2008

Phase 2a Trial of 0, 1, and 3 Month and 0, 7, and 28 Day Immunization Schedules of Malaria Vaccine RTS,S/AS02 in Malaria-Naive Adults at the Walter Reed Army Institute of Research

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Vaccine (2008) **26**, 2191–2202

available at www.sciencedirect.com

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Phase 2a trial of 0, 1, and 3 month and 0, 7, and 28 day immunization schedules of malaria vaccine RTS,S/AS02 in malaria-naïve adults at the Walter Reed Army Institute of Research☆

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Received 13 December 2007; received in revised form 14 February 2008; accepted 22 February 2008
Available online 13 March 2008

KEYWORDS
RTS,S;

Summary
Background: Immunization with RTS,S/AS02 consistently protects some vaccinees against malaria infection in experimental challenges and in field trials. A brief immunization schedule

☆ The opinions expressed herein are the private views of the authors and are not to be construed as official or as reflecting the views of the United States Army, the Department of Defense, the Centers for Disease Control and Prevention, or the Department of Health and Human Services.

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2008.02.048

Malaria;
Vaccine;
Falciparum;
Adjuvant System;
AS02;
Circumsporozoite protein;
Hepatitis B;
Antibody;
Clinical trials;
Rapid immunization;
IFN-γ;
ELISPOT

against falciparum malaria would be compatible with the Expanded Programme on Immunization, or in combination with other prevention measures, interrupt epidemic malaria or protect individuals upon sudden travel to an endemic area.

Methods: We conducted an open label, Phase 2a trial of two different full dose schedules of RTS,S/AS02 in 40 healthy malaria-naïve adults. Cohort 1 ($n = 20$) was immunized on a 0, 1, and 3 month schedule and Cohort 2 ($n = 20$) on a 0, 7, and 28 day schedule. Three weeks later, 38 vaccinees and 12 unimmunized infectivity controls underwent malaria challenge.

Results: Both regimens had a good safety and tolerability profile. Peak GMCs of antibody to the circumsporozoite protein (CSP) were similar in Cohort 1 (78 μg/mL; 95% CI: 45—134) and Cohort 2 (65 μg/mL; 95% CI: 40—104). Vaccine efficacy for Cohort 1 was 45% (95% CI: 18—62%) and for Cohort 2, 39% (95% CI: 11—56%). Protected volunteers had a higher GMC of anti-CSP antibody (114 μg/mL) than did volunteers with a 2-day delay (70 μg/mL) or no delay (30 μg/mL) in the time to onset of parasitemia (Kruskal—Wallis, $p = 0.019$). A trend was seen for higher CSP-specific IFN-γ responses in PBMC from protected volunteers only in Cohort 1, but not in Cohort 2, for ex vivo and for cultured ELISPOT assays.

Conclusion: In malaria-naïve adults, the efficacy of three-dose RTS,S/AS02 regimens on either a 0, 1, and 3 month schedule or an abbreviated 0, 7, and 28 day schedule was not discernibly different from two previously reported trials of two-dose regimens given at 0, 1 month that conferred 47% (95% CI: −19 to 76%) protection and in another trial 42% (95% CI: 5—63%). A strong association of CSP-specific antibody with protection against malaria challenge is observed and confirms similar observations made in other studies. Subsequent trials of adjuvanted RTS,S in African children and infants on a 0, 1, and 2 month schedule have demonstrated a favorable safety and efficacy profile.

Introduction

Malaria, especially malaria caused by deadly *Plasmodium falciparum* infection, is most threatening to individuals without pre-existing anti-malaria immunity. The largest vulnerable populations are infants and women during their first pregnancy living in endemic lands [1]. But malaria-naïve individuals exposed to epidemic malaria or upon travel to malaria-endemic regions are also at risk of severe disease and death [2,3].

Protection against this mosquito-borne disease is simple in concept, but difficult in practice. The present public health crisis, demonstrated by the fact that more than three children die from malaria every minute [4], is further exacerbated by the inadequate use of proven control measures, such as selective use of house-hold DDT for indoor residual spraying [5], judicious intermittent presumptive treatment [6], and sleeping under insecticide-treated bed nets to avoid nocturnal exposure [7]. The global spread of multiple drug-resistant *P. falciparum* [8] has sharply limited the choice of chemoprophylactic drugs and has worsened the chronic shortages of affordable, effective treatment drugs [9].

A malaria vaccine would be a critically important addition to the present arsenal of malaria prevention measures. Although many candidate vaccines are in development [10], only the RTS,S antigen formulated with either the AS01 or the AS02 Adjuvant System consistently confers partial protective immunity against infection by the *P. falciparum* parasite in malaria-naive ([11—13], Kester, unpublished) and malaria endemic populations ([14,15], Polhemus, unpublished), and in one trial, reduced clinical and severe malaria in young African children for 18 months [15,16]. Most recently, RTS,S/AS02 given in an Expanded Programme on Immunization compatible schedule at 10, 14, and 18 weeks of age was shown for the first time to protect infants against infection and clinical malaria for a 3-month period [17].

At the time the presently reported trial was conducted, we had evaluated three doses of adjuvanted RTS,S in Phase 2a trials in malaria naïve adults using either a 0, 1, and 9 month [10] or a 0, 1, and 6 month schedule [11], but not on the shorter schedules presented here. Importantly, RTS,S/AS02 had also displayed a promising safety and tolerability profile. We had also previously conducted two Phase 2a trials in which we evaluated the preliminary efficacy of two doses of RTS,S/AS02 given at 0 and 1 months in malaria-naïve adults [11,12]. We undertook the present trial to determine if improved efficacy might be achieved by the administration of three doses of RTS,S/AS02 when given on one of two briefer schedules of immunization; either at 0, 1, and 3 months or at 0, 7, and 28 days.

Material and methods

Ethics

The trial was conducted according to Good Clinical Practices under a protocol approved by the Human Use Review Committee of the Walter Reed Army Institute of Research and by the US Army Surgeon General's Human Subjects Research Review Board, Fort Detrick, Maryland under US Food and Drug Administration Investigational New Drug application BB-6043. Written informed consent was obtained prior to screening and enrollment.

Participants

Healthy malaria-naïve civilian and military adult men and women, age 18—45 years, were recruited by non-coercive

means through the Walter Reed Army Institute of Research (WRAIR) Clinical Trials Department, Silver Spring, Maryland. Standard measures undertaken to avoid coercion or the appearance thereof included the careful review and approval of all financial incentives for study participation by the Institutional Review Boards, the provision to each study subject of an independent point of contact in the Office of Human Subjects Protection, and the requirement that each subject pass a written comprehension test indicating understanding of all study procedures and the proviso that participation was voluntary and that the subject was free to withdraw at any time without requirement to give an explanation.

