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Phase I Dose Escalation Safety and Immunogenicity Trial of *Plasmodium falciparum* Apical Membrane Protein (AMA-1) FMP2.1, Adjuvanted with AS02A, in Malaria-Naive Adults at the Walter Reed Army Institute of Research

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Phase I dose escalation safety and immunogenicity trial of *Plasmodium falciparum* apical membrane protein (AMA-1) FMP2.1, adjuvanted with AS02A, in malaria-naïve adults at the Walter Reed Army Institute of Research☆

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Abstract

We report the first safety and immunogenicity trial of the *Plasmodium falciparum* vaccine candidate FMP2.1/AS02A, a recombinant *E. coli*-expressed protein based upon the apical membrane antigen-1 (AMA-1) of the 3D7 clone formulated with the AS02A adjuvant. We conducted an open-label, staggered-start, dose-escalating Phase I trial in 23 malaria-naïve volunteers who received 8, 20 or 40 μg of FMP2.1 in a fixed volume of 0.5 mL of AS02A on a 0, 1, and 2 month schedule. Nineteen of 23 volunteers received all three scheduled immunizations. The most frequent solicited local and systemic adverse events associated with immunization were injection site pain (68%) and headache (29%). There were no significant laboratory abnormalities or vaccine-related serious adverse events. All volunteers seroconverted after second immunization as determined by ELISA. Immune sera recognized sporozoites and merozoites by immunofluorescence assay (IFA), and exhibited both growth inhibition and processing inhibition activity against homologous (3D7) asexual stage parasites. Post-immunization, peripheral blood mononuclear cells exhibited FMP2.1-specific lymphoproliferation and IFN-γ and IL-5 ELISPOT assay responses. This is the first PfAMA-1-based vaccine shown to elicit both potent humoral and cellular immunity in humans. Encouraged by the potential of FMP1/AS02A to target host immunity against PfAMA-1 that is known to be expressed by sporozoite, hepatic and erythrocytic stages, we have initiated field trials of FMP2.1/AS02A in an endemic population in the Republic of Mali.

Keywords: Apical membrane antigen; Processing inhibition assay; FMP2.1; AS02A; Malaria; Vaccine; Falciparum; Recombinant protein; Growth inhibition assay

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1. Introduction

Malaria kills three children every minute, and afflicts hundreds of millions of people each year [1]. The spread of drug-resistant parasites, the lack of affordable effective antimalarial drugs, and the inadequate use of insecticide-impregnated bednets and household pesticides makes urgent the quest to develop and deploy an effective malaria vaccine [2].

The *Plasmodium falciparum* protein known as "apical membrane antigen-1 (PfAMA-1)" is expressed in the sporozoite [3], hepatic [4,5] and erythrocytic stages [6], and this provides the rationale for a vaccine strategy that targets host humoral and cellular immune responses against this protein therefore targeting these three phases of parasite development.

PfAMA-1 is a promising candidate vaccine antigen that has not yet been evaluated for protective efficacy in clinical trials. Evidence exists that such a strategy is worthwhile. Natural exposure to *P. falciparum* infection is associated with both B-cell and T-cell responses to recombinant PfAMA-1 and to PfAMA-1 peptides [7–9]. Naturally acquired antibody to PfAMA-1 in endemic populations is associated with protection from falciparum malaria [10,11]. Immunization of New World monkeys with recombinant PfAMA-1 formulated with Freund's adjuvant has conferred significant protection against homologous *P. falciparum* challenge [12,13], but limited protection when formulated with adjuvants intended for human use such as Montanide or AS02A [Barnwell, unpublished]. Several PfAMA-1 vaccines are in development, but none have been tested for clinical efficacy [14–20]. A recent Phase I clinical trial of a recombinant PfAMA-1 antigen adjuvanted with alhydrogel showed that this formulation elicited functional antibodies that, after affinity purification, exhibited growth inhibition of *P. falciparum in vitro* [21].

At the Walter Reed Army Institute of Research (WRAIR) Pilot Bioproduction Facility, we produced a recombinant protein, designated FMP2.1 [22], representing a major portion of the ecto-domain of PfAMA-1 from the 3D7 clone of *P. falciparum*. FMP2.1 was formulated in the GlaxoSmithKline proprietary adjuvant system, AS02A, which had been shown to enhance the immunogenicity and efficacy of the circumsporozoite-based malaria antigen, RTS,S [23–28]. RTS,S formulated with AS02A (RTS,S/AS02A) has protected approximately 41% of malaria-naïve humans against challenge with *P. falciparum* sporozoites [25]. RTS,S/AS02A efficacy in a field trial was 35% (95% CI 22–47; $p < 0.0001$) for protection against first clinical episodes and 49% (95% CI 12–71; $p = 0.02$) for protection against severe malaria during an 18 month period in young African children [27,28].

Immunization of rabbits with a pre-clinical lot of FMP2.1 formulated with AS02A-induced functional antibodies with strong growth inhibitory and processing inhibition activity in *in vitro* assays [29,30]. Evaluation of clinical grade (cGMP, current Good Manufacturing Practice) FMP2.1 formulated with AS02A in a standard rhesus monkey model demonstrated that the vaccine was safe, minimally reactogenic, and able to induce potent cellular and humoral immune responses, including growth inhibitory antibodies against homologous *P. falciparum* 3D7 parasites (Stewart, unpublished).

One strategy to augment the partial protective efficacy of the RTS,S/AS02A vaccine is to combine it with one or more antigens independently proven to limit or prevent parasitemia such as FMP2.1 [31]. Accordingly, we conducted the present Phase 1 dose-escalation trial at the WRAIR Clinical Trials Center in malaria-naïve adults to establish a preliminary safety and immunogenicity profile before proceeding to clinical trials of FMP2.1/AS02A in endemic populations in the Republic of Mali.

