A Recombinant Blood-Stage Malaria Vaccine Reduces *Plasmodium falciparum* Density and Exerts Selective Pressure on Parasite Populations in a Phase 1–2b Trial in Papua New Guinea

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The malaria vaccine Combination B comprises recombinant *Plasmodium falciparum* ring–infected erythrocyte surface antigen and 2 merozoite surface proteins (MSP1 and MSP2) formulated in oil-based adjuvant. A phase 1–2b double-blind, randomized, placebo-controlled trial in 120 children (5–9 years old) in Papua New Guinea demonstrated a 62% (95% confidence limits: 13%, 84%) reduction in parasite density in children not pretreated with sulfadoxine-pyrimethamine. Vaccinees had a lower prevalence of parasites carrying the MSP2-3D7 allelic form (corresponding to that in the vaccine) and a higher incidence of morbid episodes associated with FC27-type parasites. These results demonstrate functional activity of Combination B against *P. falciparum* in individuals with previous malaria exposure. The specific effects on parasites with particular *msp*2 genotypes suggest that the MSP2 component, at least in part, accounted for the activity. The vaccine-induced selection pressure exerted on the parasites and its consequences for morbidity strongly argue for developing vaccines comprising conserved antigens and/or multiple components covering all important allelic types.

An effective malaria vaccine will represent a cost-effective and sustainable addition to the currently available malaria control interventions [1]. Anti–asexual blood-stage vaccines are aimed at reducing parasite growth and multiplication in the blood and, hence, the occurrence or severity of symptoms [2]. Such vaccines should reduce morbidity and mortality due to malaria in the most susceptible groups (i.e., children <5 years old and pregnant women) living in areas where malaria is endemic.

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Informed consent was obtained in the local language from the village leaders and from all subjects' parents. The trial was performed within the guidelines of the declaration of Helsinki and its amendments and was approved by the Papua New Guinea Medical Research Advisory Committee, the Bancroft Centre Research Ethics Committee, and the Walter and Eliza Hall Institute of Research Ethics Committee.

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Here, we report a trial of a vaccine against asexual blood stages of *Plasmodium falciparum.* The vaccine comprises merozoite surface protein (MSP) 1, the 3D7 allele of MSP2, and the ring-infected erythrocyte surface antigen (RESA). Laboratory and animal studies indicate that immune responses against these antigens induce protective immune responses (reviewed in [3]). Longitudinal seroepidemiologic studies conducted by the Papua New Guinea Institute of Medical Research [4] also found a negative correlation between baseline levels of naturally acquired immune responses to these antigens and subsequent malaria morbidity [5–7].

A series of safety and immunogenicity trials performed in malaria-naive Australians, first with the adjuvant (Montanide ISA720) alone [8] and then using different administration and dose regimens of the vaccine formulation (Combination B) [9], showed that the product was safe and immunogenic. There was no precedent for a trial of a malaria vaccine comprising only blood-stage antigens in a previously exposed population, and no decisive test of efficacy, short of a field trial, was possible. Immune responses to the vaccine were considered to be likely to modify the course of infection without necessarily preventing it; therefore, the validated artificial challenge protocols used for pre-erythrocytic vaccines were not appropriate. The phase 2a trial showed that the vaccine did not modify the course of infection in malaria-naive individuals [3], but we considered this to be more likely to happen in semi-immune children, in whom immunization may boost immune responses primed by natural

exposure. Furthermore, in the field trial, there was an opportunity for immune responses to the vaccine to be boosted by subsequent infection. This was not possible in the phase 2a trial because of early drug treatment following the laboratory challenge.

A phase 1 trial in immune adult volunteers living in an area of Papua New Guinea where malaria is highly endemic indicated that Combination B was also safe in this population and did not predispose to serious malaria after subsequent infection [10]. These results led us to proceed with a phase 1–2b trial in the same area to assess pilot efficacy in children 5–9 years of age. We did not want to embark on a large-scale trial without any indication that the vaccine might work; therefore, we designed a trial with parasite density, which was assessed every 2 weeks over a 10-week period (weeks 8–18), as a surrogate marker of protection. Parasitologic outcomes are appropriate measures for testing asexual blood-stage vaccines that are intended to reduce parasite multiplication. Parasite density was expected to be a statistically more powerful outcome measure than the incidence of morbid episodes, making it possible to study efficacy with only a small sample size. For safety purposes, clinical episodes were monitored over an extended period (up to week 76), and this allowed a preliminary assessment of the vaccine's impact on morbidity as a secondary outcome.

A polymerase chain reaction (PCR) technique was used to determine prevalence, incidence of new infections, and parasite genotypes. All blood samples collected during the trial were genotyped using the highly polymorphic *msp2* locus as a marker. MSP2 alleles fall into 2 families (FC27 and 3D7) defined by a central dimorphic region flanked by conserved regions [11]. Only the 3D7 form of MSP2 was included in Combination B; thus, if the vaccine was efficacious, specific effects were expected on parasites belonging to this allelic family.

