

A Population-Based Clinical Trial with the SPf66 Synthetic *Plasmodium falciparum* Malaria Vaccine in Venezuela

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A phase III malaria vaccine trial in 13 villages in an endemic area, South Venezuela, compared incidence rates of *Plasmodium falciparum* and *Plasmodium vivax* infections in 1422 vaccinated and 938 nonvaccinated subjects over 18 months. The SPf66 vaccine was given in three doses, on days 0, 20, and 112. Vaccination was complete in 976 subjects (68.7%). Minor side effects requiring no treatment were reported by 123 (12.6%), with an apparent increase in frequency from the first to the third vaccine dose. No autoimmune evidence was observed in a sample of subjects. Antibodies against SPf66 were present at low titers in 24.7% of tested subjects before vaccination, increasing to 53.6% after the second dose and to 73.6% after the third dose; 26.4% of subjects initially seronegative never seroconverted. The SPf66 malaria vaccine showed a protective efficacy of 55% (95% confidence interval, 21%–75%) against *P. falciparum* and of 41% (19%–57%) against *P. vivax* malaria.

The operational difficulties and the limitations of the available tools for malaria control, further compromised by the progressive resistance of parasites to drugs and of vectors to insecticides, have given room to explore new strategies for the prevention of malaria transmission.

A remarkable effort has been placed on malaria vaccine development. Since the first attempts to immunize humans with irradiated sporozoites [1], several different approaches have been pursued, targeting different parasite stages and antigens, either chemically synthesized or developed by genetic engineering. As a result, several parasite proteins or fragments have been characterized; some have been shown to confer protective immunity in animal models [2, 3] and human studies [4–7]. Synthetic and recombinant candidate subunit vaccines based on the *Plasmodium falciparum* circumsporozoite protein have been tested in challenge and

field studies [5, 7]. Although these vaccines were found to be safe and to induce antibody response, they failed to demonstrate a solid protective efficacy.

The SPf66 hybrid malaria vaccine, developed in Colombia, is a polymeric synthetic protein with amino acid sequences of three *P. falciparum* merozoite proteins linked by the Asn-Ala-Asn-Pro motif derived from the sporozoite [4]. This vaccine evokes immune response against the asexual stages of the erythrocytic phase of *P. falciparum* and has been shown to be safe and immunogenic in human trials [3, 8, 9]. Clinical field studies initiated after the one reported here have confirmed the experimental evidence, showing a 40%–82% protective efficacy against *P. falciparum* in certain defined age groups [10–12].

Here we report the results of the first population-based phase III trial with the SPf66 synthetic malaria vaccine conducted in an endemic area.

Materials and Methods

Study area and population. The study was done in 13 small villages, covering a population of 3526 inhabitants in the municipality of Las Majadas, northeastern Bolívar State, South Venezuela. The climate is typical of a rainy tropical savanna, with a mean annual temperature of 26–28°C, average annual rainfall of ~2000 mm, an altitude of 50 m above sea level, and wind velocity of 4 km/h. A wet season extends from May to October and a dry season from November to April [13]. The peak of malaria transmission occurs during the months of December through March. According to the National Malaria Control Program, the mean annual incidence of *P. falciparum* malaria for

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Informed consent was obtained from subjects, and all procedures were approved by an Ethical Committee representing the Ministry of Health, Universidad Central de Venezuela, and Universidad de los Andes.

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this region during the 5 years before the trial was 100/1000 population. *P. falciparum* malaria represented ~25% of the cases reported. Asymptomatic parasitemia is seldom observed, nor is resistance to chloroquine chemotherapy. The main malaria vectors identified in the area were *Anopheles darlingi*, *Anopheles oswaldoi*, and *Anopheles albicans*.

The eligible population for the study comprised all persons >11 years old living in the study area ($n = 2439$). A population census was done through household visits recording age, sex, occupation, literacy, religion, pregnancy, length of residence in the area, mobilization habits, type of dwelling, use of antimalarial drugs in the last 2 months, use of antimosquito nets, and medical history from all household members. The census was updated during the study by three cross-sectional observations to derive person-time of exposure among the participants and to collect blood and urine samples for immune response and antimalarial self-medication tests.

