INFECTION AND IMMUNITY, June 2005, p. 3677–3685 Vol. 73, No. 6
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.6.3677–3685.2005

Phase 1 Clinical Trial of Apical Membrane Antigen 1: an Asexual Blood-Stage Vaccine for *Plasmodium falciparum* Malaria

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Received 21 December 2004/Returned for modification 2 February 2005/Accepted 8 February 2005

Apical membrane antigen 1 (AMA1), a polymorphic merozoite surface protein, is a leading blood-stage malaria vaccine candidate. A phase 1 trial was conducted with 30 malaria-naïve volunteers to assess the safety and immunogenicity of the AMA1-C1 malaria vaccine. AMA1-C1 contains an equal mixture of recombinant proteins based on sequences from the FVO and 3D7 clones of *Plasmodium falciparum*. The proteins were expressed in *Pichia pastoris* and adsorbed on Alhydrogel. Ten volunteers in each of three dose groups (5 μg, 20 μg, and 80 μg) were vaccinated in an open-label study at 0, 28, and 180 days. The vaccine was well tolerated, with pain at the injection site being the most commonly observed reaction. Anti-AMA1 immunoglobulin G (IgG) was detected by enzyme-linked immunosorbent assay (ELISA) in 15/28 (54%) volunteers after the second immunization and in 23/25 (92%) after the third immunization, with equal reactivity to both AMA1-FVO and AMA1-3D7 vaccine components. A significant dose-response relationship between antigen dose and antibody response by ELISA was observed, and the antibodies were predominantly of the IgG1 isotype. Confocal microscopic evaluation of sera from vaccinated volunteers demonstrated reactivity with *P. falciparum* schizonts in a pattern similar to native parasite AMA1. Antigen-specific in vitro inhibition of both FVO and 3D7 parasites was achieved with IgG purified from sera of vaccinees, demonstrating biological activity of the antibodies. To our knowledge, this is the first AMA1 vaccine candidate to elicit functional immune responses in malaria-naïve humans, and our results support the further development of this vaccine.

The worldwide incidence of malaria is estimated by the World Health Organization to be approximately 300 to 500 million clinical cases annually, with more than 90% of these cases occurring in sub-Saharan Africa (1). The majority of the estimated 3 million deaths from malaria occur in children less than 5 years of age, accounting for nearly 25% of child mortality in Africa. Of the four species of *Plasmodium* that infect humans, *Plasmodium falciparum* is responsible for most malaria-related deaths. The humanitarian and economic costs of malaria are enormous. According to estimates, the annual incremental costs of malaria prevention and treatment are estimated to be $2.5 and 4 billion for 2007 and 2015, respectively (23). There are a limited number of drugs available for the treatment and prevention of malaria, and for those, resistance is becoming an increasing problem. For these reasons, the global effort to fight malaria has increased substantially in recent years, with vaccine development becoming a high priority.

The life cycle of *P. falciparum* is comprised of multiple stages, including an asexual blood stage characterized by repeated cycles of invasion and parasite growth in erythrocytes. This stage of parasite development is responsible for the pathological and clinical manifestations of infection, and it is thought that the semi-immunity that develops after repeated infection in those living in areas where malaria is endemic is mediated in part by antibodies to blood-stage parasites. Apical membrane antigen 1 (AMA1) is a merozoite protein that is expressed during the asexual blood stages of *P. falciparum*, and it plays a significant role in erythrocyte invasion (13, 22). AMA1 is an 83-kDa protein characterized by eight intramolecular disulfide bonds and is located in the apical micronemes of the merozoite (7, 9, 16).

Prior to invasion of the erythrocyte, the protein is cleaved, and a 66-kDa product is exported to the surface of the merozoite (6, 10, 15). Vaccination with recombinant AMA1 has been demonstrated to induce protection against homologous parasite challenge in both rodent and monkey models of malaria infection (2, 4, 11, 14). We have previously reported that our recombinant 62-kDa AMA1 protein based on the FVO strain of *P. falciparum* was immunogenic and protected *Aotus vociferans* monkeys against challenge with homologous parasites (19).

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Previous studies of rodent models of malaria have demonstrated that protection induced by immunization with AMA1 was parasite strain specific (4). In addition, sequence polymorphism of the AMA1 gene has been demonstrated among circulating strains of *P. falciparum* (3, 12, 17). In this context, we have previously shown by enzyme-linked immunosorbent assay (ELISA) that rabbits immunized with a single AMA1 allele produce antibodies that preferentially recognize the homologous AMA1 antigen and showed greater inhibition of homologous parasites in an in vitro growth inhibition assay (11). In contrast, when rabbits were immunized with a mixture of two different AMA1 proteins, comparable responses were elicited to both antigens. For these reasons, we have designed the AMA1 vaccine to contain equal mixtures of the recombinant AMA1 proteins derived from the FVO and 3D7 clones of *P. falciparum* to better react with the diverse parasite strains present in areas of endemicity. In this paper, we report that this new vaccine formulation, designated AMA1-C1, adjuvanted with Alhydrogel, is safe and immunogenic when administered to healthy, malaria-naïve adult volunteers in a phase 1 clinical study. This is the first study of humans with a recombinant AMA1 protein formulation that has elicited antibodies with biological activity against malaria parasites as judged by an in vitro invasion inhibition assay.

MATERIALS AND METHODS

Vaccine preparation. The AMA1-FVO and AMA1-3D7 proteins were manufactured according to current good manufacturing practice at the Walter Reed Army Institute of Research Pilot Bioproduction Facility (Silver Spring, MD). The AMA1-C1 vaccine contains two 533-amino-acid recombinant malaria proteins based on the AMA1 sequences of the FVO and 3D7 clones of *P. falciparum*, and the manufacture and characterization of these antigens has been described (11). The recombinant proteins consist of the correctly folded ectodomain portions of the antigens, with the addition of a six-histidine C-terminal tag to allow purification of the protein. Each of these proteins was expressed in *Pichia pastoris* separately, and each product was purified using a combination of affinity, ionic, hydrophobic, and gel filtration chromatographies (11). Immediately prior to formulation, equal weights of AMA1-FVO and AMA1-3D7 were mixed, adsorbed to Alhydrogel (HCl Biosector, Denmark), and subsequently vialed by the Pharmaceutical Development Section, National Institutes of Health. The formulation was supplied in single-dose vials as a cloudy suspension, without stabilizers or preservatives, in a sterile saline solution. Three lots of clinical grade vaccine were prepared containing either 5 μg, 20 μg, or 80 μg of AMA1-C1 and 800 μg of Alhydrogel per 0.5-ml dose. Potency studies of mice using each lot of vaccine stored at 2 to 8°C were conducted every 6 months and confirmed that all lots of vaccine were stable and fully potent throughout the entire course of the trial.

