

A PRELIMINARY EVALUATION OF A RECOMBINANT CIRCUMSPOROZOITE PROTEIN VACCINE AGAINST *PLASMODIUM FALCIPARUM* MALARIA

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ABSTRACT

Background The candidate vaccines against malaria are poorly immunogenic and thus have been ineffective in preventing infection. We developed a vaccine based on the circumsporozoite protein of *Plasmodium falciparum* that incorporates adjuvants selected to enhance the immune response.

Methods The antigen consists of a hybrid in which the circumsporozoite protein fused to hepatitis B surface antigen (HBsAg) is expressed together with unfused HBsAg. We evaluated three formulations of this antigen in an unblinded trial in 46 subjects who had never been exposed to malaria.

Results Two of the vaccine formulations were highly immunogenic. Four subjects had adverse systemic reactions that may have resulted from the intensity of the immune response after the second dose, which led us to reduce the third dose. Twenty-two vaccinated subjects and six unimmunized controls underwent a challenge consisting of bites from mosquitoes infected with *P. falciparum*. Malaria developed in all six control subjects, seven of eight subjects who received vaccine 1, and five of seven subjects who received vaccine 2. In contrast, only one of seven subjects who received vaccine 3 became infected (relative risk of infection, 0.14; 95 percent confidence interval, 0.02 to 0.88; $P < 0.005$).

Conclusions A recombinant vaccine based on fusion of the circumsporozoite protein and HBsAg plus a potent adjuvant can protect against experimental challenge with *P. falciparum* sporozoites. After additional studies of protective immunity and the vaccination schedule, field trials are indicated for this new vaccine against *P. falciparum* malaria. (N Engl J Med 1997;336:86-91.)

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PLASMODIUM *falciparum* malaria causes more than 2 million deaths annually,¹ and there is currently no effective vaccine to prevent it. The bite of an infected anopheles mosquito introduces sporozoites into the microvasculature, which are carried to the liver. After invading hepatocytes, sporozoites develop into merozoites capable of infecting erythrocytes.

The circumsporozoite protein is considered to be the principal antigen on the surface of sporozoites. Epitopes found on this antigen react with antibodies

that inhibit the invasion of hepatocytes by sporozoites and induce cellular responses that kill sporozoite-infected liver cells.² Complete immunity against infection rarely develops from natural exposure, but immunization with radiation-attenuated sporozoites affords full protection.³ This vaccine strategy is not practical, since it requires repeated exposure to hundreds of infected, irradiated mosquitoes over a period of 6 to 10 months, and sporozoites cannot be cultured in vitro. Nonetheless, these findings revealed a critical role for the circumsporozoite protein in the development of immunity against sporozoite challenge and led to its development as a candidate vaccine.^{4,5} In clinical trials, however, the circumsporozoite protein is poorly immunogenic, and few subjects have been protected.⁶ To address these issues, we created a hybrid in which the circumsporozoite protein fused to hepatitis B surface antigen (HBsAg) was expressed together with unfused HBsAg. The resulting hybrid was significantly more potent than previous circumsporozoite-protein formulations.⁷ We hypothesized that more potent adjuvants could improve the efficacy of the vaccine. We therefore conducted a clinical trial to determine the safety and efficacy of three formulations of circumsporozoite-protein vaccines against *P. falciparum*.

METHODS

Subjects

Forty-six subjects who had not been exposed to malaria (age, 18 to 45 years) were recruited by noncoercive means under a protocol approved by an institutional review board. Potential risks associated with participation in the study, including those associated with a malaria challenge, were discussed at the time of recruitment. Written informed consent was obtained from each subject before enrollment, and subjects were permitted to drop out of the study at any time without prejudice. Subjects were excluded if they had undergone splenectomy; had any cardiovascular, hepatic, or renal abnormalities; were allergic to any antimalarial drugs; were immunodeficient or pregnant; or had conditions that would increase the risk of an adverse outcome from malaria.

