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Plasmodium falciparum circumsporozoite vaccine immunogenicity and efficacy trial with natural challenge quantitation in an area of endemic human malaria of Kenya

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It has been hypothesized that antibody induced by Plasmodium falciparum *circumsporozoite protein vaccine would be effective against endemic human malaria. In a malaria endemic region of Kenya, 76 volunteers, in 38 pairs sleeping adjacently, were immunized with subunit circumsporozoite protein Asn-Ala-Asn-Pro tetrapeptide repeat-pseudomonas toxin A, or hepatitis B vaccine. After quinine and doxcycycline, volunteers were followed for illness daily, parasitemia weekly, antibody, T-lymphocyte responses, and treated if indicated.* Anopheles *mosquitoes resting in houses were collected, and tested for* P. falciparum *antigen, or dissected for sporozoites and tested for blood meal ABO type and* P. falciparum *antigen. Vaccine was safe, with side-effects similar in both groups, and immunogenic, engendering IgG antibody as high as 600 μg* ml^{-1}*, but did not increase the proportion of volunteers with T-lymphocyte responses. Estimation of* P. falciparum *challenge averaged 0.194 potentially infective* Anopheles *bites/volunteer/day. Mosquito blood meals showed no difference in biting intensity between vaccine and control groups. Both groups had similar malaria-free survival curves, cumulative positive blood slides, cumulative parasites* mm^{-3}*, and numbers of parasites* mm^{-3} *on first positive blood slide, during three post-vaccination observation periods. Every volunteer had* P. falciparum *parastiemia at least once. Vaccinees had 82% and controls 89% incidences of symptomatic parasitemia (*P=*0.514, efficacy 9%, statistical power 95% probability of efficacy <50%). Vaccine-induced anti-sporozoite antibody was not protective in this study. Within designed statistical precisions the present study is in agreement with efficacy studies in Colombia, Venezuela and Tanzania.*

Keywords: Human malaria; malaria vaccine; *Plasmodium falciparum*; immunology; entomology

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Natural antibody to sporozoite antigen increasing with transmission intensity and age has been interpreted as supporting the hypothesis that vaccine induced antibody would protect against *P. falciparum* malaria[1,2]. Following demonstration that humans could be protected from sporozoite challenge by subunit sporozoite vaccines ((Asn-Ala-Asn-Pro)$_{15}$ Asn-Val-Asp-Pro)$_2$=R32, with relatively low levels of antibodies against the central repeat region of *P. falciparum* circumsporozoite protein[3,4] second generation vaccines were designed to elicit higher levels of antibody. It may be hypothesized that supra-normal levels of antibodies to sporozoites could protect, even though natural levels are not correlated with the prevalence of parasitemia or time to onset of parasitemia[5]. Increased T-lymphocyte responses to sporozoite epitopes may protect, since circumsporozoite protein repeat has a T-lymphocyte epitope[6], and endemic adults have T-lymphocyte responses to an epitope of the circumsporozoite protein repeat[7]. Efficacy in an endemic population with circumsporozoite repeat (Asn-Ala-Asn-Pro)$_3$-tetanus toxoid is unclear[8].

Immunogenicity and small-scale efficacy trials in malaria naive volunteers identified R32-Toxin A vaccine as suitable for further evaluation[9]. This vaccine produced high sporozoite antibody levels in non-immune volunteers, was safe, and immunogenic, nearly all vaccinees seroconverting[10]. One of eight volunteers was protected from challenge with laboratory infected mosquitoes, even though titers fell to relatively low levels at the time of challenge.

This endemic field trial was performed because of higher and more sustained antibody levels in response to vaccination with circumsporozoite repeat in a malaria endemic[11] than in naive[3] volunteers and because wild mosquitoes have[12] and release[13] fewer sporozoites than laboratory[14] mosquitoes.

MATERIALS AND METHODS

Study site

The study area, in western Kenya, near Lake Victoria, has been a site of community-based medical research studies[15] and has been described[15–17]. The incidence of *P. falciparum* alone or with *P. malariae* or *P. ovale* was 98.1% between 1980 and 1983[18].

Study design

This was a prospective, placebo-controlled, randomized, paired, double-blinded, active-case-seeking study. *P. falciparum* malaria incidence has been 90%[5], and 89% over 4 months[7], therefore a vaccine 50% effective would give an incidence of 45% in the experimental group. In this instance, the number of volunteers needed in each group for significance of 0.05 and power of 0.95 in a 2-tailed test is 29[19]. In previous studies in this area the drop-out rate was 15% over 8 months[5,7], 1.87% per month or 22.5% per 12 months.

Recruitment, pairing, randomization, anti-malarial clearance, treatment and follow-up

From a census[17], of five villages near a clinic, an update was made of males between the ages of 18 and 30 years. One hundred and twenty-seven prospective volunteers answered a questionnaire; 110 met criteria; 76 volunteers were enrolled. Volunteers and household family members were offered free medical care during the study and for any potential adverse effect of vaccine or medicines. Inclusion criteria: male, age 18–30 years, in good health, willing to reside in the study area and participate for 12 months, use no bed nets or other malaria prophylaxis during the study, and human immunodeficiency virus-1 (HIV) antibody test negative[20]. Exclusion criteria: evidence of cardiac, pulmonary, renal or immunologic disease, or antibody to HIV.

