Preclinical Vaccine Study of *Plasmodium vivax* Circumsporozoite Protein Derived-Synthetic Polypeptides Formulated in Montanide ISA 720 and Montanide ISA 51 Adjuvants

Myriam Arévalo-Herrera, Omaira Vera, Angélica Castellanos, Nora Céspedes, Liliana Soto, Giampietro Corradin, and Sócrates Herrera*

Instituto de Inmunología del Valle, Universidad del Valle, Cali, Colombia; Malaria Vaccine and Drug Development Center, Cali, Colombia; Fundación Centro de Primates, Cali, Colombia; Biochemistry Department, University of Lausanne, 1066 Epalinges, Switzerland

Abstract. *Plasmodium vivax* circumsporozoite (CS) protein is a leading malaria vaccine candidate previously assessed in animals and humans. Here, combinations of three synthetic polypeptides corresponding to amino (N), central repeat (R), and carboxyl (C) regions of the CS protein formulated in Montanide ISA 720 or Montanide ISA 51 adjuvants were assessed for immunogenicity in rodents and primates. BALB/c mice and *Aotus* monkeys were divided into test and control groups and were immunized three times with doses of 50 and 100 µg of vaccine or placebo. Antigen-specific antimalarial antibodies were determined by enzyme-linked immunosorbent assay, immunofluorescent antibody test, and IFN-γ responses by enzyme-linked immunosorbent spot (ELISPOT). Both vaccine formulations were highly immunogenic in both species. Mice developed better antibody responses against C and R polypeptides, whereas the N polypeptide was more immunogenic in monkeys. Anti-peptide antibodies remained detectable for several months and recognized native proteins on sporozoites. Differences between Montanide ISA 720 and Montanide ISA 51 formulations were not significant.

INTRODUCTION

Malaria is a devastating tropical disease affecting 100 countries worldwide. *Plasmodium falciparum* and *Plasmodium vivax* are the predominant parasite species responsible for 300 to 500 million clinical cases per year. *Plasmodium falciparum* infection causes the death of approximately three children each minute in Africa.¹ Although great efforts have been made to develop an effective malaria vaccine and more than 70 malaria vaccine candidates have been under investigation for the last decade, only the RTS,S a *P. falciparum* vaccine candidate based on the circumsporozoite (CS) protein is ready to enter phase III clinical trials.^{2–5}

In the case of *P. vivax*, the second most prevalent malaria species, only two parasite antigens, the sexual *P. vivax* surface protein 25 (Pvs25) expressed during the sporogonic cycle and the CS protein expressed during the pre-erythrocytic phase of the cycle, have undergone phase I clinical trials.^{5–7} Further development of these *P. vivax* vaccine candidates has been difficult mainly because of limited resources and the generalized misconception that *P. vivax* is a benign malaria species with minor epidemiological importance.⁸ Moreover, the lack of continuous parasite cultures seriously impedes the discovery of more parasite antigens with vaccine potential.⁹

The CS protein has been selected because it is a multifunctional protein abundantly expressed on the membrane surface of *Plasmodium* sporozoites as well as during the parasite liver phase.¹⁰ The CS protein has been shown to be involved in the parasite's hepatocyte invasion process^{11,12} and specific antibodies produce a precipitation reaction on the parasite surface that abrogates the parasite invasion capacity.^{13,14} Moreover, the CS protein is predominantly recognized by sera and cells of individuals naturally exposed to malaria in endemic areas or from animals and humans rendered immune by experimental immunization with radiation-attenuated sporozoites.^{15–18}