2. Materials and methods

2.1. Vaccine

FMP2.1 antigen (Lot 0971), representing amino acids #83-531 of the *P. falciparum* (clone 3D7) AMA-1 protein, was manufactured according to current good manufacturing practices (cGMP) at the Walter Reed Army Institute of Research Bioproduction Facility at Forest Glen, Maryland [22]. The final FMP2.1 product was a single dose vial containing approximately 43 μg of lyophilized protein. Just prior to immunization, the lyophilized protein was mixed with AS02A such that approximately 8, 20 or 40 μg of FMP2.1 was delivered in a final volume of 0.5 mL of AS02A. The adjuvant AS02A (Lot AS02A012A9), manufactured by GlaxoSmithKline Biologicals (Rixensart, Belgium), consists of an oil-in-water emulsion, and the immunostimulants 3-deacylated monophosphoryl lipid A (MPL) (GSK Biologicals, Rixensart, Belgium) and QS21 derived from *Quillaja saponaria* (Antigenics Inc., Lexington, MA, USA).

2.2. Ethics and monitoring

This clinical study was conducted under a protocol reviewed and approved by the Human Use Review Committee of the Walter Reed Army Institute of Research and by the Human Subjects Research and Review Board of the Surgeon General of the U.S. Army at Fort Detrick, Maryland. The study protocol was submitted to the U.S. Food and Drug Administration for review as part of Investigational New Drug application BB-IND #11140. The study was monitored for regulatory compliance and data quality assurance by the United States Army Medical Material and Development Activity, Fort Detrick, Maryland and by GlaxoSmithKline Biologicals, Rixensart, Belgium. After obtaining written informed consent, volunteers were screened by history, physical examination and laboratory testing to determine their eligibility for enrollment.

2.3. Protocol

The study was an open-label, staggered-start, dose-escalating Phase 1 trial intended to determine the safety, reactogenicity and immunogenicity of three intramuscularly administered doses of FMP2.1/AS02A in healthy malaria-naïve adults at the WRAIR Clinical Trials Center.

Volunteers were sequentially assigned to one of three dosage groups (Groups A, B, and C). Group A received approximately 8 μg FMP2.1 in 0.5 mL AS02A; Group B, approximately 20 μg FMP2.1 in 0.5 mL AS02A; and Group C, approximately 40 μg FMP2.1 in 0.5 mL AS02A.

2.3.1. Participants

Participants were adult males and females 18–45 years of age. Exclusion criteria included history of malaria, previous receipt of a malaria vaccine, splenectomy, known or suspected immunosuppression, use of systemic steroids, recent receipt of any investigational or non-registered drug or vaccine, simultaneous participation in any other clinical trial, receipt of immunoglobulin or any blood product transfusion within 3 months of study start, abnormal screening laboratories (CBC, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), or positive serum beta-human chorionic gonadotrophin), serologic evidence of active hepatitis B or C infection, antibody to HIV, or any other clinically significant acute or chronic disease that might confound the interpretation of study results.

2.3.2. Immunization procedures

Vaccine was administered by injection into the deltoid of the non-dominant arm on a 0, 1, and 2 month immunization schedule beginning with Group A. There was a minimum 14-day interval between group immunizations to allow for safety evaluation of the previous group. Follow-up evaluations occurred on days 1, 2, 3, 7, and 14 after each immunization and at 2, 4, and 6 months after the third immunization.

2.3.3. Adverse events

The volunteers were observed for 30 min after each immunization for evidence of anaphylaxis. The presence of solicited local and general signs and symptoms, including measurement of oral temperature, were assessed after each immunization and 1, 2, 3 and 7 days post-immunization. The solicited injection site adverse events were pain, redness and swelling. Solicited general adverse events were fever, gastrointestinal complaints, fatigue, headache, malaise, myalgia and joint pain. In addition to the solicited signs and symptoms, investigators recorded any other adverse events occurring within a 28-day follow-up period (day of immunization and 27 subsequent days) as unsolicited adverse events. Adverse events were assessed for intensity. Injection site pain was graded as 0 = absent, 1 = painful on touch, 2 = painful when limb is moved, and 3 = spontaneously painful. Solicited symptoms were graded as 0 = normal, 1 = easily tolerated, 2 = interferes with normal activity, and 3 = prevents normal daily activity. Additional grading scales were applied to visible swelling or redness at the injection site; 0 = none, 1 = 0–20 mm, 2 = 20–50 mm, and 3 = >50 mm, and to oral temperature; 0 = <37.5 °C, 1 = 37.5–38 °C, 2 = 38–39 °C, and 3 = >39 °C.

Serious adverse events (SAEs) were reported from enrollment until study completion 6 months after final immunization. SAEs were defined as any untoward medical occurrence that resulted in death, significant disability, hospitalization, incapacity, or required intervention to prevent such outcomes.

Biochemical (ALT, AST, and Cr) and hematological (Hb, hct, WBC, and PLTs) laboratory parameters were measured at screening, and on days of immunization, 2 weeks after each immunization and at 2, 4 and 6 months after the third immunization.

2.4. Serology

2.4.1. Anti-FMP2.1 ELISA

IgG antibody to the test antigen was measured by enzyme-linked immunosorbent assay (ELISA) in all volunteers at baseline, 2 weeks after each immunization and 2, 4, and 6 months after the third immunization. IgG ELISAs were performed, using FMP2.1 as the capture antigen, in serial two-fold dilution, and the titer defined as the serum dilution required to yield an optical density of 1.0 in our assay.

