Phase 1 Study of Two Merozoite Surface Protein 1 (MSP1\textsubscript{42}) Vaccines for Plasmodium falciparum Malaria

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Abbreviations: See section at end of manuscript.

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ABSTRACT

Objectives: To assess the safety and immunogenicity of two vaccines, MSP1\textsubscript{42}-FVO/Alhydrogel and MSP1\textsubscript{42}-3D7/Alhydrogel, targeting blood-stage Plasmodium falciparum parasites.

Design: A Phase 1 open-label, dose-escalating study.

Setting: Quintiles Phase 1 Services, Lenexa, Kansas between July 2004 and November 2005.

Participants: Sixty healthy malaria-naïve volunteers 18–48 y of age.

Interventions: The C-terminal 42-kDa region of merozoite surface protein 1 (MSP1\textsubscript{42}) corresponding to the two allelic forms present in FVO and 3D7 P. falciparum lines were expressed in Escherichia coli, refolded, purified, and formulated on Alhydrogel (aluminum hydroxide). For each vaccine, volunteers in each of three dose cohorts (5, 20, and 80 μg) were vaccinated at 0, 28, and 180 d. Volunteers were followed for 1 y.

Outcome Measures: The safety of MSP1\textsubscript{42}-FVO/Alhydrogel and MSP1\textsubscript{42}-3D7/Alhydrogel was assessed. The antibody response to each vaccine was measured by reactivity to homologous and heterologous MSP1\textsubscript{42}, MSP1\textsubscript{19}, and MSP1\textsubscript{33} recombinant proteins and recognition of FVO and 3D7 parasites.

Results: Anti-MSP1\textsubscript{42} antibodies were detected by ELISA in 20/27 (74%) and 22/27 (81%) volunteers receiving three vaccinations of MSP1\textsubscript{42}-FVO/Alhydrogel or MSP1\textsubscript{42}-3D7/Alhydrogel, respectively. Regardless of the vaccine, the antibodies were cross-reactive to both MSP1\textsubscript{42}-FVO and MSP1\textsubscript{42}-3D7 proteins. The majority of the antibody response targeted the C-terminal 19-kDa domain of MSP1\textsubscript{42}, although low-level antibodies to the N-terminal 33-kDa domain of MSP1\textsubscript{42} were also detected. Immunofluorescence microscopy of sera from the volunteers demonstrated reactivity with both FVO and 3D7 P. falciparum schizonts and free merozoites. Minimal in vitro growth inhibition of FVO or 3D7 parasites by purified IgG from the sera of the vaccinees was observed.

Conclusions: The MSP1\textsubscript{42}/Alhydrogel vaccines were safe and well tolerated but not sufficiently immunogenic to generate a biologic effect in vitro. Addition of immunostimulants to the Alhydrogel formulation to elicit higher vaccine-induced responses in humans may be required for an effective vaccine.

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The *Plasmodium falciparum* parasite is responsible for at least 300 million cases of malaria each year [1], and more than one million of these cases result in death [2]. Approximately 90% of these deaths, the majority in children under 5 y of age, occur in Africa [3,4]. The clinical symptoms and pathology associated with *P. falciparum* infection are associated with the cyclical invasion of erythrocytes, intracellular parasite multiplication, and release of parasites by rupture of the infected cells. A vaccine that interrupted this cycle of infection could reduce both mortality and morbidity secondary to *P. falciparum* infection and would be a valuable resource in the fight against this disease.

Over time, people living in endemic areas develop immunity to severe disease due to *P. falciparum* as a result of repeated infection [5,6]. This acquired immunity is mediated, in part, by blood-stage parasite-specific antibodies [7–9]. Thus, parasite proteins expressed during the blood-stage have been proposed to be good candidates for inclusion in a vaccine. The aim of an asexual blood-stage vaccine is to elicit immune responses that slow or inhibit parasite multiplication to prevent morbidity, severe disease, and death in residents of malaria-endemic areas, primarily young children and infants. *P. falciparum*’s merozoite surface protein 1 (MSP1) is synthesized as a ~200-kDa polypeptide. MSP1 is processed at, or just prior to, merozoite release from the erythrocyte into smaller fragments that form a noncovalently associated complex [10]. The C-terminal 42-kDa cleavage product of MSP1 (MSP142), a major candidate for a blood-stage malaria vaccine, is composed of two regions: MSP133 and MSP119 [11]. The MSP133 portion is dimorphic. Although the sequence similarity between the two forms of MSP133 is surprisingly low (47% identity) within each dimorphic type, the sequence is highly conserved. The MSP119 domain is largely conserved between parasite strains [12,13]. Four commonly observed amino acid substitutions have been identified in MSP119 in laboratory lines with several additional polymorphisms in field isolates [13,14]. The MSP142 proteins of the FVO and 3D7 *P. falciparum* parasite lines cover both dimorphisms in MSP133 and the more common antigenic diversity in MSP119. The inclusion of both MSP142-FVO and MSP142-3D7 proteins in a combination vaccine would, in large part, address the concerns of generating protective immune responses to a polymorphic parasite protein.

In studies of natural infection, the majority of the B cell epitopes have been localized to the highly conserved MSP119 domain [15,16], and the epitopes that induce proliferation of T cell subsets specific for MSP142 have been localized to the dimorphic region of the molecule MSP133 [15,16]. A combination MSP142-FVO and MSP142-3D7 vaccine may be essential to prime for, or to boost immune responses, that would ultimately result in optimal immune responses in endemic areas.

