Published in final edited form as:

Vaccine. 2011 May 9; 29(20): 3640–3645. doi:10.1016/j.vaccine.2011.03.015.

Long term stability of a recombinant *Plasmodium falciparum* AMA1 malaria vaccine adjuvanted with Montanide® ISA 720 and stabilized with Glycine

Daming Zhu^{*}, Holly McClellan, Weili Dai, Elizabeth Gebregeorgis, Mary Anne Kidwell, Joan Aebig, Kelly M. Rausch, Laura B. Martin¹, Ruth D. Ellis, Louis Miller, and Yimin Wu
Laboratory of Malaria Immunology and Vaccinology, National Institute of Allergy and Infectious Disease, National Institutes of Health, 5640 Fishers Lane, Rockville, MD, USA 20852

Abstract

Plasmodium falciparum apical membrane antigen 1 (AMA1) is an asexual blood-stage vaccine candidate against the malaria parasite. AMA1-C1/ISA720 refers to a mixture of recombinant AMA1 proteins representing the FVO and 3D7 alleles in 1:1 mass ratio, formulated with Montanide® ISA 720 as a water-in oil emulsion. In order to develop the AMA1-C1/ISA720 vaccine for human use, it was important to determine the shelf life of this formulation. Previously it was found 267mM glycine stabilized the proteins in Montanide® ISA 720 formulations for a short period of time at 2-8°C[25], we now test the long term stability of AMA1-C1 at 10 and 40 µg/ml formulated with Montanide® ISA 720 with 50 mM glycine as a stabilizer. Stability of AMA1-C1/ISA720 at different time points following formulation (0, 5, 12 or 18 months) was evaluated by determining the mean particle size (diameter of the mean droplet volume), total protein content by a Modified Lowry assay, identity and integrity using western blot and SDS-PAGE. Our results showed that the mean particle size of these emulsions increased over time, whereas protein content, as determined by an ELISA method using a monoclonal antibody against penta-his, decreased over time. For the 10 µg/ml AMA1-C1/ISA720 vaccine, the protein content with was 6.5 ± 2.2 µg/ml, and for the 40 µg/ml AMA1-C1/ISA720 vaccine, the protein content was only 8.2 ± 2.3 µg/ml after 18 months of storage at 2-8°C. These results suggest that the integrity of the protein was affected by long-term storage. The results of the present study indicate that the AMA1-C1/ISA720 emulsion was unstable after 12 months of storage, after which AMA1-C1 proteins were partially degraded.

Keywords

Montanide® ISA 720; AMA1; Malaria; Vaccine

^{*}Correspondence: Daming Zhu, Laboratory of Malaria Immunology and Vaccinology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5640 Fishers Lane, Room 1118, Rockville, MD 20852, USA. Phone: (301) 402-7957; FAX: (301) 480-1962; dzhu@niaid.nih.gov.

¹Current address: Novartis Vaccines Institute for Global Health. S.r.l., Siena, Italy.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Malaria is one of most deadly infectious disease in the world, with an estimate of nearly 1 million deaths per year, most of which are African children [1]. *Plasmodium falciparum* has been found to be the most virulent species to infect humans and accounts for most deaths [1-2]. A vaccine that could reduce the impact of infection with *P. falciparum* would be valuable ammunition in the fight against this disease. Apical membrane antigen 1 (AMA1), a surface protein expressed during the asexual and sporozoite stages of *P. falciparum*, is a leading blood-stage vaccine candidate and has been tested in a number of clinical trials adjuvanted with Alhydrogel[®], AS02A, or AS01B [3-6]. All vaccine formulations were found to be safe and immunogenic, induced functional antibodies with moderate parasite growth-inhibition activity (GIA). The addition of the Toll-Like Receptor 9 agonist CPG 7909 to the AMA1-C1/Alhydrogel[®] formulation enhanced induction of the anti-AMA1 antibody by 14 fold, and dramatically increased GIA up to 96% inhibition [7]. However, CPG 7909 is a novel adjuvant that has primarily been used in terminally-ill cancer patients, and a search for an alternative adjuvant is warranted.

Montanide[®] ISA 720 (ISA 720) is a squalene-based water-in-oil adjuvant that induces high antibody titers in several animal species and in human malaria, HIV and cancer clinical trials [5, 8-22], possibly due to the slow-release capability of the inert water-in-oil emulsion and immune stimulating effects of its components [23-24]. However in an early human trial, AMA1 malaria antigen formulated in Montanide[®] ISA 720 lost potency over the course of the trial [16]. Subsequently, we attributed the loss of vaccine potency to instability of the antigen in the ISA 720 formulation, and showed that the addition of glycine or glycylglycine to the formulation could prevent AMA1 modifications over a short period of time [25]. However, the long term stability of AMA1 formulated in ISA 720 has not been evaluated.