Volunteers were asked to pair themselves to sleep in the same house. Paired volunteers were assigned to two groups of similar ages and proportions of hemoglobin AS. Vaccine was randomly assigned. Houses were inspected to ascertain that they were the customary sleeping houses of the community. Reed mats provided uniform ceilings for mosquito resting and collection. Interiors were divided by a wall or curtain into separate sleeping areas. Every 7 days volunteers switched bed positions in houses.

Volunteers were given quinine sulfate 650 mg thrice daily for 3 days and doxcycycline 100 mg twice daily for 7 days, before each of the three vaccinations. After the last vaccination, volunteers were given quinine sulfate 650 mg thrice daily for 3 days and doxycycline twice daily for 28 days to eradicate blood stage and hepatic stage parasites. Clinical workers observed swallowing of medicines. For slide-proven malaria, sulfadoxine-pyrimethamine (Fansidar) was prescribed and swallowed in the presence of clinic staff. Chloroquine and amodiaquine were not prescribed. Volunteers were advised to avoid chloroquine and amodioquine, available without prescription, and obtain medicines only from the study clinic.

Follow-up was carried out for 12 months, 6 months after the last vaccination. Each morning, a clinical worker asked if the volunteer felt sick, took the axillary temperature and asked of any medicine taken. Symptoms of malaria were considered to be: fever; chills; sweats; headache; cough; or diarrhea. If the volunteer felt ill with a symptom of malaria, then a blood slide was made. If the volunteer was moderately ill or worse, he was taken to the clinic by the clinical worker for history and physical examination and tests including malaria blood slide read in the clinic. Symptomatic malaria was defined as the presence of one or more of the above symptoms plus a blood slide positive for *P. falciparum*. Volunteers were given treatment for malaria if they had both a symptom consistent with malaria and a positive blood slide. After each vaccination, volunteers wrote down side-effects.

Vaccine

The experimental vaccine was recombinant (Asn-Ala-Asn-Pro$_{15}$ Asn-Val-Asp-Pro)$_2$-Leu-Arg (R32LR)[21,22] covalently linked to purified *Pseudomonas aeruginosa* toxin A 9. Each dose consisted of R32LR peptide (175 μg), and toxin A (225 μg)[10] or recombinant hepatitis B vaccine (Energix B, SmithKline Beecham) control. Malaria (Swiss Serum and Vaccine Institute) and hepatitis vaccines were sent separately to Kenya. Vaccines were kept and transported at +4°C, in the dark, until use.

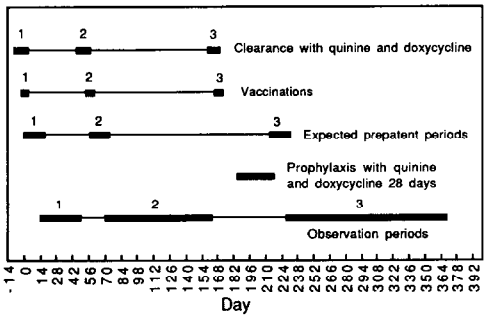

Figure 1 Study intervals. The expected prepatent period after clearance, or prophylaxis, is 14 days

Clinical parasitology, pharmacology, hematology and chemistry

Scheduled blood slides, in duplicate, were made every 7 days[23] from 2 weeks before until 52 weeks after the first vaccination and the number of parasites mm^{-3} was determined. Symptomatic blood slides were examined at the clinic[24]. For a slide to be considered positive, at least five asexual stage parasites had to be seen. These slides were later confirmed by others in a laboratory and results returned to the clinic. If a slide was read as negative in the clinic and later found to be positive, the volunteer was treated. There were no slides read as positive in the clinic that were negative on confirmation. Unless otherwise stated, parasitemia refers only to *P. falciparum*, and not *P. malariae* or *P. ovale*. The tabulation of blood slides for the purpose of vaccine efficacy was begun 14 days after the day of completion of doxycycline administration (not with the day after the completion of immunization), to avoid including any recrudescent parasites. Thus, observation periods 1 and 2 began 14 days after each of the first two vaccines were given (*Figure 1* and *Figure 4a,b*), and observation period 3 began 60 days after the third vaccine was given (study day 230, *Figure 1* and *Figure 4c*).

Serum doxycycline was assayed by reverse phase high-pressure liquid chromatography on samples from the 9th, 10th or 11th day of prophylaxis[25]. Urine was collected every 4 weeks, frozen and analyzed for aminoquinolines[26,27].

Clinical hematology, urinalysis and chemistry tests were performed at initial screening, monthly for 12 months and when clinically indicated.

Humoral and cellular immunology

Levels of anti-*P. falciparum* circumsporozoite (CS) antibody were determined by enzyme-linked immunosorbent assay (ELISA)[28] with capture antigen R32LR. Standard sera containing 9.8 μg of IgG antibody per ml, specific to R32 from a previously vaccinated volunteer[3,29] and test sera, gave co-parallel curves of absorbance vs concentration when tested against R32LR[30]. Positive was defined as the mean plus 3 S.D. of non-immune sera without exposure to malaria. Rise in titer was defined as a level 3 S.D. above the pre-immunization level for that individual. Immunofluorescent assay for antibody to sporozoites was performed before the first and after the third vaccination[11].

Lymphocyte proliferation was performed 4–8 days before the first vaccination, 1 month before and immediately before the third vaccination, and 1 month, 2 months and 6 months after the third vaccination[7].