Vaccine

The RTS,S/AS02 vaccine has been described in detail [18]. Just prior to immunization, GMP lyophilized RTS,S antigen was mixed with GMP AS02 Adjuvant System. The final 0.5 mL dose of RTS,S/AS02 contained 50 μg of RTS,S, 50 μg of MPL, 50 μg of QS21 and 25 μg of thimerosal in an oil-in-water emulsion. With the exception of the presence of thimerosal (25 μg/dose), this vaccine Adjuvant System is identical to the thimerosal-free formulation of RTS,S known as RTS,S/AS02A now in clinical trials.

Trial design

The primary objective of this open-label, Phase 2 clinical trial of RTS,S/AS02 was to assess the safety, reactogenicity, and efficacy of RTS,S/AS02 in two different three-dose schedules in malaria-naïve adults. The secondary objective was to assess the humoral and cell-mediated immune responses induced by RTS,S/AS02 when administered in the two different regimens. The trial consisted of two phases: an immunization phase followed by a challenge phase. During the immunization phase, subjects were recruited and then sequentially enrolled into one of two cohorts; Cohort 1 enrollment was completed before Cohort 2 enrollment began. Cohort 1 ($n = 20$) received RTS,S/AS02 on a 0, 1, and 3 month schedule, and Cohort 2 ($n = 20$) received RTS,S/AS02 on a 0, 7, and 28 day schedule. The immunization phase of both cohorts finished at the same time. Just prior to the challenge phase, 12 additional subjects were recruited to serve as non-immunized infectivity controls. Homologous 3D7 *Plasmodium falciparum* sporozoite challenge was then offered 3 weeks after the receipt of final immunizations to all subjects who had received three doses of vaccine, and to the infectivity controls.

Trial conduct

After obtaining written informed consent, volunteers underwent a medical interview, physical examination and laboratory screening with complete blood count, serum biochemistries (creatinine, alanine aminotransferase, and aspartate aminotransferase), and serologic tests to characterize their hepatitis B, hepatitis C, and HIV status. Volunteers were excluded from enrollment if they had a history of malaria, travel to a malaria-endemic area within the previous 12 months, or previous receipt of a malaria vaccine. Additional exclusion criteria were pregnancy, lactation, known or suspected alcohol or drug abuse, history of anaphylaxis following any vaccination, recent or planned administration of blood, blood products, surgery, participation in any concurrent research trial or clinically significant pulmonary, cardiovascular, hepatic or renal disease. Women were tested for pregnancy by serum β-HCG determination before enrollment, each immunization and malaria challenge, and were required to avoid pregnancy until two months after malaria challenge.

Assessment of safety

The safety of the vaccine was measured by assessment of reactogenicity and of hematological and biochemical parameters. After each injection of the vaccine, volunteers were observed for 30 min and again at 1, 2, and 3 days post-vaccination. During these 4 days, a standardized questionnaire was used to capture solicited local symptoms (redness, swelling or pain at the injection site) and solicited systemic symptoms (fever, headache, fatigue, gastrointestinal symptoms, myalgias, malaise, and joint pains). In addition, all unsolicited adverse events were recorded that were reported during the 30 days after each immunization.

Local adverse events were graded according to specific criteria. Pain at the injection site was graded as follows; 0 (absent), 1 (painful on touch), 2 (painful when limb is moved), or 3 (spontaneously painful). Redness and swelling were independently measured at the greatest surface diameter and assigned numbers as follows; 0 (0 mm), 1 (>0—20 mm), 2 (>20—50 mm), or 3 (>50 mm). Fever was defined according to oral temperature as follows: 0 (<37.5 °C), 1 (37.5—38 °C), 2 (>38—39 °C), or 3 (>39 °C). Other systemic symptoms were graded as follows: 0 (normal), 1 (easily tolerated), 2 (interferes with normal activity), and 3 (prevents normal activity).

Serious adverse events were captured throughout the trial. Hematologic and biochemical tests were performed on days 0, 14, 28, 42, and 98 for Cohort 1; on days 0, 7, 21, 28 and 42 for Cohort 2; and for all volunteers on day of challenge and 134 days post day of challenge.

Immunogenicity

Blood for analysis of humoral and cellular immune responses was obtained before the first immunization and at scheduled time points during the trial.

Serology

Anti-HBsAg and anti-CSP repeat region (anti-R32LR) antibody titers were measured on days 0, 14, 28, 42, and 98 for Cohort 1; on days 0, 7, 21, 28 and 42 for Cohort 2; and on day of challenge and 134 days post challenge for each cohort. Antibodies against the circumsporozoite protein central region's tandem tetrapeptide repeat epitopes were measured by ELISA using recombinant R32LR as the capture antigen as previously described [19—21]. Seropositivity was defined as ≥1.0 μg/mL of anti-CSP antibody. Antibodies against the HBsAg carrier matrix were measured using a commercial kit and were expressed in milli-international units per milliliter (mIU/mL) [22]. Seropositivity was defined as >3.3 mIU/mL of anti-HBsAg antibody, and seroprotection was defined as

>10 mIU/mL of anti-HBsAg antibody. Pre-challenge serum samples obtained on the day of sporozoite challenge along with pre-immune serum samples were analyzed by an indirect fluorescence antibody (IFA) assay using 3D7 strain air-dried sporozoites, as previously described [21].

Cell-mediated immunity

Heparinized peripheral blood was obtained before immunization and at 2 weeks after the third dose of vaccine. PBMCs were isolated by gradient centrifugation on Ficoll-Hypaque (ICN Biomedicals Inc., Aurora, OH) and were stored in liquid nitrogen until used.

Culture medium (CM) consisted of RPMI 1640 supplemented with 8 mm GlutaMAX™ (Invitrogen, Carlsbad, CA, USA), 50 μg/mL penicillin, 50 μg /mL streptomycin, 0.1 mm non-essential amino acids, 1 mm sodium pyruvate (all from Life Technologies; Grand Island NY), 0.042 mm 2-mercaptoethanol (Sigma Chemicals, St. Louis, MO) and 2.5—5% FBS (Hyclone, Logan UT) (enzyme-linked immunospot 'ELISPOT' assays) or 5% human AB serum (lymphoproliferative assays).