2.4.2. Sporozoite and merozoite immunofluorescence assay (IFA)

Blood stage late schizonts were fixed with methanol and immunofluorescence assays (IFA) were performed as described previously [22]. Briefly, 2% fetal bovine serum containing PBS was used as a diluent and serial dilutions starting from 1:2 to 1:25,600 were tested. FITC-conjugated secondary antibodies diluted 1:500 (Southern Biotech, Birmingham, AL, USA) were used and slides were mounted in SlowFade® Antifade Kit with DAPI (4′,6′-diamidino-2-phenylindole) (Molecular Probes, Inc. Eugene, OR, USA). Sporozoite IFA utilized an acetone fixed preparation of sporozoites and was performed with two pre-immunization and post-third immunization serum samples from Group C. The methodology was similar to the blood stage IFA except the mounting solution did not contain DAPI. An Olympus BX50 microscope equipped with a mercury epifluorescence lamp, a 100× (oil) objective and a multi filter cube was used to observe the fluorescence.

2.4.3. Growth invasion/inhibition assay (GIA)

Sera were tested for growth inhibitory effects against homologous 3D7 and heterologous FVO *P. falciparum* parasites in a one-cycle static assay [32]. Three time points for each volunteer were evaluated: baseline, 2 weeks after second immunization, and 2 weeks after third immunization. The parasites were cultured for 2 days in heat inactivated, dialyzed

sera (20%, v/v) at a 0.2% initial parasitemia (trophozoites) and 4% hematocrit in triplicate 150 μL static culture volumes in 48-well plates. Cultures were harvested and stained with Hoechst dye 33342 (Molecular Probes, Eugene, OR, USA) and the number of new trophozoites were counted in 40,000 erythrocytes by flow cytometry. Inhibition was calculated from final parasitemias as inhibition = (control-test)/control, where control was the final parasitemia with pre-immune serum, and was expressed as a percent.

2.4.4. Processing inhibition assay (PIA)

We have described an immuno-chemical correlate of anti-AMA-1 antibodies that measures the ability of these antibodies to inhibit the natural proteolytic processing of PfAMA-1 on the merozoite surface [29,30]. We conducted a parallel GIA and PIA on sera collected 2 weeks post third immunization. The PIA was performed in a 48-well plate format contained 80 μL of purified late-stage schizonts of the 3D7 strain of *P. falciparum* at a concentration of 1×10^7 mL^{-1} and 20 μL test serum. The plate was gassed with 5% CO_2 and incubated at 37 °C until > 90% of schizonts ruptured. The parasites were then collected by centrifugation and analyzed by Western blot with biotin labeled polyclonal anti-AMA-1 and biotin labeled monoclonal antibody against the C-terminus of AMA-1, mAb 28G2dc1. This antibody was a kind gift of Dr. Alan W. Thomas, Biomedical Primate Research Center, Rijswijk, The Netherlands. The PIA ratio was calculated as band intensities of the 10-kDa/(10-kDa + 20-kDa) AMA-1 specific bands on the Western blot [29]. The GIA plate contained 80 μL of 0.5% parasitemia schizonts + normal human RBC at 4% hematocrit and 20 μL of test serum in triplicates. Plates were gassed and incubated overnight at 37 °C; the percent invasion was determined as described above, except the ring stages were stained by SYBR® Green (Molecular Probes, Eugene, OR) dye for 1 h.

2.4.5. Cell-mediated immunity

For cellular reactivity of FMP2.1-immune cells we measured lymphoproliferation as well as IFN-γ and IL-5 secretion. Briefly, cryopreserved and thawed peripheral blood mononuclear cells (PBMC) obtained at pre- and post-third immunization were re-suspended in RPMI plus additives and 5% human AB serum. For proliferation, cells were cultured at a concentration of 2×10^5 cells/well in triplicates in 96-well round bottom microtiter plates in the presence of FMP2.1 protein (10 μg/mL), PHA (2 μg/mL), or medium control. After 5 days cultures were pulsed with ^{3}H-TdR (1 μCi/well) and harvested after an additional 16 h. Incorporation of radioactivity was measured by scintillation spectrometry and results are expressed as counts per minute (cpm). Stimulation indices (SI) were determined according to the following formula:

$$\mathrm{SI} = \frac{\text{cpm in experimental culture}}{\text{cpm in control cultures}}.$$

IFN-γ and IL-5 responses were evaluated by the ELISPOT assay. One hundred microlitres of cell suspensions (2×10^6 mL^{-1} for IFN-γ and 4×10^6 mL^{-1} for IL-5) were cultured in duplicates or triplicates in the presence of 0, 0.1, 1.0 or 10 μg/mL FMP2.1 or 0.4 μg/mL PHA in ELISPOT wells coated previously with 100 μL/well PBS containing 10 μg/mL anti-IFN-γ or 15 μg/mL anti-IL-5 (Mabtech, Mairemont, OH, USA). After 18 h of culture in a 37 °C, 5% CO_2 humidified atmosphere, the plates were washed with PBS, and 100 μL of 1:100 dilution of appropriate biotinylated detecting antibody (Mabtech) were added per well, and plates were left for 2 h at room temperature. After washing, 100 μL of a 1:1000 dilution of streptavidin–alkaline phosphatase conjugate (Mabtech) were added per well and the plates were left for an additional 1.5 h at room temperature. The plates were then washed and the ELISPOTs were developed by the addition of alkaline phosphatase substrate (Moss Laboratories). The number of spots was counted with the aid of an Immunospot Image Analyzer (Cellular Technology, Cleveland, OH). Results are expressed as the mean ± S.E.M. of triplicate wells for IFN-γ and duplicate wells for IL-5.