During the past few years, we have been developing a *P. vivax* CS-based vaccine.¹⁹ Vaccine formulations based on the

synthetic polypeptides corresponding to different regions of the CS protein and Montanide ISA 720 adjuvant have been proven to be safe, well tolerated, and highly immunogenic in a previous phase I vaccine trial conducted in Colombian naive volunteers.⁶ The phase I trial was conducted using escalating vaccine doses (10, 30, and 100 µg/dose) of three synthetic domains corresponding to the amino (N), central repeat (R), and carboxyl (C) regions of the CS protein formulated individually in Montanide ISA 720. Because it has been considered or suggested that the central repeat domain, which is highly immunogenic, represents an immune “smoke screen”, the rationale of that first trial was to determine the immunogenicity of each of the three protein domains independently. All three proteins induced high titers of specific antibodies that cross-reacted with *P. vivax* sporozoites and the production of interferon-gamma (IFN-γ) both in *Aotus* monkeys and in humans.^{6,20} Additionally, antibodies to the three regions were able to partially block sporozoite invasion.^{6,7}

We designed a pre-clinical trial in mice and monkeys to assess here the immunogenicity of a combination of the three peptides formulated in Montanide ISA 720 or in Montanide ISA 51. Both adjuvants have been previously used extensively in clinical trials addressed to test vaccines against malaria and other infectious pathogens.^{7,21–24}

MATERIALS AND METHODS

Vaccine formulation. The N protein comprises amino acids 20–96 of CS protein and constitutes a 76-mer peptide, the R peptide is a hybrid 48-mer peptide, type I or common sequence (VK210) that contains three repeats of the GDRADGQPA motif, and collinearly linked to a universal T-helper epitope from the tetanus toxin²⁵ and the 71-mer C protein is composed of amino-acid residues 301–372 of the CS protein. The C-terminal protein containing 4-Cys was oxidized according to Verdini and others.²⁶ Peptides were synthesized under good laboratory practices (GLP) conditions using solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry.²⁷ The mass and purity of the peptides were assessed by high performance liquid chromatography and mass spectrometry and was higher than 85%.⁶ Proteins were lyophilized, packaged, and both

*Address correspondence to Sócrates Herrera, Malaria Vaccine and Drug Development Center, Carrera 37 - 2Bis No. 5E - 08, Cali, Colombia. E-mail: sherrera@inmuno.org

sterility and pyrogenicity were tested. The three (N, R, and C) proteins were mixed in concentrations of 50 and 100 μg for each peptide for a final concentration of 150 or 300 $\mu\text{g}/\text{dose}$, and then 24 hrs before each immunization, the mixture was separately emulsified in two adjuvants: Montanide ISA 720 or Montanide ISA 51 (Seppic Inc., Paris, France) and stored at 4°C according to manufacturer recommendations. Saline solution (Baxter, Deerfield, IL) was emulsified with the same adjuvants and used as placebo. Both vaccine and placebo were emulsified as described earlier.^{6,7}

Ethical guidelines. The study protocol was approved by the Animal Ethical Committee of Universidad del Valle, and animals were housed and handled following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunogenicity studies in BALB/c mice. Twenty-four BALB/c mice 3–5 weeks of age were randomly selected and distributed to test and control groups of six animals each that were used to test the immunogenicity of the synthetic proteins. The experimental groups (A and B) were injected in the base of the tail three times at 3-week intervals with 50 μg of each individual peptide, for a final dose of 150 μg in a volume of 0.3 mL. The mixture of the three peptides was emulsified in either Montanide ISA 720 (Group A) or Montanide ISA 51 (Group B). The first immunization dose (given on Day 0) contained the peptides N and C only with the rational of preventing the potential immuno-dominance the B-cell epitopes contained within the R peptide. Immuno-dominance of this fragment has been previously observed in both natural conditions and in experimental potency test studies.^{6,20} The two boosting vaccine doses in contrast, contained all three (N, R, and C) peptides. The control groups (Groups C and D) were injected with the same immunization schedule and route, with saline

solution (0.9%) emulsified with the two adjuvants, respectively (Figure 1). Approximately 150 μL of blood were collected from the tip of the tail on Day 0 and before each immunization. Sera was obtained by centrifugation and kept at –70°C until use.