CSP peptides. Synthetic 15-mer peptides, p34 (a.a.316—330, NEEPSDKHIKE YLNK), p35 (a.a.321—335, DKHIKEYLNKIQNSL), p36 (a.a.326—340, EYLNKIQNSLSTEWS), p37 (a.a.331—345, IQNSLSTEWSPCSVT), p47 (a.a.351—365 QVRIKPGSANK PKDE) and p50 (a.a.371—385 DIEKKICK MEKCSSV) were used at the indicated concentrations. In addition, three longer peptides, P2 (EEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGI QVRIKPGSAN), P4 (KPKDELDYANDIEKKICKMEKCSSVFNVV SSIGL) and P5 (a 12 amino acid extension of P4) (GIQVRIKPGSANKPKDELDYANDIEKKICK MEKCSSVFNVVNSSIGL) that together represent the entire C-terminal non-repeat region of CSP incorporated into RTS,S were used at the indicated concentrations.

Lymphoproliferation assay. PBMC at 2×10^6 mL were added in 100 μL CM to the wells of 96 well U-bottom plates. The cells were then stimulated with RTS,S at 10 μg/mL or peptides P2 or P5 at 20 μg/mL, added in 100 μl CM/well. Control cultures were stimulated with PHA (positive control; 4 μg/mL) or medium alone (negative controls). The plates were then incubated at 37 °C for 48 h with PHA or 120 h with Ag; 1 μCi ^{3}H-Thymidine was then added to all of the wells and the plates were incubated at 37 °C for an additional 16 h. Subsequently, the cells were harvested onto glass fiber filters and the amount of incorporated ^{3}H-thymidine was measured using a beta-counter. Stimulation indices were reported relative to the negative controls.

Ex vivo ELISPOT assays. PBMC at 3×10^6/mL were added in 100 μl CM to triplicate wells of a 96 well U bottom plate. The cells were stimulated with RTS,S at 10 μg/mL, peptides P2, P4 or P5 at 20 μg/mL or PHA at 2 ug/mL (positive control), added in 100 μL of CM or CM alone as negative control. The plates were then incubated at 37 °C for 24 h. Subsequently, the cells were transferred to the corresponding wells of ELISPOT plates that had been pre-coated with an IFN-γ-specific capture antibody (Mabtec, Sweden) and the latter were incubated at 37 °C for an additional 18—20 h. The cells were then decanted and the plates were washed with PBS and a biotinylated IFN-γ-specific detection antibody was added for a 2 h reaction at room temperature. AP-streptavidin (Mabtec, Sweden) was added for a further 1½ h reaction at room temperature. Following this step, the plates were then washed and the spots were developed by the addition of substrate (Bio-Rad; Hercules, CA). The spots were counted on an IPLab analyzer (Scan-analytics; Fairfax, VA).

Cultured (long-term) ELISPOT assays. PBMC at 2×10^6/mL were added in 100 μl CM to the wells of 96 well U bottom plates. Peptide pools containing 15-mer CSP peptides at 20 μg/mL were added in 100 μl to each well and the cell cultures were incubated at 37 °C for 12 days. Control cultures were incubated with CM alone. The two-peptide pools consisted of the following CSP peptides; pool #1 (p34 + p36 + p47) and pool # 2 (p35 + p37 + p50). After 12-day incubation, the cells were harvested, washed and 100 μL of 10^6 cells/mL of CM was added to the wells of pre-coated 96 well ELISPOT plates (see above). The cells that had been cultured with peptide pool were re-stimulated with individual peptides at 20 μg/mL at 37 °C for an additional 18 h. The ELISPOTs were developed as described above for the ex vivo ELISPOT assay.

Efficacy

Protective efficacy was assessed by conducting a standardized challenge of vaccinated subjects and infectivity controls with the bites of five malaria-infected mosquitoes as previously described [11,23]. Cloned, chloroquine-sensitive *P. falciparum* (3D7 strain) parasites were expanded from a master seed lot and were used to infect laboratory-reared, specific pathogen-free *Anopheles stephensi*. A randomization of all subjects eligible and willing to undergo challenge, including controls, occurred one day prior to challenge to ensure that subjects were distributed evenly within and between the two days of challenge. For each volunteer, five mosquitoes were allowed to feed for 5 min, after which they were dissected to determine if they had taken a blood meal and had a minimum 2+ salivary gland score for sporozoites. If required, additional mosquitoes were allowed to feed until five infected mosquitoes with 2+ salivary scores had fed on each volunteer. Starting on day 5 after challenge, volunteers were followed daily for symptoms and had a daily Giemsa-stained thick blood film obtained and examined for the presence of asexual malaria parasites. All blood smears were examined for at least 200 oil immersion fields. Any symptomatic volunteer whose initial 200 fields were negative had an additional 1000 fields examined. For symptomatic, undiagnosed volunteers, additional blood smears were prepared and read every 6—8 h. Volunteers who developed malaria were treated with a standard oral dose of chloroquine (total 1500 mg base given in divided doses; 600 mg initially, followed by 300 mg given 6, 24, and 48 h later) under direct observation.

Data handling and statistics

Trial data for safety, reactogenicity, humoral immunogenicity, and efficacy was entered into a database, verified and locked prior to conduct of analyses and completion of the final trial report. Cell-mediated immune responses and supplemental serology testing was performed in blinded-

Table 1 Total cohort demographics

Characteristic	Parameter	Cohort 1, $n=20$	Cohort 2, $n=20$	Infectivity cohort, $n=12$	Total cohort, $n=52$
Age (years)	Mean (S.D.)	30 (7)	35 (7)	37 (6)	34 (8)
Gender	Male	60%	55%	58%	58%
Race	White	40%	45%	50%	42%
	Black	55%	50%	50%	52%
	Oriental	5%	0%	0%	2%
	Other	0%	5%	0%	2%
Seropositive anti-HBsAg	$\geq$ 3.3 mIU/mL	55%	45%	75%	54%
Seroprotected anti-HBsAg	$\geq$10 mIU/mL	50%	45%	75%	52%

fashion and reported later. Fisher's exact test was used to compare the incidence of malaria between the two treatment cohorts. A Kaplan—Meier analysis was performed on time to onset of parasitemia, and testing done between the two treatment cohorts using the log-rank statistic. The non-parametric Kruskal—Wallis test and Dunn's post-test comparison were applied for post-hoc analyses of association between immune responses and efficacy using Sigma Stat 3.5 (Systat Software, Inc., San Jose, CA, USA). All statistical tests were two-sided with an alpha = 0.05. No corrections of *p* values were made for multiple comparisons.

Results

Trial conduct

The trial was conducted between May 2000 and February 2001. The vaccine cohorts and infectivity controls were similar with regards to age, sex, ethnic group and anti-HBsAg titer seroprotected status, i.e., anti-HBsAg antibody >10 mIU/mL (Table 1). Of the 52 subjects enrolled, four (one from Cohort 1 and three from Cohort 2) dropped out before their final trial visit. Of the 50 subjects who underwent malaria challenge, all either remained malaria free for greater than 1 month after challenge or completed a course of directly observed antimalarial treatment. No dropouts were due to an adverse event (Fig. 1).

