IMMUNOGENICITY AND PROTECTIVE EFFICACY OF RECOMBINANT VACCINE BASED ON THE RECEPTOR-BINDING DOMAIN OF THE *PLASMODIUM VIVAX* DUFFY BINDING PROTEIN IN *AOTUS* MONKEYS

MYRIAM ARÉVALO-HERRERA,* ANGÉLICA CASTELLANOS, SYED S. YAZDANI, AHMAD RUSHDI SHAKRI,
CHETAN E. CHITNIS, ROSALIE DOMINIK, AND SÓCRATES HERRERA

*Institute of Immunology, Universidad del Valle, Cali, Colombia; Malaria Vaccine and Drug Development Center, Cali, Colombia;
Malaria Research Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India;
Family Health International, Durham, North Carolina*

Abstract.   Invasion of human erythrocytes by *Plasmodium vivax* requires interaction between Duffy binding protein (PvDBP) and the Duffy blood group antigen. The receptor-binding domain of PvDBP lies in a conserved N-terminal, cysteine-rich region, region II (PvRII). PvRII is a valuable malaria subunit vaccine candidate for asexual blood stages. We have evaluated in *Aotus* monkeys the immunogenicity and protective efficacy of recombinant PvRII formulated in Freund's and Montanide ISA720 adjuvants. Specific antibody titers were determined by an enzyme-linked immunosorbent assay after each of three doses of 50 µg of protein administered by the subcutaneous route. Immunization with PvRII formulated in Freund's adjuvant yielded higher antibody titers than immunization with the Montanide ISA720 formulation and offered partial protection. Although the Montanide ISA720 formulation was immunogenic, it did not provide any protection. Given the immunogenicity and partial protection observed, further studies are needed to optimize the PvRII vaccine formulation with adjuvants suitable for human use.

INTRODUCTION

Over the last two decades, important efforts have been made to identify and test malaria vaccine candidates that could be used to diminish the enormous socioeconomic and health burden that malaria represents for disease-endemic communities in tropical countries.[1] *Plasmodium vivax* is the second most prevalent human malaria parasite worldwide, accounting for approximately 20% of malaria cases globally and 80–90% of cases in Asia, Oceania, and the Americas.[2] Because of its lower prevalence, lower rates of mortality, and the lack of methods for continuous *in vitro* culture, limited resources have been made available for *P. vivax* malaria vaccine research.

The *P. vivax* Duffy binding protein (DBP) represents a valuable malaria subunit vaccine candidate for asexual blood stages. The protein is a conserved antigen expressed on the merozoite surface and it has been demonstrated to be a ligand for parasite invasion of the erythrocyte through a specific interaction with the Duffy blood group antigen (Fy), recently renamed the Duffy antigen receptor for chemokines (DARC) because of its role in chemokine binding.[3] DARC is an indispensable receptor for *P. vivax* invasion of human erythrocyte, and individuals lacking DARC (i.e., Fy-negative individuals) are naturally protected from *P. vivax*–induced clinical malaria.[4] The immune blockage of this receptor-ligand interaction through the induction of antibodies to PvDBP is therefore likely to protect humans against *P. vivax* blood infection.

PvDBP belongs to a family of erythrocyte binding proteins that includes the *P. falciparum* sialic acid binding protein, also known as 175 kD erythrocyte binding antigen, *P. knowlesi* DBP (PkDBP), and *P. knowlesi* β and γ proteins. The receptor-binding domains of these proteins lie in conserved, N terminal, cysteine-rich regions (region II) that are found in all of these proteins. Region II of PvDBP (PvRII) contains ap-

proximately 350 amino acids with 12 conserved cysteine residues.[3,5]

Because of its potential use as a subunit malaria vaccine, methods to produce recombinant PvRII in its functional conformation by expression in *Escherichia coli* followed by *in vitro* refolding and purification have been recently established.[6] Recombinant PvRII is recognized by antibodies of malaria-exposed individuals,[7,8] and immunization studies have indicated that the protein is highly immunogenic in rabbits, and mice, and elicits high-titer binding inhibitory antibodies.[6,9]

Following natural exposure to *P. vivax* infection, individuals residing in malaria-endemic areas develop antibodies that block binding of PvDBP to DARC-positive erythrocytes.[8] Studies in Papua New Guinea also show that cellular and humoral immune responses to PvRII develop progressively with age in residents from *P. vivax*–endemic areas, suggesting they are implicated in acquired immunity to *P. vivax*.[8,10,11] Importantly, antibodies raised against the homologous binding domain of PkDBP (PkDBP-RII) block the invasion of human Duffy-positive erythrocytes by *P. knowlesi in vitro*, providing further support for developing a recombinant vaccine based on PvRII.[12]

Rabbits immunized with PvRII formulated in Freund's adjuvant, as well as mice immunized with PvRII formulated with the human compatible adjuvants Montanide ISA720 and ASO2A, develop high-titer binding inhibitory antibodies that block the binding of Duffy positive erythrocytes to COS-7 cells expressing PvRII on their surface.[6,9] However, the protective efficacy of immunization with PvRII against parasite challenge has not yet been demonstrated. In the present study, we assess the immunogenicity and protective efficacy of PvRII formulated in Freund's adjuvant and the human adjuvant Montanide ISA 720 against blood-stage challenge with *P. vivax*.