Study design. An open-label, dose-escalating phase 1 clinical trial with healthy adult volunteers was designed to evaluate the safety, reactogenicity, and immunogenicity of the AMA1-C1 malaria vaccine formulated on Alhydrogel. This study was performed under an investigational new-drug application (BB-IND-10944) approved by the U.S. Food and Drug Administration. The protocol, amendments to the protocol, informed consent form, advertisements, and other study-related documents were approved by the Committee on Human Research (Johns Hopkins Bloomberg School of Public Health Institutional Review Board) and the National Institute of Allergy and Infectious Diseases Institutional Review Board.

Volunteers. Thirty healthy volunteers, 18 to 50 years of age, were recruited from the metropolitan Baltimore area. Written informed consent was obtained, and the volunteers were required to pass an informed consent comprehension evaluation prior to enrollment. Volunteers were excluded if they had any of the following: evidence of clinically significant systemic disease; pregnancy or breast feeding; serological evidence of human immunodeficiency virus infection, chronic hepatitis B, or hepatitis C; current medication with corticosteroids or immunosuppressive drugs; immunization with a live vaccine in the previous 4 weeks; prior malaria infection; previous receipt of a malaria vaccine; travel to a country where malaria is endemic during the past 12 months; or planned travel to a country where malaria is endemic during the course of the study. Ten volunteers in each of three dose groups (5 μg, 20 μg, and 80 μg of AMA1-C1) were vaccinated by a 0.5-ml intramuscular injection in alternate arms on study days 0, 28, and 180. For safety purposes, injections for each cohort of 10 volunteers were staggered so that three volunteers were vaccinated 1 week before the remaining seven volunteers. All volunteers in a lower-dose cohort had to have completed up to study day 35 prior to initial vaccination of the higher-dose cohort. Escalation to the next higher dose required approval by an independent safety monitoring committee. All females had a urine β human chorionic gonadotrophin test at screening and immediately prior to each vaccination.

Assessment of safety and tolerability. Following each vaccination, the volunteers were observed for 30 min and then evaluated 1, 3, 7, and 14 days after vaccination for evidence of local and systemic reactogenicity. Local adverse events included erythema, induration, and tenderness at the site of injection. Solicited systemic adverse events included fever (oral temperature ≥38°C), headache, nausea, malaise, myalgia, and arthralgia. Volunteers recorded local and systemic reactogenicity daily, as well as their oral temperature three times daily, on diary cards for 13 days following each vaccination. An abbreviated history and physical examination were performed at each follow-up visit. All abnormal signs and symptoms were considered adverse events. Each adverse event was graded for severity and assigned causality relative to the study vaccine. Severity was graded as either mild (easily tolerated), moderate (interfered with activities of daily living or required medication), or severe (prevented activities of daily living). Erythema or induration at the injection site was graded as follows: mild (>0 to ≤20 mm in diameter), moderate (>20 to ≤50 mm), or severe (>50 mm). A complete blood count and white blood cell differential, as well as serum creatinine and aspartate aminotransferase (AST) concentrations, were performed immediately prior to each vaccination, as well on the 3rd and 14th days following vaccination.

Antibody measurement by ELISA. Measurement of anti-AMA1 antibodies was performed according to a standardized ELISA protocol. Briefly, 96-well ELISA plates were coated with 100 ng/well of either AMA1-FVO or AMA1-3D7 protein at 4°C overnight in coating buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate). After the plates were blocked with diluent buffer consisting of 5% skim milk (Difco, Inc., Detroit, MI) in Tris–phosphate-buffered saline (Tris-PBS, pH 7.4), the sera were diluted in diluent buffer, added to antigen-coated wells in triplicate, and incubated for 2 h at room temperature. After extensive washing, the plates were incubated with goat anti-human immunoglobulin G (IgG) conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) in diluent-blocking buffer (5% skim milk in PBS) for 2 h at room temperature. Bound antibodies were visualized by adding *p*-nitrophenyl phosphate Sigma 104 substrate (Sigma Chemical Co., St. Louis, MO). The absorbance at 405 nm was read using a SPECTRAmax 340PC microplate reader (Molecular Devices Co., Sunnyvale, CA). A human anti-AMA1 standard serum was made using a pool of sera from 10 individuals residing in Mali, a region where *P. falciparum* malaria is endemic. The standard pool was assigned 10,000 ELISA units/ml, which was approximately equivalent to the reciprocal of the dilution giving an optical density at a wavelength of 405 nm (OD_{405}) of 1.0. Duplicates of the serially diluted standard serum pool were included on each test plate in order to generate a standard curve. The standard curve was fitted to a four-parameter hyperbolic function, which was used to convert the absorbances of individual test sera into antibody units (SOFTmax PRO version 4; Molecular Devices Co.). Test samples that did not fall within an acceptable OD_{405} range were retested at an alternate dilution.

Antigen-specific IgG subclasses were also determined by a similar ELISA using antibody conjugates to specific IgG subclasses as secondary reagents. The alkaline phosphatase conjugates used were mouse anti-human IgG1, IgG2, IgG3, and IgG4 (Southern Biotechnology Assoc., Birmingham, AL). Prior to the assay with human sera, each secondary antibody was standardized on a homologous human myeloma-derived IgG protein, and dilutions of each secondary antibody were adjusted to give comparable reactivities on the myeloma proteins.