Immunogenicity studies in Aotus monkeys. Twenty-four healthy adult *Aotus lemurinus griseimembra* monkeys were randomly selected from a larger group of animals from the Primate Center (FUCEP) of Cali, and were assigned to test and control groups of six monkeys each. The immunized group received 100 $\mu\text{g}/\text{dose}$ of a mixture of *P. vivax* CS synthetic proteins, and the control groups received saline solution (0.9%) formulated in the same adjuvants. Both preparations were formulated in Montanide ISA 720 and Montanide ISA 51 adjuvants as described for the mice. Animals were immunized subcutaneously three times with the peptide mixtures describe previously at 0, 2, and 4 months with a total volume of 500 μL of vaccine. Sera were obtained from blood samples collected before every immunization as described elsewhere.^{6,7} Peripheral blood mononuclear cells (PBMC) were also collected before immunization for evaluation of IFN- γ by enzyme-linked immunosorbent spot (ELISpot) assay.

Serological studies. Animal sera samples were evaluated using an enzyme-linked immunosorbent assay (ELISA) as described elsewhere.²⁸ Mice and monkey sera from each group were individually tested. Briefly, antibody response was measured by ELISA using the N, R, or C proteins (1 $\mu\text{g}/\text{mL}$), as described previously.²⁸ Immunoglobulin G (IgG) antibodies were detected using phosphatase-conjugated anti-human or anti-mouse immunoglobulins (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:1,000. The enzymatic activity was developed after incubation for 45 min at room temperature with para-nitrophenyl phosphate substrate. The final reaction was read at 405 nm in a microplate reader (MRX;

FIGURE 1. Antibody response of sera from BALB/c mice vaccinated with a mixture of N, R, and C peptides of the *Plasmodium vivax* circumsporozoite (CS) protein formulated in Montanide ISA 720 or Montanide ISA 51, respectively. Each line corresponds to sera of mice immunized with a priming dose of 100 μg of peptide N and C and two boosting doses 150 μg of the three peptide (N + R + C) at 3-week intervals. Higher titers were obtained in groups of mice immunized with C-long synthetic peptides (LSP) than in those immunized with N-LSP, independently on the adjuvant used. Control groups are not shown.

Dynex Technologies, Inc., Chantilly, VA). Cut-off points were calculated as three SD above the mean absorbance value at 405 nm of sera controls. The final reaction was read at 405 nm in a microplate reader (MRX; Dynex Technologies, Inc.). Cut-off points were calculated as three SD above the mean absorbance value at 405 nm of sera controls. Controls were selected from a pool of sera from malaria-naïve (negative controls) from mice or monkeys depending on the species being evaluated. Antibody titers were established as the last test sera dilution giving absorbance values greater than the mean of normal control sera plus two SD. The ELISA titers of < 1:100 were considered negative.

To determine the recognition of the native CS *P. vivax* protein by mice and monkey sera, immunofluorescent antibody test (IFAT) was performed as described elsewhere⁶ using *P. vivax* sporozoite antigen obtained by salivary gland dissection of *P. vivax*-infected *Anopheles albimanus*. Colonized mosquitoes had been experimentally infected using an artificial membrane feeding device loaded with human blood containing *P. vivax* parasite infections, as described elsewhere.^{29,30} The IFAT were performed with sera from either mice or monkeys from control or test groups, using 2-fold sera dilutions in phosphate buffered saline (PBS) starting at 1:100. Sera dilutions were incubated on antigen slides in a moisture chamber at room temperature for 30 min. The slides were then washed three times with PBS, and goat antimouse and goat antihuman IgG FITC (KPL, Gaithersburg, MD) conjugates were used as secondary antibodies for mice and monkey samples, respectively. Slides were then incubated as described previously, mounted with 50% glycerol, and evaluated with a fluorescence microscope (Nikon, Avon, MA). Antibody titers were determined as the reciprocal of the end-point dilution that showed positive fluorescence as described previously.⁶