Methods

Vaccine preparation. Details of the vaccine components and formulation have been reported elsewhere [9, 10]. In brief, the 3 vaccine candidate antigens were produced by recombinant DNA technology within a collaborative program involving the Walter and Eliza Hall Institute of Medical Research, Queensland Institute of Medical Research, Biotech Australia, CSL, F. Hoffmann–La Roche, and Roche Products Australia. All 3 antigens were expressed in *Escherichia coli* with histidine tags to facilitate purification by nickel chelate chromotography. Two of the antigens, 190LCS.T3 (Ro 45-2067) and Ag1624 (Ro 46-2924), corresponded to parts of MSP1 and MSP2, respectively. The MSP1 antigen was the 190L fragment from the K1 parasite line, comprising the relatively conserved blocks 3 and 4 of MSP1 fused with a universal T cell epitope derived from the circumsporozoite protein of *P. falciparum* [12]. The MSP2 antigen corresponded to the near full-length MSP2 sequence of the 3D7 cloned line [13, 14]. Ag1505H (Ro 45-2164) consisted of the C-terminal 70% of RESA of the FCQ-27/PNG parasite line [15].

The adjuvant Montanide ISA720 is an oil composition containing a natural metabolizable oil and a highly refined emulsifier from the mannide mono-oleate family; the adjuvant was donated in bulk by SEPPIC [9]. All 3 antigens were supplied in separate vials at a concentration of 160 μg/mL saline–Montanide ISA720 emulsion. Prior to use, the 3 formulations were mixed and diluted with additional emulsion to give a dose of 15 μg of each antigen in a total volume of 0.55 mL. The placebo doses consisted of 0.55 mL of the adjuvant emulsion.

Study area and subjects. The study was conducted in 4 villages of the South Wosera District, East Sepik Province, Papua New Guinea. The Wosera is an area of intense and perennial malaria transmission with entomological inoculation rates (determined from indoor landing rates) averaging around 35, 12, and 10 infectious bites per person per year for *P. falciparum*, *Plasmodium vivax,* and *Plasmodium malariae,* respectively [16]. Prevalence of asymptomatic parasitemia in 5–9-year-old children approximates 57% for *P. falciparum*, 23% for *P. vivax*, and 4% for *P. malariae* [17]. Enlarged spleen rates exceed 50%, and malaria is responsible for 20%–40% of all fevers [18]. All healthy children 5–9 years old on the day of screening were eligible. Exclusion criteria included parent/child refusal, allergic predisposition, acute illness on the day of screening, chronic illness, and impaired liver or kidney function.

Study design, cohort, and randomization. The study was a double-blind, block-randomized within age group, placebo-controlled trial to assess safety (reported elsewhere) and pilot efficacy of a combination of 3 candidate antigens. Since it has been debated whether preexisting infections should be cleared before immunization, half the children were pretreated with sulfadoxine-pyrimethamine (SP; Fansidar, Roche). Children were allocated individually into 4 treatment groups of 30 each: No-SP plus vaccine (group 1), No-SP plus placebo (adjuvant alone; group 2), SP plus vaccine (group 3), and SP plus placebo (group 4). Randomization was carried out in blocks of 12 (3 of each group).

Sample size. A sample size of 120 subjects (60 each in the vaccine and placebo groups) was chosen to enable us to detect a 30% reduction of parasite density (primary outcome) with 90% power ($\alpha = .05$). The magnitude of reduction was chosen because it was slightly higher than that obtained in the Tanzanian SPf66 malaria vaccine trial, in which a 20% reduction of *P. falciparum* density corresponded to a 31% reduction in clinical episodes of *P. falciparum* malaria [19].

Enrollment and immunizations. Since the adjuvant and the vaccine formulation had never been administered to children, the enrollment and immunizations were carried out in subcohorts of 12 or 24 subjects, starting with the older age group (7.5–9 years), with 10 days of observation in between.

In the week prior to vaccination, children were screened and examined, and a sample of venous blood was obtained. According to their randomization number, the children were given either SP or a placebo (an indistinguishable sugar tablet). During week 0, they were injected intramuscularly in the left lateral thigh with the vaccine or placebo (adjuvant alone). Four weeks after the first injection, the children received a second intramuscular injection in the right lateral thigh.

Parasitologic follow-up (weeks 8–18). Finger-prick blood samples were obtained every 2 weeks from weeks 8 to 18 for deter-

mination of *P. falciparum* parasite densities and *msp2* genotypes. Giemsa-stained blood films were examined, and the numbers of parasites per 200 white blood cells (WBC) were recorded. The number of parasites per microliter was calculated from the WBC count. One hundred thick-film fields were examined before a slide was declared negative. All slides were examined independently by 3 microscopists to determine whether they were negative or positive for parasites and to determine species and density. In case of disagreement, a fourth microscopist (supervisor) examined the slide, and all 4 microscopists then had to agree on the final result.