Previous human trials have evaluated recombinant protein vaccines based on the C terminus of MSP1. The first trial evaluated fusion proteins of FVO and 3D7 forms of MSP1 with the tetanus toxoid universal T cell epitopes P30P2 formulated on Alhydrogel in a United States population [17]. These vaccines were poorly immunogenic and their administration resulted in three immediate-type hypersensitivity reactions, which halted the trial. More recent trials assessed...
recombinant MSP142-3D7 (FMP-1) formulated with GlaxoSmithKline Biologicals’ proprietary adjuvant AS02A in the United States and Africa (Kenya and Mali) [18–21]. In all populations, FMP11/AS02A was safe and immunogenic. In the malaria-naive population, biologically active antibodies and antigen reactive T cells were induced [19]. There have been no trials to date evaluating the immunogenicity of MSP142-FVO or comparing the specificity of the human responses to MSP142-FVO or MSP142-3D7 polypeptides, respectively, which correspond to the recombinant proteins are highly purified 42,173-Da and 44,236-Da identity, and integrity. MSP142-FVO and MSP142-3D7 recombinant proteins were derived from the FVO and 3D7 parasite lines of \textit{P. falciparum} with the ultimate aim of using them in a combination vaccine. Each MSP142 protein was individually formulated on Alhydrogel (Brenntag Biosector, Denmark), an aluminum hydroxide gel, to produce the vaccines, MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel. This paper compares the safety and immunogenicity of these individual formulations when tested in a healthy United States adult population.

METHODS

Participants

Sixty healthy volunteers, 18–48 y of age, were recruited from the Lenexa, Kansas area. Written informed consent was obtained prior to enrollment. Volunteers were recruited and consented using a protocol and consent form approved by the Heartland Institutional Review Board (trial site IRB), the PATH Human Subjects Protection Committee, and the NIAID IRB. Volunteers were excluded if they had any of the following: evidence of clinically significant systemic disease; obesity (body mass index $\geq$35); pregnancy or breast-feeding; serological evidence of human immunodeficiency virus infection, chronic hepatitis B or hepatitis C infection; current medication with corticosteroids or immunosuppressive drugs; immunization with a live vaccine 4 wk prior to entry or a killed vaccine 2 wk prior to entry into the study; prior malaria infection; previous receipt of a malaria vaccine; travel to a malaria-endemic country 12 mo prior to study enrollment; or planned travel to a malaria-endemic country during the course of the study. All females had a urine \(\beta\) human chorionic gonadotropin test at screening and immediately prior to each vaccination.

Intervention: Purification and Characterization of Clinical Grade MSP142 Antigens and Vaccine Preparation

The expression, refolding, and purification of \textit{E. coli}-produced MSP142-FVO (lot WRAIR0997) and MSP142-3D7 (lot WRAIR0984) antigens was performed at the Walter Reed Army Institute of Research, Pilot Bioproduction Facility, Silver Spring, Maryland in accordance with current Good Manufacturing Practices (cGMP). Each protein underwent comprehensive quality control analysis to ensure purity, identity, and integrity. MSP142-FVO and MSP142-3D7 recombinant proteins are highly purified 42,173-Da and 44,236-Da polypeptides, respectively, which correspond to the external domain of MSP142 from the \textit{P. falciparum} FVO and 3D7 lines.

EcMSP142-FVO and EcMSP142-3D7 were each expressed as histidine-tagged fusion proteins from synthetic genes, cloned into pET-24d or pET-24a (Novagen, http://www.novagen.com), respectively, and transformed into BL21 (DE3) \textit{E. coli} (Invitrogen, http://www.invitrogen.com). Following \textit{E. coli} fermentation in defined medium and isopropyl-\(\beta\)-D-thiogalactopyranoside induction, each protein was extracted from solubilized inclusion bodies, and EcMSP142-FVO underwent an initial purification by reverse-phase chromatography. This product or the extractedEcMSP142-3D7 was subjected to nickel-affinity chromatography, refolding by rapid dilution, and further purified by anion-exchange chromatography and size-exclusion chromatography (L. B. Martin, et al., unpublished data). Both antigens were supplied in sterile phosphate buffered saline (PBS [pH 7.4]) containing 0.2% polysorbate 80. For EcMSP142-FVO, the final purity (percentage in a single band by SDS-PAGE and densitometry) was $\sim$95.0\%, with a host cell protein impurity level of 0.036\% and endotoxin level (as measured by the Limulus amoebocyte lysate gel clot assay) of 0.12 endotoxin units (EU)/mg. The results for EcMSP142-3D7 were final purity $\sim$97.4\%, host cell protein impurity level of 0.096\%, and endotoxin level of 6.7 EU/mg.

Clinical grade MSP142-FVO or MSP142-3D7 recombinant protein was adsorbed to Alhydrogel and vialed by the Pharmaceutical Development Section, NIH. Three lots were prepared, containing 5, 20, or 80 $\mu$g of MSP142-FVO or MSP142-3D7, and 800 $\mu$g of Alhydrogel per 0.5 mL dose. The formulations were supplied as single-dose vials of a cloudy suspension, without additional stabilizers or preservatives, in a sterile saline solution. Each vaccine lot underwent comprehensive quality control analysis to ensure purity, identity, and integrity. The continued potency and stability of the six lots of vaccine stored at 2–8\°C were confirmed by evaluation of their immunogenicity in mice conducted every 6 mo throughout the course of the trial. Biochemical stability was evaluated annually.