2.5. Statistics and data management

Data were entered into a Microsoft Excel spreadsheet data base and analyzed using Excel statistical tools. In the case of GIA analyses, data were compared using the Wilcoxon signed-rank test (non-directional) with paired pre-immune sera. Differences between groups were compared using the Mann–Whitney-Wilcoxon rank-sum test (non-directional).

3. Results

3.1. Participant flow

The clinical portion of the study was conducted at the Clinical Trials Center at the Walter Reed Army Institute of Research from September 2003 to July 2004. Fifty-two volunteers were screened. Twenty-three were eligible, enrolled and allocated to one of the three dosage groups: Group A (8 μg FMP2.1 in 0.5 mL AS02A, $n = 8$), Group B (20 μg FMP2.1 in 0.5 mL AS02A, $n = 8$), and Group C (40 μg FMP2.1 in 0.5 mL AS02A, $n = 7$). Four volunteers did not complete the immunization series. One volunteer from Group A was withdrawn by the PI after first immunization due to poor compliance with follow-up. One volunteer from Group B was withdrawn by the PI after the first immunization due to a severe adverse event not-related to immunization. Two volunteers from Group C withdrew after the second immunization; one withdrew without explanation, the other withdrew due to Grade 3 adverse events associated with immunization. Actual versus scheduled immunizations were as follows: Group A; 22 of 24, Group B; 22 of 24, and Group C; 19 of 21. During the 6 month post-immunization period, an additional three volunteers did not complete follow-up; one in Group A and two in group C. Thus, 16 of 23 volunteers completed the study according to protocol (Fig. 1).

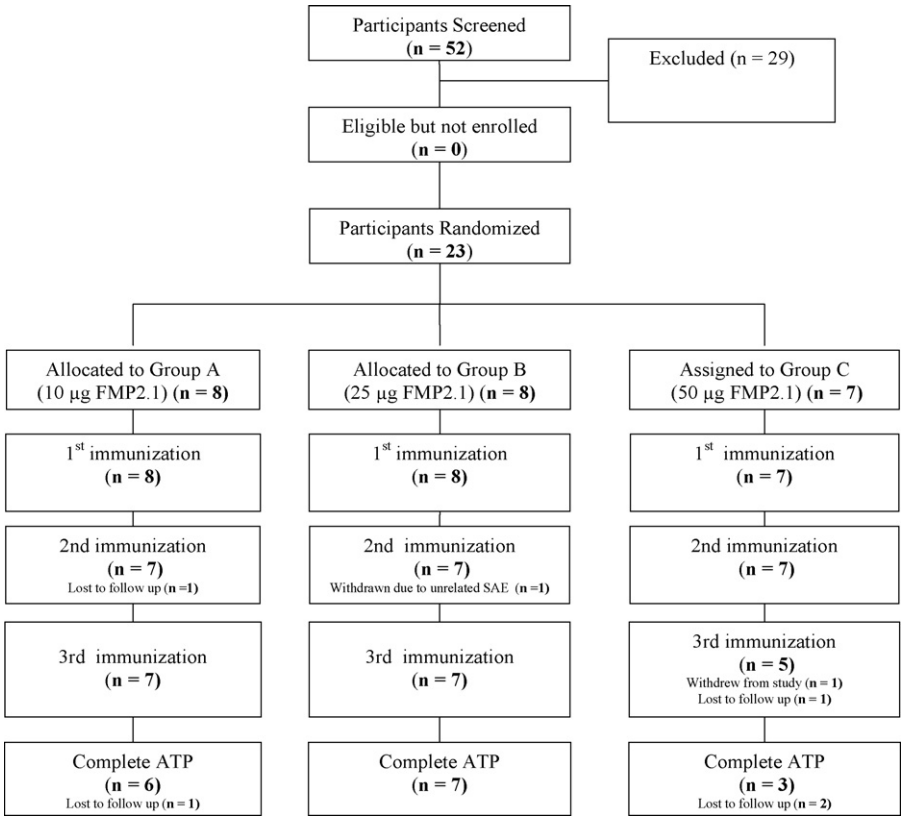

Fig. 1. Volunteer flow diagram.

3.2. Safety and reactogenicity

Adverse events (AEs) by group are summarized in Table 1. All solicited vaccine-related AEs occurred within 72 h after vaccination. Sixteen Grade 3 reactions were reported with 12 of 16 occurring in the 40 μg dosage group and the remaining 4 occurred in the 20 μg dosage group. Five of the Grade 3 reactions occurred in one individual in the 40 μg group after the second vaccination and were self-reported, as the individual did not return for follow-up during the time he was symptomatic. Local pain (43 incidents over 63 vaccinations), local swelling (10 incidents over 63 vaccinations), myalgia (14 incidents over 63 vaccinations), and headache (18 incidents over 63 vaccinations) accounted for most of the solicited AEs. Almost all adverse events resolved within the first 72 h after immunization. No clinically significant biochemical or hematological abnormalities were associated with immunization.