For each individual *(i),* we computed the geometric mean *P. falciparum* parasite density in microscopy-positive blood samples obtained at weeks 8, 10, 12, 14, 16, and 18 and denoted each as g_i. g_i was considered to be missing if all samples from weeks 8, 10, 12, 14, 16, and 18 were negative. g_i was defined a priori in the analytical plan as the primary outcome measure because it represents an independent measurement for each child that is less variable than the individual parasite density assessments. Thus the primary estimate of efficacy was calculated as follows: $1 - (V/P)$, where V is the geometric mean of the values of g_i in the vaccine group, and P is the geometric mean of the values of g_i in the placebo group. Statistical significance of the effects of vaccine and of SP pretreatment were tested by analysis of variance of the log-transformed values of g_i.

PCR–restriction fragment–length polymorphism (RFLP) analysis genotyping. For easy storage, transport, and DNA purification, 10 μL of blood pellet from the finger-prick or venous blood samples was spotted on filter papers (Isocode Stix; Schleicher & Schuell). After the filter papers were washed according to the supplier's instructions, a triangle of filter paper was submerged in *msp2* primary PCR mix. Primary and nested PCR were performed as described elsewhere [20]. All amplification products were digested with the restriction enzyme *Hinf* I. Samples containing 3D7-type alleles subsequently were digested with *Dde* I and *ScrF* I. RFLP analysis was done as described elsewhere [20]. All samples were genotyped independently without knowledge of the child's identity. The genotyping of most samples was confirmed by repeating the procedure, starting from the filter paper.

The prevalence of infection detected by PCR after vaccination was estimated from the samples obtained at weeks 8, 10, 12, 14, 16, and 18. Prevalence in vaccine recipients was compared with that in placebo recipients, using a logistic regression model with a random-effect term to allow for correlations between observations in the same child and with allowance for the effects of SP pretreatment. Separate analyses were carried out for overall PCR positivity and for each of the 2 allelic families of *msp*2.

Clinical surveillance (week 8–76). Morbidity monitoring consisted of once-a-week visits by village reporters to all children at their home, from week 8 to week 76, together with health facility-based surveillance during the same period [21]. In case of symptoms during the previous 3 days or axillary temperature of $>37.5°C$, a blood sample was obtained again for determination of parasite densities and genotypes. A clinical episode of malaria was defined as a fever (axillary temperature $>37.5°C$) or a history of fever in the previous 3 days and a *P. falciparum* parasitemia of ≥ 8000 parasites/μL. This threshold has been shown elsewhere to have adequate specificity and sensitivity in children in Wosera [22]. Further analyses considered episodes with any *P. falciparum* parasitemia.

Kaplan-Meier survival curves were used to compare attack rates of first or only episodes between the vaccine and placebo groups. Statistical significance of effects of vaccine and of SP treatment was assessed using log-rank tests. Survival analyses were also used to compare attack rates of first or only morbid episodes between the vaccine and placebo; this was done separately for the 2 allelic families of *msp*2. For each allelic family, the analysis included all episodes that satisfied the above case definitions and in which ≥ 1 *msp*2 genotype belonging to that family was detected.

Results

The study was conducted from February 1998 to September 1999. Three hundred eighty children who were between their fifth and tenth birthdays on the planned day of screening were identified from a demographic surveillance system. Fifty-two percent of the parents or guardians agreed to their children's involvement. One hundred twenty-three children were screened, 3 of whom were excluded on medical grounds. All 120 children received 2 injections within the scheduled interval and completed the parasitologic follow-up and clinical surveillance. Baseline characteristics of treatment groups 1–4 (No-SP pretreatment plus vaccine, No-SP plus placebo [adjuvant alone], SP plus vaccine, and SP plus placebo, respectively) are shown in table 1. Sizable differences among the groups in some parasitologic measures arose by chance, since there was only one baseline measurement for each child and only a small number of infected individuals in each group. All but 1 of the total of 1080 planned blood samples (weeks 0, 4, 6, 8, 10, 12, 14, 16, and 18) were obtained, corresponding to a compliance rate of 99.9%, and 7532 visits were included in the morbidity surveillance, representing 91% of the total possible.