Confocal microscopy with human sera on malaria parasites. Confocal microscopy was performed on *P. falciparum* 3D7-parasitized red blood cells (RBCs) (1 to 2% parasitemia) in the following manner. Thin smears from bulk cultures of the parasite were prepared and stored at −70°C. The slides were allowed to warm to room temperature and were fixed in methanol on dry ice for 20 min. The slides were blocked with 10% bovine serum albumin (BSA Fraction V; Sigma) in PBS for 10 min and then washed twice with PBS. Diluted preimmune (day 0; 1:100) or day 194 (1:100, 1:400, or 1:1,600) sera and a mouse monoclonal anti-AMA1 antibody (MAb 1C8; 1:400) were allowed to react with the parasite-infected RBCs for 1 h in a humidified chamber at room temperature. MAb 1C8 and a pool of sera from six volunteers with high antibody levels by ELISA (>500 U/ml) were used as single fluorescent controls. The slides were then washed twice for 10 to 15 min with PBS with 0.1% Tween 20, (Sigma Chemical Co.) and

once with PBS. A mixture of secondary antibodies, fluorescein isothiocyanate-conjugated anti-human IgG (1:800; ICN/CAPPEL, Aurora, OH) and Alexa 568-conjugated anti-mouse IgG (1:100; Invitrogen Molecular Probes, Carlsbad, CA), was applied to samples on the slides, and the slides were incubated at room temperature for 1 h. The secondary antibodies were then aspirated, and the slides were washed with PBS twice prior to examination with a confocal microscope (Leica Microsystems, Exton, PA). Fluorochromes were excited using an argon laser at 488 nm for fluorescein isothiocyanate and a krypton laser at 568 nm for Alexa 568. Images taken at the two wavelengths were collected separately and later superimposed.

In vitro parasite growth inhibition by immune IgG. The abilities of IgGs from vaccinated individuals to inhibit in vitro growth of *P. falciparum* 3D7 and FVO parasites were assessed using a standardized procedure. IgG fractions were purified from individual sera obtained on day 0 and day 194 using protein G columns (Pierce Inc., Rockford, IL); the eluted fractions were dialyzed against RPMI 1640 (Life Technologies, Gaithersburg, MD) and concentrated with centrifugal filter devices (Millipore, Billerica, MA) to a concentration of 20 mg/ml or 40 mg/ml. The purified IgGs were preadsorbed with uninfected human O^+ erythrocytes (25 μl of RBCs per 1 ml of sample) for 1 h to remove any anti-human erythrocyte immunoglobulins. Purified IgGs were sterilized by filtration through a 0.22-μm filter (Nalge Nunc, Rochester, NY) and heat inactivated at 56°C for 20 min before use in the assay. AMA1-3D7- and AMA1-FVO-specific antibody concentrations were determined for each sample of purified IgG, and all samples were aliquoted and maintained at 4°C until tested by growth inhibition assay (GIA). The GIA was performed on these samples using human erythrocytes parasitized with late trophozoite and schizont stages of *P. falciparum* prepared by Percoll gradient and/or 5% sorbitol treatment (21). Parasite growth after 40 h of culture was determined by a biochemical assay specific for parasite lactate dehydrogenase, and the results were determined by the OD_{650}. Values obtained with test IgGs were compared with those obtained with parasites incubated with a pool of malaria-naïve human control serum and with uninfected red cells. To establish the antigen specificity of growth inhibition, in some experiments, test IgGs were preincubated with various amounts of AMA1 protein for 30 min prior to conducting the GIA as described above. The results of the GIAs with the purified IgGs were expressed as percent inhibition calculated as follows: $100 - [(OD_{650}$ of infected RBCs with tested IgG $- OD_{650}$ of normal RBCs only)/(OD_{650} of infected RBCs without any IgG $- OD_{650}$ of normal RBCs only) $\times 100]$.

Statistical procedures. (i) Adverse events. The frequency of adverse events stratified by dose cohort was summarized. The adverse events of any severity, across dose groups, and across the vaccinations within each dose group were compared using generalized linear models with R version 1.9.0 (http://www.r-project.org/).

(ii) Antibody responses. The effect of antigen dose on antibody response was tested by Spearman rank correlation for days 42 and 194. To further delineate differences in antibody responses between the 5-, 20-, and 80-μg dose groups, a Kruskal-Wallis test was performed for ELISA results with sera obtained on days 42 and 194, and when found to be significant, was followed by a pairwise comparison using the Student-Newman-Keuls test. Correlation between anti-AMA1-FVO and anti-AMA1-3D7 antibody responses was assessed by the Wilcoxon signed-rank test. SPSS statistical software (version 11.0; SPSS Inc., Chicago, IL) was used for these analyses, and P values of ≤ 0.05 were considered significant. The relationship between ELISA antibody values and percent parasite growth inhibition was explored by curve fitting using Sigma Plot software (SPSS Inc.).

RESULTS

Vaccine trial. (i) Study population. Thirty volunteers (10 female and 20 male) were enrolled from July through November 2003. The mean age was 31 years (range, 21 to 48). Sixty-three percent of the volunteers identified themselves as African-American, 27% as Caucasian, and 7% as Hispanic, and 1 volunteer (3.3%) identified himself as "other/unknown." Twenty-five of 30 vaccinees (83.3%) received all three scheduled vaccinations. All 10 volunteers in the 5-μg group received the first and second vaccination as scheduled, while 9 received the third vaccination. One volunteer became pregnant 6 weeks following the second vaccination and therefore did not receive the third vaccination. All 10 volunteers in the 20-μg group received the first vaccination as scheduled, and 9 received the second and third vaccinations. One volunteer in this group was incarcerated shortly after the first vaccination and did not receive any further vaccinations. All 10 volunteers in the 80-μg group received the first vaccination as scheduled, 9 received the second vaccination, and 7 received the third vaccination. Of the three volunteers who did not receive the three scheduled vaccinations, one moved out of state unexpectedly and the other two volunteers were not vaccinated due to safety concerns unrelated to the vaccine (see below).