Intervention: Phase 1 Study Design

An open-label, dose-escalating Phase 1 clinical trial was designed to evaluate the safety, reactogenicity, and immunogenicity of the MSP142-FVO and MSP142-3D7 recombinant proteins formulated on Alhydrogel in healthy adult volunteers. Thirty volunteers received the MSP142-FVO/Alhydrogel vaccine and 30 volunteers received the MSP142-3D7/Alhydrogel vaccine (Figure 1). Rolling recruitment and enrollment took place to fill the low-dose cohorts prior to the medium-dose cohort followed by the high-dose cohort. After enrollment to a dose cohort, volunteers were alternatively assigned to either the MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel vaccine group. Ten volunteers were assigned to each of three dose cohorts (5, 20, and 80 $\mu$g) for each vaccine for a total of six groups (three groups for MSP142-FVO/Alhydrogel and three groups for MSP142-3D7/Alhydrogel). Volunteers were vaccinated with a 0.5-mL intramuscular injection in alternate arms on study days 0, 28, and 180. Escalation to the next higher dose required approval by an independent safety monitoring committee.

This study was performed under an investigational new drug application (BB-IND Number 11635) approved by the United States Food and Drug Administration. MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel were considered separate candidate vaccines contained within one protocol.
The protocol, amendments to the protocol, informed consent form, advertisements, and other study-related documents were approved by the Heartland Institutional Review Board (trial site IRB), the PATH Human Subjects Protection Committee, and the NIAID IRB.

Objectives
The goal of this Phase 1 vaccine trial was to demonstrate safety and immunogenicity of MSP\textsubscript{1\textsubscript{42}}/Alhydrogel malaria vaccines in human volunteers. The primary objective was to determine the frequency and severity of vaccine-related adverse events for each dose of vaccine. Secondary objectives assessed and compared the specific antibody response to homologous and heterologous MSP\textsubscript{1\textsubscript{42}} protein with time.

Outcomes: Assessment of Safety and Tolerability
The primary outcome was to assess the safety and tolerability of the vaccines. Following each vaccination, the volunteers were observed for 60 min and then evaluated for evidence of local and systemic reactogenicity on days 1, 3, 7, and 14 after each vaccination. Solicited systemic adverse events included fever (>37.5 °C), headache, nausea, malaise, myalgia, arthralgia, and urticaria. Volunteers were asked to keep a diary card to record oral temperature and any local or systemic adverse events daily for 6 d following each vaccination. Diary cards were collected 7 d after each vaccination. An abbreviated history and physical examination was performed at each follow-up visit. All abnormal signs and symptoms were considered adverse events. Each adverse event was graded
Outcomes: Antibody Measurement by Enzyme-Linked Immunosorbent Assay

Secondary outcomes were enzyme-linked immunosorbent assay (ELISA) measurements of specific serum antibodies to the FVO and 3D7 forms of MSP142, MSP119, and MSP133. A standardized ELISA protocol was employed for the measurement of anti-MSP142, anti-MSP119, and anti-MSP133 antibodies, representing both the FVO and 3D7 forms of the proteins, as previously described [22]. A human anti-MSP1 standard was prepared by pooling sera from ten individuals residing in Mali, a country endemic for P. falciparum malaria. The standard serum pool was assigned 10,000 ELISA units on MSP142-FVO, 5,882 ELISA units on MSP142-3D7, 2,800 ELISA units on MSP119-FVO, and 1,500 ELISA units on MSP119-3D7. An ELISA unit was approximately equivalent to the reciprocal of the dilution giving an optical density (OD) of 1.0. A volunteer was considered to be a non-responder if a specific antibody level was less than 50 ELISA units. Due to the low titer in the standard serum pool, ELISA unit values for MSP133 were not assigned.

The expression and purification of Saccharomyces cerevisiae-produced MSP119 and E. coli-produced MSP133 were carried out by MVDB. ScMSP119-FVO and ScMSP119-3D7 clones were fermented [23] and purified essentially as previously reported [24], except that the anion exchange step was excluded due to the low endotoxin levels of the yeast products. ScMSP119-FVO and ScMSP119-3D7 proteins were supplied in PBS.

E. coli BL21 (DE3) cells transformed with EcMSP133-FVO or EcMSP133-3D7 plasmids were fermented, inclusion bodies isolated, and the solubilized inclusion bodies were purified by nickel-affinity chromatography [24]. Due to the low solubility of EcMSP133-FVO, it could not be effectively purified further and was supplied in elution buffer containing 4 M guanidine. EcMSP133-3D7 was further purified by anion exchange chromatography and size-exclusion chromatography, as described for ScMSP119. EcMSP133-3D7 was supplied in PBS plus 0.2% polysorbate 80.

The purified ScMSP119 and EcMSP133 recombinant proteins were characterized by reverse-phase HPLC [25], N-terminal sequencing, electron-spray ionization mass spectrometry, SDS-PAGE (reduced and nonreduced), and immuno blot essentially as described [24]. The observed results were similar to those expected for each recombinant protein.