Efficacy on parasite density (primary outcome). The effect of the vaccine on parasite densities differed depending on whether the child was pretreated with SP. Table 2 shows the number of children with parasites during follow-up blood sampling (weeks 8, 10, 12, 14, 16 and 18) and the geometric mean parasite density in the 4 treatment groups. *P. falciparum* density was significantly reduced in the vaccinated children who did not receive SP before vaccination, compared with the density in untreated children receiving placebo (t statistic $= 2.4$; 29 *df;* $P = .024$). The vaccine efficacy estimate was 62% (95% confidence limits [95% CLs]: 13, 84). This effect was modified significantly by SP treatment (test of interaction F statistic $= 4.8$; 1.43 *df;* $P = .034$). Among SP-treated children, there was no significant difference in parasite density between the vaccine and placebo groups (t statistic $= -1.0$; 14 *df;* $P = .3$). Adjustment for baseline parasite prevalence and/or density made a negligible difference to these results.

The difference in parasite density dynamics during follow-up among the vaccine and placebo groups in the No-SP and the SP groups is shown in figure 1. Substantially fewer high parasitemias (parasite density $>500/\mu$L) occurred in the children who

Table 1. Baseline characteristics for children in a phase 1–2b trial of a recombinant blood-stage malaria vaccine administered with or without sulfadoxine-pyrimethamine (SP) pretreatment.

	Treatment group			
	No-SP		SP	
Characteristic	Placebo	Vaccine	Placebo	Vaccine
No. of enrollees	30	30	30	30
Age, mean years (SE)	7.5 (0.3)	7.4 (0.3)	7.6 (0.3)	7.6 (0.3)
Level of hemoglobin, g/L (SE)	100 (2)	101 (2)	103 (2)	101 (2)
Prevalence of parasites, %[a]	37	24	17	28
Geometric mean parasite density (95% CLs)	825 (389, 1751)	754 (106, 5380)	244 (53, 1123)	2326 (441, 12,252)
Overall prevalence, %[b]	63	37	47	43
Prevalence of 3D7-type parasites, %	50	23	33	30
Prevalence of FC27-type parasites, %	33	27	30	30

NOTE. 95% CLs, 95% confidence limits; No-SP, treatment groups receiving vaccine or placebo without SP; SP, treatment groups receiving vaccine or placebo and SP.

[a] Determined by microscopy.

[b] Determined by polymerase chain reaction.

received the vaccine formulation in the No-SP group (figure 1, *upper right*), compared with those who received placebo (3 vs. 20 slides, respectively; figure 1, *upper left*). In the SP group, few blood sample slides with high parasitemia were seen in the vaccine and placebo groups (9 and 7 slides, respectively; figure 1, *lower right and left*). The effect of the vaccine on parasite density could not be compared between infections with parasites of the FC27- or 3D7-type because of the large number of mixed infections.

Effects on prevalence, as determined by PCR. Of 360 blood samples collected from vaccine recipients (including both SP and No-SP groups) during follow-up, 57 (16%) were positive, as determined by PCR, whereas 98 (27%) of the 359 placebo samples were positive. This difference was not statistically significant when simultaneously adjusted for the effects of repeated assessment of the same children, SP treatment, and baseline positivity. However, when specific allelic families were considered, the prevalence of the 3D7 dimorphic form of *msp*2 was found to be significantly reduced, even allowing for repeated assessment of the same children and for baseline presence of 3D7 parasites ($\chi^2_1 = 4.2$; $P = .040$). In the placebo group, 78 of the samples (22%) were positive for 3D7, and, in the vaccine group, 30 of the samples (8%) were positive for this allelic family. On the other hand, the logistic models indicated that the vaccine made no difference to the prevalence of FC27-type parasites. These effects are shown in figure 2.

Among children pretreated with SP, the prevalence of both allelic families was low in both vaccine and placebo groups for the entire 18-week follow-up. We therefore could not assess whether the SP pretreatment modified the effects of the vaccine on prevalence of 3D7-type parasites.

Effects on new infection. A new infection was considered to have occurred when an *msp*2 genotype that was not detected in the same child at week 0 or 4 was found at week 8, 10, 12, 14, 16, or 18. Twenty-one new 3D7-type infections were detected in placebo recipients, and only 11 were detected in vaccinees. In contrast, only 6 new FC27-type infections were found in placebo recipients, compared with 11 in vaccinees. Twenty of the new infections were in children pretreated with SP, and 29 were in those not pretreated. Overall, this corresponded to a vaccine efficacy of only 19% (95% CLs: −23%, 49%) for preventing new infections. There was a significant difference between the allelic types in this efficacy ($\chi^2_1 = 4.1$; $P = .042$), but there was no significant effect of SP pretreatment.

Effects on first or only morbid episodes. The vaccine had no significant efficacy on the incidence of first or only fever episodes with *P. falciparum,* irrespective of whether a density cutoff was used (table 3). Similarly, using the same case definitions, there were no effects on the incidence of fever episodes with 3D7-type parasites. In contrast, when all first or only episodes with FC27-type parasites were considered, the incidence was found to be higher in vaccinees (log rank $\chi^2_1 = 6.5$; $P = .01$; figure 3). This effect was accounted for by the children who were not treated with SP (for whom log rank

Table 2. Number of children with *Plasmodium falciparum* parasites during parasitologic follow-up (weeks 8–18) in a phase 1–2b trial of a recombinant blood-stage malaria vaccine administered with or without sulfadoxine-pyrimethamine (SP) pretreatment.