Entomological background and measurement

The *Anopheles* abundance of the study area has been described[31 33]. Over 33 months, *An. gambiae* sensu latu and *An. funestus* comprised 90% and 10%, respectively, of human-biting *Anopheles*, and mean daily man-biting rates for *An. gambiae* s.l. and *An. funestus* were 3.3 and 0.6, respectively[32]. In this area, sporozoite rates by salivary gland dissection were 6.2%[34]–13.1%[32] for *An. gambiae*, and 4.9%[32] for *An. funestus*. Sporozoite rates by ELISA of undissected head and thorax samples were 15.1% and 14.2%, respectively, for *An. gambiae* s.l. and for *An. funestus*[35], and, in a nearby site 8.2% and 6.1% for these species[33]. The threshold of ELISA sensitivity is about 125 sporozoites per mosquito[36]. Sporozoite burdens in East Africa for *An. gambiae* and *An. funestus*, were reported to be 2000–4000 per salivary gland[37]. In western Kenya *A. gambiae* had a geometric mean of 962 and *A. funestus* 874 sporozoites[12]. In western Kenya, 86.6% of naturally infected *An. gambiae* transmitted, into sucrose or blood, a geometric mean of 3.84 sporozoites (range 1–34), about 3% of the total sporozoites in salivary glands[13]. In contrast, laboratory reared *An. gambiae* contained 808–13905 sporozoites, and 44.1% of infected laboratory *An. gambiae* transmitted a geometric mean of 4.5 (maximum 369) sporozoites *in vitro*, with which sporozoite loads were not a predictor of the number of sporozoites ejected[14]. The entomological inoculation rate (EIR), calculated as the monthly man biting rate times the sporozoite rate[38], or the average number of potentially infective *Anopheles* bites in a period of time, was during a previous study in Saradidi, 237 per year inside houses, and 190 per year outside houses[32]. The calculated time to inoculation exposure, to experience one potentially infective bite, is 1/EIR, for Saradidi, inside houses, 1.5 nights[32].

Six mornings per week, a 0.5 h collection of indoor resting mosquitoes was made by volunteers in the houses. One morning per week, experienced entomology workers made the collections. Mosquitoes were identified to species, and examined for the presence of blood. Four days per week, *Anopheles* were killed, desiccated and mosquito thoraces were tested later for *P. falciparum* antigen, by ELISA[31]. Three days per week, mosquitoes salivary glands were dissected for sporozoites[39], which were tested by ELISA for *P. falciparum* antigen[31] and mosquito blood meals were typed for blood groups A, B and O[40]. Group O blood cells were detected by agglutination in the presence of lectin H (American Dade, Miami, FL). Mosquitoes were determined to have fed on a volunteer when the blood type of the blood meal was the same as the blood type of the volunteer, in those pairs of volunteers with differing blood types. Blood meal host origin was determined by ELISA for human or cow immunoglobulin[41]. In a previous study, of *A. gambiae* s.l. and *A. funestus* collected in houses in Saradidi, 100% of blood meals were human or cow[41]. At the conclusion of the observation period (16 May 1991) 119 pyrethrum-spray-catch collections[39] were made in volunteer's houses, immediately following collections by volunteers. These supplemental collections were done to derive an estimate of the

Table 1 Age and erythrocyte characteristics of vaccine and control volunteer groups in a *Plasmodium falciparum* circumsporozoite vaccine immunogenicity and efficacy trial

	Study groups						African groups	
	Vaccine		Control		Total		Kenyans[a]	Americans[b]
	(No.)		(No.)		(No.)			
n	38	100%	38	100%	76	100%		
Age mean (S.E.)[c]	20.6	0.53	20.2	0.45				
HbAA*	30	79%	30	79%	60	79%		
HbAS*	5	13%	6	16%	11	14%	28% (Foy, 1982)	8% (Bunn, 1991)
HbAS							26% (Allison, 1954)	
A**	13	34%	6	16%	19	25%		39% (Giblett, 1991)
AB**	3	8%	4	11%	7	9%		4% (Giblett, 1991)
B**	7	18%	10	26%	17	22%		17% (Giblett, 1991)
O**	15	39%	18	47%	33	43%		50% (Giblett, 1991)
Rh(D) Pos.	38	100%	37	97%	75	99%		95+% (Giblett, 1991)
Rh(D) Neg.	0	0%	0	0%	0	0%		
G6PDNormal***	35	92%	33	87%	68	89%	72% (Allison, 1960)	
G6PDDeficient***	3	8%	5	13%	8	11%	28% (Allison, 1960)	

*χ^2=0.077, P=0.7816, *d.f.* =1. **χ^2=3.524, P=0.3188, *d.f.*=3. ***χ^2=0.559, P=0.4547, *d.f.*=1. Probability distribution z=0.31577, P>0.40. Wilcoxon's ranking/Mann–Whitney, z=−0.603, P>0.7257. [a]Luo people of same study area of Kenya. [b]Americans of African descent. [c](Mean of vaccine group −1 S.E.)>(mean of control group +1 S.E.)

percentage of total indoor resting mosquitoes that were obtained on average during morning aspiration collections by volunteers. No vector control methods were exercised in the area during the present vaccine study.

Statistical analysis

Efficacy was calculated using the formula $I=1-(I_v/I_p)$, where I=incidence of symptomatic parasitemia, I_v=incidence in the vaccine group, and I_p=incidence in the control group. Analysis of variation, χ^2, correlation coefficient, Fisher's exact test, Kaplan–Meier survival curve with log-rank test, and Student's *t*-test were used. Statistical calculations were determined with the assistance of StatView 512+™ (BrainPower, Inc., Calabasas, California, 1986).