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Study Design and Vaccines

We designed an open-label trial of three formulations of the vaccine RTS,S (SmithKline Beecham Biologicals, Rixensart, Belgium) with no placebo control. RTS,S consists of two polypeptides that spontaneously form composite particulate structures on their simultaneous synthesis in yeast (*Saccharomyces cerevisiae*). RTS is a single polypeptide chain corresponding to amino acids 207 to 395 of *P. falciparum* (3D7) that is fused to HBsAg (adw serotype). S is a polypeptide of 226 amino acids that corresponds to HBsAg. The particles were purified from yeast-cell cultures and constitute the antigen used in the formulations.

Vaccine 1 consisted of RTS,S in a formulation containing alum and monophosphoryl lipid A (designated SBAS4); vaccine 2 consisted of RTS,S in an oil-in-water emulsion (SBAS3); vaccine 3 consisted of RTS,S in this emulsion plus the immune stimulants monophosphoryl lipid A and QS21 (SBAS2). The standard dose of vaccine 1 was 1 ml, and that of vaccines 2 and 3 was 0.5 ml; each dose delivered 50 µg of RTS,S antigen. The third dose of vaccines 2 and 3 was reduced to 0.1 ml in response to adverse reactions after the second dose. Vaccines were administered intramuscularly in the deltoid region at 0, 4, and approximately 28 weeks (range, 25 to 28). All subjects who received three doses were asked to volunteer for sporozoite challenge.

Follow-up and Outcome Measures

The subjects were observed for 20 minutes after immunization and evaluated at 1, 2, 7, and 14 days. A reaction was graded as mild if it was easily tolerated, moderate if it interfered with normal activity, and severe if it prevented normal activity and required treatment. Blood was obtained for laboratory studies on the day of vaccination and 1 and 14 days later. Serum was separated and frozen at -70°C until use. Total IgG and IgG subclasses were measured by enzyme-linked immunosorbent assay (ELISA) with recombinant R32LR for antibodies against tandem-repeat epitopes and synthetic peptides for antibodies against the carboxy terminal.⁷ Quantitative measurements of IgG subclasses were performed with a modification of a murine ELISA.⁸ Standard curves were generated with human myeloma kappa-chain IgG1, IgG2, IgG3, and IgG4 (Binding Site, San Diego, Calif.), and the results were normalized against a human reference standard. Levels of antibodies against tandem-repeat epitopes were determined relative to a different circumsporozoite-specific standard; thus, sums of subclass values differ from the total IgG value determined by ELISA. Flanking-region antibody levels were reported in optical-density units, the dilution yielding an optical density of 1.000. Antibodies against HBsAg were measured by ELISA.⁹ Seroconversion was considered to have occurred if post-immunization antibody titers against circumsporozoite tandem-repeat epitopes exceeded the mean base-line values plus 2 SD. Serum samples were analyzed by an indirect fluorescence antibody assay with air-dried sporozoites.¹⁰

Peripheral-blood mononuclear cells were isolated from donor blood by gradient centrifugation on Ficoll and stored in liquid nitrogen until use. Proliferative and cytolytic assays were performed on cells obtained before immunization and after the third dose of vaccine. The cells were thawed, washed, diluted in culture medium, and dispensed into 96-well round-bottom plates. Purified recombinant antigens (RTS,S and HBsAg) and synthetic peptides from the carboxy-terminal nonrepeated amino acid sequences from the *P. falciparum* 3D7 circumsporozoite protein, including residues 317 to 360, 349 to 395, and 361 to 393, plus a putative universal helper T-cell epitope¹¹ were used to stimulate the cells for seven days. Control cultures were stimulated with phytohemagglutinin (2 µg per milliliter), irrelevant control peptide, or medium alone. Proliferative responses were measured by the uptake of tritiated thymidine, and the results calculated as stimulation indexes.