In this paper, we report the results of long term stability studies of 10 µg/mL and 40 µg/mL AMA1-C1 vaccine, formulated in Montanide[®] ISA 720 (AMA1-C1/ISA 720) with 50 mM glycine and stored at 2-8°C for up to 18 months. We also describe the methods used to assess stability over time. The study was conducted to support a Phase 1 clinical trial of AMA1-C1/ISA 720, the results of which have been previously published [12].

2. Materials and Methods

2.1 Manufacture of recombinant AMA1-FVO and AMA1-3D7

Clinical grade recombinant AMA1-FVO and AMA1-3D7 proteins were purified and characterized by the Laboratory of Malaria Immunology and Vaccinology (LMIV) (formerly known as Malaria Vaccine Development Branch (MVDB)), National Institute of Allergy and Infectious Disease, National Institutes of Health, with methods developed at LMIV [26]. Protein concentration was measured by UV spectrum at 280nm and calculated using an extinction coefficient of 1.206 or 1.205 for AMA1-FVO or AMA1-3D7, respectively. Purified AMA1-FVO and AMA1-3D7 proteins underwent extensive quality control analysis and met all the pre-set specifications on purity, identity, and integrity.

2.2 Preparation of AMA1-C1/ISA720 formulations

AMA1-FVO and AMA1-3D7 were mixed at a 1:1 mass ratio, at a final vaccine concentration of 10 or 40 µg/mL in saline with 50 mM glycine and homogenized with Montanide[®] ISA 720 (SEPPIC, Paris, France) in a 3:7 ratio (v/w). The clinical grade emulsions were prepared at the Pharmaceutical Development Service (PDS), NIH Clinical Center, by homogenizing 200 mL of antigen-adjuvant mixture in a 400-mL stainless steel sealed chamber using Omni Mixer-ES homogenizer (Catalog # ES-115, Omni International, Warrenton, VA) at 10000 rpm for three 2 minute mixings at room temperature, and were

then aliquotted to 1 mL of volume in 2 mL borosilicate glass vials (Wheaton, Millville, NJ). For stability studies, the vialled vaccines were stored at 2-8°C for the indicated time period.

Reference emulsions were prepared by vortexing vaccine components using Daigger Vortex Genie 2 vortexer (Daigger & Co., Inc., Vernon Hills, IL) in a total volume of 1 mL in 2 mL borosilicate glass vials for 30 min at maximum speed at room temperature. For Modified Lowry Assay, the reference standards were prepared at 5, 10, 20, 40, 80, and 160 µg/mL. For ELISA, reference standards were prepared at the dose concentrations of 1.25, 2.5, 5, 10, 20, 40, 80, and 160 µg/mL. Two dose concentrations of QC internal control samples at 10 µg/mL and 40 µg/mL were also prepared to confirm the accuracy of ELISA.

Placebo vials were prepared by vortexing with the saline, glycine and ISA 270 components but without addition of antigen.

2.3 Antigen extraction

Antigens were extracted from vaccine emulsions by addition of benzyl alcohol to a final concentration of 10% (v/v) [28]. The vials were vortexed at the maximum speed (Daigger Vortex Genie 2) at room temperature for 20 min, followed by centrifugation for 10 min at 16,000 g. The antigen-containing aqueous phases (~ 0.3 mL) were transferred to an Eppendorff tube for further analysis. The extraction efficiency was estimated to be ~100% by densitometry of silver-stained SDS-PAGE using a set of standards with known amounts of AMA1.

2.4 SDS-PAGE and Western blot

Approximately 250 ng of extracted AMA1-C1 were resolved on 4-20% gradient Tris-glycine SDS-PAGE gels (Invitrogen Corp) under reducing and non-reducing conditions using an XCell SureLock electrophoresis Mini-Cell apparatus (Invitrogen Corp). Extractions of freshly prepared or stored AMA1-C1/ISA 720 emulsions, at 4°C for 5, 12 or 18 months, were analyzed simultaneously on the same SDS-PAGE and visualized by silver staining.