Outcomes: Isotyping of MSP142 Antibodies Using Suspension Array Technology

Serum samples from volunteers vaccinated with MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel were assayed for IgG subclasses against the immunizing antigen using a flow cytometric suspension array assay. Serum samples were diluted 1:100 and mixed with microspheres coupled with the homologous MSP142 antigen (Luminex Corporation, http://www.luminexcorp.com) in MultiScreen plates (Millipore, http://www.millipore.com). Mouse anti-human IgG isotype antibody and a second donkey anti-mouse-IgG phycoerythrin (PE) labeled antibody (Jackson ImmunoResearch, http://www.jacksonimmuno.com) were added to develop the reaction. The mean fluorescence intensity (MFI), corresponding to the presence of specific IgG subclasses to MSP142, was detected by Luminex X-MAP with the software Bioplex (Bio-Rad, http://www.bio-rad.com).

Outcomes: In Vitro Parasite Growth Inhibition by Immune IgG and Immunofluorescence with Sera on Malaria Parasites

The ability of antibodies from vaccinated individuals to inhibit growth of P. falciparum FVO and 3D7 parasites in vitro was assessed using a standardized growth inhibition assay (GIA) as previously described [22]. To ensure that the inhibitory activity measured was specific to antibody and not other serum components, total IgG was purified from individual sera obtained on days 0, 42, and 194 using Protein G columns (Pierce, http://www.pierce.com). Anti-MSP142-FVO and anti-MSP142-3D7 ELISA units were determined for each purified IgG sample (10 mg/mL), and all samples were stored in small aliquots at 2–8 °C until tested.

Indirect immunofluorescence assay (IFA) of P. falciparum FVO or 3D7 parasitized red blood cells (RBC, 3%–7% parasitemia) stained using volunteer sera obtained on day 0 and day 194. Thin smears from bulk parasite cultures were prepared and stored at −80 °C. The FVO and 3D7 parasite smears were validated using mouse monoclonal antibodies 1G3 and 4H9/19 specific for MSP142-FVO and MSP142-3D7 proteins, respectively, and anti-mouse IgG FITC (Zymed, http://www.zymed.com). After fixation with methanol and blocking with 10% bovine serum albumin (BSA Fraction V; Sigma, http://www.sigmaaldrich.com), preimmune (day 0) and day 194 sera (diluted 1:100) were allowed to react with the parasite-infected RBC. A positive serum (diluted 1:400) and negative control (no sera) were included on each slide. Antibody bound to the parasite was detected with secondary antibody, anti-human IgG FITC (ICN/CAPPEL, Aurora, Ohio, United States). The slides were examined with an immunofluorescent microscope (Olympus, http://www.olympus.com) and photographed using a digital camera (Olympus), and the smears scored on staining pattern and fluorescence intensity: 0 = negative or diffuse staining; 1 = weak staining with characteristics of trophozoites, schizonts, or free merozoites; and 2 = bright staining with characteristics of trophozoites, schizonts, or free merozoites. The day 194 sera with positive immunofluorescence were compared with corresponding day 0 sera for specificity of reactions to the parasitized RBC.

Sample Size

The sample size required in each treatment group was based on analysis of the human antibody responses to a number of malaria antigens that have been tested in clinical trials [26,27]. Based on the distribution of antibody responses for each of the antigens, a sample size of ten volunteers per dose cohort would permit detection of at least a 5-fold difference in antibody concentration between groups using a Mann-Whitney test, assuming a level of significance of 0.05 and a power of 0.80. Additionally, a group size of ten volunteers per dose gives 0.80 probability for detecting one or more serious...
or severe adverse events that occurred with a probability of 0.15 per volunteer.

Randomization
This was an open-label, dose-escalating clinical trial and no randomization procedure was utilized to assign volunteers to dose cohort. Temporal staggering was used to assign volunteers to dose cohorts. Volunteers were alternately assigned to receive either the MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel vaccine after enrollment in a dose cohort. Dose-escalation occurred only after safety data up to and including day 35 post-first vaccination; 7 d post-second vaccination was reviewed by the safety monitoring committee.

Statistical Methods
The frequency of adverse events was summarized and stratified by dose cohort. Dose effects (on adverse events and on antibody response) were tested for using exact two-sided Jonckheere-Terpstra tests, and comparisons across the vaccinations within each dose cohort were compared using McNemar’s test (SAS version 9.1, SAS Institute, http://www.sas.com). Tests of paired ELISA results (e.g., comparing day 0 to day 42 responses or comparing homologous to heterologous responses) were done by the Wilcoxon signed rank test using all available pairs. Confidence intervals for paired ELISA results were done by paired t-test. For the GIA, confidence intervals were done using t-distributions. Either the UNISTRAT statistical package (version 5.5) or R (version 2.4.0 of R, using the exactRankTests package, version 0.8–15) were used for these analyses, and p-values of less than or equal to 0.05 were considered significant.

RESULTS
Participant Flow
This study was conducted from June 2004 to November 2005. Sixty volunteers (26 male and 34 female) were enrolled. The age range was 18–48 y (median, 27). Nine volunteers (15%) identified themselves as African American, three (5%) as Hispanic, and 48 (80%) as Caucasian. Forty-four volunteers received all three vaccinations as scheduled (Figure 1). No volunteer was withdrawn due to a vaccine-related adverse event.