Supernatants from parallel cultures were collected after 96 hours to measure interferon-γ by ELISA (Genzyme, Cambridge, Mass.). Serial dilutions (1:2, 1:4, 1:8, and so on) of a human in-

terferon-γ standard were assayed in parallel, and concentrations calculated from the standard curve. Cytolytic T-cell studies were performed in a subgroup of six subjects selected for HLA class I types for which circumsporozoite epitopes are known; these include HLA-A2.1 (residues 331 to 350),¹² HLA-B7 (residues 300 to 308),¹³ and HLA-B35 (residues 368 to 375).¹⁴ Quadruplicate cultures were stimulated with the HLA class I-restricted peptide for 7 or 14 days in the presence of recombinant interleukin-2. Standard chromium-release assays were performed with either autologous or HLA class I-matched B-cell blasts after transformation with the Epstein-Barr virus; the target cells were pulsed with the HLA class I-restricted peptides.

The vaccine was considered to be efficacious if there was no parasitologic evidence of *P. falciparum* infection after exposure to a sporozoite challenge that caused infection in 100 percent of unimmunized control subjects. Cloned chloroquine-sensitive *P. falciparum* 3D7 parasites were increased from a master seed lot and used to infect laboratory-reared *Anopheles stephensi*. Challenge¹⁵ occurred on one of three consecutive days approximately three weeks after the third dose of vaccine. Mosquitoes harvested from the same batch fed on a subject for five minutes, and those that had become engorged with blood were dissected to quantify the viable sporozoites. The challenge continued until five infected mosquitoes had successfully fed. On day 1, 12 subjects (7 given vaccine 1, 3 given vaccine 2, and 2 given vaccine 3) and 3 unimmunized controls were challenged. Nine subjects (one given vaccine 1 and four each given vaccine 2 and 3) and three other controls were challenged on the second day, and one subject given vaccine 3 was challenged on the third day. The sporozoite burdens in mosquito salivary glands were uniformly heavy throughout the challenge period.

The subjects were examined and blood was collected for smears each morning between days 7 and 21; blood was then obtained weekly for smears for the next three weeks. The subjects were followed monthly thereafter, and blood smears were examined for malaria if symptoms developed. Subjects who remained asymptomatic and parasite-free for 60 days after challenge were considered to be protected against the disease. Giemsa-stained thick smears were routinely examined for 200 high-power fields in the case of asymptomatic subjects and were reviewed exhaustively in the case of symptomatic subjects. The subjects who became infected were treated with an oral regimen of chloroquine plus other medications as indicated by their symptoms and were followed daily until three consecutive blood smears were negative for malaria and all symptoms had resolved.

Statistical Analysis

Statistical analyses were performed with a computerized statistical program. Comparisons of vaccine efficacy and the length of time to the onset of parasitemia were performed with Fisher's exact test and the log-rank test, respectively. The relative risks and their 95 percent confidence intervals were calculated. The immune responses of the three groups of subjects were compared with the Mann-Whitney test. All P values are two-tailed.

RESULTS

Study Population

Forty-six subjects (mean age, 30.9 years) were enrolled and received at least one immunization. Fourteen subjects were randomly assigned to receive vaccine 1, 15 to receive vaccine 2, and 17 to receive vaccine 3. Five subjects assigned to receive vaccine 1 and five assigned to vaccine 2 were positive for HBsAg on entry into the study, as compared with nine subjects assigned to receive vaccine 3. Six men and 7 women received two doses of vaccine 1, 6 men and 9 women received two doses of vaccine 2, and

10 men and 3 women received two doses of vaccine 3. Twenty-seven subjects received a third dose, of whom 22 agreed to undergo sporozoite challenge. Among the subjects who did not receive three doses of vaccine, all but two did not complete the study because of problems with scheduling or noncompliance at required follow-up visits.