Vaccination. The SPf66 vaccine was synthesized at the Instituto de Inmunología, Hospital San Juan de Dios, Bogotá, Colombia, following the solid-phase peptide synthesis method described by Merrifield [14] as modified by Houghten [15]. Each dose consisted of a volume of 0.5 mL containing 4 mg/mL SPf66 peptide adsorbed onto alum hydroxide at a 2 mg/mL concentration. Details of the formulation, quality control assays, and preservation have been described elsewhere [4].

Before vaccination, community meetings were organized to explain the possible benefits and risks of the vaccine and the voluntary nature of the vaccination. All persons residing in the community who spontaneously attended an open invitation for vaccination were vaccinated ($n = 1422$). The vaccine was administered subcutaneously in the deltoid, in three doses, on days 0, 20, and 112. Because of logistics, 160 subjects received the third dose on day 156. After each dose, the participants were observed by a clinician over 45 min and were advised to contact the local health service during the following 48 h in case of any abnormal clinical manifestation. The recruitment and vaccination lasted ~4 days for each dose. All volunteers were clinically examined, and the following were excluded from vaccination: pregnant women; persons with severe health problems, mental disorders, alcoholism, or history of allergies or debilitating diseases; those receiving immunosuppressive drugs; and those with clinical symptoms suggestive of malaria. Subjects eligible for vaccination were examined by thick and thin blood smear 15 days before vaccination. Malaria cases were treated according to the National Malaria Control Program; therefore, by the time of vaccination, only persons asymptomatic and free of parasitemia were vaccinated. Pregnancy was ascertained by interview and also by a urine test (Pregna P test, Precisa, Miami) before each vaccine dose in all women >15 years of age.

After the three doses of vaccine were given, a comparison group of 938 residents included in the initial census who did not attend the vaccination day was selected; persons who had any of the exclusion criteria applied to the vaccination group were excluded.

The study sample size has an 80% power, at a 5% significance level, to detect a 50% reduction in malaria rate over a period of 1 year, assuming an expected annual *P. falciparum* malaria incidence in the unvaccinated group of 3.9%, as reported in the previous year.

Follow-up observations. The follow-up observation for vaccinated and nonvaccinated subjects extended over 14 months after the third vaccine dose. Household visits were carried out by health field workers every 2 weeks, who asked for clinical symptoms of malaria throughout the study. A blood slide was prepared for all symptomatic subjects. Also, active case-finding detection was implemented by taking thick and thin blood smears from all participants 15 days before vaccination and every 8–10 weeks after the second dose of vaccine. Passive case detection and malaria treatment as prescribed by the National Malaria Control Program was provided by a local health facility attended by rural physicians assigned exclusively to the study.

A malaria case was defined as presence of malaria parasites in the circulating blood. A new *P. falciparum* malaria episode was defined as malaria parasites in a blood smear after a period of at least 30 days free of parasitemia. All slides were examined in the field by trained personnel and then double-checked blindly at the Central Laboratory of the National Malaria Control Program and at the Laboratory for the Study of Malaria, Ministry of Health/Universidad Central de Venezuela (UCV).

Self-medication with antimalarial drugs was ascertained by interview and also by random testing of urine samples from 289 vaccinated and 180 nonvaccinated subjects. Samples were collected 6 and 12 months after the third dose and tested by thin-layer chromatography [16] to detect 4-aminoquinoline excretion. All samples were tested at UCV and at the Swiss Institute for Tropical Medicine (Basel).

Malaria chemoprophylaxis, treatment of the patient's family, presumptive treatment, and nebulization with insecticide were all suspended during the study period. Insecticide spraying with DDT every 6 months was the only malaria control intervention maintained.