Outcomes and Estimations
Safety and tolerability data. Both the MSP142-FVO/Alhydrogel and the MSP142-3D7/Alhydrogel vaccines were safe and well tolerated (Tables 1 and 2). The most common reported adverse event was pain at the injection site. The majority of injection site reactions were graded as mild (98% for the MSP142-FVO vaccine and 94% for the MSP142-3D7 vaccine), and none were graded as severe. Erythema, induration, and pruritus also occurred at low frequencies (10%–25%). The most common solicited systemic adverse events were headache and fatigue. No immediate hypersensitivity reactions were observed and no serious adverse events occurred that were attributed to the vaccine.

No effect was found between dose and severity of the highest-grade adverse event observed for each volunteer (both vaccines: \( p = 0.762 \); MSP142-FVO/Alhydrogel vaccine only: \( p = 0.764 \); or MSP142-3D7/Alhydrogel vaccine only: \( p = 0.475 \)). Additionally, no significant dose effect was found when looking at the number of local adverse events per volunteer relative to the dose (both vaccines: \( p = 0.601 \), MSP142-FVO/Alhydrogel vaccine only: \( p = 0.594 \); or MSP142-3D7/Alhydrogel vaccine only: \( p = 0.808 \)). Local reactions were more frequent following the first vaccination compared to either the second (odds = 20.5, \( p = 0.004 \)) or third vaccinations (odds = 21.5, \( p = 0.0025 \)).

One volunteer in the 80-µg MSP142-3D7/Alhydrogel vaccine group developed pain at the injection site following the third vaccination which lasted for 4 d. After the third vaccination, 8 d later, the volunteer complained of left upper extremity/shoulde pain which was rated as severe. The volunteer was evaluated in a local emergency room with a left upper extremity venous Doppler, plain film x-ray, and laboratory testing. The physical examination and test results were negative. The final diagnosis was an atypical injection site reaction/musculoskeletal pain based on the temporal association with vaccination and exclusion of other etiologies.

Antibody assessment by ELISA. Antibody levels to both the MSP142-FVO and MSP142-3D7 antigens were measured by ELISA on sera obtained on days 0, 14, 28, 42, 120, 180, 194, 270, and 364. A subset of the sera samples were assessed for antibodies recognizing regions of the MSP142 protein: anti-MSP119-FVO and anti-MSP119-3D7 antibodies (days 0, 42, 194, and 270) and anti-MSP133-FVO and anti-MSP133-3D7 antibodies (days 0 and 194).

Figure 2 shows the anti-MSP142 antibodies to the homologous protein in each dose of the MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel vaccinated groups. Elevated MSP142-specific antibody levels were observed 2 wk following the second vaccination (day 42), when compared to prevaccination levels (\( p < 0.001 \) for both vaccines tested on either homologous or heterologous antigen). These antibodies diminished over time and in many volunteers had returned to near background levels by the time of the third vaccination. After the third vaccination (day 194), 2 wk later, a recall response was observed with an increase in specific anti-MSP142 antibodies to levels greater than or equal to those observed after two vaccinations (\( p < 0.001 \) for both vaccines tested on either homologous or heterologous antigen). The elevated MSP142-specific antibody levels observed on day 194 diminished with time, but no after the third vaccination, low-level specific antibody was still detectable.

Specific antibody levels following the third vaccination were greater than those observed after the second vaccination for all dose cohorts. The number of responders in all groups also increased with eight of 30 and ten of 30 responders after two vaccinations and 20 of 27 and 22 of 27 responders after three vaccinations with MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel, respectively. The effect of vaccine dose on specific antibody responses to either MSP142-FVO or the MSP142-3D7 protein on day 42 and 194 was evaluated for both of the MSP142 vaccines. The day 42 ELISA of MSP142-FVO/Alhydrogel on the MSP142-FVO antigen was the only significant dose effect (\( p = 0.044 \)), and this effect was only just significant. For the remainder of the comparisons, no significant correlation between vaccine dose and antibody response was detected.

The MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel vaccines were equally immunogenic. Among recipients of
### Table 1. MSP142-FVO/Alhydrogel Vaccine Adverse Events

<table>
<thead>
<tr>
<th>Dose</th>
<th>Reaction Type</th>
<th>Reactiona</th>
<th>Vaccination (1)</th>
<th>Vaccination (2)</th>
<th>Vaccination (3)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Total (%)</td>
<td>Mild</td>
<td>Moderate</td>
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<tr>
<td>5 µg</td>
<td>Local reactogenicity</td>
<td>Pain</td>
<td>6/10 (60%)</td>
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<tr>
<td></td>
<td></td>
<td>Erythema</td>
<td>1/10 (10%)</td>
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<td>0</td>
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<td>Induration</td>
<td>1/10 (10%)</td>
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<td>Pruritus</td>
<td>0/10</td>
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<td></td>
<td></td>
<td>Any local</td>
<td>8/10</td>
<td>8</td>
<td>0</td>
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<tr>
<td></td>
<td>Systemic reactionsb</td>
<td>Headache</td>
<td>3/10 (30%)</td>
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<td>2</td>
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<tr>
<td>20 µg</td>
<td>Local reactogenicity</td>
<td>Pain</td>
<td>9/10 (10%)</td>
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<td>Erythema</td>
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<td>Pruritus</td>
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<td></td>
<td></td>
<td>Any local</td>
<td>9/10</td>
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<td>80 µg</td>
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<td>5/10 (50%)</td>
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<td></td>
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<td>Erythema</td>
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aSeverity was graded as mild (easily tolerated), moderate (interfered with daily activity or requiring medication), or severe (prevented daily activity). Erythema, swelling, or induration at the injection site was graded as follows: mild (>0 to ≤20 mm in diameter); moderate (>20 to ≤50 mm); severe (>50 mm).
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doi:10.1371/journal.pctt.0020012.t001
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doi:10.1371/journal.pctr.0020012.t002
Figure 2. Kinetics of Homologous Anti-MSP142 Antibody Responses Following MSP142-FVO/Alhydrogel (Left) and MSP142-3D7/Alhydrogel (Right) Vaccination

