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A Phase 1 study of the blood-stage malaria vaccine candidate AMA1-C1/Alhydrogel® with CPG 7909, using two different formulations and dosing intervals

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Abstract

A Phase 1 study was conducted in 24 malaria naïve adults to assess the safety and immunogenicity of the recombinant protein vaccine Apical Membrane Antigen 1-Combination 1 (AMA1-C1)/Alhydrogel with CPG 7909 in two different formulations (phosphate buffer and saline), and given at two different dosing schedules, 0 and 1 month or 0 and 2 months. Both formulations were well tolerated and frequency of local reactions and solicited adverse events was similar among the groups. Peak antibody levels in the groups receiving CPG 7909 in saline were not significantly different than those receiving CPG 7909 in phosphate. Peak antibody levels in the groups vaccinated at a 0,2 month interval were 2.52 fold higher than those vaccinated at a 0,1 month interval ($p=0.037$, 95% CI 1.03, 4.28). In vitro growth inhibition followed the antibody level: median inhibition was 51% (0,1 month interval) versus 85% (0,2 month interval) in antibody from samples taken 2 weeks post second vaccination ($p=0.056$).

1. Introduction

An effective blood-stage malaria vaccine has the potential to reduce the tremendous burden of morbidity and mortality from *Plasmodium falciparum*, particularly in sub-Saharan Africa. Vaccines containing the merozoite surface protein apical membrane antigen 1 (AMA1) (reviewed in [1]) have been shown to be protective in animal models and have advanced to Phase 2 trials in malaria-exposed children [2–5]. AMA1 is highly polymorphic and immunization with only one form of AMA1 may not protect against parasites expressing different AMA1 alleles [6,7]. For this reason, the AMA1-Combination 1 (AMA1-C1) vaccine contains equal mixtures of the recombinant AMA1 proteins representing the FVO and 3D7 strains of *P. falciparum*. A previous Phase 1 study showed markedly enhanced antibody responses when the novel adjuvant CPG 7909 was added to AMA1-C1/Alhydrogel®, and in vitro growth inhibition of homologous parasites as high as 96% in some volunteers [8]. A high

level of free (unbound) protein has been associated with hypersensitivity reactions in previous trials of malaria vaccines adjuvanted with Alhydrogel [9–12]. Formulation studies conducted at the Malaria Vaccine Development Branch (NIAID/NIH) indicated that binding of both AMA1 and CPG 7909 to Alhydrogel was optimized when CPG 7909 was formulated in a saline buffer [13]. In order to enhance stability of the vaccine and to allow for the addition of higher protein doses to future formulations, the CPG 7909 formulation was changed from phosphate buffer to saline. A Phase 1 study in 24 malaria naïve adults was conducted to confirm the safety and immunogenicity of the new CPG 7909 formulation (saline) compared to the previous formulation (phosphate buffered), and also explored the effect of a 0,1 month versus 0,2 month dosing schedule.

2. Materials and Methods

2.1. Study design

The study was a double-blinded, randomized controlled trial conducted at the Center for Immunization Research in Washington DC, designed to assess the safety and immunogenicity of AMA1-C1/Alhydrogel with CPG 7909 in saline and phosphate buffers, when given on 0,1 and 0,2 month dosing schedules. Eligible volunteers were randomized in a 2×2 design to receive AMA1-C1/Alhydrogel + CPG 7909 (phosphate) or AMA1-C1/Alhydrogel + CPG 7909 (saline), at a dosing interval of either 0,1 month or 0,2 months. Volunteers were randomized at the time of enrollment to one of the four groups, with all groups enrolled concurrently. The study was conducted under a protocol reviewed and approved by the Institutional Review Board (IRB) of the National Institute of Allergy and Infectious Disease (NIAID), and by the Western IRB. The study protocol was submitted to the U.S. Food and Drug Administration for review as part of Investigational New Drug application BB-IND#13228. The study was monitored for regulatory compliance and data quality by the Regulatory Compliance and Human Subjects Protection Branch of NIAID; safety and immunogenicity data were reviewed by the NIAID Data Safety and Monitoring Board. Written informed consent was obtained from all volunteers prior to screening for eligibility for participation. Participants and investigators conducting clinical and immunologic assessments remained blinded for the duration of the study.