Treatment group	No. of children with parasites[a]	Geometric mean of g_i	95% Confidence limits
No-SP			
Placebo	18	382.6	201, 728
Vaccine	13	144.6	89, 236
SP			
Placebo	6	257.2	47, 1421
Vaccine	10	689.5	163, 2911

NOTE. g_i, Individual parasite densities in blood samples obtained at weeks 8, 10, 12, 14, 16, and 18; No-SP, treatment group receiving vaccine or placebo without SP; SP, treatment group receiving vaccine or placebo and SP.

[a] Determined by microscopy.

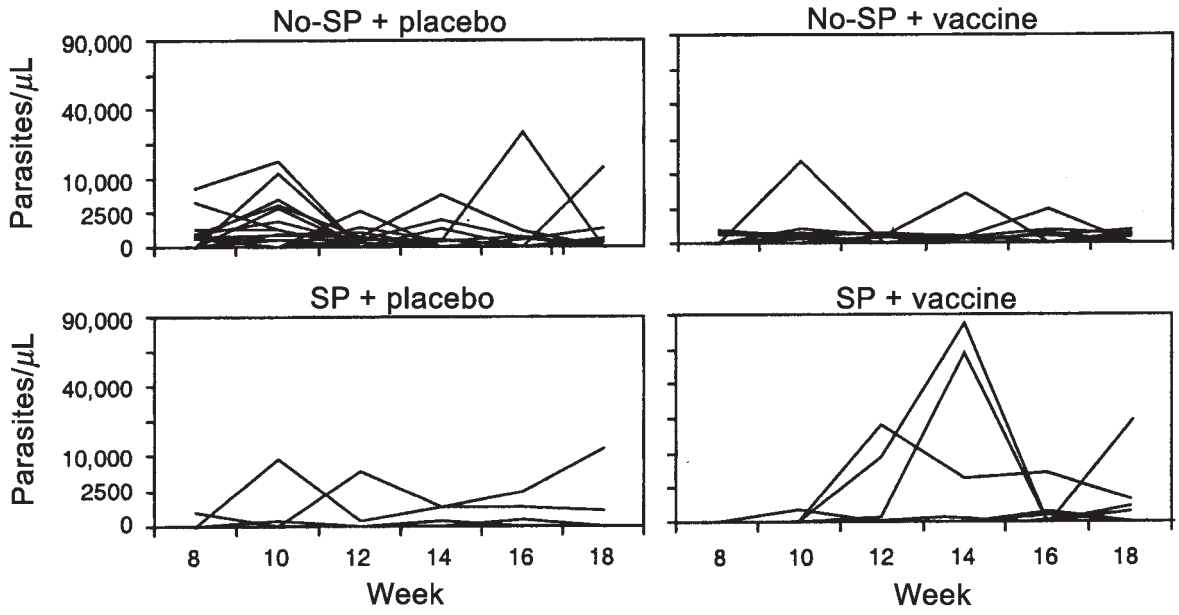

Figure 1. Parasite densities in 4 treatment groups in a phase 1–2b trial of a recombinant blood-stage malaria vaccine administered with or without sulfadoxine-pyrimethamine (SP) pretreatment. No-SP + placebo, group treated with placebo only; No-SP + vaccine, group treated with vaccine only; SP + placebo, group treated with SP and placebo; SP + vaccine, group treated with SP and vaccine. Vertical axes are on a square-root scale.

$\chi_1^2 = 7.0$; $P = .008$). There was no significant effect in the SP group ($\chi_1^2 = 1.1$; $P = .3$). The tendency was the same for the FC27-type episodes with densities of ⩾8000 parasites/μL, but the significance level was less clear-cut because of the lower number of occurrences ($\chi_1^2 = 3.6$; $P = .06$). Adjustment (using Cox regression) for the presence or allelic family of parasites at baseline made little difference to the estimated vaccination effects. Nor were these effects dependent on whether the cases were detected in the health center or in the community surveillance.