3.2.1. Serious adverse events (SAEs)

There was one SAE, and it was judged by the Principal Investigator and the Medical Monitor to be not related to

Table 1
Instances of local and systemic solicited adverse events recorded during first 7 days after immunization summarized by group and by grade

	Group A 8 μg FMP2.1 in 0.5 mL AS02A			Group B 20 μg FMP2.1 in 0.5 mL AS02A			Group C 40 μg FMP2.1 in 0.5 mL AS02A		
	Grade1	Grade2	Grade3	Grade1	Grade2	Grade3	Grade1	Grade2	Grade3
Pain	6	4	0	6	13	1	6	7	0
Redness	0	0	0	0	0	1	4	0	2
Swelling	1	1	0	2	0	1	4	1	0
Fever	0	0	0	0	1	1	2	0	2
GI	0	0	0	3	1	0	3	2	0
HA	2	0	0	3	5	0	3	2	3
Malaise	0	0	0	1	2	0	1	1	2
Myalgia	2	1	0	3	2	0	3	2	1
Fatigue	0	0	0	2	2	0	2	4	1
Arthralgia	0	0	0	0	1	0	1	1	1
Total	11	6	0	20	27	4	29	20	12

Five Grade 3 events in Group C occurred in one volunteer after the second immunization. All solicited adverse events occurred within 72 h after immunization.

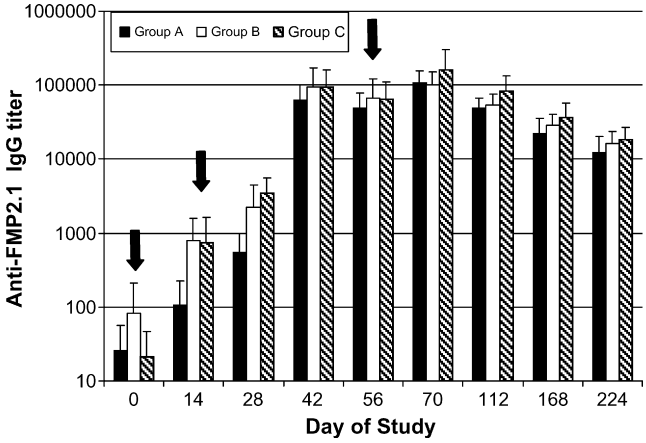

Fig. 2. Anti-FMP2.1 titers by vaccine group. Each bar represents the group average at a specific time expressed as a titer. Error bars depict one standard deviation. Immunizations occurred on Days 0, 28 and 56 as indicated by arrows.

immunization. Two days after first immunization, a female volunteer from Group B (20 μg dose group) presented with palpitations, lightheadedness, and tachycardia by electrocardiogram. An extensive cardiology evaluation determined that her symptoms and signs were consistent with a pre-existing condition (paroxysmal supraventricular tachycardia) that was deemed not causally related to immunization.

3.3. Serology

3.3.1. Anti-FMP2.1 antibody by ELISA

There was a robust three-$\log^{10}$ anti-PfAMA-1 antibody (Ab) response after two immunizations in all three vaccine groups as determined by ELISA (Fig. 2). After the second immunization, the antibody response to all three dosages was similar with no statistical differences between groups by analysis of variance. Increases in antibody titer were most pronounced after first and second immunization, with no significant increase after third immunization. During the 6 months of follow-up after the third immunization, antibody titers decayed by approximately one $\log^{10}$. All volunteers in all three dosage groups seroconverted to FMP2.1 following the second immunization. The average coefficient of variation of log titer at all subsequent time points after the second injection was less than 10% for all vaccine groups.

3.3.2. Antibody to P. falciparum merozoites

IFAs were conducted using sera collected after the third immunization for 17 volunteers. Baseline IFA titers of the pre-immune sera were 0–50. Fourteen of 16 volunteers developed a positive IFA titer ≥200. Median and range of IFA titers for each group were as follows: Group A, 300 (0–25,600); Group B, 4800 (800–12,800) and Group C, 1600 (800–6400). Fig. 3A shows the typical pattern of AMA-1 distribution, namely apical fluorescence of individual merozoites within a schizont [22].

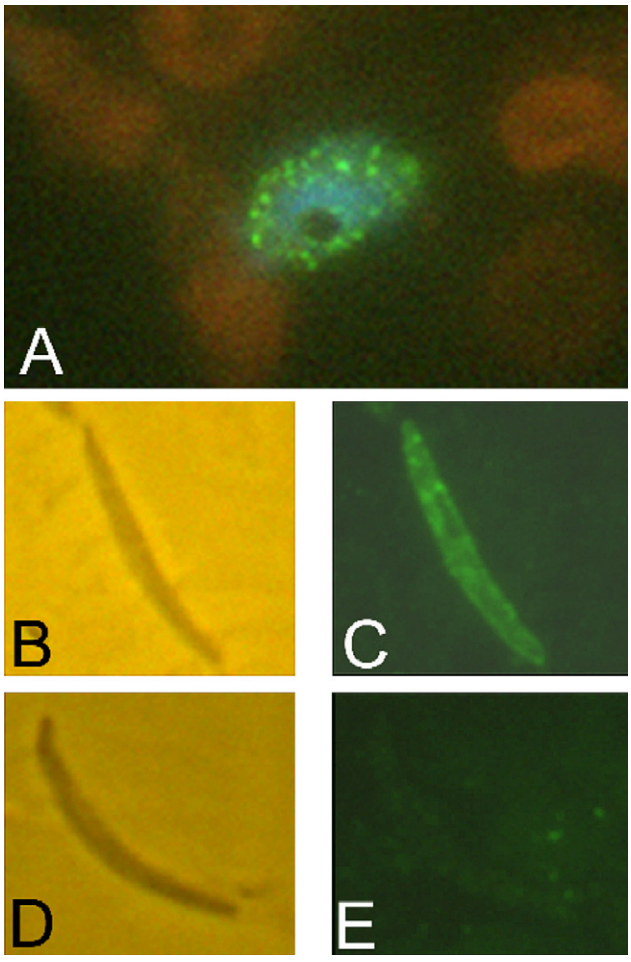

Fig. 3. Merozoite and sporozoite IFA results. (A) Blood stage schizont immuno-stained with post third immunization sera at 1:800 dilution (primary antibody). (B) Phase contrast and (C) fluorescence images of a sporozoite immuno-stained with post third immunization sera at 1:100 dilution. (D) Phase contrast and (E) fluorescence image of a sporozoite stained with pre-immune sera at 1:100 dilution.