RESULTS

Study population and follow up

Age means were similar for the vaccine and control groups (*Table 1*). The distribution of sickle trait was similar between the study population and the general population[42–44] and between the vaccine and control groups. There was no significant difference between the study population and a previously reported distribution of ABO blood types in this population of western Kenya[45]. There was no significant difference between vaccine and control groups in distribution of ABO blood types. The prevalence of glucose-6-phosphatase dehydrogenase deficiency was similar between the study population and the general population[46]. There was no significant difference between vaccine and control groups in prevalence of glucose-6-phosphatase dehydrogenase deficiency (*Table 1*).

There was no significant difference in volunteer participation between the vaccine and control groups (P>0.25, log rank test). Sixty-nine of 76 volunteers completed the study on day 367. All who left did so for personal reasons: to further their education or seek employment away from the study area.

Safety and adverse reactions

There were no serious adverse reactions to the vaccines or medicines. Adverse reactions to vaccines were few in number, mild in intensity, and brief in duration in both vaccine and control groups. There were no adverse reactions manifest by the clinical hematology or chemistry tests.

Humoral and cellular immunology

Among those receiving hepatitis vaccine control, 4 of 38 volunteers (10%) had an antibody rise to R32LR 3 S.D. above the arithmetic mean. Among those receiving malaria vaccine, 73% (28 of 38) had an antibody rise to R32 3 S.D. above pre-immunization. The geometric mean titers for the malaria group increased after each of the vaccinations and fell during the interim (*Table 2*, *Figure 2*). The geometric mean titers for the control group did not change (*Figure 2*).

Among those receiving malaria vaccine there were three patterns of antibody response: no increase, an increase with a drop toward baseline, and a sustained increase. Those not responding had lower pre-vaccination levels, and those responding had higher pre-vaccination levels, in agreement with the previous vaccine study showing greater response in those with higher natural levels[11]. In most, the third vaccination did not increase antibody levels beyond that achieved by the first or second vaccinations. The highest antibody level achieved was 600 μg ml^{-1}. Four of 38 volunteers (10%) receiving malaria vaccine had peak levels in this range. Among controls, some had low levels of antibody, unchanged during the course of the study, and some had higher levels, also unchanged.

There was an association between increase in sporozoite immunofluorescence score with increase in antibody by ELISA (n=14) and no increase in immunofluorescence with no increase in antibody by ELISA (n=9), among those receiving malaria vaccine (n=33) ($P1+P2$=0.002, Fisher's exact test), indicating peptide vaccine increased antibody to natural sporozoites, although a subset (n=10) had no increase in

Table 2 Anti-circumsporozoite antibody levels of vaccine and control volunteer groups in a *Plasmodium falciparum* malaria vaccine trial

	Vaccine				Control			
Time (weeks)	Mean[a] (mcg ml^{-1})	Minimum (mcg ml^{-1})	Maximum (mcg ml^{-1})	1 S.D. (mcg ml^{-1})	Mean[a] (mcg ml^{-1})	Minimum (mcg ml^{-1})	Maximum (mcg ml^{-1})	1 S.D. (mcg ml^{-1})
0.0	5.3	0.3	23.0	4.4	6.9	0.4	73.9	12.5
4.0	63.4	3.3	66.2	129.0	9.5	0.4	45.8	10.5
8.0	52.5	4.6	500.0	94.2	12.8	1.2	117.1	22.1
12.0	135.6	7.2	3200.0	521.2	11.3	0.7	75.4	14.3
16.0	41.8	3.0	298.2	65.4	9.0	0.6	72.3	12.8
20.0	42.7	3.5	327.0	61.2	11.3	0.4	75.5	14.9
24.0	37.1	4.7	373.1	66.3	9.0	0.3	55.8	11.8
28.0	50.8	2.0	285.4	58.0	8.1	0.4	47.8	9.8
34.0	43.2	4.6	273.3	62.6	8.3	0.3	29.2	7.8
40.0	27.1	4.2	167.4	31.9	10.0	0.4	50.4	11.5
44.0	23.4	2.8	170.9	31.8	6.7	0.4	27.7	7.3
48.0	31.1	3.2	217.9	50.5	7.2	0.6	29.0	7.3
52.0	23.7	3.8	190.1	34.5	8.2	0.7	31.3	8.0

[a]Geometric mean

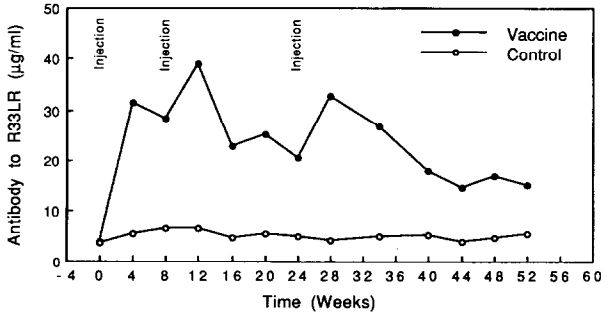

Figure 2 Geometric mean antibody levels for vaccine and control groups. At week 0 there was no significant difference between vaccine and control groups in antibody levels (P=0.417, analysis of variance). From 4 to 52 weeks there were significant differences between vaccine and control groups in antibody levels (P=0.001–0.021, analysis of variance)

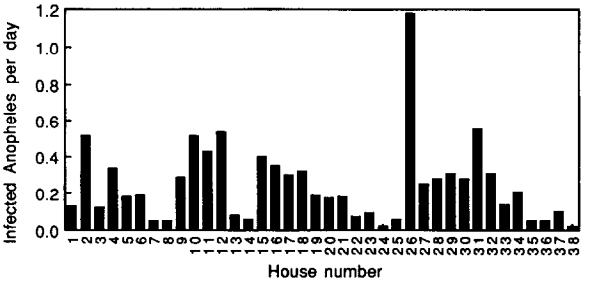

Figure 3 Infected *Anopheles* per day per house. Average of *P. falciparum* antigen positives by enzyme-linked immunosorbent assay of dried thoraces

immunofluoresence with an increase in antibody by ELISA.