Single-cell IFN- γ release in monkeys. Monkey PBMC were separated from whole blood using Ficoll Histopaque (Sigma-Aldrich, St. Louis, MO) density gradients, and were resuspended in RPMI 1640 medium (Gibco, Grand Island, NY). IFN- γ -producing cells were identified using a commercial IFN- γ ELISpot human kit (Mabtech AB, Stockholm, Sweden). Spots were counted with a spot-counting system (Scanalytics, Fairfax, VA), and results were expressed as the mean number of IFN- γ spot-forming cells (SFC) per 10^6 PBMC. Monkeys were considered responders if the number of SFC in their samples had increased from their own baseline level (before immunization on Day 0).⁶

Statistical analysis. Comparisons of interest were between adjuvants and peptides. Antibody titers and IFN- γ production were compared among peptides and adjuvants using Wilcoxon signed-rank test. The *P* values < 0.05 were considered significant. A descriptive analysis was done to evaluate trends in humoral and cellular immune responses in each study group.

RESULTS

Immunogenicity in BALB/c mice. Mice immunized with the mixture of *P. vivax* synthetic proteins developed specific IgG antibody titers against the three domains. As observed in Figure 1, N and C peptides induced a faster and higher antibody response than the R peptide as expected, because they received the mixture of three peptides on the second and third immunization. The C peptide induced the strongest response, and antibodies reached the highest titer (10^4) to the C peptide

after the first immunization. Both adjuvants and boosting effect were observed after second and third immunization with better responses (8×10^6) when Montanide ISA 51 was used. The N polypeptide reached similar titers (10^4) after the second immunization with Montanide ISA 720 or Montanide ISA 51 formulations. The R peptide was slower to respond, the highest titer (10^4) was reached after the third immunization with Montanide ISA 720. Mouse sera were reactive with native sporozoite proteins by IFAT, and all of them reached titer of 1:640 in both experimental groups (data not shown). In general, although there were differences in the antibody titers elicited by the different peptides, there were no significant differences for the individual peptides formulated in Montanide ISA 720 or Montanide ISA 51 (*P* = 0.80). Sera from control groups did not recognize the synthetic peptides or the parasite.

Immunogenicity in *Aotus* monkeys. All vaccine formulations were immunogenic, although Montanide ISA 51 formulations induced better antibody titers to N, R, and C antigens. The ELISA titers ranged between 10^4 and 10^5 . After immunizing the monkeys with the mixture of peptides the antibody response to N protein was faster and more consistent than against R and C proteins. Whereas antibodies to the N protein reached highest titers soon after the first immunization and in most of the animals immunized with C peptides, but as expected antibodies to R reached high titers only after the second or third immunization, corresponding to priming and first boost doses for peptide R. Antibodies remained detectable for up to 8 months after the last immunization with titers of 1×10^5 . Formulations in Montanide ISA 720 also induced more consistent responses to N protein than to C fragment (Figure 2). Differences for the vaccine formulated in Montanide ISA 720 or Montanide ISA 51 were not significant (*P* = 0.83).

All sera collected after the last immunization was assessed by IFAT for their reactivity with sporozoite surface proteins. Sera of both experimental groups specifically recognized *P. vivax* sporozoites. On Day 30 post last immunization, antibody titers in monkey ranged from 1:500 to 1:2,000 for the group immunized with the Montanide ISA 51 formulation, whereas it presented lower antibody titers (1:100–1:500) in the group immunized with the Montanide ISA 720 formulation (Table 1).