3.3.3. Antibody to P. falciparum sporozoites

Sera selected from two volunteers in Group C on the basis of high post third immunization anti-FMP2.1 titers reacted with sporozoites. Post third immunization sera, but not pre-immunization sera, from both of these two individuals yielded a characteristic apical and circumferential flourescence pattern [3]. Fig. 3B–E shows representative fluorescence patterns from one volunteer's pre-immune sera (Fig. 3D and E) and post-third immunization sera (Fig. 3B and C).

3.3.4. Growth/invasion inhibition assay

Significant inhibition of merozoite invasion was induced in the static *in vitro* assay against homologous *P. falciparum* 3D7 parasites. The mean inhibition of all the pre-immune sera in Groups A, B and C was found to be 1% with a range of 4 to −7% inhibition when compared to normal control human serum, Combining all the volunteers in all three groups led to significant ($p < 0.001$) average inhibition

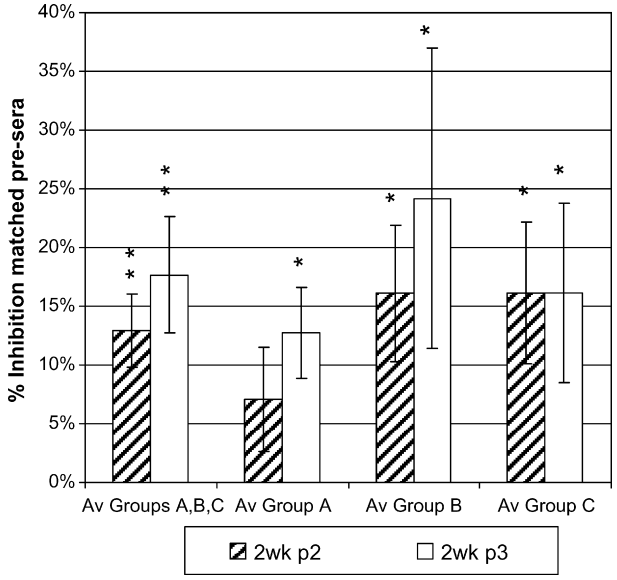

Fig. 4. Growth invasion/inhibition assay (GIA) by static culture method. *y*-Axis denotes % inhibition in growth by 1:5 diluted immune sera in comparison to matched pre-immune sera (which in turn were all within 6% of normal media controls). Comparing growth with paired pre-immune sera using the Wilcoxon signed-rank test (non-directional), $^{*}p < 0.05$ or $^{**}p < 0.001$. Error bars are standard error of the mean. Av Group = the average of all individuals within a group (6–7 volunteers) or all groups. 2wk p2 = sera collected 2 weeks post second immunization; 2wk p3 = sera collected 2 weeks post third immunization. There were no significant differences between any groups (or between any 2wk p2 or 2wk p3 or pooled groups) using the Mann–Whitney-Wilcoxon rank-sum test (non-directional, all $p > 0.1$).

after both second and third immunization (average inhibition of 13 and 17%, respectively) compared to matched pre-immunization controls (Fig. 4). Within groups, there was a trend toward higher average inhibition after the third immunization and higher average inhibition with increased antigen (20 and 40 μg dosage groups compared to the 8 μg dosage group) but this did not reach statistical significance using the Mann–Whitney-Wilcoxon rank-sum test (non-directional, all $p > 0.1$). When the same serum samples were tested against FVO parasites, a heterologous strain of *P. falciparum*, no inhibition of invasion was observed (data not shown). The persistence of inhibitory antibody was not determined.

There was a positive correlation between the ELISA titers and GIA activity of individual serum samples. The coefficient of correlation squared (R^2) was 0.38. When a single outlier that gave 82% inhibition was eliminated, R^2 increased to 0.75 (Fig. 5A).

3.3.5. Processing inhibition assay

We measured the PIA activity of 17 serum samples collected 2 weeks post third immunization and 2 pre-immune controls. The average PIA ratio for pre-immune controls was 0.13. The average and range for vaccine groups were; Group A 0.26 (0.19–0.36), Group B 0.22 (0.16–0.32), and Group C 0.21 (0.14–0.43). There was no significant difference between the three vaccine groups. The PIA activity positively correlated with GIA activity with an R^2 of 0.585