Immune response. Antibody tests against native *P. falciparum* proteins were done before vaccination by indirect immunofluorescence assay (IFA). Titers $\geq 1:80$ were considered positive. Paired blood samples collected on the first day of vaccination, 30 days after the second dose, and 1, 6, and 12 months after the third dose were stored at -80°C and processed simultaneously at the end of the study. Antibodies to SPf66 polymer were titered by ELISA as described [17]. A cutoff point was established as the mean optical density + 3 SD of the results obtained testing a large number of serum samples from persons who had never lived in an endemic area.

Autoimmune responses were also evaluated by testing serum samples collected from a random sample of participants before vaccination and 1 month after completing the third dose. Tests included determination of IgG, IgA, IgM, and C3 and C4 complement components (Kallestad QM 300 protein analysis system; Sanofi Diagnostics Pasteur, Chaska, MN) by nephelometry [18]; complement B factor by radial immunodiffusion [19]; IgE by RIA (Quanticlone IgE Kit; Sanofi) [20]; antinuclear antibodies by immunofluorescence (Quantafluor substrate slides Hep.2; Sanofi); circulating immune complexes by ELISA [21]; and rheumatoid factor by nephelometry [18].

Statistical analysis. Baseline characteristics of vaccinated and unvaccinated subjects were compared. Mean age, sex distribution, and proportions of participants with the different characteristics, including antibody response, were calculated. Differ-

ences between proportions were evaluated by χ^2 and differences between means by Student's *t* test [22]. A significance level of .05 was used.

P. falciparum and *Plasmodium vivax* malaria incidences were calculated monthly, taking persons at the end of the study and also person-time of follow-up in each group as denominators. Incidence rates were examined according to age, sex, ethnic group, occupation, place of residence, and malaria history. To compare the incidence of malaria after vaccination between the vaccinated and unvaccinated groups adjusting for a dissimilar malaria risk at baseline, rate ratios were calculated for the incidence during the 12 months subsequent to the third dose of vaccination in relation to that observed during an equivalent calendar period just before vaccination, separately for *P. falciparum* and *P. vivax*. The after- to before-vaccination incidence ratio of each group was used to derive vaccine efficacy (VE) as $VE = 1 - (R_v/R_{nv})$, where R_v and R_{nv} are ratios of the incidence after vaccination to the incidence before vaccination for the vaccinated and nonvaccinated groups, respectively [23]. χ^2 significance tests and 95% confidence intervals of the estimates were calculated. This procedure was required to have a proper assessment of the effect of immunization on malaria incidence taking into account that the two groups were not randomized to be vaccinated and presented with different characteristics of risk of infection.

Results

Of the 1422 subjects who received the first vaccine dose, 976 (68.7%) completed the full three-dose vaccination schedule, 362 (25.5%) received two doses, and 84 (5.9%) received just the first vaccine dose. Noncompliance with vaccination was attributed to absence from the community on the day of vaccination, pregnancy, and other exclusion criteria. No statistical difference was observed regarding age, sex, or occupation distributions between the vaccinated and comparison groups. Mean age of participants was 32.7 years, and ~58% were male. Occupations with a high risk of exposure to infection, such as farmer, fisher, hunter, and miner, were reported by 55% of controls and 51% of vaccinated subjects. Most participants (79.7%) were mestizos. The proportion of subjects identified as Amerindian was greater among those not vaccinated than among vaccinated subjects (14.9% vs. 6.9%; $P < .01$).

Persons receiving vaccination lived predominantly in localities of higher risk of transmission than nonvaccinated subjects. However, the history of malaria as reported during the interviews was similar for both study groups. Also, the proportions of subjects reporting use of antimalarial drugs during the follow-up period did not differ statistically between the two groups (10% vs. 12%). Urine tests for aminoquinolines were positive in 13.8% of nonvaccinated and in 11.7% of vaccinated subjects ($P > .05$). Analysis of records from the National Malaria Control Program indicated that annual *P. falciparum* malaria incidences during the 5 years before the study increased progressively from 0.6% to 6.3% among un-

Table 1. Side effects of SPf66 vaccine according to number of doses.