Western blots (250 ng per lane) were performed on nitrocellulose membrane (Invitrogen Corp.) and probed with the monoclonal antibodies 1G4 that recognizes AMA1 domains I/II; 2C2 that recognizes an unknown epitope of AMA1, and 4G2 that recognizes a conformational epitope of AMA1. Briefly, after transfer, the membranes were blocked with 3% skim milk in Tris-buffered saline (TBS), followed by incubation with individual mAbs. The membranes were then incubated with goat anti-mouse (or rat for 4G2) immunoglobulin G (IgG)-alkaline phosphatase conjugate (KPL Labs, Gaithersburg, MD). After extensive washing in TBS/0.05% Tween 20, blots were developed by incubation with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT substrate, KPL Labs) according to the manufacturer's instructions.

The silver stained gel and Western blot membranes were scanned with a Laser Densitometer (Molecular Dynamics) and the intensity of bands were analyzed by ImageQuant software (GE Health Care) [29]. The relative quantity of the extracted proteins intensity of extract at each time point was calculated as [relative quantity of the extracted proteins intensity = (intensity of extract/intensity of initial) × 100].

2.5 Particle size analysis

Vaccine emulsions were analyzed for particle size distribution using a Mastersizer 2000 laser diffraction-based particle size analyzer with an attached Hydro 2000S sample dispersion unit (Malvern Instruments Inc.). ISA 720 emulsions were dispersed in an oil phase consisting of Marcol 5 paraffin oil (Exxon Co., U.S.A.) and 2% (v/v) Montanide 80

surfactant (SEPPIC, Inc.) flowing through the particle size analyzer. Approximately 200 μL per emulsion was added to the dispersion unit to obtain laser obscuration in a range suitable for each reading. The system was flushed with Marcol 5 paraffin oil between all readings.

2.6 Modified Lowry Assay

The total protein concentration of ISA 720 vaccines was determined by a modified Lowry protein assay (Pierce) that included additional steps for phase separation of the ISA 720 emulsion. In short, 40 μL each of reference standards and stability samples of the ISA 720 vaccines were mixed with 40.6 μL of 20% SDS (Quality Biological) and 72 μL of saline (Baxter) in a 1.5 mL microcentrifuge tube, followed by addition of 650 μL of Modified Lowry reagent and 325 μL of Folin-Ciocalteu reagent (diluted 1:6 with distilled water, Gibco) according to the manufacturer's instructions. The samples were incubated at room temperature for 10 minutes, and then mixed for 10 seconds using a Vortex Genie 2 at maximum speed. Following vortexing, 325 μL of 1-butanol (Sigma-Aldrich) were added to separate the emulsion phase. Each sample was immediately mixed at the maximum speed and was incubated at 55°C for 30 minutes. After centrifuging samples at 16,000 g for one minute, the aqueous layer of each sample was transferred to a new tube. Finally, 700 μL of the aqueous layer from each sample and 100 μL of reagent blank solution (final composition 5.2% SDS, 11.5% saline, and 83.3% Modified Lowry reagent) were mixed in a cuvette and the absorbance at 750 nm was measured using a spectrophotometer (GE Healthcare). A placebo was used as a blank to zero the instrument. The protein concentrations of test ISA 720 vaccines were derived from a linear regression of absorbance readings from the reference standard ISA 720 vaccines. Each reference standard or test sample was tested in triplicate.

2.7 Competitive ELISA

A competitive ELISA was developed for the measurement of total protein concentration of ISA 720 vaccines. Briefly, a 96-well flat bottom ELISA plate (Dynex Technologies, Inc.) was coated with 200 μL of 0.0625 $\mu\text{g}/\text{mL}$ AMA1-3D7 in coating buffer (15 mM Sodium Carbonate/35 mM Sodium Bicarbonate) and was incubated overnight at 4°C. The plate was washed three times with washing buffer (1 $\times$ TBS/0.1% Tween-20) and blocked with blocking solution (5% skim milk/1 $\times$ TBS) for 2 hr at 37°C. After washing, 10 μL of standard or vaccine extraction, 90 μL of BSA dilution buffer and 100 μL of 100 ng/mL mouse anti-Penta-His mAb (Qiagen) were added to each well sequentially, the plate was then incubated at 37°C for 1.5 hours. After washes, 200 μL of 160 ng/mL of HRP-conjugated goat-anti-mouse IgG (Thermo Scientific) was added to each well and the plates was incubated for another 1.5 hr at 37°C. After final washes, 150 μL of TMB (KPL Inc.) was added to the wells and color development was read at 650 nm by a spectrometer (Spectra Max, Molecular Devices) after 60 min incubation at room temperature.

Standard curves were generated at a range of 2.5 to 80 $\mu\text{g}/\text{mL}$, i.e. 2.5, 5.0, 10, 20, 40, and 80 $\mu\text{g}/\text{mL}$ AMA1-C1 with the correlation coefficient of linear regression greater than 0.99. The concentrations of test antigen were calculated from the linear regression equations.