MATERIALS AND METHODS

Recombinant protein. The recombinant protein was expressed in *E. coli,* refolded, and purified in its functional con-

——————
* Address correspondence to Myriam Arévalo-Herrera, Malaria Vaccine and Drug Testing Center, Cra 35 No. 4A-53, Cali, Colombia. E-mail: marevalo@immuno.org

formation as described previously.[6] Briefly, a plasmid containing the gene encoding the *P. vivax* DBP was used as a template to amplify the gene fragment encoding the PvRII region (amino acids 194–521) fused to hexa-histidine (6-His) at the C-terminal end. The polymerase chain reaction product was digested and cloned as a *Nco* I-*Sal* I fragment, then expressed in the vector pET28a+ (Novagen, Madison, WI). The resultant plasmid was used to transform *E. coli* and expression of recombinant PvRII was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Recombinant PvRII was purified from inclusion bodies under denaturing conditions by metal affinity chromatography, refolded by the method of rapid dilution, and purified to homogeneity by ion exchange chromatography as previously described.[9]

***Aotus* monkeys and immunization.** *Aotus lemurinus griseimembra* monkeys from the Primate Center in Cali, Colombia were used for this study. Adult, naive male and female animals were selected for weight (> 800 grams), age (> 2 years old), and presence of normal liver and kidney functions before immunization. The animal experimental protocol was reviewed and approved by the Animal Ethical Committe of the Universidad del Valle. The study was conducted in compliance with National Institutes of Health guidelines for animal use.

A total of 24 monkeys (6 per group) were arbitrarily assigned to two experimental groups (A and C) and two controls groups (B and D). Group A was immunized with 50 μg of recombinant PvRII (rPvRII) formulated in completed Freund's adjuvant and incomplete Freund's adjuvant in a 50: 50 ratio. Group C was immunized with rPvRII formulated in Montanide ISA 720 in a 7:3 antigen:adjuvant ratio, as recommended by the adjuvant manufacturer (Seppic, Inc., Paris, France). Groups B and D were injected with distilled water containing no protein and emulsified with Freund's adjuvant and Montanide ISA-720, respectively. Each animal was immunized by subcutaneous injection at five different sites on the back on days 0, 30, and 60.

Hematologic parameters (hemoglobin level, hematocrit, liver and kidney functions, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase) were evaluated before and during the immunization process to check the safety of the vaccine candidate. After challenge, if animals developed a hematocrit below 25%, they were treated with a combination of sulfadoxine-pyrimethamine.[13]

Antibody responses. Plasma fractions were separated by centrifugation from whole blood collected from immunized and control monkeys before each vaccination and before challenge. Plasma fractions were kept at −70°C until used for detection of specific antibodies to PvRII using an enzyme-linked immunosorbent assay (ELISA) antibodies to parasites using an immunofluorescence assay (IFA).

Enzyme-linked immunosorbent assay. Plates (Maxisorp; Nunc, Roskilde, Denmark) were coated for two hours at 37°C with 100 μL/well of rPvRII at a concentration of 2 μg/mL in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The plates were then blocked with 2.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4, overnight at 4°C, washed with PBS 1× pH 7.2 tween 20 at 0.05% and incubated for one hour at 37°C in duplicate with 10-fold or two-fold serial dilutions of plasma from immunized *Aotus* monkeys containing 1% BSA. The plates were washed with PBS 1× pH 7.2 tween 20 at 0.05% incubated for one hour with peroxidase-conjugated rabbit anti-*Aotus* IgG (pro-

duced at the Malaria Vaccine and Drug Development Center, Cali, Colombia and conjugated at Absea Biotechnology Ltd., Beijing, People's Republic of China) diluted 1:1,000, and incubated with 100 μL of the substrate peroxidase solution. Absorbance was read at 630 nm (OD_{630}) in a multi-channel spectrophotometer (MRX; Dynex Technologies, Inc., Chantilly, VA). To define the cut-off value, we tested the antigen using sera from 10 *Aotus* monkeys with no history of malarial infection or vaccination. The ELISA results were considered positive when absorbance of the test sera was greater than or equal to the mean value of *Aotus* control plasma OD_{630} plus 3 standard deviations.

Enzyme-linked immunosorbent assay–based binding assay. To study the ability of *Aotus* antibodies to block binding of PvRII an ELISA-based binding assay was carried out as described elsewhere.[14] Briefly, rPvRII at a concentration of 0.1 μg/mL was pre-incubated for one hour at room temperature with five-fold serial dilutions of individual *Aotus* serum samples from days 60 and 90 after immunization in duplicate and allowed to bind to ELISA microplates coated with recombinant Duffy antigen at a concentration of 1 μg/mL. Fifty percent inhibition titers were determined from curves obtained by plotting percentage binding at different dilutions of sera compared with binding with no sera.