Single-cell IFN- γ production in monkeys. The PBMC from immunized monkeys produced significant amounts of IFN- γ upon stimulation with some of the synthetic domains as determined by ELISpot. Cells from monkeys immunized with the synthetic polypeptides formulated in Montanide ISA 51 produced higher amounts of IFN- γ after stimulation with N protein than with C and R antigens. In general, a lower IFN- γ response was observed in PBMC cultures from monkeys immunized with the proteins formulated in Montanide ISA 720 (Figure 3). However, the differences of IFN- γ production in response to both adjuvant formulations did not present statistical significance (*P* = 0.07). The specificity of IFN- γ production was demonstrated by negative results obtained from a control group. All cells stimulated with PHA released high levels of IFN- γ .

DISCUSSION

Here, we report the immunogenicity of two vaccine formulations based on synthetic polypeptides containing defined domains of the *P. vivax* CS protein, emulsified in two adjuvants currently being assessed with malaria vaccine candidates

FIGURE 2. Antibody response of sera from *Aotus* monkeys vaccinated with a mixture of N, R, and C peptides of the *Plasmodium vivax* circumsporozoite (CS) protein formulated in Montanide ISA 720 or Montanide ISA 51, respectively. Groups of six monkeys were primed with 100 µg of each N and C peptide and two boosting doses of 300 µg of the mixed peptide (N + R + C) at 0, 2 and 4 months. Antibody titers were followed 6 and 8 months post immunization. Enzyme-linked immunosorbent assay (ELISA) titers < 1:100 were considered negative.

intended for human use. We had the opportunity to test similar vaccine formulations in mice and monkeys, and later on in humans.³¹ The reproducibility of the data obtained here with previous studies⁶ using similar synthetic protein fragments is encouraging for further vaccine testing. In preliminary studies we had tested the immunogenicity of the individual synthetic protein fragments individually, both in animals and humans,^{6,20} with the rationale of selecting the most immunogenic and functionally active CS protein domain. Because all three fragments were immunogenic and antibodies partially blocked *in vitro* parasite invasion to hepatic cells, we decided here to assess the immunogenicity of the three peptides, giving some advantage to the N and C protein fragments. This rationale is supported by the fact that the native CS protein of all *Plasmodium* species contain a highly repetitive (R) central domain covering approximately 50% of the protein sequence.¹⁷ Although antibodies to the R domain are capable of blocking sporozoites invasion into hepatocytes, it is considered that a high immunodominance of

this domain may represent an immune “smoke screen” developed by the parasite to survive. In this case, a vaccine based in the full length sequence would not be desirable.

Additionally, because the regions flanking this R domain contain functional stretches (RI and RII regions) that participate in parasite invasion to the host cells,³² it is likely that at least in *P. vivax* other protein regions may be immunogenic and effective in eliciting protective immune responses. The *P. falciparum* RTS,S vaccine candidate has been constructed with sequences representing the R and the C region of the *P. falciparum* CS² known to contain strong B- and T-cell epitopes, respectively.³³ Apparently, for the RTS,S construct the N region of that protein was not considered important; however, in the case of *P. vivax* we have evidence to believe that the N fragment has greater vaccine potential.³⁴

The high immunogenicity displayed by the N region of the CS here, is in agreement with previous serological studies performed before, not only with *P. vivax*³⁵ but more recently also with *P. falciparum*,³⁶ where the N region induced better immunogenicity under natural conditions. Additionally, it reproduces the finding of a former preclinical study in *Aotus* monkeys where N fragment was more immunogenic.²⁰ Although those studies indicated good immunogenicity of the three fragments when delivered individually, formulated in Montanide ISA 720 using a three dose immunization regimen, the N fragment induced better responses. Recently, it has been observed that the N fragment of the CS of *P. berghei* contains an aminoacid sequence located in the highly conserved RI that requires cleavage before parasite invasion of the hepatocyte. Cleavage occurs extracellularly on the sporozoite surface when parasites contact target cells and the sulfation level of liver heparan-sulfate proteoglycans (HSPGs) were identified as the trigger of this event.³⁷ In addition, recent studies indicated that antibody response specific for the fragment 65–110 are associated with protection.³⁶