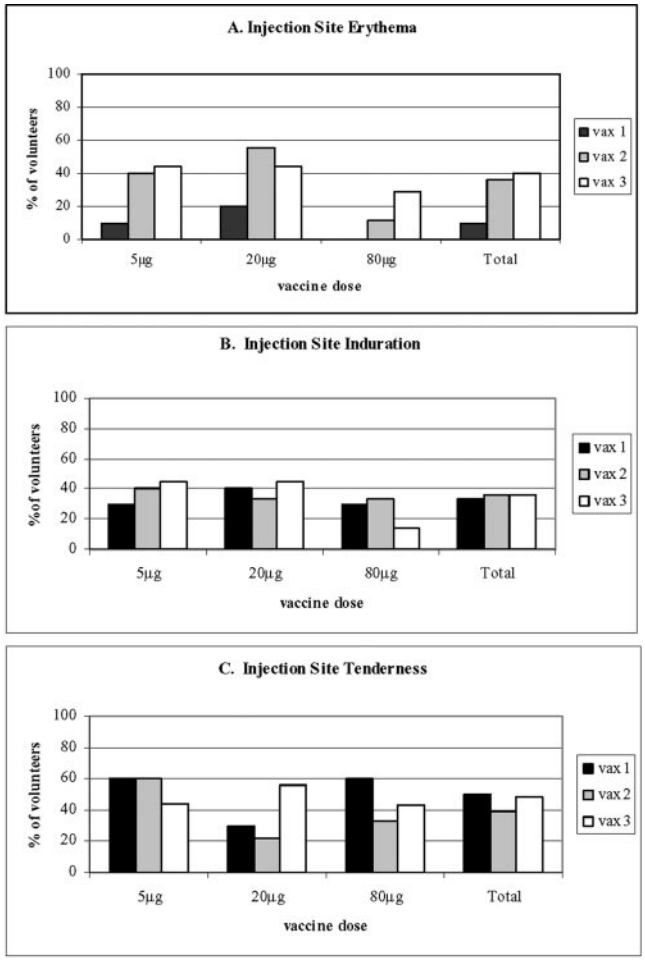

FIG. 1. Local reactogenicity in recipients of the AMA1-C1 vaccine. The percentages of volunteers who experienced injection site erythema (A), induration (B), or tenderness (C) after the first (black bar), second (gray bar), and third (white bar) immunizations with 5 μg, 20 μg, or 80 μg of AMA1-C1 are presented.

(ii) Safety. All vaccinations were well tolerated (Fig. 1). There were no statistically significant differences among the proportions of volunteers with an injection site reaction of any severity, either between dose groups or between vaccinations within each dose group. All reported local and solicited adverse events were graded as either mild or moderate in severity. Neither the frequency nor the severity of local adverse events increased with the dose or with subsequent vaccination. No serious adverse events occurred that were definitely, probably, or possibly related to vaccination. No hypersensitivity

reactions were observed, and no changes in vital signs occurred during the 30-min postvaccination observation period or during follow-up.

Headache was the most commonly observed systemic reaction and occurred more commonly after the first vaccination (five volunteers) than after subsequent vaccinations (two volunteers after the second vaccination; three volunteers after the third vaccination). Headache occurred more commonly in volunteers who received the 20-μg dose of AMA1-C1 (seven volunteers) than in volunteers who received the 5-μg dose (one volunteer) or the 80-μg dose (two volunteers). All of the reported headaches were mild in severity, except for one that was reported as moderate. Nausea was the next most frequently reported adverse event and was reported by two volunteers after the first vaccination and by two volunteers after the second vaccination. No volunteer reported nausea after the third vaccination. Malaise and myalgia were the next most common reactions, occurring in three volunteers each. Malaise was reported only after the first vaccination and only by volunteers in the 5-μg dose cohort. Myalgia was typically localized to one body part and not generalized. A volunteer in the 80-μg dose cohort was noted to have a mild elevation in AST level after the third vaccination. The volunteer's AST level rose from 50 U/liter on the day of vaccination to 75 U/liter (1.5 times above the upper limit of laboratory normal) 3 days after the third vaccination. The volunteer was completely asymptomatic, and the AST level returned to normal 14 days after the third vaccination. As there was no apparent etiology, it was classified as possibly related to vaccination due to the timing of the event.

Two volunteers in the 80-μg dose cohort did not receive their scheduled third vaccination because of medical reasons deemed unrelated to vaccination. One volunteer was noted to have an elevation of AST (186 U/liter; laboratory range, 10 to 59 U/liter) found on routine testing of blood drawn just prior to the second vaccination on study day 28. The laboratory result was not available until the day following vaccination. The volunteer was seen again on day 31 for scheduled safety laboratory tests, at which time the AST was found to be 797 U/liter (grade 4 toxicity). Screening for hepatitis C virus antibody and hepatitis B virus surface antigen had been performed 15 days prior to the first vaccination, and both were negative, as were tests repeated using blood collected on study day 31. However, testing of retained frozen serum collected on study day 0 (prior to vaccination) was positive for hepatitis C virus by quantitative PCR. The volunteer remained asymptomatic during the elevation in AST. Due to the diagnosis of acute hepatitis C virus infection, this event was deemed unrelated to the study vaccine. The volunteer was referred to a hepatologist, and no further vaccinations were administered.

The second volunteer in the 80-μg dose cohort who did not receive the third vaccination had experienced an episode of exercise-induced bronchospasm 30 days after receiving the second vaccination, which was deemed unrelated to vaccination. The volunteer had denied having a history of asthma and/or respiratory disease during screening but later admitted to a remote history of childhood asthma. The volunteer was followed for the duration of the trial for safety assessments and remained healthy.

Immune responses to AMA1. (i) Anti-AMA1 IgG antibodies. Antibody levels against the constituent AMA1 alleles in the vaccine were measured by ELISA at different time points following vaccination with AMA1-C1. Antibody responses to AMA1-FVO and AMA1-3D7 were measured separately to study the contribution of each allele to the overall antibody response. In the ELISA employed to measure antibody responses, the results were expressed as antibody units corresponding to a reciprocal dilution of serum that gives an OD of 1.0 rather than the commonly used endpoint dilution method. Using this standardized ELISA procedure, antibody responses in the sera of vaccinated individuals were undetectable until 2 weeks after the second vaccination, on day 42 (Fig. 2).