Discussion

This field trial with an asexual blood-stage malaria vaccine provided strong evidence that Combination B vaccine is efficacious in reducing parasite densities (the primary outcome) in children who are not pretreated with SP. This is the largest reduction (62%) in parasite density that has yet been achieved in a malaria vaccine trial in malaria-endemic areas. It is a rather conservative estimate of the reduction in parasite load, since it is based only on the reduction in density in parasite-positive

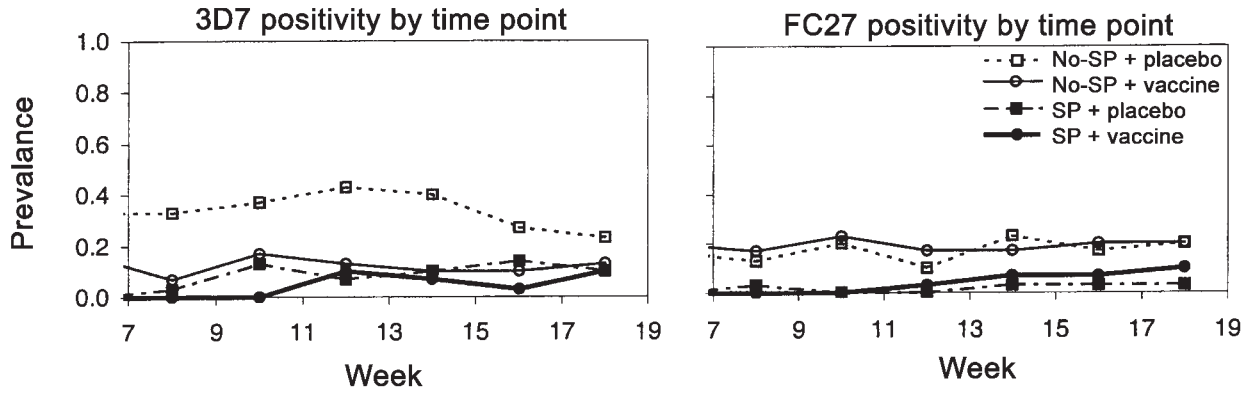

Figure 2. Prevalence, by time point, of 3D7- and FC27-type parasites in 4 treatment groups in a phase 1–2b trial of a recombinant blood-stage malaria vaccine administered with or without sulfadoxine-pyrimethamine (SP) pretreatment. No-SP + placebo, group treated with placebo only; No-SP + vaccine, group treated with vaccine only; SP + placebo, group treated with SP and placebo; SP + vaccine, group treated with SP and vaccine. Vertical axes are on a square-root scale.

Table 3. Number of children with symptomatic first or only morbid episodes during the clinical surveillance period (weeks 8–76) of a phase 1–2b trial of a recombinant blood-stage malaria vaccine administered with or without sulfadoxine-pyrimethamine (SP) pretreatment.

	Treatment group			
	No-SP		SP	
Variable	Placebo	Vaccine	Placebo	Vaccine
Fever and ≥8000 parasites/μL				
FC27-type parasites	8	15	7	10
3D7-type parasites	7	11	7	8
Any *Plasmodium falciparum* parasites	11	18	12	15
Fever and >0 parasites/μL				
FC27-type parasites	11	21	7	11
3D7-type parasites	12	12	10	9
Any *Plasmodium falciparum* parasites	18	22	14	18

NOTE. No-SP, treatment group receiving vaccine or placebo without SP; SP, treatment group receiving vaccine or placebo and SP.

blood films. It does not take into account the reduced number of positive blood films in the No-SP plus vaccine group, compared with the No-SP plus placebo groups (35 vs. 48, respectively). Nor does it consider the reduced number of children with detectable parasites in the SP plus vaccine group at any time during follow-up (13 vs. 18, respectively).

The randomization of half the children to be pretreated with SP, a long-acting drug combination with a long elimination period, had a substantial effect on the trial. This treatment reduced the number of patent infections during the first period of the study and, therefore, reduced the power to compare vaccine and placebo within the treatment arm.

Although highly effective in reducing parasite density, Combination B had no significant effect on the overall number of clinical malaria episodes. There was even a tendency for vaccinated children to have a higher incidence of disease in the year following immunization. This observation raises some concerns and deserves some comments. The sample size was small. Since the present trial was designed to provide an indication of a vaccine effect on parasite density and to acquire information on safety as a prelude to a large morbidity trial in younger children, we did not have the power to assess morbidity with confidence. A trial of vitamin A supplementation in children 6 months to 4 years old in the present trial site [23] found very similar reductions in malaria morbidity rates and parasite densities, and this provided the rationale for using parasite density as primary outcome. However, although high parasite densities are generally associated with malaria morbidity in areas where malaria is endemic, the association is by no means invariant [22, 24, 25]. In another malaria vaccine trial, the effects of the vaccine on parasite densities were strongly age dependent, but those on morbidity were not [19].

The present study demonstrates a specific activity of Combination B vaccine against parasite growth and multiplication, the putative mechanism for anti–asexual blood-stage vaccines. The demonstration of a specific effect of the vaccine against the development of 3D7-type infections also indicates that the activity of Combination B is due, at least in part, to the MSP2 component, which seems to protect children against infections with homologous parasites. This does not preclude a contributing role of the MSP1 and RESA components in the observed effects.