Placebo extracts, saline and the formulation buffer were included in each plate as negative controls.

Each sample including standard, test sample and negative controls was tested in triplicate.

2.7 Vaccine potency assays

Animal studies were performed in compliance with National Institutes of Health guidelines and under the auspices of an Animal Care and Use Committee approved protocol. The

vaccine potency assays were carried out in female BALB/c mice. For potency assay design, the stored 10 and 40 µg/mL of AMA1-C1/ISA 720 vaccines (test vaccines) were compared to two freshly formulated, thus considered to be fully potent, reference vaccines with equal dose concentrations. Both test and reference vaccines are serially diluted to 3 dose concentrations that give low (~10%), median (~50%), and high (~90%) immune response. The assay is conserved to be internally valid if the antibody titers induced by both test and reference vaccines give a significant dose response correlation by Spearman Rank analysis. The test vaccine is considered to be fully potent if the antibody titer induced by middle dose (located at the mid-point of the dose-response curve) of the test vaccine is statistically indistinguishable, by Mann-Whitney U test, from that induced by the reference vaccine [30]. Each animal (10 animal per group) received two 250 µL intraperitoneal immunizations, in a 4-week interval, of 0.03, 0.1 or 0.3 µg of AMA1-C1 derived from the 10 and 40 µg/mL of AMA1-C1/ISA 720 vaccines diluted in a placebo formulation consisting of saline, 50 mM glycine and ISA720 after storage at 0, 4, 8, or 14 months at 4°C. A set of freshly formulated 10 and 40 µg/mL vaccines were diluted in the same manner to equivalent dose concentrations and served as reference controls. Sera were collected 4 weeks post-secondary immunization and serum antibodies to AMA1-FVO or AMA1-3D7 were assayed by ELISA [31].

3. Results

3.1 Emulsion particle size

The particle size distributions of the 10 and 40 µg/mL of AMA1-C1/ISA 720 vaccines at T=0 and following storage at 2-8°C for 5, 12 or 18 months were analyzed by a Mastersizer 2000 laser diffraction-based particle size analyzer. The volume median diameter D (0.5) (the diameter where 50% of the distribution is above and 50% is below), D (0.9) (90% of the volume distribution is below this value) and the volume weighted mean (VWM, mean droplet volume) were measured. The particle size of these emulsions increased over time (Table 1 and Fig. 1), though still within the pre-determined specifications of VWM=0.5-1.8 µm, and D/VWM<2.2.

3.2 AMA1-C1 dose concentration

Modified Lowry assay was used to determine the AMA1-C1 dose concentration because the method allows extraction of the antigen from the water-in-oil emulsion without compromising the integrity of AMA1-C1 protein (Zhu D et al unpublished data)

As indicated by the results, the change in total protein concentrations extracted from the 40 µg/ml AMA1-C1 vaccine formulation at various storage time points was within a pre-set specification ($\pm 15\%$), ranging from 37 ± 1.6 µg/ml to 43.1 ± 4.7 µg/ml. For the 10 µg/ml AMA1-C1 vaccine, however, the concentration was 11.4 ± 2.7 µg/ml at T=0 and decreased to 7.5 ± 2.5 µg/ml at T=5 month, and became undetectable for the samples at 12 months and after. (Table 2).

A more sensitive quantification method, a competition ELISA using an anti-penta-His mAb, was developed to determine the protein concentration in the vaccine extracts at T=5 and 18 months. No significant difference was detected between the expected value and tested value for both vaccine doses stored for 5 months at 4°C. However, at T=18-months, only 6.5 ± 2.2 µg/ml was detected for the 10 µg/ml vaccine and 8.2 ± 2.3 µg/ml for the 40 µg/ml vaccine. (Table 2) (Fig. 3), indicating the loss of the hexa-his epitope.

Consistent with results in the competition ELISA, the loss of AMA1-C1 protein-specific epitopes was also observed in extracts from vaccines stored over time. As shown in Figure 2, the intensity of AMA1-C1 protein bands resolved by SDS-PAGE and visualized by silver

staining and AMA1-C1 specific mAbs decreased. The relative amount of the AMA1-C1 protein band was quantified by densitometry (Fig 2C, 2D). By T=18 months, the majority of the AMA1-C1-specific epitopes were less than 50% of their initial levels.