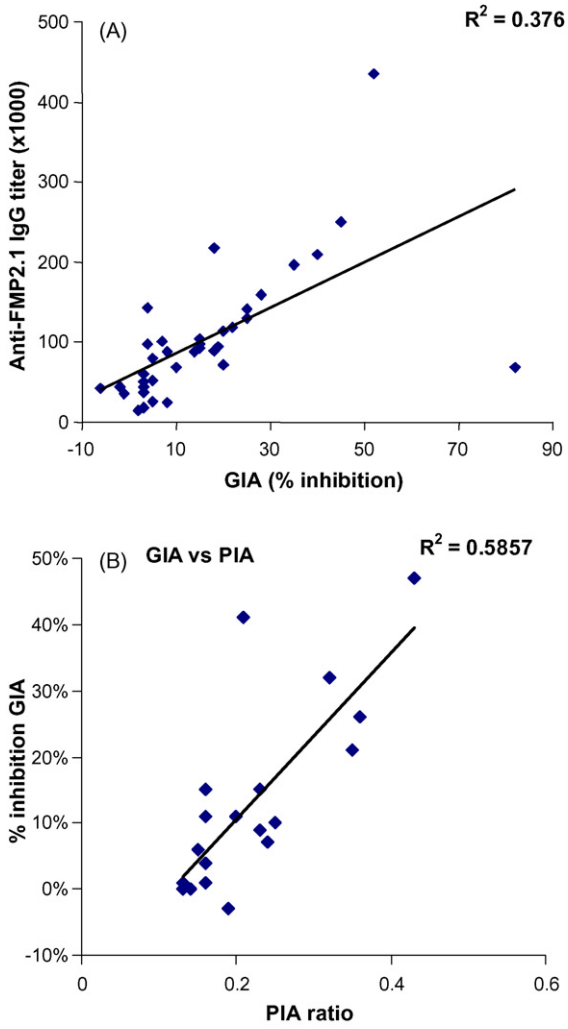

Fig. 5. (A) Correlation of ELISA and GIA. The ELISA end-point titers × 1000 (*y*-axis) were plotted against the corresponding percent inhibition in the static one-cycle GIA (*x*-axis) [30]. Data include 37 serum samples collected 2 weeks post second and 2 weeks post third immunization of the vaccine. (B) Correlation of GIA and PIA. The PIA ratio (*x*-axis) was plotted against the corresponding percent inhibition in the static one-cycle GIA (*y*-axis) [27]. Data include 17 serum samples collected 2 weeks post third immunization and two pre-immunization controls.

(Fig. 5B). The R^2 correlation between PIA versus ELISA and GIA versus ELISA was 0.471 and 0.479, respectively (graphs not shown).

3.3.6. Cell-mediated immunity

Significant lymphocyte proliferation was measured in all three groups after third immunization with stimulation indices ranging between 5 and 96 (data not shown). IFN-γ production was detected in cultures stimulated with FMP2.1 at concentrations ranging from 0.1 to 10 μg/mL; however, the most robust response was elicited with 10 μg/mL of FMP2.1 (Fig. 6) across all three groups. Means and ranges for IFN-γ were similar, that is 578 (65–995), 765 (195–995) and 750 (615–905) spots per 10^6 PBMC for Groups A, B and C, respectively. We also detected IL-5 in cultures of FMP 2.1-

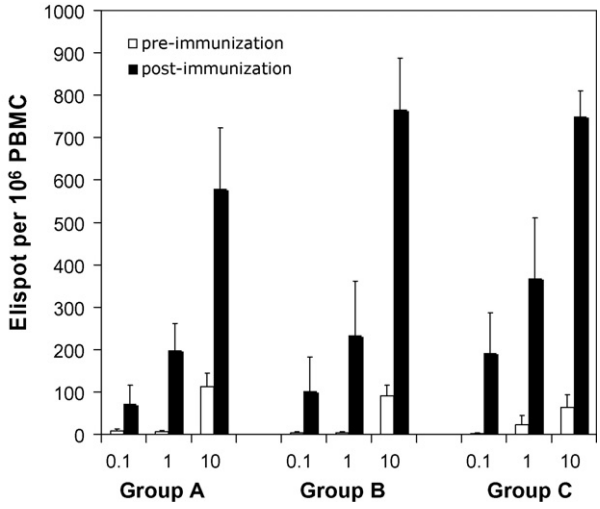

Fig. 6. IFN-γ response by ELISPOT. IFN-γ response by ELISPOT at baseline (pre-immunization) or post third dose immunization (post-immunization) by vaccine group in response to *in vitro* stimulation with 0.1, 1.0, or 10 μg of FMP2.1. Error bars represent standard error of the means (mean + S.E.M.).

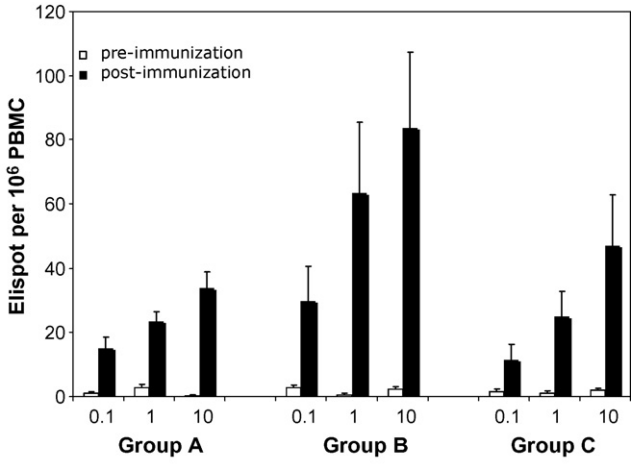

Fig. 7. IL-5 response by ELISPOT. IL-5 response by ELISPOT at baseline (pre-immunization) or post third dose immunization (post-immunization) by vaccine group in response to *in vitro* stimulation with 0.1, 1.0, or 10 μg of FMP2.1. Error bars represent standard error of the means (mean + S.E.M.).

immune PBMC, and the responses were likewise FMP2.1 concentration-dependent with 10 μg/mL eliciting maximum cytokine production (Fig. 7). IL-5 responses were of lesser magnitude than that seen for IFN-γ, with means and ranges of 34 (14–50), 84 (11–154) and 47 (15–113) spots per 10^6 PBMC for Groups A, B and C, respectively.

4. Discussion

This is the first immunization of humans with FMP2.1/AS02A. The vaccine was reactogenic but generally well-tolerated with no vaccine-related serious adverse events. The vaccine proved to be highly immunogenic inducing both humoral and cellular responses. In addition, the vaccine induced invasion and PfAMA-1 proteolytic processing-inhibitory antibodies against homologous *P. falciparum* parasites.