Lymphocyte responses to R32LR were determined in the 11th or 12th month of the study, using fresh, non-cryopreserved, lymphocytes. Results were available on 52 of 76 volunteers (68%), four of whom had positive responses. Two were from the control group, and two from the vaccine group. Among these four volunteers, there was no association between lymphocyte response and post vaccination antibody level. Among all volunteers, there was no association between lymphocyte response and mean change in antibody level after each of the three vaccinations (P=0.964, 0.820. 0.870, ANOVA). The malaria vaccine group did not have an increase in the proportion of volunteers responding to the antigen as a T-lymphocyte epitope, in comparison with the placebo group. There was no correlation between age and stimulation index at end of study (r= − 0.055, n=52). T-lymphocyte responses to R32LR were assayed in the 1 week before the first vaccination, using fresh lymphocytes. Technical difficulty prevented completion of the pre-vaccination assay.

Entomology and parasitologic challenge

From May 1990 to May 1991, 10.9% of 18138 desiccated *Anopheles* females were positive for *P. falciparum* antigen by ELISA. Of these, *A. gambiae* comprised 66.5%, *A. funestus* 32.1% and *A. arabiensis* 1.4%. 7.75% of 7842 *Anopheles* females were *P. falciparum* sporozoite positive, by ELISA, after salivary gland dissections. Therefore, 71.1% of ELISA-positive desiccated *Anopheles* were potentially infective (i.e. had *P. falciparum* sporozoites in salivary glands). Plasmodial circumsporozoite antigen exists in anophelene gut, thorax, and salivary glands during the structural development of the incipient sporozoite, before and possibly after, the sporozoite is functionally infective. The presence of antigen alone, in thorax or in salivary gland, by ELISA, does not equate to infectivity. Furthermore, if <125 sporozoites per mosquito are present, the ELISA may be negative, being below the ELISA test threshold. Visual dissection for the presence of sporozoites was performed and this material tested for *P. falciparum* circumsporozoite antigen by ELISA.

For desiccated mosquitoes, the total number of *P. falciparum* ELISA-positive *Anopheles* caught was 1971. The calculated average of infected *Anopheles* collected per volunteer was 0.137 per day. The average number of infected *Anopheles* captured per day among the study houses ranged from 0.02 to 1.18 (*Figure 3*). The mean, minimum and maximum number of infected *Anopheles* per house per day varied for each of the three observation periods (*Table 3*).

Of 18138 mosquitoes 63.2% blood fed by visual inspection. Of 4029 blood-fed mosquitoes for which blood was typed, 94.3% matched the blood type of one of the volunteers in the house in which the mosquito was caught, suggesting that ingress of blood fed mosquitoes from the outside was minimal. Twenty-eight of the 38 houses contained volunteers with different blood types.

Table 3 Number of *Plasmodium falciparum* sporozoite-containing mosquitos per house per day in a malaria vaccine immunogenicity and efficacy trial

Observation period[a]	Number of houses[b]	Mean	1 S.D.	Median	Minimum	Maximum
1	38	0.469	0.426	0.383	0.000	2.083
2	39	0.090	0.126	0.041	0.000	0.640
3	43	0.241	0.272	0.127	0.000	1.315

[a]1=1 Jun to 1 Jul; 2=27 Jul to 22 Oct; 3=31 Dec to 17 May. [b]*n*=Number of houses; some volunteers moved to new houses

Table 4 Symptomatic malaria in vaccine and control groups in a *Plasmodium falciparum* malaria vaccine trial

	Number of volunteers having symptomatic malaria							Number of occurrences of symptomatic malaria		
	Vaccine			Control				Vaccine	Control	
Observation period	*n*	No.	%	*n*	No.	%	*P*[a]	No.	No.	*P*[b]
1	38	9	24	38	11	29	0.795	11	12	0.384
2	38	23	61	37	22	59	0.888	39	44	0.250
3	34	25	74	35	30	86	0.338	56	65	0.261
1+2+3	38	31	82	38	34	89	0.514	106	121	0.343

[a]χ^2 with continuity correction. [b]Paired, 1-tailed *t*-test

There was a significant positive correlation between the vaccine and control group pairs in numbers of blood fed *Anopheles* containing blood of the same type as a volunteer in a house (r^2=0.50, P<0.001). There was no difference between vaccine and control groups in the number of blood meals from a sporozoite-positive *Anopheles* associated with individual volunteers (paired *t*-test, t=0.68, *d.f.*=24, P=0.50).

Four parameters were used to estimate total malaria challenge on volunteers. A=Proportion potentially infective of desiccated ELISA-positive *Anopheles*=0.711. B=Proportion of blood fed Anopheles of the total collection=0.632. C=Average number of infected desiccated *Anopheles* collected per volunteer per day throughout entire study=0.137. D=Number of times greater the pyrethrum spray catch (PSC) collections were than the accompanying aspiration collections by volunteers (ACV); on average=2.149. PSC collections and ACV were significantly correlated (log10(PSC+1)=0.52062 × log10(ACV+1)+0.74362; r^2=0.351, P<0.001).