3.3 Vaccine potency

Potency of the vaccines after 4, 8, and 14 months of storage was determined by antibody responses in mice compared to a freshly formulated reference vaccine, thus considered to be fully potent. Mouse anti-AMA1-FVO and anti-AMA1-3D7 titers were measured by ELISA. The vaccines were fully potent at 4 and 8 months (data not show) Figure 4 shows results of the T=14 months potency assay, and there was no statistical difference in antibody titers between the AMA1-C1/ISA720 vaccines and the reference vaccine, indicating the vaccines were potent in mice.

Discussion

Montanide® ISA 720 has been shown to induce high antibody titers and specific cytotoxic T-lymphocyte (CTL) responses in a variety of animal species [10, 27]. It has been used as a vaccine adjuvant in malaria, HIV and cancer clinical trials [5, 8-22] and induced both humoral and cellular immune responses [5, 16-22]. However, a loss of potency with a pre-formulated AMA1/ISA 720 vaccine was reported due to the lack of vaccine stability [16]. After an extensive screening of the effects of antioxidants and amino acids in the formulation, Miles et al reported that glycine or glycalglycine were able to stabilize the protein by eliminated the broadening of protein band and preventing multimer formation [25]. The optimal concentration of glycine was later determined to be 50 mM after comparison of the glycine concentrations at 50, 134 and 267 mM in our laboratory (data not shown).

Due to difficulties in standardizing bedside formulation of ISA 720 emulsions, we chose to prepare and store AMA1-C1/ISA 720 with the addition of 50 mM glycine as an antigen stabilizer prior to initiating clinical trials [12]. Therefore, monitoring the stability of the pre-formulated vaccine emulsions was critical, particularly given the previous example of loss of potency with a pre-formulated AMA1/ISA 720 vaccine [16]. There are no generic methods available to evaluate the stability of ISA 720 based vaccines. In the present study, we report a set of analyses to assess the physical, chemical and biological stability of ISA720 emulsions. Appearance, sterility, integrity by particle size and SDS-PAGE with silver staining, identity by mAb recognition, and protein content by Modified Lowry Assay and ELISA were defined as stability indicating tests.

It is important to achieve a homogeneous distribution of small particles even if the smallest particles are not linked with stability [23]. The manufacturer of ISA 720 (SEPPIC, Inc.) recommends an emulsion droplet size of approximately 1 μ m diameter to achieve stability and immunogenicity. Larger droplets may cause the emulsion to prematurely collapse [25]. In our study, although the droplet size of both the 10 μ g/mL and 40 μ g/mL emulsions still met the acceptable specification of VWM at 0.5-1.8 μ m (based on the manufactures' recommendation and our experiences) after 18 month storage at 2-8°C, a significant increase, from 0.77 μ m on the day of formulation to 1.26 μ m at 18 months for 10 μ g/mL and from 0.76 μ m to 1.27 μ m for 40 μ g/mL was observed, suggesting that physical stability of emulsions changes over storage time at 2-8°C.

The AMA1-C1 proteins were also found to be unstable in the ISA720 emulsion after 18 months of storage. For the 10 μ g/ml vaccine, the protein content at the 18 month time point was below the level of detection (5 μ g/ml) in modified Lowry assay, and was significantly lower as measured by competition ELISA with anti-His mAb, and densitometry of Western

blots. For the 40 µg/ml vaccine at the same time point, the protein content was 42 ± 2.6 µg/ml by modified Lowry assay, but was about 4 fold less by competition anti-His tag ELISA and densitometry of Western blots with mAb 1G4, 2C2 or 4G2. Because the modified Lowry assay indiscriminately measures intact as well as degraded protein fragments, data by competition ELISA using anti-His mAb and densitometry of Western blots using multiple mAbs recognizing AMA1 functional and conformational epitopes are better measurements of the AMA1 antigen integrity.

In contrast to biochemical measurement for antigen and formulation stability, *in vivo* potency assay in mice, as measured by antibody ELISA, is a less sensitive method to evaluate formulation stability. Despite a considerable decrease in AMA1 antigen content after 12 months storage at 2-8°C, the vaccines induced antibody response levels comparable to a freshly formulated reference vaccine, thus considered to be fully potent. These could be due to the monoclonal antibodies used in present study recognize specific epitopes of AMA1, loss of these epitopes due to degradation or denaturation will result in loss of signal in ELISA or western blot. However, the *in vivo* potency assay measures polyclonal IgGs against multiple epitopes, it is thus less sensitive compared to biochemical tests. The vaccines remained within specifications during the course of the clinical trial, and both doses were also shown to be potent in humans [12].