Antibody ELISA units to the immunizing antigen for each individual are shown by the scatter plots. The bars represent the group arithmetic means. Volunteers were vaccinated intramuscularly on days 0, 28, and 180 (indicated by arrows) with 5 μg (top), 20 μg (middle), or 80 μg (bottom) dose of vaccine. ELISA units are expressed relative to a MSP1 standard serum pool. A response was defined as >50 ELISA units.

doi:10.1371/journal.pctr.0020012.g002
either vaccine, there was some evidence of MSP142 strain specificity in the antibody response of sera from days 42 or 194 (Figure 3); sera recognized the homologous (i.e., immunizing) antigen better than heterologous antigen at day 194 ($p < 0.001$) but not at day 42 ($p = 0.642$). However, in both cases the mean absolute difference in ELISA units is small (day 42: $-3$ units, 95% confidence interval: $-19$ to $13$; day 194: 58 units, 95% confidence interval: $10$ to $106$).

Vaccination with MSP142 generated antibodies that recognized the C-terminal domain of the MSP119 protein. The antibodies specific to MSP119 were observed following the second vaccination (day 42), increased following the third vaccination, and remained detectable 90 days after the third vaccination (day 270) (Figure 4). The only significant relationship between dose and anti-MSP119 antibody levels was for the MSP142-FVO/Alhydrogel vaccine at day 42 ($p = 0.0238$ when tested on MSP119-FVO protein and $p = 0.0378$ on MSP119-3D7 protein).

By day 42, individuals vaccinated with MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel generated similar levels of antibodies that recognized the homologous and heterologous MSP119 proteins (Wilcoxon signed rank test, $p > 0.05$). Comparison of the MSP119 antibody reactivity in sera from days 194 and 270 of the MSP142-FVO vaccine showed no difference between homologous and heterologous MSP119 proteins. But within the MSP142-3D7 vaccine, individual sera collected on days 194 and 270 recognized MSP119-3D7 (homologous) protein better than MSP119-FVO (heterologous) protein (Wilcoxon signed rank test, $p < 0.004$ for each day); the absolute difference in ELISA units was very small. For both vaccines, a strong association between MSP142 antibodies and MSP119 antibodies was observed after day 194 (Spearman rank correlation: $r > 0.94$ and $p < 0.0001$ for all homologous comparisons on days 194 and 270).

Following three vaccinations (day 194), a small increase in antibodies to the homologous MSP133 protein was observed in both the MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel vaccinated groups (Figure 5). Increased antibody response to the heterologous MSP133 protein was not observed. There was no correlation between vaccine dose and homologous anti-MSP133 antibody levels. For both vaccines, a significant association between homologous MSP142 and MSP133 antibodies was observed (MSP142-3D7/Alhydrogel Spearman rank correlation, $r = 0.89$, $p < 0.0001$ and MSP142-FVO/Alhydrogel Spearman rank correlation, $r = 0.78$, $p < 0.0001$). A correlation was also observed between homologous anti-MSP133 and anti-MSP119 antibodies (Spearman rank correlation: FVO proteins, $r = 0.73$, $p < 0.0001$ and 3D7 proteins, $r = 0.84$, $p < 0.0001$).

**IgG subclass analysis of day 194 sera.** Sera obtained at day 194 with MSP142-specific antibody units greater than 500 were analyzed by a multiplex Luminex system for the proportion of MSP142-specific IgG1, IgG2, IgG3, and IgG4 antibodies. For volunteers vaccinated with either MSP142-FVO/Alhydrogel ($n = 7$) or MSP142-3D7/Alhydrogel ($n = 8$), the majority of the MSP142 antibodies were IgG1 (Figure 6), consistent with the IgG subclass profile of an Alhydrogel-based vaccine.

**Parasite GIA.** The GIA is designed to measure the biologic function of the antibodies by determining whether anti-MSP142 antibodies can inhibit merozoite invasion into erythrocytes. Analysis of preclinical immunogenicity studies in a variety of animals has shown a positive correlation between anti-MSP142 antibody levels and inhibition of parasite growth in vitro [28] (L. B. Martin, et al., unpublished data). Immunoglobulin G (IgG) was purified from day 0 and 42 sera of the 80-μg dose cohorts. Subsequently, IgG was also purified from days 180 and 194 sera of all volunteers with an anti-MSP142 ELISA level on day 194 greater than 500 antibody units ($n = 7$ from the MSP142-FVO/Alhydrogel and $n = 8$ from the MSP142-3D7/Alhydrogel). The IgG was tested for activity in the GIA using both the FVO and 3D7 *P. falciparum* lines.