Vaccines containing only one allelic type of a polymorphic antigen may induce immune responses that select for parasites expressing alternative alleles. Such selection has been demonstrated recently with a conjugate *Streptococcus pneumoniae* vaccine [26] and is a major concern for *Haemophilus influenzae* type b vaccination [27]. A similar selection of alternate proteins after vaccination has been shown experimentally in the *Plasmodium knowlesi* system [28]. The relationship between the 2 allelic families of MSP2 is not one of simple competition, since

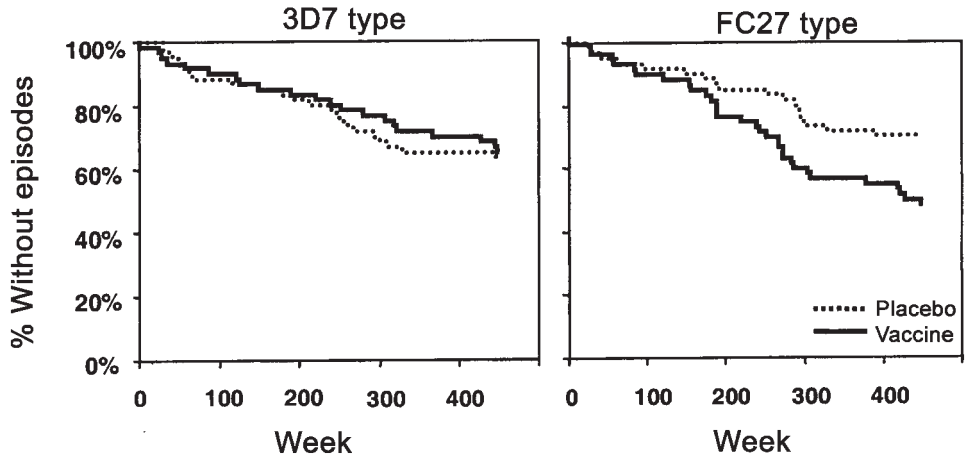

Figure 3. Kaplan-Meier survival curves comparing positive episodes of 3D7- and FC27-type parasites in subjects 8–60 weeks after treatment with placebo or a recombinant blood-stage malaria vaccine.

they tend to occur together more often than expected, at least in asymptomatic individuals [29, 30]. Hence it was difficult to predict what might be the effect of inclusion of only one allele of MSP2 in a subunit vaccine, particularly since the MSP2 component also contained the conserved region of the protein.

A previous case-control study suggested that *msp*2 FC27-type infections are more virulent than those of 3D7-type infections in the study area [31]. Vaccination with 3D7 appears to have favored the FC27-type parasites, contributing to an increased rate of FC27-associated morbidity in vaccinated children. It is possible that 3D7 parasites play a role in the natural control of densities of FC27-type infections and that, by reducing the load of 3D7 parasites, the vaccine interfered with such cross-protection.

To our knowledge, this study is the first demonstration of vaccine-induced selection of malaria parasites in an area where the disease is endemic and this study highlights the necessity to monitor closely such trials by molecular means. The results of this study imply that, for this particular vaccine-development program, further vaccine formulations must include both allelic families of MSP2 and these results generally argue for the development of vaccines comprising conserved antigens and/or multiple components covering all important allelic types.