4.1. Safety and reactogenicity

There were apparent differences among the dosage groups in numbers of vaccine-associated adverse events in this small study, with the higher dose groups having more solicited adverse events. The duration of adverse events was similar for all the groups with the majority resolving within 72 h of immunization. However, because all the groups received the same dose of adjuvant in their vaccine formulation, any real differences in reactogenicity by dosage group would be attributable to the amount of antigen. It should be noted however, that the pattern of the 16 Grade 3 adverse events has been well documented in a small percentage of volunteers who have received other AS02A vaccine formulations, so the finding is not unique to the FMP2.1/AS02A formulation [24,25]. There were no vaccine-related clinical laboratory abnormalities.

4.2. Antibody responses

After the second immunization, there was no significant difference in antibody response between dose groups. Following three immunizations, all three dosage groups had high antibody levels by ELISA that persisted for 6 months after the last immunization. Two immunizations appear to be enough to induce a maximal antibody response, but since a third immunization was given, its impact on antibody kinetics is unclear.

The potential for FMP2.1-specific antibodies to inhibit parasite invasion of erythrocytes *in vivo* was demonstrated by the finding of functionally active antibody by both GIA and PIA in all dose groups against homologous *P. falciparum* 3D7 parasites. It is important to note that the GIA was done at 1:5 dilution, hence anti-FMP2.1 antibody levels *in vivo* would be higher and presumably more active. The trend toward greater inhibition in the GIA with higher doses of antigen did not reach statistical significance, perhaps due to small group sizes and variability in inhibition among individuals in each group. The percent growth invasion inhibition assay (GIA) also positively correlated with the ELISA and with the processing inhibitory assay (PIA) activity of anti-FMP2.1, consistent with our hypothesis that antibodies are an important component of PfAMA-1 immunity and that the processing inhibition may be causally related to inhibition of invasion [29,30]. Although GIA and PIA are not established as a correlate of protection in humans, these functional humoral response assays are likely to play an important role in the evaluation of future trials of this and other PfAMA-1 vaccines, as well as other vaccines exhibiting these or similar activities. The relevance of the absence of activity against heterologous *P. falciparum* FVO parasites in a GIA

assay remains to be determined in field trials. The *P. falciparum* FVO and 3D7 PfAMA-1 ecto-domains differ at 24 amino-acid positions which represents the two most distant haplotypes reported [33].

The demonstration that FMP2.1/AS02A-induced antibodies recognized sporozoites by IFA further suggests a potential role of this vaccine in inducing antibodies that might interfere with the invasion of sporozoites into liver hepatocytes.

4.3. Cellular immunity

The cell-mediated immune (CMI) results indicate that FMP2.1/AS02A produces a strong Th1 and a somewhat weaker Th2 response. Given the expectation that antibody may play a dominant role in controlling blood stage infection, these CMI responses suggest adequate T cell help for the B cell response. Furthermore, the CMI response may have implications beyond support of antibody response. T lymphocytes from volunteers immunized with irradiated *P. falciparum* sporozoites recognize PfAMA-1, and may contribute to sterile immunity against virulent sporozoite challenge by targeting falciparum-infected hepatocytes [4]. In AMA-1 vaccinated mice challenged with *P. chabaudi*, Xu et al. demonstrated that AMA-1-specific T-cells played a role in controlling parasitemia independent of antibody activity [34]. Taken together, these observations suggest that T-cells induced by FMP2.1/AS02A might act against PfAMA-1 expressed in pre-erythrocytic as well as on blood stages [4,5].

4.4. PfAMA-1 vaccine design

Blood stage malaria antigens, including PfAMA-1, exist as diverse alleles that elicit varying degrees of cross-reactive antibody. Sero-epidemiologic evidence [35], and the limited GIA data presented here, suggest a PfAMA-1-based vaccine may elicit allele-specific antibodies. Consequently, in addition to monovalent PfAMA-1 vaccines [14–17,21] there are also bivalent PfAMA-1 vaccines in development in an effort to broaden protective immune responses [18,19,21]. With FMP2.1/AS02A, we intend to determine if high titer antibodies elicited against sporozoite and asexual stages, as well as potent anti-PfAMA-1 cellular responses, might act against diverse alleles. For this reason, initial field trials of FMP2.1/AS02A will include allelic genotyping endpoints to determine its ability to protect against parasites bearing homologous and heterologous alleles of PfAMA-1 [36].

4.5. Conclusion

This pilot study has established the initial safety, reactogenicity and immunogenicity profile for FMP2.1/AS02A, a PfAMA-1-based vaccine that elicited potent humoral and Th1-biased cellular immune responses. Further studies are already underway to evaluate this vaccine in volunteers living in malaria-endemic regions. Thus, a Phase 1B trial in children is now ongoing in Bandiagara, Mali, where subsequent trials are planned to determine its safety and efficacy in children [37].

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Conflict of interest: W.R. Ballou, J.D. Cohen, E. De-Kock, Marie-Claude Dubois and O. Ofori-Anyinam are employees of GlaxoSmithKline Biologicals, the manufacturer of the AS02A adjuvant described in this report. S. Dutta, D.E. Lanar and L.A. Ware hold patents for the FMP2.1 vaccine antigen described in this report. The other authors declare that they have no conflict of interests. *Funding:* This study was funded by the Malaria Vaccine Development Program, US Agency for International Development, Washington, DC and by the Military Infectious Diseases Research Program, Fort Detrick, MD. *Previous disclosure:* Presentation in part as Abstract #924 at the 53rd Annual Meeting of the American Society of Tropical Medicine and Hygiene held October 2004 in Miami, Florida.

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