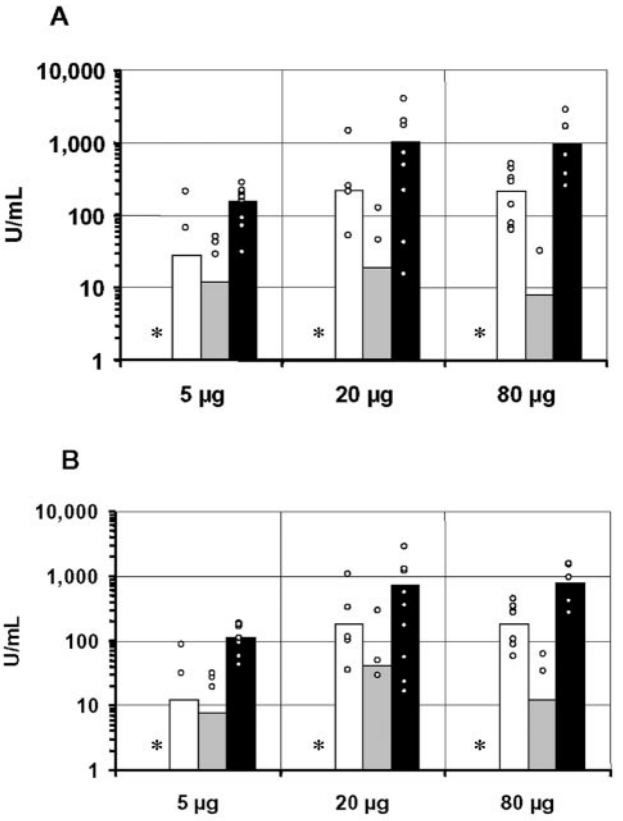

FIG. 2. Immunological responses to AMA1 allelic variants in recipients of the AMA1-C1 vaccine. Antibody units against AMA1-3D7 (A) and AMA1-FVO (B) measured by ELISA assays in sera collected on day 0 (day of vaccination 1; *), day 42 (14 days postvaccination 2; white bars), day 180 (day of vaccination 3; gray bars), and day 194 (14 days postvaccination 3; black bars). Samples with ELISA unit values below the limit of detection were assigned values of zero. * indicates that the mean for the cohort was zero. The bars represent the arithmetic mean antibody units against AMA1-3D7 (A) and AMA1-FVO (B) for the dose cohort: 5 μg/vaccination (n = 10), 20 μg/vaccination (n = 9), and 80 μg/vaccination (n = 7). The circles represent the individual unit values of responders.

Two weeks after the second vaccination, 2 of 10 (20%) individuals who received 5 μg of antigen, 5 of 9 (55%) who received 20 μg of antigen, and 8 of 9 (89%) who received 80 μg of antigen had detectable antibody responses to AMA1-3D7 and were classified as responders; the corresponding frequencies of responders for AMA1-FVO were 20%, 55%, and 78%, respectively, for the 5-μg, 20-μg, and 80-μg groups (Table 1). There was a significant dose-response relationship for AMA1-

TABLE 1. ELISA antibody values in sera from volunteers on selected days postvaccination

Volunteer no.	Vaccine dose (μg)	ELISA value for plate antigen: AMA1-3D7				AMA1-FVO			
		Day 0	Day 42	Day 194	Day 364	Day 0	Day 42	Day 194	Day 364
1	5	0[a]	0	161	22	0	0	190	31
2		0	0	93	19	0	32	92	29
3		0	0	192	12	0	0	169	17
4		0	0	215	53	0	0	93	38
5		0	211	176	110	0	89	112	92
6		0	0	91	12	0	0	16	0
7		0	0	31	12	0	0	42	0
8		0	0	72	12	0	0	57	0
9		0	68	218	48	0	0	179	29
10		0	0	281	77	0	0	179	53
Mean[b] ± SE		0	28 ± 21	153 ± 25	38 ± 11	0	12 ± 9	113 ± 20	29 ± 9
12	20	0	0	218	89	0	36	173	78
13		0	0	0	0	0	0	23	13
14		0	257	1,760	171	0	333	1,192	143
15		0	53	729	188	0	101	568	216
16		0	0	43	18	0	0	55	22
17		0	53	500	114	0	0	353	79
18		0	211	2,000	227	0	112	1,272	200
19		0	1,440	4,084	235	0	1,060	2,816	355
20		0	0	16	0	0	0	16	0
Mean ± SE		0	203 ± 140	937 ± 419	105 ± 30	0	165 ± 105	649 ± 284	111 ± 37
21	80	0	77	NA[c]	NA	0	58	NA	NA
22		0	0	0	NA	0	0	9	NA
23		0	67	377	27	0	89	414	26
24		0	449	1,688	333	0	287	1,496	239
25		0	512	2,864	109	0	455	1,568	116
26		0	322	672	NA	0	269	944	NA
27		0	294	257	44	0	347	268	46
28		0	63	NA	38	0	108	NA	60
29		0	140	NA	NA	0	33	NA	NA
Mean ± SE		0	206 ± 56	897 ± 386	97 ± 49	0	175 ± 48	712 ± 237	87 ± 33

[a] Samples with ELISA values below the limit of detection in the assay were assigned a value of 0.
[b] For calculation of arithmetic mean values, samples with less than the minimum detectable concentration were assigned a value of zero.
[c] NA, not available.

3D7- and AMA1-FVO-specific responses in the 5-μg, 20-μg, and 80-μg groups on day 42 (Spearman rank correlation: $\rho = 0.56$, $P = 0.002$ for AMA1-3D7; $\rho = 0.59$, $P = 0.001$ for AMA1-FVO).

Five months following the second vaccination (day 180), antibody levels declined and became undetectable in 8 of 15 responders (53%) for the AMA1-3D7 protein and 6 of 14 responders (43%) for AMA1-FVO (Fig. 2). Two weeks after the third vaccination, 23 of 25 (92%) individuals in the three-dose groups who received a third vaccination boosted their antibody levels against AMA1-3D7 and AMA1-FVO antigens. Antibody responses from the 5-μg, 20-μg, and 80-μg groups ranged from undetectable to 4,084 U (arithmetic means, 153, 1,041, and 978 U, respectively) (Fig. 2A). The corresponding range for AMA1-FVO was undetectable to 2,816 U/ml, with mean values of 113, 649, and 712 U, respectively, for the 5-μg, 20-μg, and 80-μg groups (Fig. 2B).