Estimate of total challenge=$A \times B \times (C+(D \times C))$= 0.711 × 0.632 × (0.137+(2.149 × 0.137))=0.194 potentially infective bites per volunteer per day, or one infective bite every 5.2 days.

There was no significant difference in the distribution of *P. falciparum* sporozoite-infected mosquitoes per house among the blood types O, A, B and AB for vaccine (P=0.597) or control (P=0.565, ANOVA). There was no significant difference in the distribution of *P. falciparum* sporozoite-infected mosquitoes with matching blood type per volunteer among the blood types O, A, B and AB for vaccine (P=0.384) or control (P=0.381, ANOVA).

Antimalarial pharmacology

Serum doxycycline levels ranged in the vaccine group (n=34) from 0.196 to 2.975 μg ml^{-1} (mean ± 1 S.D. =0.797 ± 0.555 μg ml^{-1}), and in the control group (n=35) from 0.145 to 2.686 (mean ± 1 S.D.= 0.795 ± 0.606 μg ml^{-1}), between expected published peak (5 μg ml^{-1}) and trough (1 μg ml^{-1}) levels[47]. There were no positive blood slides during the 14 days following the last dose of doxycycline, the expected prepatent period, had an infective bite been received on the first day following cessation of doxycycline. Fifteen of 78 volunteers, each on only one occasion, had a positive urine test for aminoquinoline, indicating compliance (1.6% of a possible 912 occasions) in taking only those medicines prescribed in the clinic, not chloroquine or amodiaquine available commercially.

Clinical and parasitological outcome

During the three postvaccination observation periods, the number of volunteers who developed symptomatic malaria in the vaccine group and control groups were comparable, and there were no significant differences by χ^2 analysis (*Table 4*). The number of occurrences of symptomatic malaria during each of the three postvaccination observation periods in the vaccine and control groups were comparable, and there were no significant differences by paired *t*-test (*Table 4*). Efficacy was calculated according to the formula $I=1-(I_v/I_p)=1-(31/38)=0.09=9\%$. The mean number of fever occurrences from any cause, defined as a temperature 3 S.D. above the mean, was 1.3 (range 0–5, n=38) for the vaccine group and 1.7 (range 0–5, n=36) for the control group. There was no significant difference between the two groups of paired volunteers in the number of fever occurrences (paired 2-tailed *t*-test, $t=-1.247$, *d.f.*=35, P=0.2208).

None of the volunteers died during the study. None of the volunteers had severe malaria (defined as a positive blood slide for malaria with seizure, loss of consciousness, or hypotension (blood pressure <80 systolic), cerebral malaria (defined as a positive blood slide with sustained coma)), a seizure, loss of consciousness, or change in mental status.

All volunteers had at least 1 positive blood slide for *P. falciparum* during each of the three observation periods after each of the three vaccinations, except 1 vaccine and 1 control in the first observation period (*Figure 4*a), both of whom had positive slides at the next clinic visit for

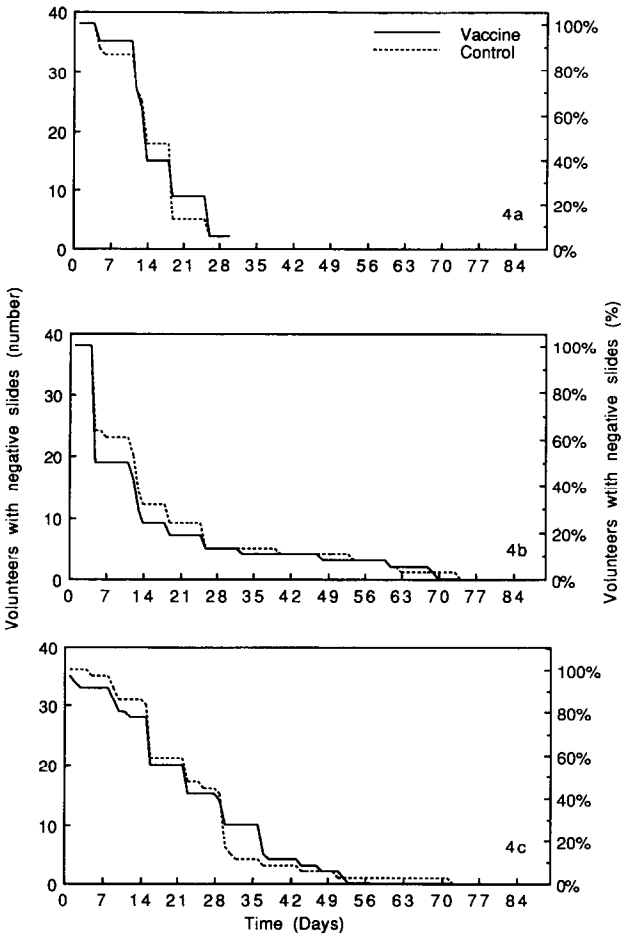

Figure 4 Kaplan–Meier actuarial curves. The tabulation of blood slides for the purpose of vaccine efficacy was begun 14 days after the day of completion of doxycycline administration (not with the day after the completion of immunization), to avoid including any recrudescent parasites. Thus, observation periods 1 and 2 began 14 days after each of the first two vaccines were given (study days 14 and 70, see Fig. 1). Observation period 3 began 14+28=42 days after the third vaccine was given (study day 230, see Fig. 1). (a) First observation period. Log rank t=0.03169, P>0.25, not significant. (b) Second observation period. Log rank t=0.5585, P>0.25, not significant. (c) Third observation period. Log rank t=0.6629, P>0.25, not significant