Safety

Both vaccine regimens had a good safety and tolerability profile. Pain at the injection site was the most commonly reported local adverse event over the 4-day follow-up period in both cohorts and over all doses (Table 2). There was no instance of grade 3 pain, and 90% of the instances of pain completely resolved within the 4-day follow-up period. Redness and swelling at the injection site was less frequent, and instances of grade 3 redness or swelling were less than 10% and less than 5%, respectively. Headache was the most common systemic adverse event. Overall, there were more systemic adverse events in Cohort 1 than in Cohort 2. There were only two instances of grade 3 solicited systemic adverse events; both occurred in Cohort 2, and only one, an instance of fatigue, was deemed to be related to immunization. In Cohort 1, eight subjects reported causally related unsolicited adverse events versus four subjects in Cohort 2. No clinically significant laboratory abnormalities were detected after administration of any dose. There were no instances of allergic reaction. One serious adverse event occurred 7 days after the first immunization, a hospital admission after a motor vehicle accident, and was deemed unrelated to immunization.

Immunogenicity

Anti-CSP antibody titers

At baseline, Cohorts 1 and 2 were equivalent with regards to IgG-specific anti-CSP antibody GMCs (0.5 and 0.7 μg/mL, respectively). One volunteer in Cohort 2 was seropositive (26.2 μg/mL anti-CSP antibody) despite a history of no malaria or previous receipt of a malaria vaccine; all others were seronegative. After second dose, all subjects in both cohorts were seropositive (i.e., >1.0 μg/mL) and remained so at 134 days after the sporozoite challenge (Fig. 2a). In both cohorts, GMC anti-CSP antibody increased after each vaccine dose. On DOC, Cohorts 1 and 2 were similar with regards to IgG-specific, anti-CSP antibody GMCs (72.7 μg/mL; 95% CI: 41.1—128.5 versus 64.4 μg/mL; 95% CI: 39.9—104.3). At 134 days after DOC, anti-CS antibody GMCs had similarly declined to 19.7 and 13.5 μg/mL, with broadly overlapping confidence intervals, and resulting in estimates of an antibody half-life of approximately 8 weeks for each cohort. Analysis of anti-CSP antibody concentrations categorized by trial cohort and by seropositivity status for hepatitis B virus (i.e., >3.3 mIU/mL anti-HBsAg) for all four groups at baseline, day of challenge and 134 days post challenge showed broadly overlapping confidence intervals, suggesting no significant effect of baseline anti-HBsAg antibody on the anti-CSP antibody response to RTS,S/AS02 (Fig. 2b). There were nine subjects in Cohort 1 and seven subjects in Cohort 2 who were naïve to both HBsAg and CS at baseline. In Cohort 1, antibody responses to CS were seen in 89% (eight of nine) of subjects 2 weeks after the first dose (GMC 5.8 μg/mL). In Cohort 2, antibody responses to CS were seen in 100% (seven of seven) of subjects 2 weeks after the second dose (GMC 44.6 μg/mL).

Anti-HBsAg antibody titers

At baseline, volunteers were evenly distributed according to seroprotection status for hepatitis B antibody (>10 mIU/mL

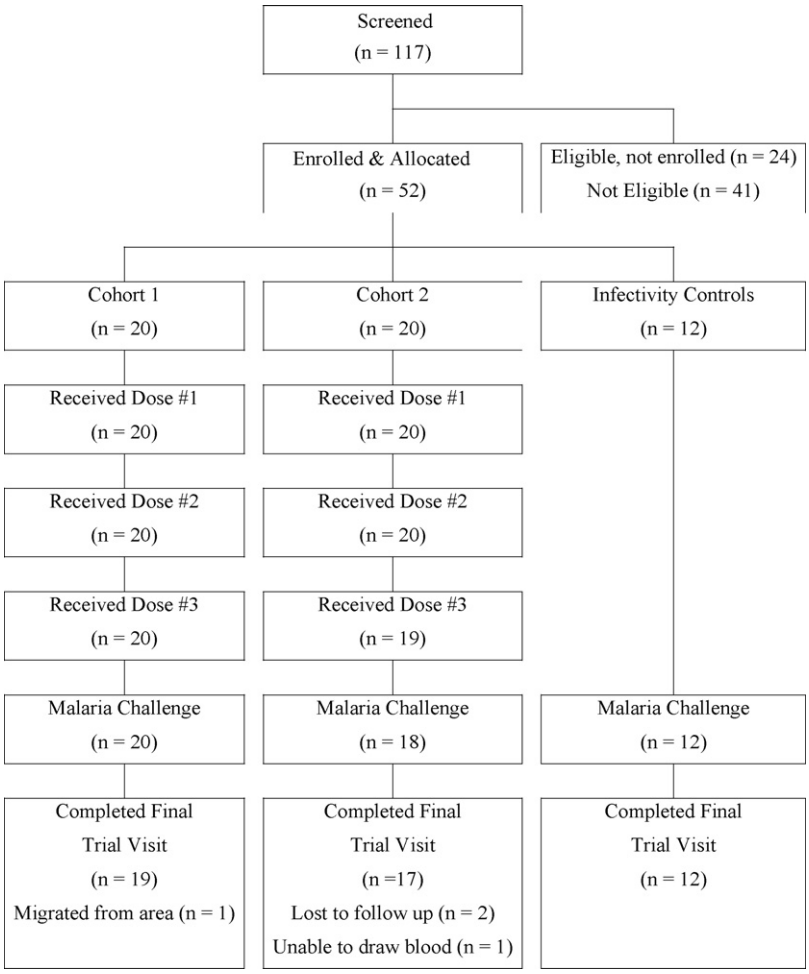

Figure 1 Trial flow.

anti-HBsAg) between Cohort 1 (50%) and 2 (45%) (Table 1). By day 42 (post dose 2 in Cohort 1, and post dose 3 in Cohort 2), 100% of volunteers were seroprotected and remained seroprotected at 134 days after the sporozoite challenge. Antibody levels were higher in Cohort 1 than in Cohort 2 at day of challenge and 134 days later, but 95% confidence intervals overlapped at both time points. On day of challenge, the GMC for Cohort 1 was 41,797 mIU/mL (95% CI: 21,352—81,821) versus a GMC for Cohort 2 of 5072 mIU/mL (95% CI: 958—26,844). Similarly, 134 days later, GMC for Cohort 1 was 17,448 mIU/mL (95% CI: 8414—36,164) versus a GMC for Cohort 2 of 8908 mIU/mL (95% CI: 3132—25,332) (Fig. 3). There were nine subjects in Cohort 1 and seven subjects in Cohort 2 who were naïve to both HBsAg and CS at baseline. In Cohort 1, seroprotective antibody responses to HBsAg were seen in 56% (five of nine) of subjects 2 weeks after the first dose (GMC 64 mIU/mL). In Cohort 2, seroprotective antibody responses to HBsAg were seen in 100% (seven of seven) of subjects 2 weeks after the second dose (GMC 150 mIU/mL).