A relationship was found between antigen dose and antibody response to AMA1-FVO 2 weeks after the third vaccination (Spearman rank correlation: $\rho = 0.43$, $P = 0.032$), but did not reach statistical significance for responses to AMA1-3D7 (Spearman rank correlation: $\rho = 0.38$, $P = 0.062$) at the same time point. Pairwise comparisons of the antibody responses in the 5-, 20-, and 80-μg dose groups revealed that only the responses to the 5-μg dose were significantly lower than those observed with the 80-μg group for both AMA1-3D7 and AMA1-FVO (Student-Newman-Keuls test; $P < 0.05$).

By day 364, the last study day, antibody levels had declined. At this time point, 7 of 10 vaccinees tested in the 5-μg dose group, 8 of 9 vaccinees tested in the 20-μg group, and 5 of 5 vaccinees tested in the 80-μg group still had detectable anti-AMA1 antibodies.

Each serum sample was tested by ELISA against both AMA1-3D7 and AMA1-FVO proteins to determine whether both allelic variants present in the vaccine would be recognized. The antibody units to AMA1-3D7 test antigen correlated strongly with units to AMA1-FVO 2 weeks after both the second and third vaccinations (Wilcoxon signed-rank test; $P < 0.05$ for 2 weeks after the second vaccination; $P < 0.01$ for 2 weeks after the third vaccination). This indicates that both allelic variants were comparably recognized by antisera from responders.

In addition to total IgG responses, the IgG subclass distribution of anti-AMA1 antibodies to AMA1-3D7 and AMA1-

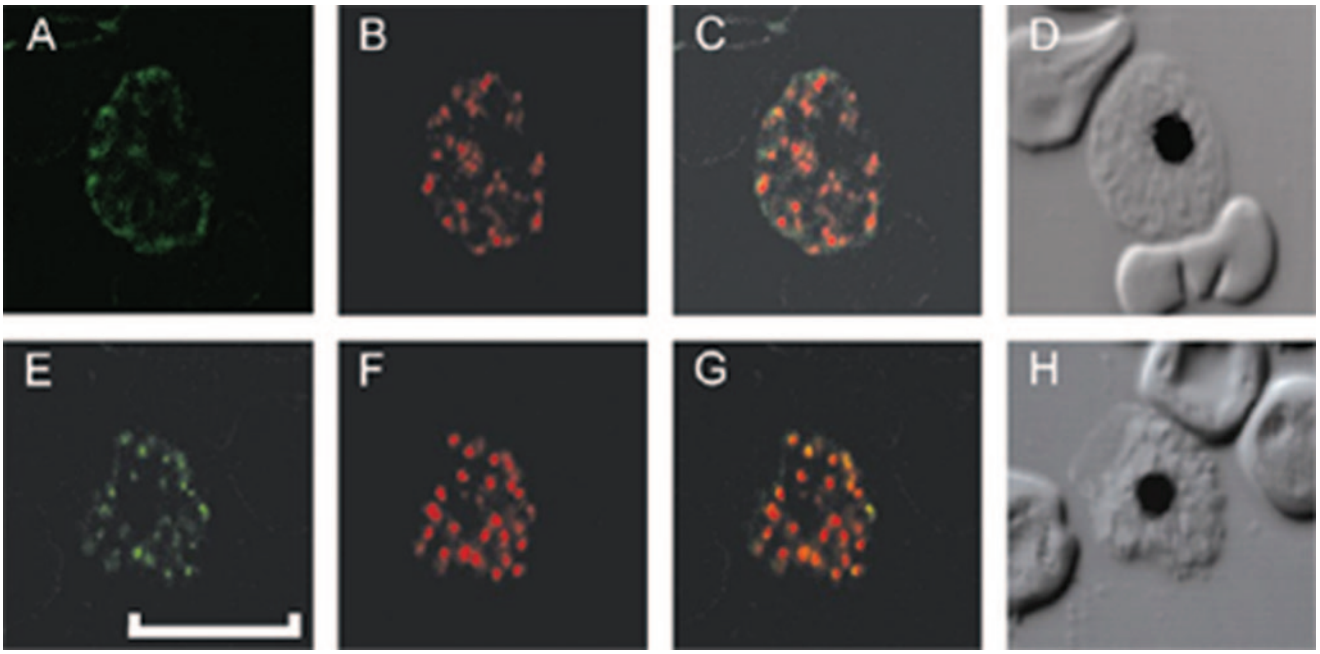

FIG. 3. AMA1-C1 vaccine induces antibodies that recognize the native AMA1 protein on parasites. Representative confocal micrographs illustrating indirect immunofluorescent antibody detection and localization on *P. falciparum* FVO parasites with anti-AMA1 monoclonal antibody 1C8. Sera collected on days 0 (A to D) and 194 (E to H) were from an individual with anti-AMA1-FVO ELISA titers of 2,816 U on day 194. The sera were tested at a dilution of 1:400, and anti-AMA1 monoclononal antibody 1C8 was diluted 1:1,600. Shown are green fluorescence, representing the vaccinee's antibody against AMA1 (A, E); the corresponding red fluorescence, representing the mouse monoclonal anti-AMA1 antibody (B, F); and the merged images (C, G) and bright-field micrographs (D, H) of the test sera. A 10-μm scale bar is included in panel E.

FVO was evaluated using specific anti-subclass antibodies. Among responders, the sera had predominantly IgG1 AMA1-specific antibodies. Two individuals from the 80-μg dose group also had low levels of anti-AMA1 IgG3 antibodies. There were no detectable IgG2 or IgG4 antibodies.

(ii) Confocal microscopy with human sera on malaria parasites. The abilities of immune sera to recognize native AMA1 on *P. falciparum* 3D7 parasites were evaluated using confocal microscopy. Sera from three individuals with high ELISA antibody units (>1,000 U/ml for both AMA1-FVO and AMA1-3D7) 2 weeks after the third vaccination were tested to determine the binding distribution of anti-AMA1 antibody on parasitized erythrocytes. As seen in Fig. 3, the fluorescence patterns were very similar to those observed with a mouse anti-AMA1 MAb 1C8 and colocalized to the same structures.