2.2. Participants

Participants were healthy adults age 18–50. Exclusion criteria included prior malaria infection, recent or planned travel to a malaria endemic area, recent use of malaria prophylaxis, and pre-existing autoimmune disease. Subjects were required to be in good general health, without known significant medical conditions or significant medical history, and were required to have normal results for screening laboratories: complete blood count, alanine aminotransferase (ALT), and creatinine; no serologic evidence of hepatitis B, hepatitis C, or human immunodeficiency virus infection; and negative anti-double stranded DNA (dsDNA) as a marker for autoimmune disease. Urine pregnancy testing was performed at screening as well as prior to each vaccination for females.

2.3. Vaccines

The AMA1-C1/Alhydrogel vaccine, which is vialled separately from CPG 7909, is a 1:1 combination of recombinant proteins representing the FVO and 3D7 alleles of AMA1. AMA1 antigens were expressed in the yeast *Pichia pastoris*, and 80 µg doses of AMA1-C1/Alhydrogel® were manufactured under cGMP conditions as previously described [6,14]. The AMA1-C1/Alhydrogel used in this trial was supplied in single dose vials containing 1.0 mL of 160 µg/mL sterile saline solution. cGMP CPG 7909 drug substance was supplied by Coley Pharmaceutical Group (Wellesley, MA) under a clinical trials agreement. CPG 7909 is a short synthetic ODN with a sequence of 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3' with all

nucleotides linked with phosphorothioate bonds. AMA1-C1/Alhydrogel + CPG 7909 was prepared as a point of injection formulation up to 6 hours before vaccination, as described below. Characterization and quality control were performed as previously described [13,15]. CPG 7909 in phosphate buffer was supplied in multi-dose vials (each containing 1 mL of 10 mg/mL solution) and CPG 7909 in saline was supplied in single dose vials (each containing 0.08 mL of 10 mg/mL solution). For the groups receiving CPG in phosphate, 0.11 mL of CPG 7909 was withdrawn from the multi-dose vial and added to a single dose vial of AMA1-C1/Alhydrogel and gently mixed. When ready to administer, 0.55 mL was withdrawn into a syringe, and the vaccine injected. For the groups receiving CPG in saline, 0.7 mL of AMA1-C1/Alhydrogel was withdrawn and added to the single dose CPG 7909 (saline) vial. As with the phosphate formulation, after mixing, 0.55 mL was withdrawn into a syringe and the vaccine was injected. For both groups the vaccine was administered no more than 6 hours after mixing and was kept at 4°C until administered. Vaccines were given by intramuscular injection into the deltoid muscle on Days 0 and 28 or 0 and 56, with the second vaccination given in the alternate arm. A 0.55 mL dose of AMA1-C1/Alhydrogel + CPG 7909 corresponded to 80 µg of AMA1-C1 and 545 µg of CPG 7909 (phosphate), and 80 µg of AMA1-C1 and 564 µg of CPG 7909 (saline). The difference in mixing methods was due to the multi-dose vial for the phosphate formulation and the single dose vial for the saline formulation. The resulting differing amounts of CPG in the two vaccines were considered to be within the range of measurement error and to not be sufficient to affect the immune response.

The study randomization code was kept by the study pharmacist, who prepared the vaccines. The vaccines were visually indistinguishable, and were administered by the study nurse. Unblinding occurred after all clinical and immunologic assessments were complete.