None of the IgG samples purified from volunteer sera either on day 42 or 194 showed growth inhibitory activity higher than 20% (unpublished data) when tested at an IgG concentration in the assay of 2.5 mg/mL (approximately 25% of in vivo levels). Purified IgG from day 194 samples with an
ELISA concentration of >500 units were also tested at a concentration of 5 mg/mL (ELISA units in the assay ranged from 200–1,400 units) and there was small but significant activity (GIA on FVO parasites: mean 11.9, 95% confidence interval: 9.4 to 14.4; GIA on 3D7 parasites mean: 3.6, 95% confidence interval 2.1 to 5.1). Significant correlations were obtained between the level of anti-MSP142 antibody on day 194 and percent growth inhibition of both the FVO and 3D7 parasite lines (Spearman rank correlation, $r = 0.56$, $p = 0.0177$ and $r = 0.8394$, $p = 0.0001$, respectively).

Parasite IFA. The ability of 53 of 54 volunteer’s sera obtained at day 0 and day 194 to recognize the native MSP1 protein on *P. falciparum* FVO and 3D7 parasites was evaluated. Sera from all individuals (diluted 1:100) were evaluated for intensity of staining and the staining pattern of parasitized RBC.

Of the samples tested, minimal diffuse fluorescence was detected in the day 0 serum samples. After three vaccinations with MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel, half of the day 194 serum samples (27 of 54 sera on FVO parasites and 24 of 54 on 3D7 parasites) exhibited positive fluorescence staining of trophozoites (characteristic surface pattern) and schizont (morphology of grape-like clusters) blood-stage parasites as well as surface pattern of free merozoites (unpublished data). Regardless of the vaccine, the immune sera recognized the homologous and the heterologous native parasite antigen similarly (Wilcoxon signed rank test, $p =$...
Figure 6. IgG Subclass Analysis of Antibodies Generated by Vaccination with MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel

Day 194 serum samples from seven volunteers vaccinated with MSP142-FVO/Alhydrogel and eight with MSP142-3D7/Alhydrogel were assayed for IgG subclasses against the immunizing antigen. Serum samples were diluted 1:100 and mixed with beads coupled with homologous MSP142 antigen. Mouse anti-human IgG isotypes and donkey anti-mouse-IgG PE labeled was added to develop the reaction. The mean fluorescence intensity of specific IgG subclasses to MSP142 is presented.
doi:10.1371/journal.pctr.0020012.g006

0.6509). Intensity of staining in the IFA correlated with MSP142 ELISA units at day 194 (Spearman rank correlation, \( r = 0.7628 \) with \( p < 0.0001 \) for FVO parasites and ELISA, and \( r = 0.7726 \) with \( p < 0.0001 \) ELISA for 3D7 parasites and ELISA).

DISCUSSION

Interpretation

This study compared the safety and immunogenicity of two dimorphic forms of the MSP142 antigen in humans. The results of this trial demonstrate both MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel are safe when administered to healthy adult malaria-naïve volunteers. The safety profile of these blood-stage vaccines supports the further development of a vaccine containing both proteins, referred to as MSP142-C1.

An important secondary objective was to compare the ability of the MSP142-FVO and MSP142-3D7 vaccines to elicit specific antibody responses in malaria-naive individuals after primary immunization and revaccination. For both MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel groups, no antibody response to the homologous immunogen was detected 2 wk after the first vaccination, but an increase in specific antibody was observed in volunteers after the second and third immunizations of either vaccines. These observations suggest that the initial immunizations induced B cell memory and revaccination boosted memory B cell responses. The proportion of responders in all dose cohorts increased markedly after each vaccination.

Generalizability

MSP142-FVO and MSP142-3D7 polypeptides provide multiple B cell and T cell epitopes [15,16], and their amino acid compositions differ by greater than 50%, predominantly in the N-terminal 33-kDa region. The highly conserved C-terminal 19-kDa domain of MSP142 is preferentially recognized by antibodies. Antibodies to MSP119 prevent invasion and growth of the parasites in erythrocytes [29–31], and are also associated with protection against severe malaria [32–37]. Several T cell epitopes have been mapped to the dimorphic N-terminal 33-kDa region [16].

Preclinical studies showed overall antibody responses to MSP142-FVO were greater than MSP142-3D7. Although antibodies generated in response to vaccination cross-reacted with both forms of the protein, they preferentially recognized the homologous protein by ELISA and the homologous parasite by GIA (L. B. Martin, et al., unpublished data). In contrast, data from this clinical trial showed MSP142-FVO/Alhydrogel and MSP142-3D7/Alhydrogel were equally immunogenic, and the antibodies elicited by each vaccine recognized the homologous and heterologous MSP142 recombinant proteins and native parasite proteins qualitatively similarly.

Antibody levels to MSP142 protein correlated with antibody levels to both the MSP119 and to MSP133 proteins. These data indicate that both the conserved 19-kDa domain and the dimorphic 33-kDa region of MSP142 are recognized by B cells and stimulate antibody responses. However, the majority of the antibodies induced by vaccination were targeted to the conserved domain, MSP119. The biological impact of the low levels of anti-MSP133 antibodies to the homologous protein is unknown. These data suggest that there is little difference in the antibody response generated by the dimorphic forms of MSP142. The data from this study are in contrast to that obtained following vaccination with FMP1/AS02A where the resulting antibodies preferentially recognized the homologous antigen in malaria-naïve [19] and malaria-experienced [20,38] adults.