Sporozoite IFA

At baseline, the Cohort 2 volunteer seropositive for anti-CS antibody by ELISA also had a positive IFA against air-dried homologous parasites of 1:6400, all other volunteers were negative (<1:50). On day of challenge, median IFA titer was 1:3200 for both groups, and the distribution closely overlapped for both cohorts (Fig. 4). The Cohort 2 volunteer with the initial titer of 1:6400 had the identical IFA result after immunization which did not change after immunization.

Lymphoproliferation

PBMCs obtained 2 weeks after the 3rd dose from the Cohort 1 and Cohort 2 subjects demonstrated marked increases in proliferative activity over baseline. There were no differences in mean stimulation indices of responses to RTS,S or peptides P2 or P5 between Cohorts 1 and 2 at baseline or post 3rd dose (Table 3).

1.1.1. INF-γ responses measured by ELISPOT

IFN-γ responses elicited from antigen-specific T cells have been considered as one of the key mechanisms involved in mediating protective immunity to pre-erythrocytic stage Plasmodia parasites. In this trial we measured RTS,S- and CSP peptide-specific IFN-γ responses in an ex vivo ELISPOT assay that likely indicates responses of effector T cells as well as CSP peptide-specific IFN-γ responses following a long-term culture of PBMC that might indicate responses of

Table 2 Solicited adverse event summary

Event	Intensity	Cohort 1, ($n = 60$ doses)		Cohort 2, ($n = 59$ doses)	
		n	(%)	*n*	(%)
Local					
Pain	All	47	78.3	49	83.1
	Grade 3	0	0	0	0
Redness	All	8	13.3	12	20.3
	> 50 mm	3	5.0	5	8.5
Swelling	All	3	5.0	5	8.5
	> 50 mm	1	1.7	2	3.4
Systemic					
Arthralgia	All	12	20.0	4	6.8
	Grade 3 and PB/SU	0	0	0	0
Fatigue	All	10	16.7	8	13.6
	Grade 3 and PB/SU	0	0	1	1.7
Gastrointestinal	All	5	8.3	5	8.5
	Grade 3 and PB/SU	0	0	0	0
Headache	All	18	30.0	16	27.1
	Grade 3 and PB/SU	0	0	0	0
Myalgia	All	13	21.7	5	8.5
	Grade 3 & PB/SU	0	0	0	0
Fever	All	11	18.3	4	6.8
	Grade 3 and PB/SU	0	0	0	0

Local and systemic Instances of solicited adverse events associated with each immunization by cohort. ''All'' denotes all grades. ''PB/SU'' indicates instances deemed probably or suspected to be related to immunization.

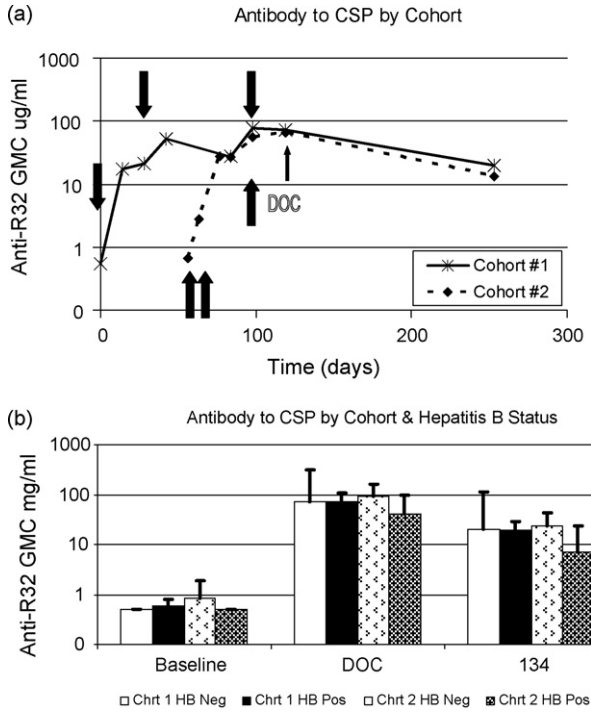

Figure 2 (a) Antibody to CSP by cohort. (Cohort 1 and 2 are aligned for day of challenge. large arrows mark immunization dates.) (b) Antibody to CSP by cohort and by hepatitis B status at baseline. (Bars depict group geometric means. Error bars denote upper limit of the 95% confidence interval.) DOC: day of sporozoite challenge.

any induced memory T cell populations [24].

INF-γ responses measured by ex vivo ELISPOT. PBMCs obtained at baseline or 2 weeks after the 3rd dose were assayed for IFN-γ production following in vitro stimulation with either RTS,S or one of three long peptides (P2, P4, or P5) that together represent the entire C-terminus of the CSP (Table 3). RTS,S-recalled responses in PBMC-immune cultures were higher than those recalled by individual peptides. The mean spots per million (MSPM ± S.D.) for RTS,S for Cohort 1 of 200 ± 107 and Cohort 2 of 220 ± 133 were higher than those elicited by individual peptides. For P2 and P5, elicited responses were equivalent in both cohorts, but for P4, values were lower in Cohort 1 (33 ± 37) than in Cohort 2 (88 ± 104) ($p = 0.03$).

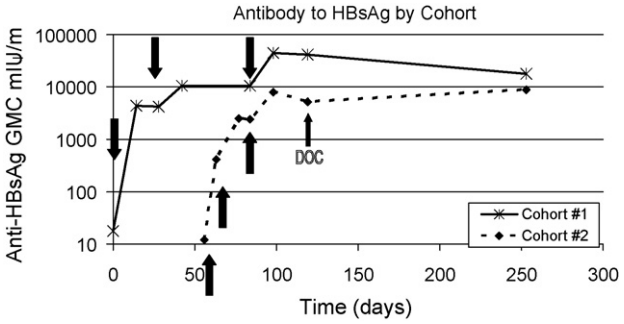

Figure 3 Antibody to HBsAg by cohort. Cohort 1 and 2 are aligned for day of challenge. Data points represent group mean anti-HBsAg antibody concentrations. DOC: day of sporozoite challenge. Large arrows mark immunization dates.