2.4. Safety

Volunteers were observed for 30 minutes after each vaccination to evaluate immediate adverse events and were given diary cards to record events occurring during the first week after vaccination. The diary cards were used as a memory prompt and were reviewed with volunteers at follow up visits, when adverse events were recorded. Subjects were seen at 3, 7, and 14 days after each vaccination, and then approximately monthly for a total of 7 months (to study Day 210). Solicited adverse events included injection site pain, erythema, and induration, fever, headache, nausea, myalgia, arthralgia, and rash. Pain and solicited adverse events other than fever and urticaria were graded as follows: 0=absent/none, 1=easily tolerated, 2=interferes with daily activity or treatment given, 3=prevents daily activity. Unless otherwise specified, non-solicited adverse events were graded as 0=none, 1=no effect on activities of daily living and no treatment given, 2=partial limitation in activities of daily living or treatment given, 3=activities of daily living limited to <50% of baseline or medical evaluation required. Injection site erythema, swelling, and induration were graded based on the maximum diameter as follows: mild = > 0 to ≤ 20mm, moderate = 21 - ≤ 50mm, and severe = > 50mm. Hematological (hemoglobin, white blood cell counts, and platelets) and biochemical (ALT and creatinine) laboratory parameters were measured at screening and on days of immunization, and 3 and 14 days after each vaccination; hematological parameters were also checked 7 days after each vaccination. Anti-dsDNA was checked as a marker for autoimmunity at screening, at first vaccination, 14 days after the second vaccination, and at the end of the study. All adverse events were graded for severity and relationship to study product. Serious adverse events (SAEs) were defined as any adverse event resulting in death, life threatening, requiring hospitalization, resulting in disability or incapacity or congenital anomaly or birth defect, or any other event which required intervention to prevent such outcomes.

2.5 Immunogenicity

The standardized methodology for performing the enzyme-linked immunosorbent assay (ELISA) and the growth inhibition assay (GIA) have been described previously [8,16]. AMA1-FVO and AMA1-3D7 allele specific IgG antibody levels were assessed by ELISA at baseline for each vaccination, two weeks after each vaccination (Days 14 and 42 for the 0,1 month groups and Days 14 and 70 for the 0,2 month groups), and at Days 90 and 210. Samples for ELISA were also obtained at Day 28 for the 0,2 month groups. The minimum detection level of the assay was 1.28 µg/ml and all data less than 1.28 µg/ml were assigned a value of 0.64 µg/ml for statistical analysis. GIA was performed using purified IgG from two weeks post second vaccination (study Day 42 for 0,1 month groups, Day 70 for 0,2 month groups) to assess biologic activity of the induced antibody against *P. falciparum* FVO and 3D7 parasites. In this assay, purified antibody was added to the parasite cultures at approximately the same concentration as present in the corresponding serum sample (10 mg/mL in GIA well).

2.6. Statistics

For the post vaccination samples, the anti-AMA1-FVO and anti-AMA1-3D7 antibody concentrations were very similar (concordance 0.989, 95% confidence interval (0.985,0.992)), where concordance is measured by using log transformed values for all study days with the random marginal agreement coefficient with the squared difference function [17]. Thus, the arithmetic average of the anti-AMA1-FVO and anti-AMA1-3D7 concentrations was used as the response of each subject at each time point. A stratified Wilcoxon Mann Whitney (WMW) test was used for significance testing for the responses to the saline vs. phosphate formulations, stratified by dosing interval (0,1 month and 0,2 month). Tests were done for responses two weeks after the second dose for each group (peak response), and for Day 210. Ratios of geometric means with 95% confidence intervals were calculated using the Hodges-Lehman method [18] on the stratified WMW tests. A similar analysis was used to compare the 0,1 month vs. 0,2 month dosing, stratified by phosphate and saline for both the average anti-AMA1 response and the GIA. All non-missing observations were used for each test, but for the graphic representation of geometric mean antibody over time (Figure 2), subjects who were missing any antibody values were not included. To model the relationship between anti-AMA1 antibodies (X) and growth inhibition (Y), we used a Hill function:

$$Y = \frac{100\left(\frac{X}{b}\right)^a}{1 + \left(\frac{X}{b}\right)^a}$$

where a is the Hill coefficient, and b is the Ab_{50} (amount of antibody needed to give 50% growth inhibition). We fit the model by nonlinear least squares. The analyses were done using SAS version 9.1, R Version 2.7.2, and Stat Xact Procs Version 7.