Immunofluorescence assay. The IFA was performed using air-dried smears of asexual blood stages of the *P. vivax* Salvador I strain derived from an infected *Aotus* monkey. Parasites were purified using a modification of a method described elsewhere.[15] Briefly, whole infected blood was collected and leukocytes were removed by passage through a loosely packed CF 11 cellulose powder column previously washed with RPMI 1640 medium. The effluent was centrifuged at 500 × *g* for 10 minutes at room temperature and resuspended at a 50% hematocrit. We examined thick and thin Giemsa-stained blood films to reconfirm parasite stage. The parasite fraction was enriched using a 47% Percoll gradient and the top cell layer was removed, washed three times with RPMI medium without serum and cultured for 40 hours using the candle jar culture. The IFA slides were prepared with mature schizonts and were stored at −70°C until use. After de-hemoglobinization and fixation in acetone for five minutes, slides were incubated with test plasma diluted 1:50 in PBS for one hour and washed with PBS. Fluorescein-conjugated affinity-purified rabbit anti-*Aotus* IgG (produced at Malaria Vaccine and Drug Development Center and conjugated at Absea Biotechnology, Ltd.) was added, and incubated for one hour at room temperature. After additional washings with PBS 1× pH 7.2, the slides were overlaid with 30% glycerol and examined with an ultraviolet epifluorescence microscope.

Parasite challenge and parasitemia follow-up. One month after last immunization (on day 90), all monkeys were intravenously challenged with 10^5 *P. vivax* Salvador I blood-stage parasites. *Plasmodium vivax* Salvador I was kindly provided by Dr. William Collins (Centers for Disease Control and Prevention, Atlanta, GA) and maintained as a frozen stabilate in liquid nitrogen until use. An *Aotus* monkey was infected with the thawed parasites and used as a parasite donor for the challenge studies. We initially assessed parasitemia by thick blood smear until it was detectable. The parasitemia was then quantified by scoring the frequency of infected erythrocytes in thin blood smears stained with Giemsa. Parasitemia was checked three times a week for 39 days (i.e., up to day 129)

when all animals were treated with 25 mg of sulfadoxine-pyrimethamine.

Statistical analysis. We evaluated three efficacy outcomes: prepatent period, maximum parasitemia, and total parasitemia estimated as area under the curve (AUC). The AUC was calculated using the trapezoid rule. Samples that tested positive for parasitemia by thick smear that were not quantifiable by thin smear were treated as 0% in the AUC calculation. Differences between experimental and control groups (A versus B, C versus D) were tested using a two-sided Wilcoxon rank sum test (Stata version 8.0; Stata Corporation, College Station, TX).

RESULTS

Antibody responses to rPvRII. We demonstrated that immunization of *A. l. griseimembra* monkeys with rPvRII induced high-titer specific antibodies (Table 1). Substantial increases in the specific anti-rPvRII end point ELISA titers ($2–5.6 \times 10^6$) were observed in all animals immunized (day 90) with rPvRII formulated in Freund's adjuvant (group A). Animals immunized with PvRII formulated in Freund's adjuvant developed approximately four-fold higher endpoint titers than animals immunized with PvRII formulated in Montanide ISA 720 ($0.6–20 \times 10^4$, group C). No reactivity against PvRII was found in the corresponding control groups (B and D). Sera from three monkeys from group A (079*032, 301*630, and 276*614) showed reactivity to the parasite, as shown by the IFA at a serum dilution of 1:50.

Inhibition of binding of PvRII to DARC by *Aotus* sera. Sera obtained on day 60 from monkeys immunized with rPvRII formulated in Freund's adjuvant showed the highest binding inhibition titers with 50% inhibition titers ranging from 1:1,650 to 1:46,200 (Table 2). High-binding inhibition titers in the Freund's group (group A) are associated with a longer pre-patent period (Table 3). The 50% binding inhibition titers for the Montanide ISA 720 group (group C) ranged from < 1:10 to 1:99 on day 60 with moderate increases on day 90. However, these values were much lower than those observed for the Freund's adjuvant group (group A) on day 60 and were insufficient to provide any protection. Due to small

amounts of sera available, day 90 sera from group A could not be tested in binding inhibition assays.

Parasite challenge and parasitemia follow-up. As shown in Table 3 and Figure 1, all animals from both the experimental and control groups became infected after parasite challenge. Overall pre-patency was longer and parasitemia levels were lower for vaccinated monkeys in group A compared with control group B. Five of the six animals in group B had a pre-patent period of seven days by thick blood smear, whereas in experimental group A, all animals were negative (Table 2). The median number of days to patency was 7 days for group B and 16 days for group A (Table 4). The median peak parasitemia was 0.25% for group B and 0.1% for group A. The median AUC for group B was nearly four times greater than the median AUC for group A (Table 4). However, only the differences in pre-patency distributions between groups A and B reached statistical significance ($P < 0.05$).