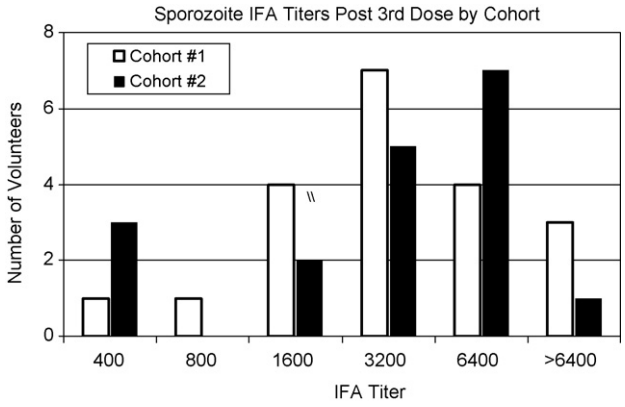

Figure 4 Sporozoite IFA titers by cohort on day of challenge. Bar graphs depict numbers of individual with a positive IFA against air-dried homologous 3D7 strain *P. falciparum* sporozoites. Titer is the greatest dilution yielding a positive IFA result.

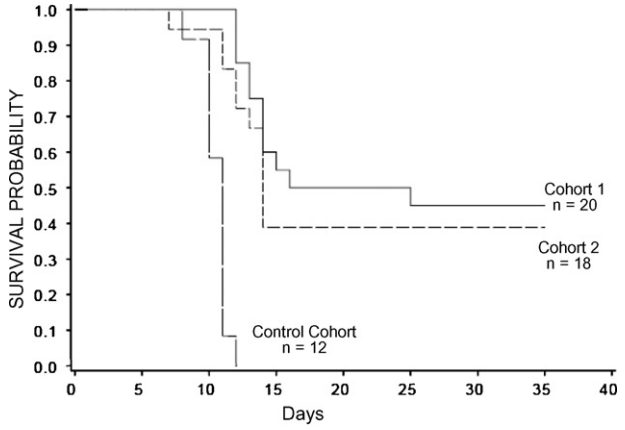

Figure 5 Kaplan—Meier plot of time to malaria by cohort. Challenge performed on day 0. Survival probability = Parasitemia free probability. Parasitemia end point determined by expert light microscopy.

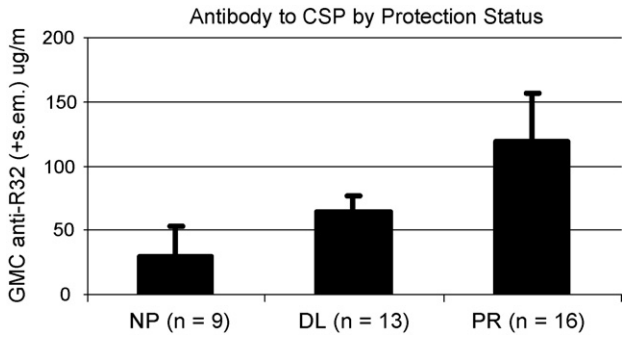

Figure 6 Antibody to CSP on day of challenge by protection status. Protection status is in comparison to concurrent infectivity controls: NP = ''No Protection,'' i.e. no delay in time to infection; DL = ''delay,'' i.e. prepatent period > 14 days; PR = ''Protected,'' sterile immunity, i.e. did not develop parasitemia. Bars depict geometric mean anti-R32 antibody concentration. Error bars are standard error of the mean.

INF-γ reponses by cultured ELISPOT. INF-γ responses elicited by CSP peptides during the long-term culture showed rather high background activity at baseline for all six CSP peptides in both cohorts. In all cases there were no significant differences in MSPM responses for each peptide between the cohorts (Table 3). The individual SPM responses to specific CSP peptides varied greatly among the immunized subjects (data not shown).

Efficacy

A total of 50 subjects participated in the malaria challenge: 20 in Cohort 1, 18 in Cohort 2, and 12 infectivity controls. Both of the immunization schedules conferred a statistically significant degree of protection from malaria challenge. In Cohort 1, 9 of 20 were protected (vaccine efficacy (v.e.) 45%; 95% CI: 18—62, $p = 0.012$ by Fisher's exact) and in Cohort 2, 7 of 18 were protected (v.e. 39%; 95 CI: 11—56%, $p = 0.024$

Table 3 CMI responses on day of challenge by cohort

	Cohort 1			Cohort 2		
	Antigen	Pre	Post	Pre	Post	*p*-Values
Index	RTS,S	7 (7)	35 (39)	6 (10)	43 (44)	0.57
	P2	3 (3)	6 (10)	1 (1)	9 (13)	0.38
	P5	1 (1)	5 (8)	1 (0)	10 (13)	0.18
Ex	RTS,S	56 (123)	200 (107)	41 (107)	220 (133)	0.60
vivo	P2	7 (9)	45 (73)	7 (22)	50 (66)	0.81
ELISPOT/10^6	P4	8 (14)	33 (37)	9 (20)	88 (104)	0.03*
PBMC	P5	15 (33)	54 (59)	27 (90)	106 (108)	0.07
Cultured	p34	80 (115)	81 (106)	133 (177)	68 (86)	0.69
ELISPOT	p35	127 (162)	341 (471)	175 (191)	251 (316)	0.50
SFC/10^6	p36	149 (235)	259 (307)	227 (362)	336 (504)	0.57
PBMC	p37	93 (135)	145 (236)	140 (171)	111 (165)	0.62
	p47	68 (102)	71 (123)	120 (152)	76 (83)	0.90
	p50	101 (145)	136 (232)	113 (172)	138 (194)	0.97

Pre: preimmunization. Post: DOC. Values are means (standard deviation). *p*-Values compare DOC data for Cohorts 1 and 2 by Student's *t*-test for $\alpha = 0.05$.Stimulation indices are in comparison to media controls, thus are not always ''1'' at baseline.

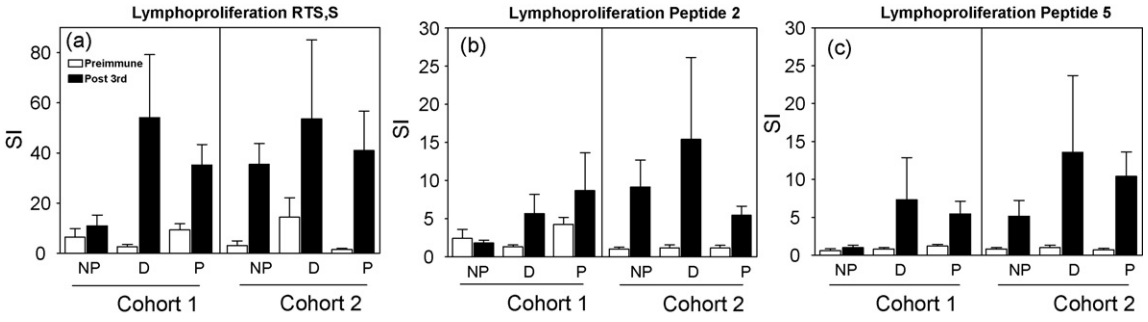

Figure 7 Lymphoproliferative responses and protection status. Panels depict lymphoproliferative responses to RTS,S (a) and to CSP peptides P2 (b) and P5 (c) by vaccine cohort and by protection status. Bars represent group means. Error bars are standard error of the mean. NP = not protected. D = significant delay in onset of parasitemia. P = protected.

by Fisher's exact). For subjects who developed parasitemia (unprotected), the median time to infection was 14.0 days for Cohort 1, 13.0 days for Cohort 2, compared to only 11.0 days for the control cohort. Log-rank tests of time to infection also showed benefit in both vaccine cohorts, with $p < 0.001$ for each vaccine cohort compared to control, but no difference between cohorts in time to infection ($p = 0.51$) (Fig. 5).