3. Results

3.1 Safety

Forty-three volunteers were screened for participation and 24 volunteers were enrolled. Vaccinations began in May 2007, and all vaccinations were complete by August 2007. One volunteer in the saline 0,2 month group withdrew from participation prior to the second dose for personal reasons. Two additional volunteers withdrew subsequent to second vaccination for personal reasons, one in the phosphate 0,1 group and one in the phosphate 0,2 month group. A fourth volunteer in the phosphate 0,2 month group was lost to follow up after the second vaccination. No volunteers were withdrawn due to adverse events. Adverse events related to vaccinations were all mild or moderate except for one episode of severe injection site erythema

in a volunteer in the saline 0,2 month group that lasted for 6 days following second vaccination (Table 1 and Table 2). The maximum diameter of the erythema was 7 cm and there was no associated swelling. Unsolicited adverse events judged to be possibly related to vaccination were diarrhea and vertigo (mild) and one vasovagal response which was moderate in severity. No serious adverse events occurred. All volunteers had a decrease in absolute neutrophil count (ANC) at day 3 following first vaccination (mean decline $1363 \pm 191/\text{mm}^3$). All but two volunteers who received a second vaccination had a decrease in ANC at day 3 following second vaccination (mean decline $1341 \pm 247/\text{mm}^3$). In only one volunteer did the decrease in ANC result in a grade 1 neutropenia ($1000\text{--}1499/\text{mm}^3$). In addition, another volunteer had a grade 1 decrease in platelets ($125,000\text{--}135,000/\text{mm}^3$) 3 days after vaccination. The neutropenia and thrombocytopenia had resolved by the next visit at 7 days post-vaccination. Frequency of local reactions and solicited adverse events was similar among the groups. No rashes related to vaccination occurred. No volunteer developed a positive anti-dsDNA or any clinical signs of autoimmunity.

3.2 Immunogenicity

Peak antibody responses in the 4 groups are shown in Figure 1 and responses over time are shown in Figure 2. Peak antibody responses in the combined saline groups were not significantly different from those in the combined phosphate groups (the ratio of geometric mean responses [RR] was estimated as 0.90, i.e., saline response was 0.90 of the phosphate response; 95% CI 0.43, 1.60; $p=0.559$), and were also not significantly different at Day 90 or Day 210 (RR=0.92, 95% CI 0.42, 1.41; $p=0.676$; and, RR=1.22, 95% CI 0.74, 1.82; $p=0.391$ respectively). Peak responses in the combined groups vaccinated at a 0,2 month interval were 2.52 fold higher than those vaccinated at a 0,1 month interval (95% CI 1.03, 4.28; $p=0.037$). Responses declined rapidly in both dosing interval groups and were not significantly different at Day 210 (RR=1.61, 95% CI 0.97, 2.68; $p=0.084$).

Regardless of buffer or dosing interval, the growth-inhibitory activity judged by GIA followed the antibody level of the samples (Figure 3). Growth inhibition of 3D7 parasites ranged from 30–93% (median 51%) (0,1 month interval) and from 31–97% (median 85%) (0,2 month interval) with antibody obtained 2 weeks post second vaccination ($p=0.056$). Results for FVO parasites were slightly less, with a range of 20–81% (median 33.5%) (0,1 month interval) and 24–90% (median 60%) (0,2 month interval) using antibody obtained 2 weeks post second vaccination ($p=0.023$). The Ab_{50} for the 3D7 parasites was estimated as $162.7 \mu\text{g/mL}$ (95% CI 149.4, 175.9) and for FVO parasites was $210.4 \mu\text{g/mL}$ (95% CI 191.3, 231.6).

4. Discussion

Both formulations of AMA1-C1/Alhydrogel with CPG 7909 were well tolerated, with only one severe local adverse event of short duration reported and no volunteers withdrawn from vaccinations due to adverse events. This is in contrast to a previous Phase 1 study of AMA1-C1/Alhydrogel with CPG 7909 in a phosphate buffer, where local and solicited adverse events were more severe when CPG 7909 was added to the formulation and several volunteers were withdrawn due to adverse events [8]. The excellent safety profile in this study increases confidence in the tolerability of the vaccine. Nearly all volunteers had transient decreases in neutrophil counts after first and second vaccinations, with one decrease reaching Grade 1. Transient fluctuations in white blood cell counts and platelet count are expected adverse events with CPG 7909 and other TLR 9 agonists [19,20], and are likely due to redistribution of leukocytes as part of the immune response. No clinical events associated with these fluctuations have been reported to date and, given the rapid return to baseline, their clinical significance is likely to be minimal. There were no laboratory or clinical signs of autoimmunity, which is a theoretical concern with the use of CPG oligonucleotides.