Side effect	Dose		
	1 (n = 1420)	2 (n = 1338)	3 (n = 976)
Induration	0.3	0.3	5.6
Pain	0.7	0.5	0.4
Erythema	0.1	0.4	0.5
Local pruritus	0.1	0.8	3.5
Generalized pruritus	0	0.1	0.5
Bronchospasm	0	0	0.2
Other	0	0.3	0.3

NOTE. Data are % with side effect.

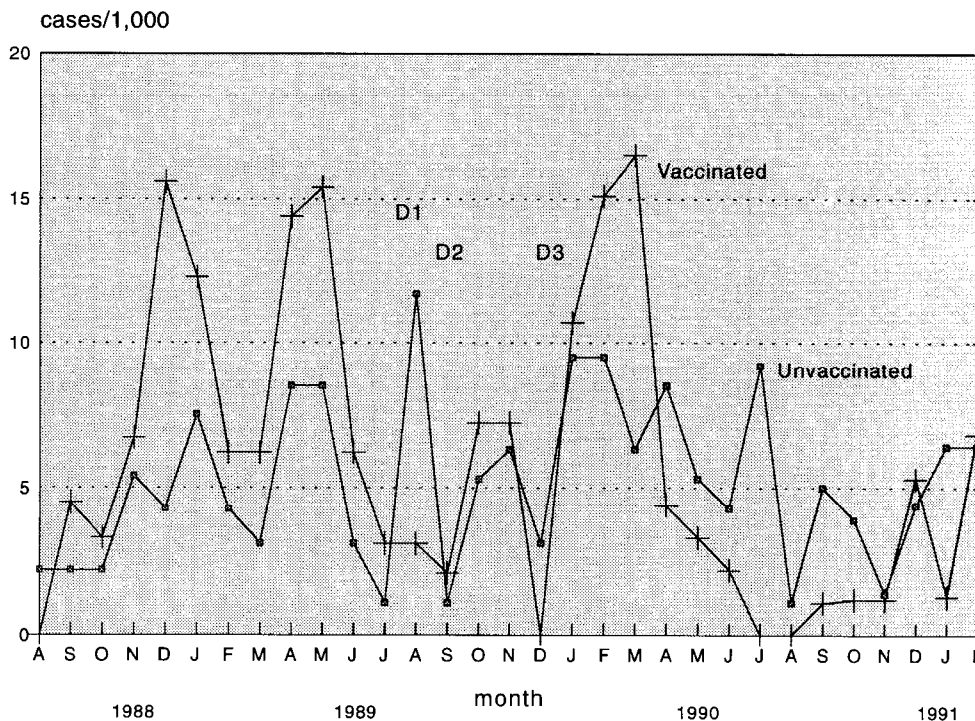
vaccinated subjects and from 0.8% to 8.3% among vaccinees. Corresponding rates for *P. vivax* malaria increased from 1.5% to 11.3% for controls and from 0.9% to 20.4% among vaccinees.

Side effects. Minor side effects requiring no treatment were reported in a small proportion of subjects (table 1). Local pain, induration, and pruritus were the most common reactions observed. Contralateral induration and local erythema were more frequent after the third dose. Five women presented with systemic pruritus outside of the injection site, and 1 case of bronchospasm was reported, occurring 15 min after vaccination in an apparently healthy woman. The frequency of systemic reactions was significantly higher in women. No case of delayed reaction was observed. No generalized reaction was reported for subjects receiving one or two vaccine doses.

Autoimmune tests. No abnormalities were seen in the comparison of results of the various autoimmunity serum marker tests done before vaccination and 30 days after application of the third dose in a sample of 43 vaccinated subjects, suggesting that the vaccine was safe in this respect. None of the immunologic parameters analyzed showed statistically significant variation. In 1 vaccinated subject, the level of C3 and C4 decreased, while values for factor B and circulant immune complexes were normal.