Safety

No clinically important abnormal laboratory values were detected after the administration of any dose. All initial doses were well tolerated, causing mild discomfort at the site of the injection. In contrast, the second doses of vaccines 2 and 3 produced more reactions. Four subjects had severe symptoms. In one of these, a subject with a history of migraines who received vaccine 3, an uncomplicated headache developed one week after the first dose. Three other subjects (two given vaccine 2 and one given vaccine 3) had constitutional symptoms, including pain, malaise, feverishness, headache, and myalgias, within 24 hours after receiving the second dose. Laboratory results were unremarkable and did not suggest a cause for their symptoms. We did not give the two subjects with the most symptoms (one given vaccine 2 and one given vaccine 3) a third dose, and we reduced the third dose to 0.1 ml for the remaining subjects assigned to receive vaccine 2 or 3. All third doses were well tolerated.

Immunogenicity

Antibodies against circumsporozoite tandem-repeat epitopes developed in all subjects who received two or more doses (Fig. 1). The levels peaked after the second dose, declined between the second and third doses, and then returned toward maximal levels after the third dose. There was considerable individual variability in antibody responses, and the small sample size precluded a meaningful analysis of the effect of preexisting hepatitis B immunity on antiparasite responses. However, when considered as a group after two or three doses, the responses to vaccines 2 and 3 were significantly greater than those to vaccine 1 ($P < 0.02$).

In contrast, the responses to HBsAg were clearly affected by preexisting hepatitis B immunity. Whereas all subjects who were negative for HBsAg before immunization seroconverted after receiving a single dose of vaccine 2 or 3, most such subjects required two doses of vaccine 1 to seroconvert and three doses to achieve maximal responses. On the other hand, subjects who were positive for HBsAg before immunization had nearly maximal responses after the first dose, and subsequent doses resulted in minimal increases in the response. Mean HBsAg titers on ELISA after three doses of any formulation exceeded 10^5 mIU per milliliter, regardless of whether there was preexisting immunity to hepatitis B.

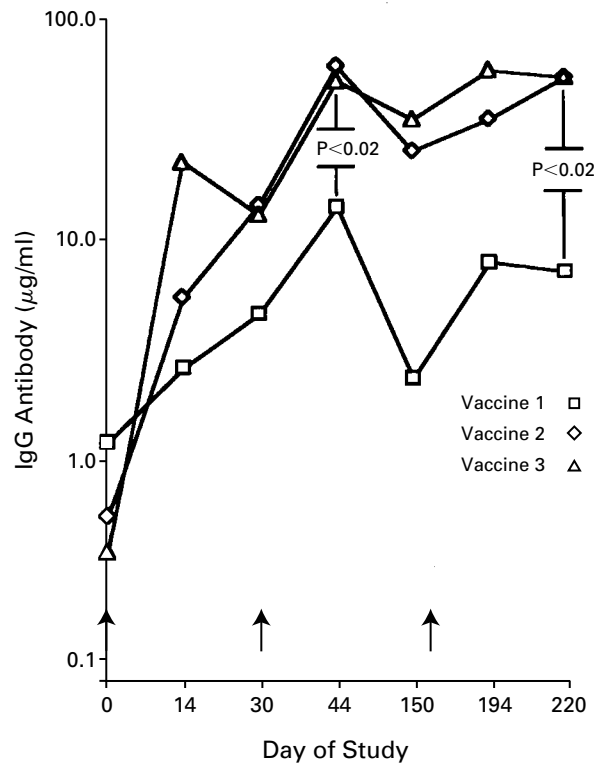


Figure 1. Geometric Mean Responses of Antibody against Circumsporozoite Tandem-Repeat Epitopes after the Administration of the Vaccines.

Total IgG was measured by ELISA with recombinant R32LR for antibodies against tandem-repeat epitopes. Vaccine was administered on day 0, at week 4, and at approximately week 28 (range, 25 to 28). The P values were calculated by the Mann-Whitney test. Arrows indicate vaccine administration.