While the safety profile for CPG 7909 as an adjuvant is limited, novel formulations and/or adjuvants are likely to be needed to induce high antibody levels for malaria proteins, which are generally poor immunogens in humans. The only malaria vaccine shown to have a consistent benefit in young children in Africa, RTS,S, is a novel formulation (virus-like particle) combined with a novel adjuvant (AS02 or AS01) [21,22]. Adjuvants such as CPG 7909, which induces a Th1 biased response, may be of particular use with malarial antigens [23]. Additional clinical studies in malaria exposed populations and in children should proceed in a cautious step-wise fashion, to expand the safety base for CPG adjuvants in the target population and to demonstrate benefit against clinical outcomes.

AMA1-C1/Alhydrogel® + CPG 7909 in saline was shown to have similar immunogenicity as the AMA1-C1/Alhydrogel® + CPG 7909 buffered with phosphate, and will be used in future clinical trials. Peak responses were superior when the vaccines were given on a 0,2 month versus 0,1 month dosing schedule, although given the small numbers in each individual dose group (n=6) it is possible that there may be a difference in kinetics between the phosphate and saline groups which the study was not powered to detect. However, studies in animals and humans also support a longer vaccination interval as more likely to induce higher antibody levels and cellular responses [24–27]. AMA1 specific antibody levels dropped rapidly after vaccination. It is possible that, when used in malaria endemic areas, infection will boost antibody responses. However, a longer lasting high level antibody response is more likely to be protective. Future studies will examine the use of a two month initial dosing interval, with a boost at 6 or 12 months.

The in vitro growth-inhibitory activity in this study was the highest in our human vaccine trials with AMA1 or other antigens to date. However, the range of antibody responses and corresponding growth inhibition was wide, with some volunteers only achieving 30–40% inhibition. While growth inhibition activity greater than 60% was associated with protection in *Aotus* monkeys vaccinated with MSP1₄₂ [28], the extent to which in vitro growth-inhibitory activity correlates with in vivo parasite multiplication rates, or with protection in the field, remains to be demonstrated. Furthermore, inhibition against heterologous parasites is markedly less than that against homologous parasites [8], and the extent to which this combination vaccine would protect against the highly diverse AMA1 variants in the field is also unknown. The addition of other proteins, either to more broadly cover AMA1 polymorphism or to broaden the individual response to various antigens, may be an effective strategy for future investigation.

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Figure 1. Geometric means and individual antibody responses for each dose group
 Geometric mean of the (arithmetic) average FVO and 3D7 antibody level and individual responses, two weeks after second vaccination for each group. A=Saline 0,1 Month, B=Phosphate 0,1 Month, C=Saline 0,2 Month, D=Phosphate 0,2 Month.

Figure 2. Geometric mean ELISA antibody responses by vaccine group and dosing interval
Antibody levels over time by vaccine group and dosing interval. Black lines connect the geometric means of the average anti-AMA1-FVO and anti-AMA1-3D7 antibody concentration for each time point. Gray lines connect individual responses. Vaccinations are shown by arrows, at either 0,1 month (Figure 2A) or 0,2 months (Figure 2B). Number in each group are: saline 0,1 month dosing interval, n=6; saline 0,2 month dosing interval, n=5; phosphate 0,1 month dosing interval, n=5; phosphate 0,2 month dosing interval, n=4.

○ Saline 0,1 Month ▽ Saline 0,2 Month
 ○ Phosphate 0,1 Month ▽ Phosphate 0,2 Month

Figure 3. In vitro growth inhibition for homologous parasites

In vitro growth inhibition for 3D7 parasites plotted against ELISA antibody response. Data points shown are peak responses (at Day 42 or Day 70).