Protection and immunity

We assumed that any vaccine effect would be directed against either the sporozoite or hepatic stages, and thus would not affect blood stage parasite growth. Therefore, we attributed any delay in the prepatent period as indicative of a vaccine effect in reducing the number of hepatic merozoites and hence prolonging the time to reaching the threshold of detectability by light microscopy of parasitemia. This approach has been well described using quantitative PCR-based asexual parasite detection [25]. Therefore, as a *post hoc* analysis, we further categorized the malaria challenge outcomes in the vaccinated volunteers compared to infectivity controls; non-protected volunteers developed parasitemia before Day 14, delayed volunteers developed parasitemia on or after Day 14 (mean + 2 standard

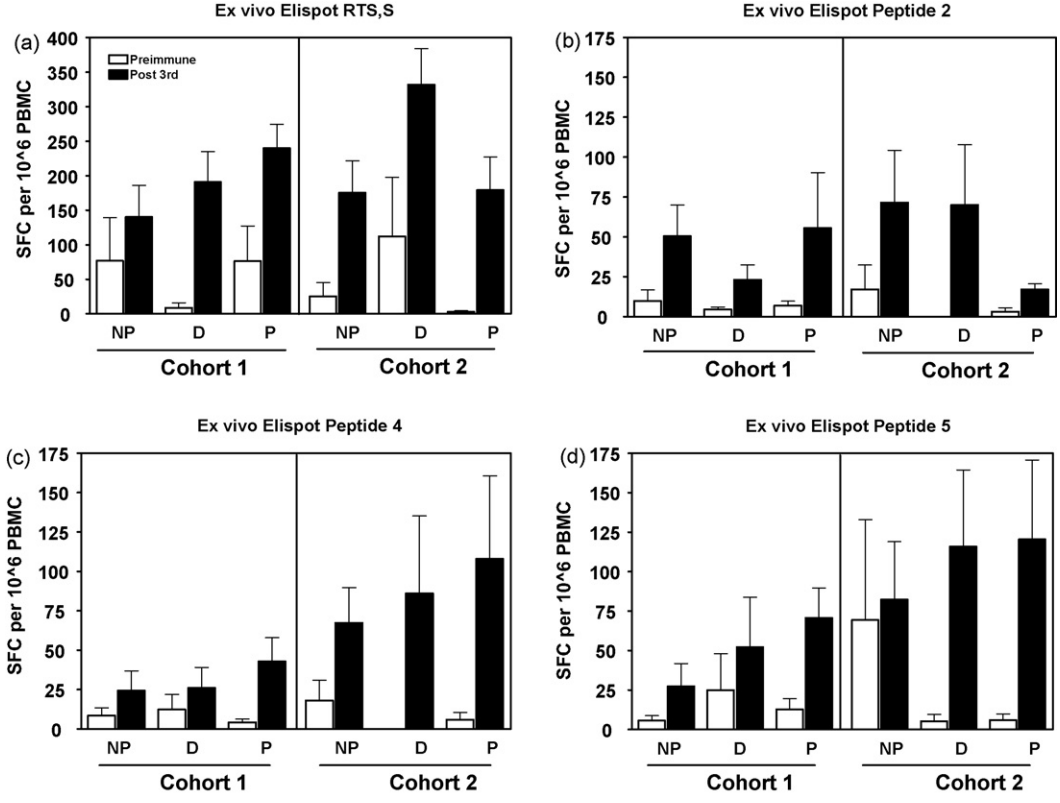

Figure 8 Ex vivo interferon-γ ELISPOT responses and protection status. Ex vivo interferon-γ ELISPOT responses to RTS,S and CSP peptides P2, P4 and P5 by cohort and by protection status. Panels depict responses to RTS,S (a), P2 (b), P4 (c) and P5 (d) by vaccine cohort and by protection status. Bars represent group means. Error bars are standard error of the mean. NP = not protected. D = significant delay in onset of parasitemia. P = protected.

deviations of prepatent period for the infectivity controls), and protected volunteers did not develop parasitemia.

Antibody and protection. Geometric mean antibody concentrations in the non-protected group ($n = 8$) were 29.6 μg/mL, in the delayed group ($n = 14$) 67.5 μg/mL, and in the protected group ($n = 16$) 113.7 μg/mL. These differences were significant for all vaccinees in Cohort 1 and Cohort 2 (Kruskal—Wallis, $p = 0.019$) (Fig. 6).

Cellular immunity and protection. For lymphoproliferation assays in Cohort 1, greater responses were generally seen in the delayed and protected versus non-protected volunteers. This pattern was not apparent in Cohort 2 (Fig. 7). Similarly, greater mean ex vivo IFN-γ ELISPOT responses were generally seen for individual assays and for the mean of the maximum responses in the delayed and protected versus the non protected volunteers in Cohort 1, but not for Cohort 2 (Fig. 8). The high background IFN-γ responses elicited during the long-term ELISPOT assay made the analysis of the response patterns less clear. However, the mean of individual maximum IFN-γ responses showed a trend toward greater responses in the delayed and protected volunteers in Cohort 1, but not in Cohort 2.

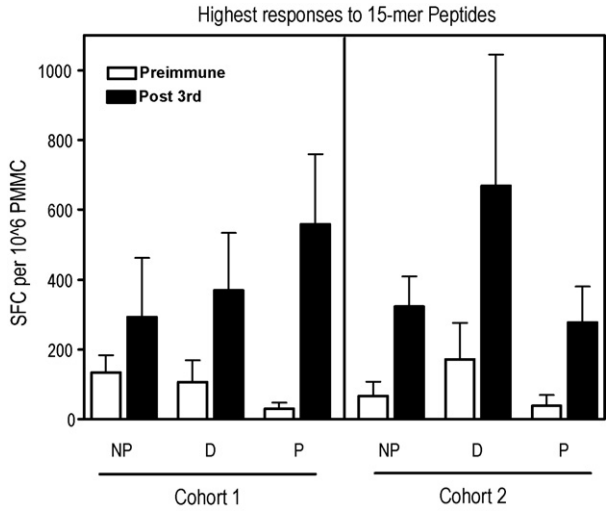

Figure 9 Cultured interferon-γ ELISPOT responses to peptides and protection status. Panel depicts the mean of each individual's highest responses to a panel of 15-mer peptides representing the CSP C-terminal region. Bars represent group means. Error bars are standard error of the mean. NP = not protected. D = significant delay in onset of parasitemia. P = protected.