In group C, all animals developed detectable parasitemia by thick blood smear by day 7 or day 9 (Table 3). All animals from the corresponding control group (group D) had detectable parasitemias on day 7. The median number of days to patency was eight days for group C and seven days for group D (Table 3). The median peak parasitemia was 0.1% for group C and 0.2% for group D. The median AUC for group D was nearly twice as high as the median AUC for group C (Table 4). In contrast to groups A and B, which were immunized with Freund's adjuvant, the differences between experimental and control groups C and D immunized with Montanide ISA 720 was not as large. No statistically significant differences were observed between groups C and D.

Other clinical outcomes. Animals immunized with Freund's adjuvant developed granulomas, but the severity of the toxic reactions was smaller than that observed in studies using other malaria proteins, as well as in a pilot study using the same protein.

A slight decrease in the levels of hemoglobin was observed in three (monkey 355*782, 9.7 g/dL; 301*630, 11.1 g/dL; and 079*032, 12.4 g/dL) of the six animals immunized with the rPvRII formulated in Freund's adjuvant at day 90 in comparison with only one monkey that showed a similar decrease in the control group (077*552, 9 0.6 g/dL). However, these changes remained in the normal range (13.1–23.4 g/dL) and were not clinically significant. In contrast, animals immunized with the rPvRII formulated in Montanide ISA-720 had normal hemoglobin levels during the immunization period. We observed normal leukocyte values in the groups immunized with Montanide, but the number of leukocytes increased in some of the animals immunized with Freund's adjuvant.

All animals in this trial were subjected to liver and kidney function tests to determine the safety of the vaccine, particularly when formulated in Montanide ISA-720. No clinically significant differences in hematologic, liver, or kidney parameters were observed during the immunization period in either experimental or control group.

DISCUSSION

Duffy binding protein is a parasite ligand used by *P. vivax* for invasion of human erythrocytes through a specific interaction with DARC on the red blood cell surface. Because of the potential of the receptor-binding domain, PvRII of DBP

TABLE 1
Kinetics of rPvRII-DBP-specific antibodies in *Aotus* monkeys*

			Day of bleedings			
			ELISA†			IFA
Group	Monkey code	0	30	60	90	90
A	301*630	0	1,000	1,800	5,200	Neg
rPvRII-DBP	079*032	0	1,000	1,400	5,000	Neg
Freund	355*782	0	1,000	1,600	4,000	Neg
	383*845	0	500	1,200	2,000	50
	276*614	0	500	1,400	5,600	50
	378*513	0	500	4,800	2,000	50
C	119*853	0	5	8	6	Neg
rPvRII-DBP	565*377	0	10	50	100	Neg
Montanide	531*108	0	8	16	20	Neg
	358*363	0	5	50	50	Neg
	120*339	0	10	80	200	Neg
	353*126	0	50	80	100	Neg