More degradation was found in the higher antigen dose (40 µg/mL) when compared to the lower antigen dose (10 µg/mL). This may be due to the higher amount of protein in the emulsion, the more polar and nonpolar groups present in the dispersed phase of emulsion, which will modify the global hydrophilic/lipophilic balance (HLB), and decrease the stability of the emulsion [23]. This result suggests that different doses of vaccine may have different shelf lives although they were formulated with the same antigen.

In summary, we found that while the AMA1-C1/ISA720 emulsions remained within specifications during the clinical trial (within 12 months post vaccine manufacture), the emulsions were unstable at longer storage times, with partial degradation of AMA1-C1 after 12-18 months of storage at 2-8°C. Combinations of particle size analysis, SDS-PAGE, Western blot, a modified Lowry assay and competitive ELISA methods are likely to be more sensitive methods for determining protein degradation than animal potency assays.

Acknowledgments

We thank Dr. David Narum and the Process Development Unit for coordinating the production of recombinant proteins AMA1-FVO and AMA1-3D7, Lynn Lambert and Dr. Kazutoyo Miura and their teams for performing the mouse potency study.

References

1. WHO. World Malaria Report. 2008. <http://www.who.int/malaria/wmr2008/malaria2008.pdf>
2. Goodman AL, Draper SJ. Blood-stage malaria vaccines - recent progress and future challenges. *Ann Trop Med Parasitol*. 2010 Apr; 104(3):189–211. [PubMed: 20507694]
3. Remarque EJ, Faber BW, Kocken CH, Thomas AW. Apical membrane antigen 1: a malaria vaccine candidate in review. *Trends Parasitol*. 2008; 24(2):74–84. [PubMed: 18226584]
4. Malkin E, Diemert DJ, McArthur JH, Perreault JR, Miles AP, Giersing BK, et al. Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *Infection and Immunity*. 2005; 73:3677–3685. [PubMed: 15908397]
5. Roestenberg M, Remarque E, de Jonge E, Hermsen R, Blythman H, Leroy O, et al. Safety and immunogenicity of a recombinant *Plasmodium falciparum* AMA1 malaria vaccine adjuvanted with Alhydrogel, Montanide ISA 720 or AS02. *PLoS One*. 2008; 3:e3960. [PubMed: 19093004]