Vaccine Efficacy

Twenty-two subjects who received three doses of vaccine agreed to a challenge with *P. falciparum* sporozoites, eight given vaccine 1 and seven each given vaccines 2 and 3. Six unimmunized subjects served as controls, and parasitemia developed in all six 11 to 13 days after sporozoite challenge. Malaria developed in seven of eight subjects given vaccine 1, with a mean prepatent period of 12.6 days (range, 11 to 18). Five of seven subjects given vaccine 2 became infected, with a mean prepatent period of 15.2 days (range, 14 to 19). The length of time to the onset of malaria was significantly longer among those given vaccine 2 than among the controls ($P < 0.01$ by the log-rank test). In contrast, only one of seven subjects given vaccine 3 became infected, for an estimated vaccine efficacy of 86 percent (relative risk of infection, 0.14; 95 percent confidence interval, 0.02 to 0.88; $P < 0.005$). Subjects who were asymptomatic and parasite-free for 60 days after challenge remained so more than 6 months after challenge.

Protected subjects tended to have higher antibody titers against tandem-repeat epitopes than those in whom malaria developed, and the only subject given vaccine 3 who became infected had a poor antibody response (Table 1). On the day of challenge, antibody titers against tandem-repeat epitopes measured by ELISA correlated well with indirect fluorescence antibody responses (coefficient of correlation, 0.86), indicating that these antibodies accounted for most of the reactivity against intact sporozoites. IgG1 and IgG2 accounted for nearly all the antibody against tandem-repeat epitopes, whereas IgG3 and IgG4 responses were minimal (data not shown). Antibodies against carboxy-terminal epitopes were more common and pronounced in subjects given vaccine 2 or 3, but the titers were not predictive of protection. Geometric mean antibody titers against tandem-repeat epitopes or intact sporozoites did not differ significantly between the groups receiving vaccine 2 or 3 who underwent sporozoite challenge (Table 2). After three doses, a majority of all subjects had evidence of immunologic priming to one or more RTS,S epitopes, as evidenced by proliferative responses or interferon- γ production in response to antigen stimulation, but the presence of circumsporozoite-specific cellular responses did not predict protection (data not shown).

Among the small subgroup of subjects selected on the basis of HLA class I typing (four of whom were protected and two of whom were not), there was no clear evidence of cytolytic T-cell activity in cultures of peripheral-blood mononuclear cells obtained after immunization, as measured by lysis of peptide-labeled HLA class I-restricted target cells.

TABLE 1. TITERS OF ANTIBODIES AGAINST CIRCUMSPOROZOITE TANDEM-REPEAT EPITOPES AND INTACT SPOROZOITES AT THE TIME OF SPOROZOITE CHALLENGE.

VACCINE AND SUBJECT No.	TOTAL IgG* $\mu\text{g/ml}$	IFA TITERT	PREPATENT PERIOD‡ days
Vaccine 1			
1	1.38	<50	11
2§	43.19	3,200	>60
4	6.22	800	13
8	4.35	800	12
9	4.09	50	10
10	5.88	800	13
11§	20.87	3,200	18
13	7.10	3,200	11
Vaccine 2			
16§	101.23	12,800	14
22	122.99	25,600	>60
25	365.70	51,200	>60
27§	10.31	100	14
28§	15.48	800	19
29	35.32	3,200	14
30	43.58	12,800	15
Vaccine 3			
32§	283.00	51,200	>60
34	22.37	3,200	>60
35	5.22	800	11
38§	595.00	102,400	>60
41	40.33	800	>60
42§	79.71	12,800	>60
44§	18.54	3,200	>60

*Total IgG was measured by ELISA.

†The end-point titer (greatest dilution that yielded a positive fluorescence above the control levels) of the indirect fluorescence antibody assay (IFA) is shown.

‡The prepatent period is the interval between challenge and the detection of parasitemia.

§This subject was positive for HBsAg at base line.

TABLE 2. GEOMETRIC MEAN SPOROZOITE ANTIBODY LEVELS AMONG THE SUBJECTS WHO UNDERWENT SPOROZOITE CHALLENGE.