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References

1. World Health Organization. Investing in health research and development. Report of the Ad Hoc Committee on Health Research Relating to Future Intervention Options. Geneva: World Health Organization, **1996**: TDR/gen/96.1.
2. Hoffman SL, Rogers WO, Carucci DJ, Venter JC. From genomics to vaccines: malaria as a model system. Nat Med **1998**;4:1351–3.
3. Lawrence GW, Cheng Q, Reed C, et al. Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. Vaccine **2000**;18:1925–31.
4. Alpers MP, al-Yaman F, Beck HP, et al. The Malaria Vaccine Epidemiology and Evaluation Project of Papua New Guinea: rationale and baseline studies. P N G Med J **1992**;35:285–97.
5. al-Yaman F, Genton B, Anders R, et al. Assessment of the role of the humoral response to *Plasmodium falciparum* MSP2 compared to RESA and SPf66 in protecting Papua New Guinean children from clinical malaria. Parasite Immunol **1995**;17:493–501.
6. al Yaman F, Genton B, Kramer K, et al. Assessment of the role of naturally acquired antibody levels to *Plasmodium falciparum* merozoite surface protein–1 in protecting Papua New Guinean children from malaria morbidity. Am J Trop Med Hyg **1996**;54:443–8.
7. al-Yaman F, Genton B, Taraika J, Anders R, Alpers MP. Association between cellular response (IL-4) to RESA/Pf155 and protection from clinical malaria among Papua New Guinean children living in a malaria endemic area. Parasite Immunol **1997**;19:249–54.
8. Lawrence GW, Saul A, Giddy AJ, Kemp R, Pye D. Phase I trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. Vaccine **1997**;15:176–8.
9. Saul A, Lawrence G, Smillie A, et al. Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. Vaccine **1999**;17:3145–59.
10. Genton B, al-Yaman F, Anders R, et al. Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. Vaccine **2000**;18: 2504–11.
11. Smythe JA, Peterson MG, Coppel RL, Saul AJ, Kemp DJ, Anders RF. Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. Mol Biochem Parasitol **1990**;39:227–34.
12. Holder AA. The precursor to major merozoite surface antigens: structure and role in immunity. Prog Allergy **1988**;41:72–97.
13. Smythe JA, Coppel RL, Day KP, et al. Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2. Proc Natl Acad Sci USA **1991**;88:1751–55.
14. Sturchler D, Berger R, Rudin C, et al. Safety, immunogenicity, and pilot efficacy of *Plasmodium falciparum* sporozoite and asexual blood-stage combination vaccine in Swiss adults. Am J Trop Med Hyg **1995**;53:423–31.
15. Culvenor JG, Day KP, Anders RF. *Plasmodium falciparum* ring–infected erythrocyte surface antigen is released from merozoite dense granules after erythrocyte invasion. Infect Immun **1991**;59:1183–7.
16. Hii JL, Smith T, Mai A, et al. Spatial and temporal variation in abundance of *Anopheles* (Diptera:Culicidae) in a malaria endemic area in Papua New Guinea. J Med Entomol **1997**;34:193–205.
17. Genton B, al-Yaman F, Beck HP, et al. The epidemiology of malaria in the Wosera area, East Sepik Province, Papua New Guinea, in preparation for vaccine trials. I. Malariometric indices and immunity. Ann Trop Med Parasitol **1995**;89:359–76.
18. Genton B, al-Yaman F, Beck HP, et al. The epidemiology of malaria in the Wosera area, East Sepik Province, Papua New Guinea, in preparation for vaccine trials. II. Mortality and morbidity. Ann Trop Med Parasitol **1995**;89:377–90.
19. Alonso PL, Smith TA, Armstrong-Schellenberg JR, et al. Duration of protection and age-dependence of the effects of the SPf66 malaria vaccine in African children exposed to intense transmission of *Plasmodium falciparum*. J Infect Dis **1996**;174:367–72.
20. Felger I, Irion A, Steiger S, Beck HP. Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. Trans R Soc Trop Med Hyg **1999**;93(Suppl 1):3–9.
21. Genton B, Smith T, Baea K, et al. Malaria: how useful are clinical criteria for improving the diagnosis in a highly endemic area? Trans R Soc Trop Med Hyg **1994**;88:537–41.
22. Smith T, Genton B, Baea K, et al. Relationships between *Plasmodium falciparum* infection and morbidity in a highly endemic area. Parasitology **1994**;109:539–49.
23. Shankar AH, Genton B, Semba RD, et al. Effect of vitamin A supplementation on morbidity due to *Plasmodium falciparum* in young children in Papua New Guinea: a randomised trial. Lancet **1999**;354:203–9.

24. Greenwood BM, Bradley AK, Greenwood AM, et al. Mortality and morbidity from malaria among children in a rural area of The Gambia, West Africa. Trans R Soc Trop Med Hyg **1987**;81:478–86.
25. Delley V, Bouvier P, Breslow N, et al. What does a single determination of malaria parasite density mean? A longitudinal survey in Mali. Trop Med Int Health **2000**;5:404–12.
26. Obaro SK, Adegbola RA, Banya WA, Greenwood BM. Carriage of pneumococci after pneumococcal vaccination. Lancet **1996**;348:271–2.
27. Lipsitch M. Vaccination against colonizing bacteria with multiple serotypes. Proc Natl Acad Sci USA **1997**;94:6571–6.
28. David PH, Hudson DE, Hadley TJ, Klotz FW, Miller LH. Immunization of monkeys with a 140 kilodalton merozoite surface protein of *Plasmodium knowlesi* malaria: appearance of alternate forms of this protein. J Immunol **1985**;134:4146–52.
29. Felger I, Tavul L, Kabintik S, et al. *Plasmodium falciparum:* extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. Exp Parasitol **1994**;79:106–16.
30. Paul RE, Brockman A, Price RN, et al. Genetic analysis of *Plasmodium falciparum* infections on the northwestern border of Thailand. Trans R Soc Trop Med Hyg **1999**;93:587–93.
31. Engelbrecht F, Felger I, Genton B, Alpers M, Beck HP. *Plasmodium falciparum:* malaria morbidity is associated with specific merozoite surface antigen 2 genotypes. Exp Parasitol **1995**;81:90–6.