* rPvRII-DBP = recombinant *Plasmodium vivax* Duffy binding protein; ELISA = enzyme-linked immunosorbent assay; IFA = immunofluorescence assay.
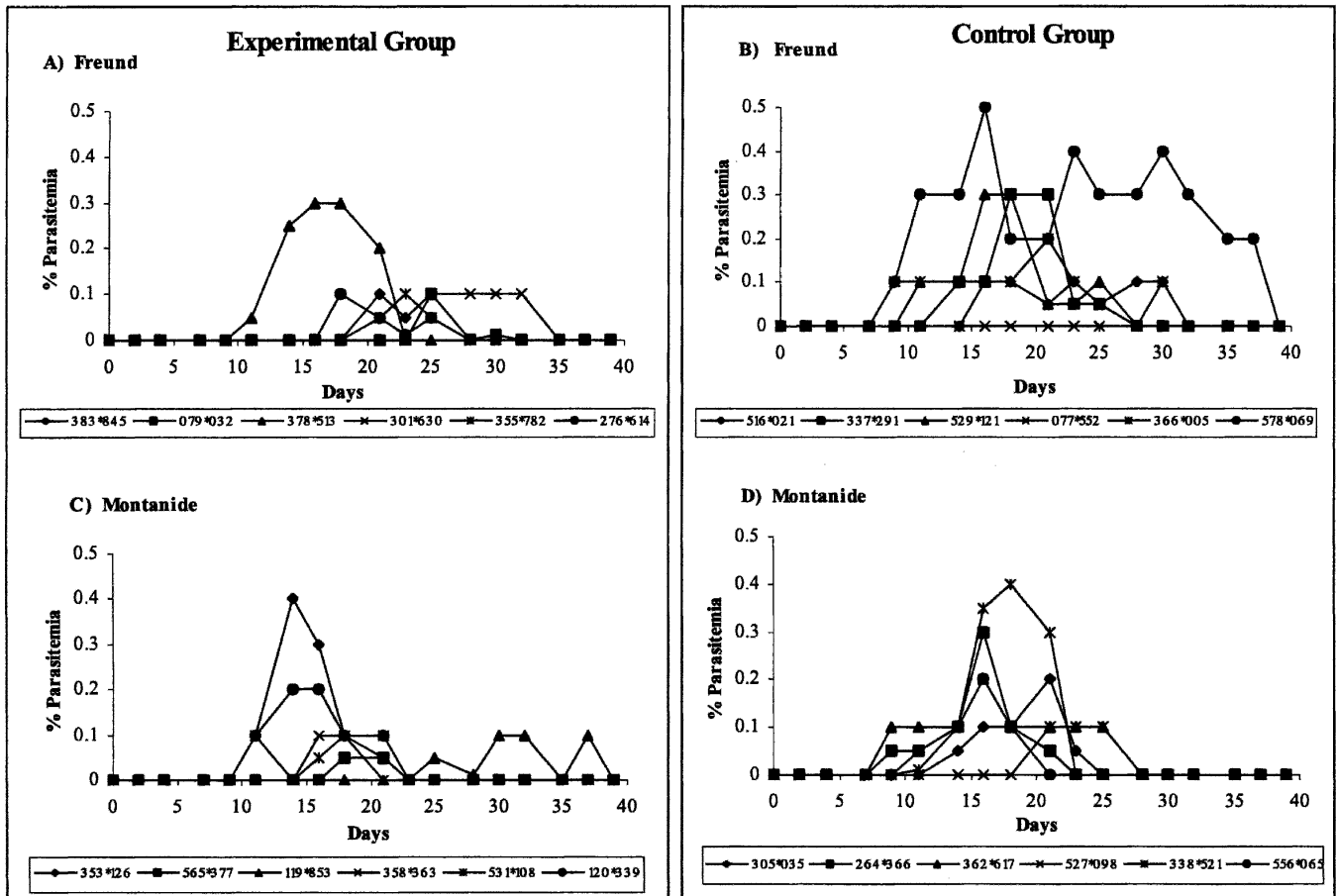† Values are reciprocal dilution titers $\times 10^3$.

FIGURE 1.   Course of *Plasmodium vivax* infection in *Aotus* monkeys immunized with recombinant *P. vivax* Duffy binding protein.

as a vaccine candidate for human use, we assessed the immunogenicity and protective efficacy of rPvRII formulated in both Freund's adjuvant and Montanide ISA 720. Freund's adjuvant commonly yields high-titer antibodies in animal studies and was thus used as a positive control. Montanide ISA720 has been successfully used in multiple malaria clinical trials in humans with very limited or no adverse events.[16]

TABLE 2
Fifty percent inhibition titers of sera from individual *Aotus* monkeys immunized with rPvRII-DBP*

| Group | Monkey code | Day of bleedings† | |
		60	90
A	301*630	42,600	ND
rPvRII-DBP	079*032	2,049	ND
Freund	355*782	17,172	ND
	383*845	1,840	ND
	276*614	1,650	ND
	378*513	1,808	ND
C	119*853	< 10	16
rPvRII-DBP	565*377	35	214
Montanide	531*108	51	253
	358*363	50	89
	120*339	99	155
	353*126	63	366

* rPvRII-DBP = recombinant *Plasmodium vivax* Duffy binding protein; ND = not determined.
† Values are reciprocal dilution titers.

We found that the Freund's formulation of the protein was more immunogenic than the formulation with Montanide ISA-720. More importantly, vaccination with rPvRII formulated in Freund's adjuvant partially protects *Aotus* monkeys from experimental parasite challenge. All parameters examined were strongly suggestive of efficacy. However, except for the longer pre-patency period in the rPvRII-Freund's group, the differences between vaccinated and control groups were not statistically significant due to the small sample sizes.

Immunization of *Aotus* with rPvRII formulated in Freund's adjuvant not only substantially delayed the appearance of patent parasitemia at a statistically significant level ($P = 0.004$), but also decreased the magnitude of parasitemia in most animals. These results are consistent with those of an earlier pilot study in which we observed complete protection in three of five *Aotus* monkeys immunized with 100 μg of rPvRII formulated in Freund's adjuvant. In both experiments, immunization with this diluted formulation of Freund's adjuvant induced granuloma, a toxic effect that will not be acceptable in humans. Here, we have observed cutaneous lesions such as inflammation at the sites of inoculation, and granuloma formation without further complications. Bacteriologic examination failed to demonstrate the presence of acid-fast microorganisms and other opportunistic microorganisms.