Immunogenicity. The prevalence of native fluorescent antibodies to *P. falciparum* before vaccination was 54.9% for the vaccine group and 51.9% for the nonvaccinated subjects ($P < .05$). No significant change in these prevalences was observed after vaccination. Of the 929 tested vaccinated subjects, 24.7% had anti-SPf66 antibodies at low titers (1:100–1:400) before vaccination. Sixty days after the second vaccine dose, 53.6% of the study population showed anti-SPf66 antibodies, 76.6% of samples with titers of <1:400. Thirty days after completing the three-dose vaccination, 60.4% of subjects were positive to antibodies against the peptide. In this group, 552 (80.9%) were classified as low responders, with titers of <1:400.

Figure 1. Monthly incidence of *P. falciparum* malaria among vaccinated and unvaccinated persons, covering 1 year before vaccination up to end of study, Las Majadas, 1988–1991.



Of subjects initially negative for antibodies to SPf66, 26.4% never seroconverted and 48.4% seroconverted after the second and third doses, with titers falling to <1:100 by the end of follow-up. One year after the third dose, 25.2% of those showing seroconversion still had positive titers.

Protective efficacy. Figure 1 shows monthly *P. falciparum* malaria incidences for both study groups. There was a significant decrease in incidence starting 3 months after the third dose of vaccine. During the 12 months immediately after the third dose, the incidence of *P. falciparum* malaria was 4.5/1000 person-months at risk for the vaccinated group and 5.6/1000 person-months for the nonvaccinated group. Protective efficacy conferred by the vaccine was calculated by taking the ratio of the incidence during the follow-up period to the baseline incidence for the same amount of time before vaccination as a way to control for seasonal variation in incidence. The vaccinated group had a mean incidence before vaccination of 7.9/1000 person-months, while the nonvaccinated group had a mean incidence of 4.4/1000 person-months (table 2). On the basis of these estimates, the vaccine protective efficacy for those receiving the complete series of three doses was calculated to be 55.1% (95% confidence interval, 21%–75%; $P < .01$). Protective efficacy calculated for subjects receiving only two doses of vaccine was ~67%, with wide and nonsignificant confidence intervals because of the small numbers of subjects and cases in this group.

The incidence of *P. falciparum* malaria was higher, but not statistically significant, for subjects who did not seroconvert after immunization (3.8%) than for those who seroconverted (1.3%). Among nonvaccinated subjects, the incidence of ma-

laria did not differ between those negative and positive for native SPf66 antibodies.

Monthly *P. vivax* malaria incidences covering 1 year before vaccination and the follow-up period are shown in figure 2. Conversely to *P. falciparum* malaria, incidences for *P. vivax* increased after vaccination in both groups, mostly in the last quarter of the follow-up year. For the vaccinated group, the incidences during the 12 months preceding and following vaccination were 16.3/1000 and 30.7/1000 person-months, respectively. Corresponding figures for the nonvaccinated group were 9.1/1000 and 28.9/1000 person-months (table 3). The vaccine's protective effect against *P. vivax* malaria was estimated as 41% (95% confidence interval, 19%–57%). No further adjustment to estimate vaccine efficacy was considered necessary, since incidences were compared within the same group of subjects before and after vaccination.

Table 2. Cumulative incidence of *P. falciparum* malaria during 12 months preceding and following the complete series of three doses of vaccine.

	Vaccine	Control
Before vaccination		
Cases/person-months	89/11,289	49/11,171
Rate/1000 person-months	7.9	4.4
After vaccination		
Cases/person-months	46/10,223	56/9904
Rate/1000 person-months	4.5	5.6