ANTIBODY*	VACCINE 1		VACCINE 2		VACCINE 3	
	MEAN	RANGE	MEAN	RANGE	MEAN	RANGE
Total IgG ($\mu\text{g/ml}$)	7.00	1.38–43.19	52.63†	10.31–365.70	52.98†	5.22–595.00
IgG1 ($\mu\text{g/ml}$)	1.55	0.06–14.49	12.65†	0.94–95.00	15.54†	1.06–233.00
IgG2 ($\mu\text{g/ml}$)	2.13	0.38–6.78	4.19	0.50–18.28	8.20†	0.77–28.14
IFA	654	<50–3200	4755	100–51,200	6400	800–102,400

*IgG antibodies against circumsporozoite tandem-repeat epitopes were measured on the day of challenge. The end-point titer of the indirect fluorescence assay (IFA) of antibodies against intact sporozoites is shown.

† $P < 0.05$ for the comparison with vaccine 1 by the Mann-Whitney test.

DISCUSSION

We have developed an immunogenic recombinant circumsporozoite vaccine that protects adults who have never been exposed to malaria against experimental challenge with *P. falciparum* sporozoites. The process required more than a decade, during which our group and others have systematically evaluated numerous candidate formulations.^{6,16-24} Until now, the only strategy that predictably protected humans was immunization with irradiated sporozoites.²⁵ This impractical model established the basis for current sporozoite vaccine strategies, including the identification of the circumsporozoite protein as a leading candidate vaccine.^{2,26} The RTS,S vaccine contains circumsporozoite-protein central tandem-repeat epitopes and carboxy-terminal epitopes that provide targets for both antibody and cellular responses. The tandem-repeat epitopes are highly conserved among *P. falciparum* isolates and are recognized by antibodies that neutralize the infectivity of sporozoites for liver cells.²⁷ In preclinical adjuvant screening studies, the SBAS2 formulation (vaccine 3) proved superior for inducing strong antibody responses and strong antigen-specific delayed hypersensitivity in primates and proliferative and cytolytic T-cell responses in mice.

Pre-erythrocytic immunity is an all-or-none phenomenon, and experimental sporozoite challenge provides a powerful tool for screening vaccine candidates. The response is very clear, for if a single sporozoite completes its development in the liver, clinical malaria ensues. Plasmodia are superbly adapted to humans, and in the field, pre-erythrocytic immunity develops rarely if at all.²⁸ This observation is consistent with the concept that certain pathogens or antigens are not recognized as dangerous by the immune system and fail to induce protective immune responses.²⁹ Indeed, developing liver schizonts express abundant circumsporozoite protein, and except in the setting of hyperimmunization with attenuated sporozoites, schizont-infected hepatocytes fail to elicit inflammatory infiltrates.³⁰

Our study demonstrated that strong adjuvants were required, but comparison of the efficacy of SBAS2 (vaccine 3) with that of SBAS3 (vaccine 2) suggested that strong antibody responses to tandem-repeat epitopes alone were insufficient to confer protection. Effective adjuvants such as those in SBAS2 may also provide signals required to up-regulate costimulatory molecules on antigen-presenting cells, induce expression of molecules that permit these cells to travel to target tissues, or induce production of cytokines that mediate protection.³¹

We have not yet identified the cellular mechanisms that explain the protection obtained with the SBAS2 formulation, but it might provide an optimal stimulus for T cells recruited after challenge to eliminate liver-stage parasites by enhanced local release

of interferon- γ .³² The period between the administration of the reduced third dose of vaccine and sporozoite challenge was less than a month, but the absence of constitutional symptoms after immunization makes it unlikely that protection was mediated by nonspecific transiently circulating cytokines. Although cellular studies are incomplete, there is no evidence to date that the vaccine protected subjects through mechanisms involving cytolytic T cells. In the light of the proliferative responses or the production of interferon- γ in response to RTS,S epitopes in a number of subjects, the role of CD4+ T-cell responses must be further investigated.³³

Many important questions remain to be addressed before the full potential of this vaccine is known. In particular, expanded safety studies will be needed and efficacy against heterologous challenge must be determined in the field. The history of humanity's struggle with this disease is a sobering reminder of the hard work that still lies ahead.

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APPENDIX

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