With the aim of assessing the protective efficacy of a vaccine formulation composed of rPvRII and an adjuvant suitable for human use, we selected Montanide ISA720. In pre-

TABLE 3
Parasitemia follow-up in *Aotus* monkeys*

Group	Monkey code	Day 0	7	9	11	14	16	18	21	23	25	28	30	32	35	37	39	Peak†	AUC
A rPvRII-DBP Freund	301*630	–	–	–	–	–	–	–	+	+	0.1	0.1	0.1	0.1	0	0	0	0.1	0.95
	079*032	–	–	–	–	–	–	–	+	+	0.1	0	0.01	0	0	0	0	0.1	0.27
	355*782	–	–	–	–	–	+	+	0.05	0.1	0.05	0	0	0	0	0	0	0.1	0.45
	383*845	–	–	–	–	–	+	+	0.1	0.05	0.1	0	Dead					0.1	0.6
	276*614	–	–	–	+	+	+	0.1	0.05	0.01	0.05	0	0	0	0	0	0	0.1	0.52
	378*513	–	–	+	0.05	0.25	0.3	0.3	0.2	0	0	0	0	0	0	0	0	0.3	2.6
B PBS Freund	077*552	–	+	+	+	+	+	+	+	+	+	0.1	0	0	0	0	0	0.1	0.2
	516*021	–	+	+	+	+	0.1	0.1	0.05	0.1	0.05	0.1	0.1	0	0	0	0	0.1	1.35
	337*291	–	+	+	+	0.1	0.1	0.3	0.3	0.05	0.05	0	0	0	0	0	0	0.3	2.18
	529*121	–	–	+	0.1	0.1	0.3	0.3	0.05	0.05	0.1	0	0	0	0	0	0	0.3	2.33
	366*005	–	+	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.05	0	0	0	0	0	0	0.2	1.98
	578*069	–	+	0.1	0.3	0.3	0.5	0.2	0.2	0.4	0.3	0.3	0.4	0.3	0.2	0.2	0.2	0.5	8.45
C rPvRII-DBP Montanide	119*853	–	–	+	+	+	+	+	+	+	0.05	0.01	0.1	0.1	0	0.1	0.1	0.1	0.8
	565*377	–	–	+	+	+	+	0.05	0.05	0	0	0	0	0	0	0	0	0.05	0.25
	531*108	–	–	+	+	+	0.05	0.1	0.1	0	0	0	0	0	0	0	0	0.1	0.6
	358*363	–	+	+	0.1	0	0.1	0.1	0	0	0	0	0	0	0	0	0	0.1	0.7
	120*339	–	+	+	0.1	0.2	0.2	0.1	0.1	0	0	0	0	0	0	0	0	0.2	1.65
	353*126	–	+	+	0.1	0.4	0.3	0.1	0.05	0	0	0	0	0	0	0	0	0.4	2.23
D PBS Montanide	527*098	–	+	+	+	+	+	+	0.1	0.1	0.1	0	0	0	0	0	0	0.1	0.7
	305*035	–	+	+	+	0.05	0.1	0.1	0.2	0.05	0	0	0	0	0	0	0	0.2	1.18
	556*065	–	+	+	0.05	0.1	0.2	0.1	0	0	0	0	0	0	0	0	0	0.2	1.03
	338*521	–	+	+	0.01	0.1	0.35	0.4	0.3	0	0	0	0	0	0	0	0	0.4	2.73
	264*366	–	+	0.05	0.05	0.1	0.3	0.1	0.05	0	0	0	0	0	0	0	0	0.3	1.45
	362*617	–	+	0.1	0.1	0.1	0.2	0.1	0.1	1.01	0.1	0	0	0	0	0	0	0.2	2.05

* Parasitemia by thick smear is reported as positive (+) or negative (–) whereas parasitemia by thin smear is reported as a percentage. AUC = area under curve; rPvRII-DBP = recombinant *Plasmodium vivax* Region II—Duffy binding protein; PBS = phosphate-buffered saline. All other results for days 0–7 were negative.
† Maximum percentage.

vious studies, this adjuvant has been used with rPvRII to immunize mice[9] and with other proteins to immunize monkeys.[17] Similar to the results found in the mice study,[9] we report that *Aotus* sera raised against rPvRII are able to block the binding of rPvRII to recombinant DARC *in vitro.* In the group immunized with the Montanide ISA720 formulation, the binding inhibition titers were related to the end point titers, with both titers increasing from day 60 to day 90. In the group immunized with the PvRII-Freund's formulation, Monkey 301*630 showed the highest 50% binding inhibition titer (1:42,600), which was associated with a delay in patency (day 21). However, the antibody titers achieved could not block the development of parasitemia in monkeys directly challenged by inoculation with live *P. vivax* parasites.

High antibody titers are necessary for effective blocking of parasite invasion and growth *in vivo*. Monkey 378*513, which developed the earliest patency in the Freund's group (group A), had a low binding inhibition titer on day 60. Only half of the sera from group A (Freund's group) could recognize native PvDBP in the parasite by IFA, whereas none of the sera from group C (Montanide group) could recognize native PvDBP. Inability to detect PvDBP in merozoites may be due to technical difficulties in the IFAs given the small amounts of PvDBP present. Since immunoglobulin subtyping reagents are not available for *Aotus* monkeys, it was not possible to study any possible correlation of immunoglobulin subtypes with protection.