the second vaccination. The highest *P. falciparum* parasitemia in any volunteer was 63054 mm^{-3}. Survival curves for the proportion of volunteers free of *P. falciparum* on blood slide after the first, second and third vaccinations showed no difference between the vaccine and control groups by log rank test (*Figure 4a–c*). Blood slide results for the two groups of paired volunteers in the three observation periods are show in *Table 5*. There was no significant difference in the mean number of days until the first positive slide (patency), the total number of positive slides, the cumulative number of *P. falciparum* mm^{-3} (parasite densities), or mean number of *P. falciparum* mm^{-3} on first positive blood slide. Twenty-eight (36.8%) of 76 volunteers had *P. malariae* alone or mixed parasitemia for a total of 61 times. Twenty-two (28.9%) of 76 volunteers had *P. ovale* alone or mixed parasitemia for a total of 29 times. The prevalences of *P. falciparum*, *P. malariae* and *P. ovale* are shown (*Table 6*).

There was no significant correlation between antibody level at weeks 0, 8, 12, 16, 20, 24, 28, 34, 40, 44, 48 and 52, or the mean over weeks 0–52 or 34–52, or the change in antibody level from weeks 0–4, 8–12 or 24–28, and time in days to first positive blood slide, cumulative number of positive blood slides, or cumulative number of parasites mm^{-3} during the third observation period from weeks 34 to 52. Correlation between mean antibody levels over weeks 0–52 and number of days to first positive blood slide was r=0.3.

There was no difference between volunteers with and without T-lymphocyte responses in time to first positive slide, total number of positive slides or cumulative parasitemia.

DISCUSSION

This field study was designed to determine the safety and efficacy of a recombinant alum adjuvanted subunit vaccine which induces antibodies against the repeat region of the *P. falciparum* circumsporozoite protein, in a malaria-experienced population. This vaccine was safe, with a rate of side-effects comparable to that of recombinant hepatitis B vaccine.

The vaccine induced high levels of antibody in a subpopulation, higher than seen previously in non-immune or in semi-immune volunteers. In this endemic population, responders to this vaccine had higher pre-immunization natural antibody levels than non-responders, as was observed in a similar population no longer living in a malaria endemic area[11]. Antibody levels of vaccines decreased gradually over 12 months. There was an association between having an increase in antibody by ELISA and immunofluoresence, indicating reaction with native sporozoites, but there remained a subset (10 volunteers) who had antibody by ELISA but not by immunofluoresence. In volunteers immunized with (NANP)3-tetanus toxoid or recombinant R32tet32, although there was a correlation between level of IgG in protected human volunteers and inhibition of sporozoite invasion of cultured HepG2A-16 hepatoma cells, IgG from protected human volunteers did not block sporozoite invasion of human hepatocytes[48]. Sporozoites can develop into hepatic schizonts in the presence of antibody to R32[49]. High levels of antibodies reacting with sporozoites by immunofluorescence inhibit entry and development of sporozoites in hepatocyte culture by only 82–88% and have been found in the presence of blood stage *P. falciparum* infection, suggesting that a single antigen vaccine may not be adequate[50]. Mouse antibody against the R32 recombinant peptide has a delayed inhibitory effect on development of sporozoites in liver cells[49]. Repeat epitopes may induce a distracting B-lymphocyte production of non-protective antibody[51,52]. In the present study the level of antibody did not correlate with the number of positive slides, time to onset of parasitemia or total burden of parasites.

The vaccine did not increase the small proportion mounting a T-lymphocyte response. The present result is consistent with a report that NANP repeat is a T-lymphocyte epitope on the circumsporozoite protein[6], but recognized by only a portion of the population (4 of 52=7.7%), in agreement with previous results from the same area (2 of 28=7.1%)[7]. The repeat region is conserved among widely separated regions[53]. Such conservation may reflect the low frequency of individuals whose T-lymphocytes recognize the repeat. Polymorphisms in T-lymphocyte epitopes lie outside the repeat region[54,55]. Although exposure to sporozoites

Table 5 Time to first parasitemia, number of episodes of parasitemia, aggregate numbers of parasites, and parasite density in first episode of parasitemia in vacccine and control volunteer groups in a *Plasmodium falciparum* malaria vaccine trial

Observation period	Vaccine				Control				P^a
	n	Mean	Min	Max	*n*	Mean	Min	Max	
No. days to first positive blood slide for *P. falciparum*									
1	37	15	4	25	37	14	4	25	0.366
2	38	14	4	69	37	17	4	73	0.198
3	34	26	2	85	35	23	4	71	0.252
No. positive blood slides for *P. falciparum*									
1	38	2	0	4	38	2.2	0	4	0.452
2	38	5	0	12	37	5.4	1	13	0.399
3	34	7	1	18	35	6.4	1	14	0.139
No. *P. falciparum* mm^{-3}									
1	38	1470	0	22687	38	794	0	6118	0.169
2	38	1441	0	27918	37	1471	2	13757	0.496
3	34	4350	58	36802	35	6217	3	62049	0.214
No. *P. falciparum* mm^{-3} on first positive blood slide									
1	36	297	1	6405	36	169.7	1	1705	0.249
2	37	155	2	4971	37	390	2	5659	0.183
3	34	582	2	8080	35	791	3	13015	0.332

[a]Paired, 1-tailed *t*-test

Table 6 Prevalence of *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale* in vaccine and control volunteer groups in a malaria vaccine trial