(iii) In vitro growth inhibition assay. To determine whether antibodies elicited by immunization had biologic activity against *P. falciparum* parasites, we tested the abilities of sera from selected vaccinees to inhibit in vitro growth of the *P. falciparum* FVO and 3D7 parasite clones. To minimize the possibility of nonspecific in vitro inhibition of parasite growth by the sera and to ensure that the inhibitory activity was attributable to the antibodies alone, we used IgG purified from each serum sample and compared the inhibition with that obtained with prevaccination IgG from the same individual. Significant inhibition of both *P. falciparum* 3D7 and FVO parasite growth was achieved with 4 of 22 sera tested, with inhibitions ranging from 14% to 54%; prevaccination IgG showed <10% inhibition (Table 2). Overall, there was a statistically significant correlation between percent inhibition and ELISA units (Spearman rank correlation: $\rho = 0.63$, $P < 0.001$ for 3D7, and $\rho = 0.42$, $P < 0.01$ for FVO). To confirm that the inhibition was AMA1 specific, IgG fractions from selected individuals were tested for reversal of parasite inhibition by preincubation with AMA1 antigen. Inhibitory activity was reversed to background levels in sera from 2 weeks after the third vaccination in the two individuals tested. These observations provide evidence of the induction of biologically functional and antigen-specific AMA1 antibodies after vaccination with AMA1-C1.

To determine the correlation between the concentration of anti-AMA1 antibodies and the degree of growth inhibition, each sample was tested at final IgG concentrations of 5 mg/ml and 10 mg/ml, and the antigen-specific ELISA values were determined for each. This correlation could be represented by a hyperbolic curve function for both AMA1-3D7-specific antibodies ($R^2 = 0.797$; $P < 0.0001$) and AMA1-FVO-specific antibodies ($R^2 = 0.337$; $P < 0.0001$) (Fig. 4). The hyperbolic curve is defined by the following function: percent inhibition = $100 \times [\text{ELISA units}/(A + \text{ELISA units})]$, where the constant A represents the ELISA units corresponding to 50% inhibition and where the maximum theoretical inhibition is 100%. Using the best-fit hyperbolic curves for all samples, the predicted antibody concentrations required to achieve 50% growth inhibition were calculated to be 3,623 ± 360 U for AMA1-3D7 and 4,104 ± 567 U for AMA1-FVO.

DISCUSSION

The results of this trial demonstrate that the AMA1-C1 blood-stage malaria vaccine is safe when administered to adult

TABLE 2. In vitro growth inhibition of *P. falciparum* by immune sera from vaccine recipients

Volunteer no.	Anti-AMA1 antibody (U/ml)[a,b] for parasite strain		% Inhibition[c] for parasite strain			
			3D7		FVO	
	3D7	FVO	Day 0	Day 194	Day 0	Day 194
25 (80 μg)[d]	1,975	1,450	0	54	3	50
24 (80 μg)	1,646	909	−2	30	4	17
26 (80 μg)	633	553	3	15	6	14
15 (20 μg)	613	506	0	22	1	18
17 (20 μg)	608	390	−5	9	−4	2
Others (n = 17)	7 to 308	3 to 206	−12 to 5	−6 to 11	−4 to 7	−4 to 6

[a] Serum samples were collected on day 194, 2 weeks after the third vaccination.
[b] Total IgG was purified from individual sera and concentrated to 40 mg/ml for the growth inhibition assay. The final concentration of IgG in the ELISA wells was 10 mg/ml. The ELISA unit values shown are the amounts of anti-AMA1 antibody in purified IgG added to test wells.
[c] Percent inhibition of parasite growth compared to wells with equivalent concentrations of normal human serum.
[d] AMA1-C1 vaccine dose is indicated in parentheses.

malaria-naïve volunteers. AMA1-C1 was formulated with Alhydrogel, an aluminum-based adjuvant similar to that used in licensed hepatitis B and diphtheria-tetanus toxoid vaccines. One of the more difficult challenges in vaccine development is to achieve the appropriate balance between vaccine immunogenicity and reactogenicity. The AMA1-C1 candidate vaccine induced little local or systemic reactogenicity in vaccinated volunteers. Local reactogenicity accounted for the most commonly reported adverse events. The majority of injection site adverse events were graded as mild (78%), and none were reported as severe or serious. There was no statistically significant increase in adverse events with dose escalation or after repeated vaccinations. The acceptable safety profile of the AMA1-C1 vaccine is encouraging, and further development of this blood-stage vaccine in areas of endemicity is warranted.

While assessment of the safety of the vaccine formulation was the primary goal of this study, an important secondary objective was to determine the ability of the AMA1-C1 vaccine to elicit humoral immune responses in malaria-naïve individuals after primary vaccination and to boost memory responses with revaccination. While there was no detectable antibody response to either AMA1-3D7 or AMA1-FVO after the first dose of AMA1-C1 in malaria-naïve volunteers, a significant antibody response was detected in volunteers after the second and third vaccinations with 20 μg or 80 μg of AMA1-C1. A positive relationship between antigen dose and antibody response was observed. Revaccination clearly boosted antibody responses, as antibody levels that had declined to baseline 5 months after the second vaccination increased rapidly after the third vaccination to levels that were well above those observed with prior vaccination. The proportion of responders in the 5- and 20-μg dose groups increased significantly after the third vaccination, with 23 of 25 vaccinees responding after three vaccinations. Taken together, these data indicate that AMA1-C1 vaccination in malaria-naïve individuals resulted in immunological sensitization, and reimmunization boosted the antibody response.