Table 1

Local Reactogenicity

	Vaccination #1				Vaccination #2			
	Total	Mild	Moderate	Severe	Total	Mild	Moderate	Severe
AMAI-Cl/Alhydrogel+CPG 7909 (Phosphate) 0, 1 Month								
Pain	6/6	5	1	0	5/6	1	4	0
Erythema	0/6	0	0	0	1/6	1	0	0
Swelling	1/6	1	0	0	2/6	2	0	0
Induration	0/6	0	0	0	0/6	0	0	0
AMAI-Cl/Alhydrogel+CPG 7909 (Saline) 0, 1 Month								
Pain	6/6	4	2	0	5/6	3	2	0
Erythema	3/6	3	0	0	1/6	1	0	0
Swelling	3/6	3	0	0	2/6	2	0	0
Induration	0/6	0	0	0	1/6	1	0	0
AMAI-Cl/Alhydrogel+CPG 7909 (Phosphate) 0, 2 Month								
Pain	4/6	4	0	0	4/6	3	1	0
Erythema	0/6	0	0	0	0/6	0	0	0
Swelling	0/6	0	0	0	1/6	1	0	0
Induration	0/6	0	0	0	0/6	0	0	0
AMAI-Cl/Alhydrogel+CPG 7909 (Saline) 0, 2 Month								
Pain	4/6	3	1	0	5/5	2	3	0
Erythema	1/6	1	0	0	2/5	0	1	1
Swelling	1/6	1	0	0	2/5	2	0	0
Induration	0/6	0	0	0	0/5	0	0	0

Table 2

Systemic Reactogenicity

	Vaccination #1				Vaccination #2			
	Total	Mild	Moderate	Severe	Total	Mild	Moderate	Severe
AMAI-CI/Alhydrogel+CPG 7909 (Phosphate) 0, 1 Month								
Fever	1/6	0	1	0	1/6	1	0	0
Headache	3/6	0	3	0	2/6	2	0	0
Malaise	2/6	1	1	0	3/6	2	1	0
Myalgia	1/6	1	0	0	0/6	0	0	0
Nausea	1/6	1	0	0	0/6	0	0	0
Neutropenia	0/6	0	0	0	0/6	0	0	0
Thrombocytopenia	0/6	0	0	0	1/6	1	0	0
AMAI-CI/Alhydrogel+CPG 7909 (Saline) 0, 1 Month								
Fever	0/6	0	0	0	0/6	0	0	0
Headache	2/6	1	1	0	3/6	2	1	0
Malaise	1/6	1	0	0	0/6	0	0	0
Myalgia	0/6	0	0	0	1/6	1	0	0
Nausea	0/6	0	0	0	0/6	0	0	0
Neutropenia	0/6	0	0	0	0/6	0	0	0
Thrombocytopenia	0/6	0	0	0	0/6	0	0	0
AMAI-CI/Alhydrogel+CPG 7909 (Phosphate) 0, 2 Month								
Fever	0/6	0	0	0	1/6	1	0	0
Headache	1/6	0	1	0	0/6	0	0	0
Malaise	0/6	0	0	0	2/6	2	0	0
Myalgia	0/6	0	0	0	2/6	2	0	0
Nausea	0/6	0	0	0	0/6	0	0	0
Neutropenia	0/6	0	0	0	0/6	0	0	0
Thrombocytopenia	0/6	0	0	0	0/6	0	0	0
AMAI-CI/Alhydrogel+CPG 7909 (Saline) 0, 2 Month								
Fever	0/6	0	0	0	0/5	0	0	0
Headache	1/6	1	0	0	1/5	0	1	0
Malaise	0/6	0	0	0	1/5	1	0	0
Myalgia	0/6	0	0	0	0/5	0	0	0

Fever (oral) grade 1=100.4° – 101.5°, grade 2 = 101.6° – 102.4°, grade 3 = ≥102.4°F; neutropenia grade 1 = 1000–1499, grade 2 = 500–999, grade 3 = <500, grade 4 = <500 cells/mm³ with fever; thrombocytopenia grade 1 = 125,000 – 135,000, Grade 2 = 100,000 – 124,000, Grade 3 = 100,000 – 124,000, Grade 4 = <20,000 cells/mm³.

	Vaccination #1			Vaccination #2		
Nausea	0/6	0	0	0	0/5	0
Neutropenia	0/6	0	0	0	1/5	1
Thrombocytopenia	0/6	0	0	0	0/5	0