Species	Vaccine		Control		Combined	
	n	%	*n*	%	*n*	%
P. falciparum	604	93.5	571	94.2	1175	93.8
P. malariae	14	2.2	13	2.1	27	2.2
P. ovale	4	0.6	8	1.3	12	1.0
P. falciparum+P. malariae	14	2.2	9	1.5	23	1.8
P. falciparum+P. ovale	9	1.4	5	0.8	14	1.1
P. malariae+P. ovale	0	0.0	0	0.0	0	0.0
P. falciparum+P. malariae+P. ovale	1	0.2	0	0.0	1	0.1
Total any *P. falciparum*	628	97.2	585	96.5	1213	96.9
Total	646	100.0	606	100.0	1252	100.0

boosts immunity initiated by a sporozoite vaccine in mice[56], exposure to natural sporozoites after the third vaccination did not appear to increase the proportion of volunteers with a T-lymphocyte response. *P. berghei* sporozoites do not prime mouse T-lymphocytes for circumsporozoite epitopes[57], which appears to be the case for *P. falciparum* sporozoites in humans, based on the 7% prevalence of T-lymphocyte response to R32LR in this study.

This vaccine did not protect this population against malaria. All volunteers acquired parasitemia. Vaccine and control groups had the same malaria-free survival curves, number of positive slides for malaria, parasite densities. This vaccine had protected 1 of 8 non-immune American vaccinees from sporozoite challenge, using 5 laboratory infected *An. stephensi* mosquitoes, at least 50% of mosquitoes infected with at least 2+(10–100 sporozoites per gland)[10], with a correlation between immunofluorescence and antibody by ELISA[10]. With this vaccine there had been no correlation between serum inhibition of sporozoite invasion of hepatoma cells with human protection[10]. Close follow-up and early treatment may have precluded the development of severe malaria, so a vaccine effect on this could not be determined.

This study provides an indirect comparison of experimentally infected laboratory mosquitoes with natural field exposure. The entomological challenge in numbers of *P. falciparum* sporozoite-infected mosquitoes per volunteer was 0.194 per day, an average of one infectious bite every 5.2 days. Five laboratory infected mosquitoes at once have been used in laboratory efficacy studies[3,4,10]. It may be thought that the malaria challenge is high in western Kenya, and too severe a test for vaccine efficacy. However, in this study, there was a 70-fold range in challenge, 3.5–245 *P. falciparum* infected mosquitoes collected per house. It was important to conclude that absence of malaria was not due to absence of infected mosquitoes.

The SPf66 vaccine has the sequence CGDELEAETQ NVYAAP NANP YSLFQKEKMVLP NANP PANKK NAG (NANP=Asn-Ala-Asn-Pro)[58]. Immunization of rabbits with SPf66 induced antibody to (Asn-Ala-Asn-Pro)[40], but not of monkeys nor human volunteers[58]. SPf66 has been reported 33.6% effective (95% confidence interval 18.8–45.7%), against a first episode of endemic malaria, with blood slides made every 4–8 weeks, in a semi-immune human population in Colombia[59], statistically in agreement with the present study (for asymptomatic parasitemia efficacy was 0%,

for symptomatic parasitemia efficacy was 9%, statistical power 95% probability of efficacy <50%). Antibody levels to Asn-Ala-Asn-Pro did not correlate with incidence[59]. It is not clear to what extent two non-contiguous copies of (Asn-Ala-Asn-Pro) in SPf66 contributed to immunogenicity or efficacy. SPf66 was reported to be 55% effective (95% confidence interval 21–75%), with blood slides made every 4–8 weeks, in Venezuela[60], also statistically consistent with the present study (for asymptomatic parasitemia efficacy was 0%, for symptomatic parasitemia efficacy was 9%, statistical power 95% probability of efficacy <50%). SPf66 was reported to be 31% effective (95% confidence interval 0–52%), with blood slides made only if temperature was 37.5°, in Tanzania[61], also statistically consistent with the present study (for asymptomatic parasitemia efficacy was 0%, for symptomatic parasitemia efficacy was 9%, statistical power 95% probability of efficacy <50%). In the present study, visits to ascertain symptoms were made every day rather than waiting for patients to present, and blood slides were made every 1 week rather than every 4–8 weeks, and with such frequent active seeking the observation remains that every volunteer of both vaccine and control groups had a parastiemia at least once, and both vaccine and control groups had similar incidences of symptomatic parasitemia.

The SPf66 vaccine was tested in the Gambia and found to have a protective efficacy of 8% (95% confidence interval −18%–29%, $P=0.50$)[62], in statistical agreement with the present study showing protective efficacy against symptomatic parasitemia of 9% (statistical power 95% probability of efficacy <50%), and also in statistical agreement with the study in Tanzania showing protective efficacy of 31% (95% confidence interval 0–52%)[61].

With R32LR, in an immunigenicity study in semi-immune volunteers, with vaccination at 0, 8 and 16 weeks, in Thailand, anti-circumsporozoite antibodies rose significantly, but fell[63]. Minimal rainfall precluded natural malaria challenge, leaving the question of vaccine efficacy unresolved in that study[62].

Newer candidate vaccines may induce both humoral and cellular immunity, with better adjuvants, presentation and delivery of antigens against multiple parasite stages.

This present study in malaria-endemic semi-immune volunteers was useful for comparison with studies in animals and non-immune volunteers, in the absence of definitive laboratory assays of protective immunity. It showed that high levels of anti-circumsporozoite repeat antibody, induced by this antigen, with this adjuvant, were ineffective in preventing malaria in this endemic population, exposed to a range of intensity of natural challenge.

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