This study suggests that to achieve protection, antibodies to

TABLE 4
Characteristics of distributions of percentage parasitemia outcomes measured by group*

Adjuvant	Immunogen	Parasitemia	Median	Minimum	Maximum	*P* vs control
Freund	Group A rPvRII-DBP	Prepatency	16	9	21	0.004
		Peak	0.1	0.1	0.3	0.149
		AUC	0.56	0.27	2.6	0.200
	Group B PBS	Prepatency	7	7	9	
		Peak	0.25	0.1	0.5	
		AUC	2.08	0.2	8.45	
Montanide	Group C rPvRII-DBP	Prepatency	8	7	9	0.056
		Peak	0.1	0.05	0.4	0.173
		AUC	0.75	0.25	2.23	0.229
	Group D PBS	Prepatency	7	7	7	
		Peak	0.2	0.1	0.4	
		AUC	1.31	0.7	2.73	

* rPvRII-DBP = recombinant *Plasmodium vivax* Duffy binding protein; AUC = area under percent parasitemia curve; PBS = phosphate-buffered saline.

DBP, particularly the fraction able to cross-react with the native protein, must be at sufficiently high levels to efficiently block erythrocyte invasion by *P. vivax* merozoites. The partial protection achieved in the Freund's group suggests that a potent adjuvant will be required for use in humans. PvDBP is expressed in small quantities for a limited time starting late in schizogeny and is localized intracellularly in micronemes.[18] It is likely to be on the apical surface only briefly once the invasion process is initiated. Antibodies directed against PvDBP would need to be in abundant amounts and have high binding rates and high binding affinities to efficiently block the binding of *P. vivax* merozoites to DARC.

In addition, although a monkey model is usually not used to test safety of malaria vaccine candidates, in this study we found that certain parameters such as hematologic, liver, and kidney functions could be evaluated and thus provided useful information on the effect of the protein during immunization. In this regard, the use of Montanide ISA-720 eliminated concerns related to the potential toxicity of vaccination with a protein that binds the chemokine receptor DARC on erythrocytes. Animals from group C immunized with rPvRII formulated in Montanide ISA-720 and those from group that D that only received the adjuvant did not show any detectable toxic effect.

A recent clinical study using ASO-2A adjuvant (GlaxoSmithKline, Research Triangle Park, NC) and a recombinant hybrid protein derived from the *P. falciparum* circumsporozoite protein reported high levels of antibodies that protected human volunteers from natural malaria infection.[19] Moreover, other adjuvants such as Montanide ISA-51, which are suitable for human use, can induce strong antibody responses likely to efficiently block parasite reinvasion.[20] We conclude that the rPvRII vaccine candidate is immunogenic, and partially protective in *Aotus* monkeys, and that it has promising potential for further use in clinical trials. However, to achieve higher levels of protective antibodies for this promising protein, new and more powerful adjuvants suitable for human need to be identified.

Authors' addresses: Myriam Arévalo-Herrera, Angélica Castellanos, and Socrates Herrera, Instituto de Inmunología de la Universidad del Valle, Calle 4B 36-00, Edificio de Microbiología, Tercer Piso, Facultad de Salud, Universidad del Valle Sede San Fernado, AA 25574, Cali, Colombia, Telephone: 57-2-558-1931, Fax: 57-2-557-0449 and Malaria Vaccine Development Center, AA 26020, Cali, Colombia, Telephone: 57-2-558-3937, Fax: 57-2-556-0141, E-mail: marevalo@ inmuno.org. Syed S. Yazdani, Ahmad Rushdi Shakri, Chetan E. Chitnis, Malaria Research Group, International Centre for Genetic Engineering and Biotechnology, Campus Aruna Asaf Ali Marg, 110067, New Delhi, India, Telephone: 91-11-2619-5007, Fax: 91-11-2616-2316. Rosalie Dominik, Family Health International, 2224 E. NC Highway 54, Durham, NC 27713, Telephone: 919-544-7040, Fax: 1-919-544-7261.

Reprint requests: Myriam Arévalo-Herrera, Malaria Vaccine and Drug Testing Center, Cra 35 No. 4A-53, Cali, Colombia, E-mail: marevalo@inmuno.org