Antigens expressed during the blood stage of *P. falciparum* infection are attractive targets for malaria vaccine development because the morbidity associated with malaria infection is a consequence of the repeated cycles of asexual replication in an individual's red blood cells. AMA1 is a leading vaccine

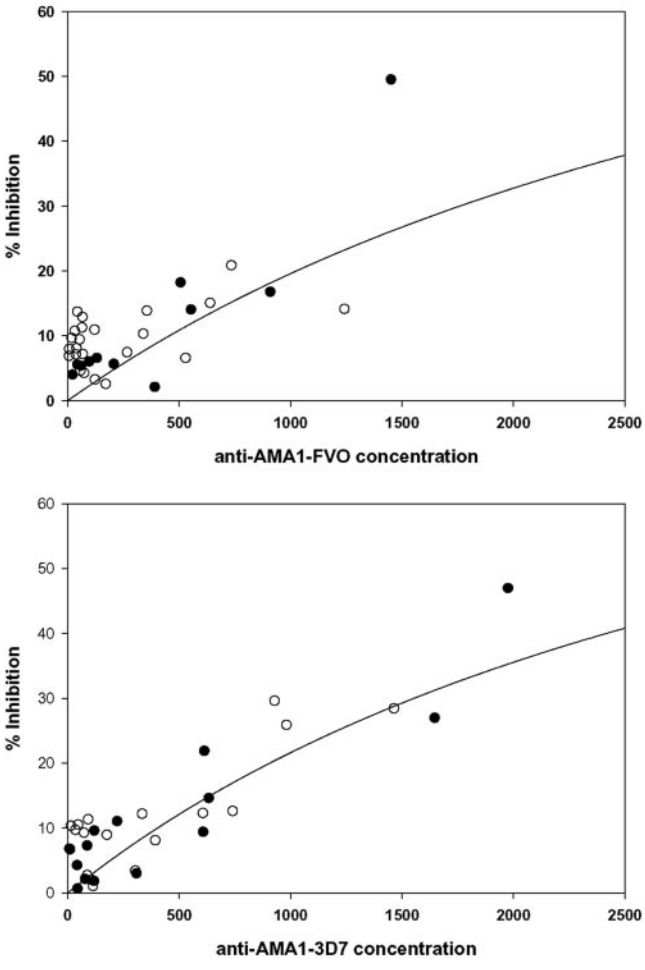

FIG. 4. Anti-AMA1-3D7 and -FVO ELISA values correlate with growth inhibition by immune sera on day 194. Total IgG was purified from each serum collected on day 194 (2 weeks postvaccination) as described in Materials and Methods. Antibody levels against AMA1-3D7 (A) and AMA1-FVO (B) in purified IgG were plotted against the corresponding in vitro growth inhibition. Samples were tested at two different final concentrations of total IgG, 5 μg/ml (open symbols) and 10 μg/ml (black symbols). A hyperbolic function was used to derive the best-fit curve shown for each antigen.

candidate because of its involvement in merozoite invasion of erythrocytes (20). Studies of people living in areas where malaria is endemic have demonstrated that antibodies to AMA1 are associated with protection from clinical disease (18) and that such antibodies, when isolated from infected individuals, can inhibit the in vitro growth of *P. falciparum* (8). Finally, protection against lethal parasitemia has been achieved in animal models with both active and passive immunization with AMA1 (2, 5).

While there is considerable experimental support for the choice of AMA1 as a vaccine candidate, the selection of this protein is potentially complicated by the fact that it displays significant amino acid variability in different field isolates. At least 70 amino acid residues have been shown to have one or more substitutions, but the impact of this polymorphism with respect to protective responses is unknown (7). To address this issue, the AMA1-C1 vaccine was designed to include AMA1 proteins based on the sequences of two relatively diverse *P. falciparum* clones: 3D7 and FVO. The AMA1 sequences from these two parasite clones differ by 25 amino acids. By incorporating both the 3D7 and FVO alleles into the AMA1-C1 vaccine, the goal was to induce a broader immune response than could be achieved by vaccination with either individual component alone.

The anti-AMA1 antibodies induced by the AMA1-C1 vaccine recognize the native parasite protein, as shown by confocal microscopy, and exhibited functional activity, as demonstrated by the in vitro parasite growth inhibition assay. To our knowledge, this is the first demonstration that vaccination of malaria-naïve humans can result in biologically functional anti-AMA1 antibodies. Moreover, these anti-AMA1 antibodies inhibited growth of the FVO and 3D7 clones of *P. falciparum* equally, suggesting that this vaccine may be able to induce humoral immune responses against a number of AMA1 variants. Although the levels of parasite growth inhibition were moderate in this study, these results are encouraging, since we believe that a further increase in antibody response should enhance the level of inhibition seen in the GIA. This hypothesis is supported by preclinical mouse, rabbit, and rhesus monkey studies, which have demonstrated a correlation between antibody levels and growth inhibition (11).

Whether an immune response to AMA1 can protect vaccinees against malaria in areas of endemicity is unknown, nor do we know the levels of cellular and humoral responses required to achieve such protection. However, we do anticipate that natural infection following vaccination would boost the immune responses of infants and children at risk of malaria. While we are also pursuing other formulations that may elicit higher vaccine-induced responses, it is possible that the immune responses we observed in this study, particularly in the 20- and 80-μg groups, may be sufficient to provide partial protection in young children in areas of endemicity. In order to test this hypothesis, the vaccine candidate must be tested in individuals exposed to infection. A phase 1 trial of AMA1-C1 formulated with Alhydrogel has begun in Mali, West Africa, to determine the safety of this vaccine for healthy, malaria-exposed adults. Once safety has been demonstrated in a region of endemicity, it will be possible to determine whether the immunogenicity of this formulation will be sufficient to protect young children exposed to natural infection.

In conclusion, in this phase 1 trial, the AMA1-C1 vaccine demonstrated an excellent safety profile and was able to induce significant immune responses in malaria-naïve individuals. Additionally, the functional properties of these antibodies were demonstrated by their ability to partially inhibit parasite growth in vitro. AMA1-C1 is therefore a promising blood-stage vaccine candidate for future clinical development.

ACKNOWLEDGMENTS

We thank Brian Murphy, cochief of the Laboratory of Infectious Diseases and Project Officer for the NO1A115444 contract, which supported this malaria vaccine trial. We are grateful to Felipe Troncoso and Sabrina Weaver for their assistance in the recruitment of volunteers and their expert technical assistance. We also thank Kazutoyo Miura for advice on data analysis, Joan Aebig for other technical assistance, and Owen Schwartz for assistance with confocal microscopy. Special thanks are due to all of the volunteers who participated in the trial.

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Editor: W. A. Petri, Jr.