The approach for formulating the vaccine as a combination of the different CS fragments was done for two reasons. First,

TABLE 1
Immunofluorescent antibody test (IFAT) antibody titers to *Plasmodium vivax* sporozoites in immunized monkeys

Adjuvant	Immunogen	Monkey code	Antibody titers*
Montanide ISA 720	Peptides mixture	272	500
		83	500
		93	500
		622	500
		122	500
		371	100
		Pool†	< 100
Montanide ISA 51	Peptides mixture	890	500
		617	500
		849	2,000
		804	2,000
		294	2,000
		792	1,000
		Control	< 100

* Monkey sera from Day 30 after the third immunization.
† Pool of normal human sera at 1:100 dilution.

FIGURE 3. Comparison of *in vitro* interferon-gamma (IFN- γ) production by *Aotus* peripheral blood mononuclear cells (PBMCs) measured by *ex vivo* enzyme-linked immunosorbent spot (ELISpot). Results show the mean values of six monkeys per group immunized with a blend of three PvCS synthetic polypeptides (N, R, and C) formulated in Montanide ISA 720 and Montanide ISA 51, respectively.

it was to determine if there was any protein fragment interference (synergy or antagonism). Although we did not test in parallel the individual peptides, previous studies appear to indicate that there was no immunological interference among these polypeptides, as immune responses were very similar to those observed when the three fragments were administered individually.²⁸ Second, we wanted to offer some advantage to the N and C regions by giving three doses as compared with the two doses of the R region. It appeared that under those conditions, the R region did not present any dominance over the N and C flanking regions.

The BALB/c mice responded better to the C fragment than to N or R regions, whereas both *Aotus* in this study and humans in former studies responded better to N fragment. It is not surprising that BALB/c mice did not respond as this is a syngeneic strain, whereas primates are outbred species; however, it indicates that this mouse strain does not reflect the responses achieved in primates, therefore in this case, *Aotus* monkeys are better predictors of the immune responses elicited by vaccine formulations intended for human use. Most preclinical vaccine studies include the use of syngeneic mouse strains that may not reflect the major histocompatibility complex (MHC) diversity to be faced with human populations. Because of the scarcity of primates and the consequent high cost, numerous groups are currently moving vaccines from mice to humans without considering the predictive value of preclinical studies. Biologically, this may be simply because of different antigen processing and epitope recognition by MHC molecules between rodents and primates.^{38,39}

For vaccine optimization, it is also important that the immunogenicity of *P. vivax* domains using Montanide ISA 720 formulations in the preclinical studies are reproducible in a phase I trial,^{6,20,34} and that Montanide ISA 51 displayed a similar or slightly better response. If not better responses could be achieved in further trials with other adjuvants, Montanide ISA 720 or Montanide ISA 51 would be accurate in terms of safety and tolerability.

The immunogenicity of *P. vivax* polypeptide using Montanide ISA 720 have been previously studied by our group as individual or mixed formulation.³⁴ There is a great consistency in the antigenicity²⁸ and immunogenicity of these peptides formulated in this adjuvant in *Aotus* monkeys and the results obtained in humans immunized with the same formulation.⁶ In both humans and monkeys the N-terminal region of this protein has always been the most frequently recognized when formulated in Montanide ISA 720. Antibodies against the native protein tested by IFAT were much higher in monkeys immunized with vaccine formulated in Montanide ISA 51 (1:2,000) than those formulated with Montanide ISA 720 (1:500). Although, in previous studies higher antibodies titers (1:800 to 1:51,200 on Day 30 post immunization) were obtained in monkeys immunized with the same vaccine formulation by IFAT. These results may be explained by batch variability.