REFERENCES

1. Richie TL, Saul A, 2002. Progress and challenges for malaria vaccines. *Nature 415:* 694–701.
2. Mendis K, Sina BJ, Marchesini P, Carter R, 2001. The neglected burden of *Plasmodium vivax* malaria. *Am J Trop Med Hyg 64:* 97–106.
3. Fang XD, Kaslow DC, Adams JH, Miller LH, 1991. Cloning of the *Plasmodium vivax* Duffy receptor. *Mol Biochem Parasitol 44:* 125–132.
4. Horuk R, Chitnis CE, Darbonne WC, Colby TJ, Rybicki A, Hadley TJ, Miller LH, 1993. A receptor for the malarial parasite *Plasmodium vivax*: the erythrocyte chemokine receptor. *Science 26:* 1182–1184.
5. Adams JH, Sim BKL, Dolan AS, Fang X, Kaslow DC, Miller LH, 1992. A family of erythrocyte binding proteins of malaria parasites. *Proc Natl Acad Sci USA 89:* 7085–7089.
6. Singh S, Pandey K, Chattopadhayay R, Yazdani SS, Lynn A, Bharadwaj A, Ranjan A, Chitnis C, 2001. Biochemical, biophysical, and functional characterization of bacterially expressed and refolded receptor binding domain of *Plasmodium vivax* Duffy-binding protein. *J Biol Chem 276:* 17111–17116.
7. Xainli J, Cole-Tobian JL, Baisor M, Kastens W, Bockarie M, Yazdani SS, Chitnis CE, Adams JH, King CL, 2003. Epitope-specific humoral immunity to *Plasmodium vivax* Duffy binding protein. *Infect Immun 71:* 2508–2515.
8. Michon P, Fraser T, Adams JH, 2000. Naturally acquired and vaccine-elicited antibodies block erythrocyte cytoadherence of the *Plasmodium vivax* Duffy Binding Protein. *Infect Immun 68:* 3164–3171.
9. Yazdani SS, Shakri AR, Mukherjee P, Baniwal SK, Chitnis CE, 2004. Evaluation of immune responses elicited in mice against a recombinant malaria vaccine based on *Plasmodium vivax* Duffy binding protein. *Vaccine 22:* 3727–3737.
10. Fraser T, Michon P, Barnwell JW, Noe AR, AlYaman F, Kaslow DC, Adams JH, 1997. Expression and serologic activity of a soluble recombinant *Plasmodium vivax* Duffy binding protein. *Infect Immun 65:* 2772–2777.
11. Xainli J, Baisor M, Kastens W, Bockarie M, Adams JH, King LC, 2002. Age-dependent cellular immune response to *Plasmodium vivax* Duffy binding protein in humans. *J Immunol 169:* 3200–3207.
12. Singh AP, Puri K, Chitnis CE, 2002. Antibodies raised against receptor-binding domain of *Plasmodium knowlesi* Duffy binding protein inhibit erythrocyte invasion. *Mol Biochem Parasitol 31:* 72–81.
13. Doberstyn EB, Teerakiartkamjorn C, Andre RG, Phintuyothin P, Noeypatimanondh S, 1979. Treatment of *P. vivax* malaria with sulfadoxine-pyrimethamine and with pyrimethamine alone. *Trans R Soc Trop Med Hyg 73:* 15–27.
14. Hans D, Pattnaik P, Bhattacharyya A, Shakri AR, Yasdani SS, Sharma M, Choe H, Farzan M, Chitnis C, 2005. Mapping binding residues in the *Plasmodium vivax* domain that binds Duffy antigen during red cell invasion. *Mol Microbiol 55:* 1423–1434.
15. Golenda CF, Li J, Rosenberg R, 1997. Continuous *in vitro* propagation of the malaria parasite *Plasmodium vivax*. *Proc Natl Acad Sci USA 94:* 6786–6791.
16. Saul A, Lawrence G, Smillie A, Rzepczyk CM, Reed C, Taylor D, Anderson K, Stowers A, Kemp R, Allworth A, Anders RF, Brown GV, Pye D, Schoofs P, Irving DO, Dyer SL, Woodrow GC, Briggs WR, Reber R, Sturchler D, 1999. Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA 720 adjuvant. *Vaccine 17:* 3145–3159.
17. Genton B, Al-Yaman F, Betuela I, Anders RF, Saul A, Baea K, Mellombo M, Taraika J, Brown GV, Pye D, Irving DO, Felger I, Beck HP, Smith TA, Alpers MP, 2003. Safety and immunogenicity of a three-component blood-stage malaria vaccine

(MSP1, MSP2, RESA) against *Plasmodium falciparum* in Papua New Guinea children. *Vaccine 22:* 30–41.

18. Aikawa M, Miller LH, Johnson J, Rabbege J, 1978. Erythrocyte entry by malaria parasites. A moving junction between erythrocyte and parasite. *J Cell Biol 77:* 72–82.

19. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, Mandomando I, Spiessens B, Guinovart C, Espasa M, Bassat Q, Aide P, Ofori-Anyinam O, Navia MM, Corachan S, Ceuppens M, Dubois MC, Demoitie MA, Dubowsky F, Menendez C, Tornieporth N, Ballou WR, Thompson R, Cohen J, 2004. Efficacy of the RTS, S/ASO2A vaccine against *Plasmodium falciparum* infection and disease in young African children: ramdomised controlled trial. *Lancet 364:* 1380–1383.

20. Kumar S, Jones TR, Oakley MS, Zheng H, Kuppusamy SP, Taye A, Krieg AM, Stowers AW, Kaslow DC, Hoffman SL, 2004. CpG oligodeoxynucleotide and Montanide ISA 51 adjuvant combination enhanced the protective efficacy of a subunit malaria vaccine. *Infect Immun 72:* 949–957.