVO at these four amino acid residues. Similar results were described previously (16); antibodies against parasites that differed at these four amino acids could cross-inhibit, although, as in our study, the inhibition appeared to be less than the inhibition observed with the homologous strain. In another study, however, in which a competitive ELISA with heterologous $MSP1_{19}$ domains was used, some rabbit sera exhibited no cross-reaction with the heterologous recombinant protein (29). Thus, despite the cross-inhibition in rabbits in the two studies, the lack of cross-reactions in other rabbits may indicate that both types of sequences are required in a vaccine.

Immunization with *E. coli* $MSP1_{42}$ elicits high-titer antibodies in *Aotus* monkeys and leads to significant protection against a lethal *P. falciparum* in vivo challenge. A strong correlation between protection and antibody titers was observed, and the

TABLE 1. Course of infection in *A. nancymai* monkeys challenged with *P. falciparum* parasites

Vaccine group	Monkey	ELISA titer[a]	Days to patency	Days to treatment[b]	Parasitemia at treatment (%)	Days to peak parasitemia	Peak parasitemia (%)	Outcome[c]
Pvs25H	2864	—	9	13	4.75	13	4.75	Virulent
	2898	—	7	12	9.35	12	9.35	Virulent
	2934	—	8	13	4.95	13	4.95	Virulent
	2938	—	7	12	14.30	12	14.30	Virulent
	2962	—	7	12	8.50	12	8.50	Virulent
	2985	—	7	12	5.30	12	5.30	Virulent
	T1170	—	7	18	0.01	13	0.50	Anemic
	Mean[d]	—	7.4	13.1	6.70	12.4	6.80	
E. coli $MSP1_{42}$	2871	223,000	Never patent	28	0	Never patent	0	Self-cured[e]
	2881	85,025	7	18	0.45	18	0.45	Anemic
	2893	107,733	9	18	0.75	18	0.75	Anemic
	2895	48,988	9	12	5.35	12	5.35	Virulent
	2974	38,863	8	18	3.85	16	3.90	Anemic
	2979	125,133	9	21	0.55	20	1.25	Anemic
	T1175	95,750	10	21	0.06	18	0.19	Anemic
	Mean[d]	89,664.3	8.6[f]	19.4	1.60	18.3[g]	1.70	

[a] The values are the reciprocals of the serum dilutions giving an optical density of 0.5 against baculovirus $MSP1_{42}$. —, not measured.
[b] If not already treated, all monkeys were treated on day 28.
[c] Course of infection: virulent, a sharply rising, uncontrolled parasitemia requiring treatment (parasitemia, >4.5%); self-cured, parasites cleared by the animal without intervention; anemic, monkey required treatment for anemia (hematocrit, <25%).
[d] Arithmetic means for each column, except for the ELISA column, in which the values are geometric means.
[e] Monkey 2871 was smear negative during the 28-day course of the challenge.
[f] Monkey 2871 was subpatent, so the arithmetic mean for the days to patency in the *E. coli* $MSP1_{42}$ group was calculated without data for monkey 2871.
[g] Monkey 2871 was subpatent, so the arithmetic mean of the days to peak parasitemia in the *E. coli* $MSP1_{42}$ group was calculated without data for monkey 2871.

variation in protection between animals can be accounted for by variation in the antibody titers ($P = 0.0004$; Spearman rank $r = -0.79$). These findings are in accordance with recent data from rodent malaria models, in which protection is also antibody dependent (31, 32). In three previous independent studies of baculovirus $MSP1_{42}$-immunized *Aotus* monkeys, a consistent pattern of protection was observed with six of seven animals (29), five of seven animals (14), and five of seven animals (30). In the control groups all four animals (29), five of seven animals (14), and six of seven animals (30) developed virulent infections. In the present study *E. coli* $MSP1_{42}$ protected six of seven monkeys, while six of seven monkeys in the control group developed virulent infections and required treatment for parasitemia. Therefore, we concluded that the *E. coli* $MSP1_{42}$ efficacy in the protection study was indistinguishable from that previously seen with baculovirus $MSP1_{42}$. In conclusion, immunization with *E. coli* $MSP1_{42}$ induces protective efficacy, and at least one of the probable effector mechanisms of that efficacy (as determined by an antibody-dependent growth inhibition assay) is a mechanism thought to be instrumental in natural immunity.

As a part of an overall strategy for malaria vaccine development based on recombinant MSP1, the following five different expression systems have been evaluated for MSP1 production: *S. cerevisiae* (18, 19), *P. pastoris* (4), baculovirus-infected insect cells (29), transgenic milk (30), and *E. coli* (this study). We concluded that *E. coli* is the optimal expression system. Both of the yeast systems failed to make full-length $MSP1_{42}$, and the efficacy of the smaller $MSP1_{19}$ fragment was less consistent (29). Although the efficacies of baculovirus-expressed $MSP1_{42}$, transgenic $MSP1_{42}$, and *E. coli* $MSP1_{42}$ appear to be indistinguishable, the yields of *E. coli* $MSP1_{42}$ are superior to those of baculovirus $MSP1_{42}$ (~30 mg/liter, compared to ~8 mg/liter), and the *E. coli* $MSP1_{42}$ development time frame is greatly reduced. As an example of the latter finding, we have been able to successfully manufacture cGMP grade material for human clinical trials of not just the FVO allele of *E. coli* $MSP1_{42}$ discussed here but also the alternate 3D7 allele (S. Singh and A. Stowers, unpublished data). Thus, the new construct, *E. coli* $MSP1_{42}$, is a viable candidate for human vaccine trials.

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