Although there are contrasting results regarding the protective role of anti-CS antibodies, it has been repeatedly shown that they are able to *in vitro* inhibit sporozoite invasion to hepatic cells,⁴⁰⁻⁴² to prevent infection in mice and monkeys injected with live sporozoites.^{43,44} Additionally, anti-CS antibodies appear to be associated with protection against natural exposure of vaccinated^{45,46} and non-vaccinated human volunteers.^{47,48} Although a recent study indicates that there appears to be no association between levels of anti-CS antibodies and protection,⁴⁹ a possible explanation for these discrepancies may be the different antigens used for antibody detection. The CS antigen is a large protein with numerous B-cell epitopes distributed along its entire sequence and therefore while antibodies to certain regions might not be associated with protection, antibodies to epitopes comprising or related to functional domains, i.e., those comprising region I within the N region may block the protein cleavage necessary for parasite invasion and are likely to be associated with protection.³⁶

Concerning cellular immune responses as assessed by IFN- γ production, there was consistency in the production of IFN- γ induce by the N fragment in monkeys and humans; production

of high levels of IFN- γ is critical to prevent the development of parasite liver stage.¹⁷ We therefore consider that an effective vaccine directed to protect against malaria pre-erythrocytic stages must elicit vigorous antibody and T cell responses.¹⁹

Monkeys and chimpanzees have been recognized as ideal models to study pre-erythrocytic malaria vaccine candidates,^{50–52} and we have extensively proven the great value of *Aotus* monkeys for malaria vaccine development.⁵² Although this model has not yet been validated, in this study we have showed that the immune responses against the *P. vivax* CS protein in *Aotus* are similar to those observed in humans immunized with the same vaccine formulation, whereas they differ from results in mice. This may be caused by different polypeptide processing and HLA recognition by these two animal species.^{38,39} Ongoing studies have showed significant homology between human and *Aotus* MHC molecules (Ampudia E, unpublished data) as well as epitope recognition in both species.

In conclusion, *P. vivax* CS individual domains have indicated good immunogenicity in mice and *Aotus* monkeys vaccinated with the three domain combination, and appear that no immunological interference is produced among the three different fragments. Despite the fact that the vaccine formulations in Montanide ISA 51 induced a trend of better immune response, no statistically significant immune response was observed. Therefore, the same vaccine formulations should be tested in humans to select the best one for further studies. Finally, care should be taken on the selection of malaria pre-erythrocytic antigen candidates for further development into clinical trials on the basis of the results obtained in animal models.

Received February 18, 2010. Accepted for publication October 7, 2010.

Acknowledgments: We acknowledge the laboratory technical support of Carolina Ramírez, veterinarian help of Gustavo Quintero and Alejandro Castellanos from FUCEP, and the editorial support of Juan Felipe Delgado and Claudia García.

Financial support: This work was supported by the National Institute of Allergy and Infectious Diseases through Tropical Medicine Research Centers NIAID/TMRC grant no. 49486, the Instituto Colombiano para el Desarrollo de la Ciencia y Tecnología 'Francisco José de Caldas' COLCIENCIAS grant no. 487-2003, and the Ministry for Social Protection/Colciencias no. 255-2004

Authors' addresses: Myriam Arévalo-Herrera, Omaira Vera, Angélica Castellanos, Nora Céspedes, Liliana Soto, and Sócrates Herrera, Instituto de Inmunología, Edificio de Microbiología, Facultad de Salud, Universidad del Valle and Malaria Vaccine and Drug Development Center, Cali, Colombia, E-mails: marevalo@inmuno.org, ovizcano@gmail.com, ncespedes@inmuno.org, lsoto@inmuno.org, and sherrera@inmuno.org. Giampietro Corradin, Biochemistry Institute, University of Lausanne, Lausanne, Switzerland, E-mail: giampietro.corradin@unil.ch.

Reprint requests: Sócrates Herrera, Malaria Vaccine and Drug Development Center, Carrera 37 - 2Bis No. 5E - 08, Cali, Colombia, E-mail: sherrera@inmuno.org.