6. Spring MD, Cummings JF, Ockenhouse CF, Dutta S, Reidler R, Angov E, et al. Phase 1/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A. *PLoS One*. 2009; 4:e5254. [PubMed: 19390585]
7. Mullen GED, Ellis RD, Miura K, Malkin E, Nolan C, Hay M, et al. Phase 1 trial of AMA1-C1/Alhydrogel plus CPG 7909: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *PLoS One*. 2008; 3:e2940. [PubMed: 18698359]
8. Collins WE, Walduck A, Sullivan JS, Andrews K, Stowers A, Morris CL, et al. Efficacy of vaccines containing rhoptry-associated proteins RAP1 and RAP2 of *Plasmodium falciparum* in Saimiri boliviensis monkeys. *Am J Trop Med Hyg*. 2000; 62(4):466–79. [PubMed: 11220762]
9. Saul A, Hensmann M, Sattabongkot J, Collins WE, Barnwell JW, Langermans JA, et al. Parasite Immunogenicity in rhesus of the *Plasmodium vivax* mosquito stage antigen Pvs25H with Alhydrogel and Montanide ISA 720. *Immunol*. 2007; 29(10):525–33.
10. Langermans JA, Hensmann M, van Gijlswijk M, Zhang D, Pan W, Giersing BK, et al. Preclinical evaluation of a chimeric malaria vaccine candidate in Montanide ISA 720: immunogenicity and safety in rhesus macaques. *Hum Vaccine*. 2006; 2(5):222–6.
11. Kaumaya PT, Foy KC, Garrett J, Rawale SV, Vicari D, Thurmond JM, et al. Phase I active immunotherapy with combination of two chimeric, human epidermal growth factor receptor 2, B-cell epitopes fused to a promiscuous T-cell epitope in patients with metastatic and/or recurrent solid tumors. *J Clin Oncol*. 2009; 27(31):5270–7. [PubMed: 19752336]
12. Pierce MA, Ellis RD, Martin LB, Malkin E, Tierney E, Miura K, et al. Phase 1 safety and immunogenicity trial of the *Plasmodium falciparum* blood-stage malaria vaccine AMA1-C1/ISA 720 in Australian adults. *Vaccine*. 2010; 28(10):2236–42. [PubMed: 20051276]
13. Lawrence GW, Saul A, Giddy AJ, Kemp R, Pye D. Phase 1 trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. *Vaccine*. 1997; 15(2):176–178. [PubMed: 9066035]
14. Toledo H, Baly A, Castro O, Resik S, Laferté J, Rolo F, et al. A phase I clinical trial of a multi-epitope polypeptide TAB9 combined with Montanide ISA 720 adjuvant in non-HIV-1 infected human volunteers. *Vaccine*. 2001; 19(30):4328–36. [PubMed: 11457560]
15. Raya NE, Quintana D, Carrazana Y, Gómez CE, Duarte CA. A prime-boost regime that combines Montanide ISA720 and Alhydrogel to induce antibodies against the HIV-1 derived multiepitope polypeptide TAB9. *Vaccine*. 1999 Jun 4; 17(20-21):2646–50. [PubMed: 10418914]
16. Saul A, Lawrence G, Allworth A, Elliott S, Anderson K, Rzepczyk C, et al. A human Phase 1 vaccine clinical trial of the *Plasmodium falciparum* malaria vaccine candidate apical membrane antigen 1 in Montanide ISA720 adjuvant. *Vaccine*. 2005; 23:3076–3083. [PubMed: 15811655]
17. Genton B, Al-Yaman F, Anders R, Saul A, Brown G, Pye D, et al. Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. *Vaccine*. 2000; 18(23):2504–11. [PubMed: 10775784]
18. Hersey P, Menzies SW, Coventry B, Nguyen T, Farrelly M, Collins S, et al. Phase I/II study of immunotherapy with T-cell peptide epitopes in patients with stage IV melanoma. *Cancer Immunol. Immunother*. 2005; (54):208–218.
19. Hu J, Chen Z, Gu J, Wan M, Shen Q, et al. Safety and immunogenicity of a malaria vaccine, *Plasmodium falciparum* AMA-1/MSP-1 chimeric protein formulated in montanide ISA 720 in healthy adults. *PLoS One*. 2008; 3(4):e1952. [PubMed: 18398475]
20. Lawrence G, Cheng Q, Reed C, Taylor D, Stowers A, Cloonan N, et al. Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. *Vaccine*. 2000; (18):1925–1931. [PubMed: 10699342]
21. Audran R, Lurati-Ruiz F, Genton B, Blythman HE, Ofori-Anyinam O, Reymond C, et al. The synthetic *Plasmodium falciparum* circumsporozoite peptide PfCS102 as a malaria vaccine candidate: a randomized controlled phase I trial. *PLoS One*. 2009; 4(10):e7304. [PubMed: 19798415]
22. Oliveira GA, Wetzel K, Calvo-Calle JM, Nussenzweig R, Schmidt A, Birkett A, et al. Safety and enhanced immunogenicity of a hepatitis B core particle *Plasmodium falciparum* malaria vaccine formulated in adjuvant Montanide ISA 720 in a phase I trial. *Infect Immun*. 2005; 73(6):3587–97. [PubMed: 15908388]

23. Aucouturier J, Dupuis S, Deville S, Ascarateil S, Ganne V. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev Vaccines*. 2002; 1:111–118. [PubMed: 12908518]
24. Aucouturier J, Ascarateil S, Dupuis L. The use of oil adjuvants in therapeutic vaccines. *Vaccine*. 2006 Apr 12; 24(2):S2–44–5. [PubMed: 16823921]
25. Miles AP, McClellan HA, Rausch KM, Zhu D, Whitmore MD, Singh S, et al. Montanide ISA 720 vaccines: quality control of emulsions, stability of formulated antigens, and comparative immunogenicity of vaccine formulations. *Vaccine*. 2005; 23(19):2530–2539. [PubMed: 15752840]
26. Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, et al. In vitro studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun*. 2002; 70(12):6948–60. [PubMed: 12438374]
27. Scalzo AA, Elliott SL, Cox J, Gardner J, Moss DJ, Suhrbier A. Induction of protective cytotoxic T cells to murine cytomegalovirus by using a nonapeptide and a human-compatible adjuvant (Montanide ISA 720). *J Virol*. 1995; 69:1306–1309. [PubMed: 7815511]
28. Miles AP, Saul A. Extraction and characterization of vaccine antigens from water-in-oil adjuvant formulations. *Methods Mol Biol*. 2005; 308:293–300. [PubMed: 16082043]
29. Miles AP, Saul A. Quantifying recombinant proteins and their degradation products using SDS-PAGE and scanning laser densitometry. *Methods Mol Biol*. 2005; 308:349–56. [PubMed: 16082047]
30. Giersing BK, Dubovsky F, Saul A, Denamur F, Minor P, Meade B. Potency assay design for adjuvanted recombinant proteins as malaria vaccines. *Vaccine*. 2006; 24(20):4264–70. [PubMed: 16767804]
31. Miura K, Orcutt AC, Muratova OV, Miller LH, Saul A, Long CA. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine*. 2008 Jan 10; 26(2):193–200. Epub 2007 Nov 20. [PubMed: 18054414]