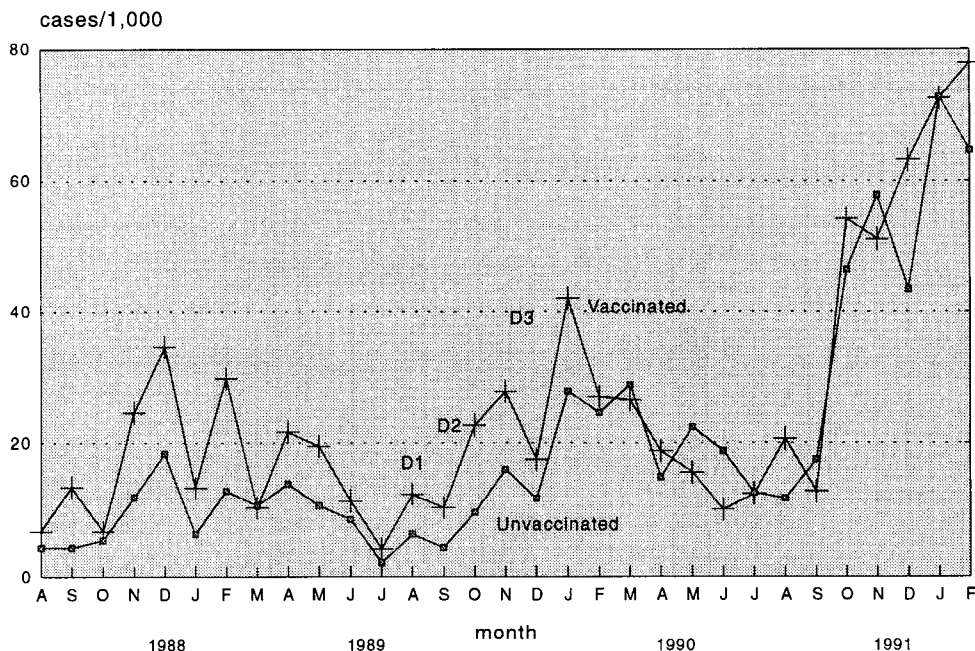


Figure 2. Monthly incidence of *Plasmodium vivax* malaria among vaccinated and unvaccinated persons, covering 1 year before vaccination up to end of study, Las Majadas, 1988–1991.

Discussion

The SPf66 vaccine was evaluated in Venezuela after promising results were obtained in initial preclinical studies in *Aotus* monkeys [3, 24] as well as in hospital and field clinical studies among Colombian military personnel [25]. The Las Majadas trial was initiated in August 1989 and corresponded to the first population trial with a synthetic malaria vaccine in a civilian population from an endemic area.

This trial was proposed with the objective of further expanding the study of safety, immunogenicity, and protection of the SPf66 vaccine in an area geographically distant from those originally studied in Colombia [10], with a different ethnic population and parasite strains and with a higher proportion of *P. vivax* infections. For ethical reasons, it was designed as an open trial, because it was thought that vaccination could delay the usual early health care-seeking behavior of symptomatic persons in this area.

Full compliance with vaccination was achieved by 68.7% of participants. Migration out of the study area accounted for

the majority of persons lost during follow-up, occurring in a similar proportion among the vaccinated and nonvaccinated groups (31.1% and 33.9%).

Although the vaccinees were not randomly selected, no statistical difference was observed regarding personal characteristics, history of malaria, consumption of antimalarial drugs during follow-up, or prevalence of prevaccination antibodies to the SPf66 polymer and to *P. falciparum* antigens. However, vaccinees lived in areas of higher malaria incidences than the controls and consequently had significantly higher *P. falciparum* and *P. vivax* malaria incidences during the year before vaccination.

The vaccine was very well tolerated. Side effects were rare and mostly at the site of injection. The effects reported were similar to those observed with other multiple-dose vaccines and with alum hydroxide as adjuvant [26]. The frequency of postvaccination effects other than local reactions was significantly higher in women (0.52% vs. 0). Local side effects were less frequent than those reported in Ecuador [12] but higher than those reported in Colombia [10, 11].

The autoimmunity serum markers studied showed absence of vaccine-induced injury. Just 1 subject showed a minor alteration of one immunologic parameter 30 days after application of the third dose. Chemical, hematologic, and autoimmune profiles reported in other studies also demonstrated that the vaccine is considered to be innocuous [4, 8, 9, 24].

Baseline immune response to *P. falciparum* was high: 24.7% of vaccinees and 28.5% of nonvaccinees recognized the SPf66 polymer, and 54.9% and 51.9%, respectively, were also positive by IFA, indicating a similar prevaccination humoral immune response in both groups. The higher sero-

Table 3. Cumulative incidence of *Plasmodium vivax* malaria during 12 months preceding and following the complete series of three doses of vaccine.