Discussion

Safety

We conducted a Phase 2a trial of RTS.S/AS02 in malaria naïve adults to define the safety and efficacy of two three-dose immunization schedules. The 0, 1, and 3 month and the shorter 0, 7, and 28 day regimens appeared to have a good safety and tolerability profile. Local adverse event rates were identical, but surprisingly, systemic adverse events were more common in the longer regimen.

Humoral immunity

The accelerated 0, 7, and 28 day regimen elicited identical anti-CSP antibody concentrations but less HBsAg-specific antibody than did the 0, 1, and 3 month regimen. In subjects that were initially seronegative to both antigens, brisk antibody responses were seen in the majority of subjects 14 days after either one or two doses of RTS,S/AS02, demonstrating that immunologic priming and recruitment of IgG-producing B cells occurs efficiently after even a single dose of this adjuvanted protein vaccine. This trial demonstrated a statistically significant association of anti-CSP antibody with prevention of parasitemia after malaria challenge. This confirms similar associations of anti-CSP antibody with protection in Phase 2a trials of adjuvanted RTS,S in malaria-naïve adults [12,13] and in a Phase 2b trial in malaria-experienced adults [14]. However, such association of anti-CS antibody with protection in Phase 2b trials of RTS,S/AS02A was not found in children [15,16] but was found in infants in malaria endemic Mozambique [17].

Cellular immunity

The two vaccination regimens elicited CSP-specific lymphoproliferative and IFN-γ responses as measured by both the ex vivo and the cultured ELISPOT assays, and with the single exception of lymphoproliferative responses to P4, did not differ in magnitude between the two cohorts (Table 3). Exploratory analyses showed that cell mediated immunity did not associate significantly with efficacy outcome in either of the two cohorts when categorized as non-protected, delayed or protected. These findings do not rule out a significant association, but demonstrate that it was not detected with the single cytokine methods employed here and the relatively small sample sizes. It is worth noting, however, that the means of the ex vivo IFN-γ ELISPOT responses to RTS,S and to peptides P4 and P5 (Fig. 8), and the maximum cultured IFN-γ ELISPOT responses to the CSP 15-mer peptides (Fig. 9) for the protected, delayed, and non-protected groups of Cohort 1 exhibited a trend such that responses in protected subjects were higher than those in delayed subjects, which in turn, exceeded those in non-protected subjects. These results suggest that the longer time between the boost immunizations in Cohort 1 may have favored the development of some degree of protective T cell immunity mediated by IFN-γ responses. In contrast, the responses of the Cohort 2 subjects did not exhibit the same trend and it is conceivable that the repeated administration of antigen/adjuvant within a short time period may have caused the responding T cells to undergo antigen-induced cell death, thus reducing overall responsiveness.

The IFN-γ responses elicited by the 15-mer peptides in the cultured ELISPOT (Table 3) were maximal for peptides p35 and p36. These peptides overlap the universal T cell epitope [26] and thus it is possible that these responses were not restricted by the HLA-DR alleles. Antigen-specific IFN-γ responses are considered a *sine qua non* of protective immunity against infectious diseases. We demonstrated previously that indeed CSP-specific IFN-γ reactivity as measured by both the ELISPOT and by intracellular cytokine staining

assay showed a strong association with protection conferred by RTS,S immunization [27]. According to recently emerging evidence, the control of both chronic viral [28] and parasitic [29] infections is likely mediated by multifunctional CD4+ and CD8+ T cells. Determination of a single cytokine, IFN-γ, might have limited our detection of the scope of a protective network of cellular immune responses. Determination of additional cytokines, such as TNF-α or IL-2, might have provided a clearer resolution of T cell reactivities induced by the two regimens of RTS,S vaccination. In future vaccine trials, be it with RTS,S or other pre-erythrocytic vaccine candidates, the approach of measuring Ag-specific T cells that produce multiple cytokines is a viable option to achieve a better understanding of vaccine outcomes and the underlying mechanisms of protective immunity so that eventually specific mechanisms could be adapted as correlates of protection.

Efficacy

In this pilot trial, immunization with three doses of RTS,S/AS02 on either a 0, 1, and 3 month schedule or a rapid 0, 7, and 28 day schedule conferred equivalent protection against homologous *P. falciparum* challenge conducted 3 weeks after the last immunization. Observed efficacy was not different from that previously reported for two doses of RTS,S/AS02 administered at a 1-month interval [12,13] failing to suggest any benefit for a third dose of RTS,S/AS02 in terms of immediate protective efficacy against an experimental challenge. However, the present and previously reported Phase 2a trials, conducted in malaria-naïve volunteers, did not address the potential benefit of a third dose of RTS,S/AS02 on the duration of efficacy.

Future development

This trial was one in series of Phase 2a trials to explore the immunogenicity and efficacy of varying dose and schedule of intramuscular RTS,S/AS02 in malaria-naïve adults at WRAIR [11—13]. Although confidence intervals were broad, three doses of RTS,S/AS02 on the accelerated 0, 7, and 28 day schedule yielded no increase in immunogenicity or efficacy over the 0, 1, and 3 month schedule. Importantly, the safety, reactogenicity and immunogenicity data directly supported the selection and subsequent establishment of a 0, 1, and 2 month schedule of administration, compatible with the Expanded Program on Immunization, for further studies in children and infants in malaria endemic regions [15—17].

Conflict of interest: Joe Cohen, Martine Delchambre, Laurence Vigneron, W. Ripley Ballou and Marie-Ange Demoite are employees of GlaxoSmithKline, the manufacturer of the RTS,S/AS02 vaccine. Nadia Tornieporth was an employee of GlaxoSmithKline at the time the trial was conducted.

Previous disclosure: Presented in part at the 50th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, Georgia 11—15 November 2001 as Abstract #335.

Financial disclosure: This trial was funded by the Medical Research and Materiel Command, Ft. Detrick, Maryland, the Malaria Vaccine Program of the United States Agency for International Development, Washington, DC and by GlaxoSmithKline Biologicals, Rixensart, Belgium.

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