Abbreviations

AMA1	<i>Plasmodium falciparum</i> apical membrane antigen 1
AMA1-C1	AMA1-FVO and AMA1-3D7 mixed in a mass ratio of 1:1

Fig. 1. Particle size analysis of AMA1-C1/ISA720 formulations. A: 10 μg/ml formulation; B: 40 μg/ml formulation. At vaccine release (—●—) or following storage at 2-8°C for: (—■—) 5 months; (—▲—) 12 months; (—◆—) 18 months.

Fig. 2. SDS-PAGE/Western blot analyses and relative intensity of AMA1-C1/ISA 720 extractions. A and C: 10 µg/mL vaccine formulation; B and D: 40 µg/mL vaccine formulation. For A and B, Lane 1, T=0; Lane 2, T=5 months; Lane 3, T=12 months and Lane 4, T=18 months. For C and D, ■ T=0; ■ T=5 months; □ T=12 months; and ■ T=18 months.

Fig. 3.

Competitive ELISA results for 5 and 18 months clinical samples. (A). standard curve of competitive ELISA. The linear detection range of the assay was from 2.5 to 80 $\mu\text{g/mL}$. The correlation coefficient of linear regression was greater than 0.99 for all assays performed. (B). ELISA results. □ Reference formulations as internal control of assay; ▒ Clinical formulations stored at 2-8°C for 5 months; ■ Clinical formulations stored at 2-8°C for 18 months. Each bar represents the average value and standard deviation of test samples from 3 independent experiments with triplicate sample loadings.

Fig. 4.

Mouse potency study of vaccines stored for 14 months at 2-8°C. Each group of 10 mice was immunized with 0.1 µg and 0.3 µg of AMA1-C1/ISA720 per mouse, respectively. This graph shows average ELISA unit of anti-AMA1-FVO and AMA1-3D7 alleles. ● Reference formulations, ○ Clinical formulations. Shown are antibody levels of mice received 0.1 µg or 0.3 µg per immunization, diluted from the Reference or Clinical formulations of 10 µg/mL and 40 µg/mL, respectively.

Table 1
Particle size distribution of AMA1-C1/ISA720 formulations at different storage time

		Time of Analysis (month)			
		0	5	12	18
10 µg/ml	VWM ^a	0.773	1.033	1.175	1.257
	D(0.5) ^b µm	0.707	0.976	1.220	1.198
	D(0.9) ^c µm	1.311	1.548	1.766	1.867
	D(v, 0.9)/VWM	1.700	1.510	1.500	1.490
40 µg/ml	VWM ^a	0.763	1.053	1.193	1.270
	D(0.5) ^b µm	0.700	1.004	1.144	1.223
	D(0.9) ^c µm	1.284	1.583	1.728	1.765
	D(v, 0.9)/VWM	1.680	1.510	1.450	1.390

^aVolume Weighted Mean, i.e., diameter of the mean droplet volume.
^bD(v, 0.5) is the particle diameter where 50% of the distribution is above and 50% is below.
^cD(v, 0.9), is the particle diameter where 90% of the volume distribution is below this value.

Table 2
Analysis of test samples using modified Lowry assay and ELISA

Detecting Methods		Time of Analysis			
		Initial	5 months	12 months	18 months
10 µg/ml	Modified Lowry ^a	11.4 ± 2.7	7.5 ± 2.5	undetectable	undetectable
	ELISA ^b	N/A	10.6 ± 0.5	N/A	6.5 ± 2.2
40 µg/ml	Modified Lowry ^a	43.1 ± 4.7	39.4 ± 1.8	37 ± 1.6	42 ± 2.6
	ELISA ^b	N/A	46.5 ± 3.9	N/A	8.2 ± 2.3

^a Modified Lowry assay was performed in triplicate, and the experiments were repeated to confirm the “undetectable” findings for 10 µg/ml samples stored at 12 and 18 month, respectively.

^b ELISA data was generated from 3 experiments, in triplicate in each experiment.

^c i.e. mean ± standard deviation. The standard deviation was calculated by the following formula:

$$\sqrt{\frac{\sum (x - \bar{x})^2}{(n - 1)}}$$

where $\bar{X}$ is the sample mean and n is the sample size.