The nature of the MSP142-specific antibody response and T cell immune response generated in the volunteers of the current study were found to be qualitatively different. Antibodies generated following vaccination with either MSP142-FVO/Alhydrogel or MSP142-3D7/Alhydrogel were highly cross-reactive in their ability to recognize the FVO or 3D7 form of MSP142 and MSP119, whereas the low-level antibody to the dimorphic MSP133 region as allele-specific. This suggests limited strain specificity in the majority of the antibody response. In contrast, cytokine ELISPOT analysis of MSP142-specific T cell responses revealed a strong preference toward the immunizing antigen with limited activation by the alternate form of MSP142 (C. Huanan, et al., unpublished data). The epitopes responsible for the cytokine production were localized to the N-terminal 33-kDa region of MSP142. This suggests that the development of a memory response following MSP142 vaccination may require the inclusion of the relevant T-helper epitopes from the dimorphic region.

While there is considerable experimental support for the choice of MSP142 as a vaccine candidate, the selection of this protein is potentially complicated by the dimorphism observed in different field isolates [39]. The levels of cellular and humoral immune responses required to protect against severe malaria are unknown. Thus, the requirements for a blood-stage vaccine to sensitize and potentiate appropriate immune responses that would protect individuals, particularly children, against malaria in areas of varying endemicity are even more speculative. Dramatically different immune responses have been reported in adult volunteers vaccinated
with FMP1/AS02A in Mali and Kenya, areas of Africa that vary in parasite transmission [21]. This suggests that in a malaria-endemic area where multiple allelic forms of the parasite are present, a combination MSP142-FVO and MSP142-3D7 vaccine (MSP142-C1) to prime for or boost immune responses may be required.

Overall Evidence
The anti-MSP142 antibodies induced by either MSP142 vaccine recognized the native parasite protein, as shown by immunofluorescence microscopy, but did not give high levels of activity in the in vitro parasite growth inhibition assay at the concentrations tested. Vaccine-induced MSP142 antibodies from preclinical studies in animals have shown substantial GIA activity, but generation of this activity requires either multiple vaccinations or formulation with other adjuvants [28,40] (L. B. Martin, et al., unpublished data). Biochemical stability studies and in vivo potency studies conducted over the course of the clinical trial have confirmed that the antigens in each of the formulations retained conformation. Therefore, it was concluded that the two MSP142/Alhydrogel formulations are not sufficiently immunogenic to generate an antibody response able to block parasite invasion of erythrocytes detectable by the in vitro growth inhibition assay. On the basis of the correlation observed, it is estimated that at least a 10-fold higher antibody response will be needed before substantial GIA activity will be observed. Thus, if in vitro parasite GIA is found to be a good predictor of vaccine-induced protection of infants and children from malaria, then enhancing the immunogenicity of Alhydrogel-formulated MSP142 will be an important step toward developing a useful vaccine. MVDB is pursuing the addition of immunostimulants to the Alhydrogel formulation of MSP142-C1, the combination of MSP142-FVO and MSP142-3D7, to elicit higher immune responses in humans.

SUPPORTING INFORMATION

CONSORT Checklist
Found at doi:10.1371/journal.pctr.0020012.sd001 (50 KB DOC).

Trial Protocol Version 7.1
Found at doi:10.1371/journal.pctr.0020012.sd002 (601 KB DOC).

Accession Numbers
EcMSP142-FVO (AY343089) and EcMSP142-3D7 (DQ414722) were expressed as histidine-tagged fusion proteins from synthetic genes from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). Also included are plasmids EcMSP142-FVO (DQ92123) and EcMSP142-3D7 (DQ92124).

ACKNOWLEDGMENTS
The Phase 1 trial was conducted at Quintiles Phase 1 Services, Lenexa, Kansas. We are grateful to all trial site personnel, particularly Carmen Letcher, for the recruitment of volunteers and their execution of the clinical trial. We would like to extend our thanks to the Molecular Biology, Fermentation, Purification, Quality Control, Formulation, Immunology, Animal, and Clinical groups at MVDB/NIADDK/NIAIDNIH. We are also indebted to Drs. Giny Fouda and Diane Taylor for assistance with the Lumines assay for antibody subclasses and to Dr. Ruth Ellis for critical review of the manuscript. Special thanks go to the volunteers who participated in the trial.

Author Contributions
EM, CAL, ET, PL, SM, LHM, AS, and LBM designed the study. EM, CAL, MF, PL, SM, AS, and LBM analyzed the data. PL was the principal investigator and enrolled subjects. EM and LBM wrote the paper. CAL, MF, SM, LHM, and AS contributed to the paper. ACO, OML, SF, HZ, and AD performed experiments and collected data. AWS, LZ, SS, NM, DLN, APM, AS, and LBM were responsible for the generation, characterization, and release of the vaccines. EM, ET, and PL had access to the primary clinical data.

Funding
This work was supported by the Intramural Program of the National Institute of Allergy and Infectious Diseases/National Institutes of Health. Conduct of the clinical trial at Quintile Phase 1 Services was supported through project management by the PATH Malaria Vaccine Initiative and with funding from the Bill and Melinda Gates Foundation.

Abbreviations
ELISA, enzyme linked immunosorbant assay; GIA, growth inhibition assay; IFA, indirect immunofluorescence assay; MSP1, merozoite surface protein 1; MSP142, C-terminal domain of MSP142; MSP535, N-terminal region of MSP142; MSP142-g, 42 kDa C-terminal region of MSP1

REFERENCES