	Vaccine	Control
Before vaccination		
Cases/person-months	184/11,289	102/11,171
Rate/1000 person-months	16.3	9.1
After vaccination		
Cases/person-months	314/10,223	286/9904
Rate/1000 person-months	30.7	28.9

prevalence of *P. falciparum* antibodies in relation to that found in other studies [11, 12] could be associated with a higher exposure to *P. vivax* and *P. falciparum* infections in this population. Of vaccinees initially negative, 26.4% remained seronegative throughout the study. However, most of the responders had low titers. Possible genetic differences could account for this finding, since the HLA-DR4 antigen carrier state has been reported to be associated with a low or nonexistent humoral immune response to the peptide [27, 28].

This study confirms previous reports showing no correlation between seroconversion and risk of *P. falciparum* infection [4], suggesting that ELISA cannot discriminate between protective and nonprotective antibodies. Markers of protective immune response induced by the vaccine should be investigated. Further studies are planned to correlate specific humoral immune responses to side effects and vaccine immunogenicity to prevaccination antibody levels.

To adjust for dissimilar malaria risks in vaccinated and nonvaccinated groups, protective efficacy was estimated by comparing changes in malaria incidence rates before and after vaccination between the two groups. The protective efficacy for *P. falciparum* infection for subjects receiving three doses was 55.1%. This is an intermediate value between results obtained in La Tola, Colombia (33.6%) [11], and La T, Ecuador (66.8%) [12].

In contrast to what was observed for *P. falciparum*, incidences for *P. vivax* increased in both groups after vaccination, particularly among the nonvaccinated group. This was reflected in a crude protective efficacy of 41% with a wide confidence interval. This unexpected result deserves special attention because this was the first SPf66 trial conducted in an area with a high *P. vivax* incidence rate, allowing for proper statistical analysis. A theoretical biologic explanation for a possible cross-protective effect is the existence of common protein sequences in both the SPf66 peptide and relevant *P. vivax* protein [29, 30] (figure 3). Also, SPf66-vaccinated persons show recognition of *P. vivax* antigen by IFA [31]. In addition, if protective *P. falciparum* nonspecific immune responses are elicited by SPf66, it could also affect other malaria parasite species.

We cannot rule out all possible biases in the interpretation of the study results, considering some limitations of the study design. The follow-up observation was similar in the two study groups. It is unlikely that the precision of malaria diagnosis was different between vaccinees and controls, since all blood slides were read blindly in a central laboratory. The increased exposure to malaria infection among vaccinees was controlled in the statistical analysis by estimating within groups malaria incidence changes over time, before and after vaccination. A crude comparison of the groups could have underestimated the protective efficacy conferred by the vaccine. The consumption of antimalarial drugs assessed by interviews and validated by urine analysis of 4-aminoquino-

RBP	K	S	L	L	E	K	E	K	K	L	K	2368-2378
83.1 SPf66	Y	S	L	F	Q	K	E	K	M	V	L	
Pv200	Y	E	L	F	H	K	K	K	L	G	G	42-52

Figure 3. Comparison of amino acid sequences of SPf66 (83.1 peptide) and of known *Plasmodium vivax* protein molecule sequences, reticulocyte binding protein (RBP, top) and Pv200 (bottom). Amino acid residue numbers are given at right.

lines showed no differences between the two groups, excluding the possibility of increased ingestion among vaccinees after complete vaccination.

The development of an efficacious synthetic vaccine opens an exciting perspective of future investigation in this field for other infectious diseases. The synthetic nature of the vaccine allows the development of a more rational and simpler approach to further improve the former vaccine design.

This was the first field trial in a civilian population with a chemically synthesized vaccine and the first with a vaccine against malaria. In the present study, SPf66 was safe and immunogenic; under the characteristics of this trial, it conferred protection of ~55% against *P. falciparum* and 41% against *P. vivax* infections. Further studies should evaluate the impact of SPf66 in different ecoepidemiologic situations. The potential public health application of a malaria vaccine could substantially contribute to the global effort for the control of malaria.

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