REFERENCES

- Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, Kabaria CW, Manh BH, Elyazar IR, Brooker S, Smith DL, Moyeed RA, Snow RW, 2009. A world malaria map: *Plasmodium falciparum* endemicity in 2007. *PLoS Med* 6: e1000048.
- Bojang K, Milligan P, Pinder M, Doherty T, Leach A, Ofori-Anyinam O, Lievens M, Kester K, Schaefer K, Ballou WR, Cohen J, 2009. Five-year safety and immunogenicity of GlaxoSmithKline's candidate malaria vaccine RTS,S/AS02 following administration to semi-immune adult men living in a malaria-endemic region of The Gambia. *Hum Vaccin* 5: 242–247.
- Brown GV, Moorthy VS, Reed Z, Mendis K, Arevalo-Herrera M, Alonso P, 2009. Priorities in research and development of vaccines against *Plasmodium vivax* malaria. *Vaccine* 27: 7228–7235.
- Vekemans J, Leach A, Cohen J, 2009. Development of the RTS,S/AS malaria candidate vaccine. *Vaccine* 27 (Suppl 6): G67–G71.
- Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, Vekemans J, Mshamu S, Lang T, Gould J, Dubois MC, Demoitie MA, Stallaert JF, Vansadia P, Carter T, Njuguna P, Awuondo KO, Malabeja A, Abdul O, Gesase S, Mturi N, Drakeley CJ, Savarese B, Villafana T, Ballou WR, Cohen J, Riley EM, Lemnge MM, Marsh K, von Seidlein L, 2008. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. *N Engl J Med* 359: 2521–2532.
- Herrera S, Bonelo A, Perlaza BL, Fernández OL, Victoria L, Lenis AM, Soto L, Hurtado H, Acuña LM, Vélez JD, Palacios R, Chen-Mok M, Corradin G, Arévalo-Herrera M, 2005. Safety and elicitation of humoral and cellular responses in Colombian malaria-naïve volunteers by a *Plasmodium vivax* circumsporozoite protein-derived synthetic vaccine. *Am J Trop Med Hyg* 73: 3–9.
- Arevalo-Herrera M, Chitnis C, Herrera S, 2010. Current status of *Plasmodium vivax* vaccine. *Hum Vaccin* 6: 124–132.
- Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM, 2007. *Vivax* malaria: neglected and not benign. *Am J Trop Med Hyg* 77: 79–87.
- Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, del Portillo HA, 2009. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect Dis* 9: 555–566.
- Ejigiri I, Sinnis P, 2009. *Plasmodium* sporozoite-host interactions from the dermis to the hepatocyte. *Curr Opin Microbiol* 12: 401–407.
- Pinzon-Ortiz C, Friedman J, Esko J, Sinnis P, 2001. The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for *Plasmodium* sporozoite attachment to target cells. *J Biol Chem* 276: 26784–26791.
- Singh AP, Buscaglia CA, Wang Q, Levay A, Nussenzweig DR, Walker JR, Winzeler EA, Fujii H, Fontoura BM, Nussenzweig V, 2007. *Plasmodium* circumsporozoite protein promotes the development of the liver stages of the parasite. *Cell* 131: 492–504.
- Gysin J, Barnwell J, Schlesinger DH, Nussenzweig V, Nussenzweig RS, 1984. Neutralization of the infectivity of sporozoites of *Plasmodium knowlesi* by antibodies to a synthetic peptide. *J Exp Med* 160: 935–940.
- McCarthy VC, Clyde DF, 1977. *Plasmodium vivax*: correlation of circumsporozoite precipitation (CSP) reaction with sporozoite-induced protective immunity in man. *Exp Parasitol* 41: 167–171.
- Clyde DF, 1990. Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies 1971–1975. *Bull World Health Organ* 68: 9–12.
- Herrington D, Davis J, Nardin E, Beier M, Cortese J, Eddy H, Losonsky G, Hollingdale M, Sztein M, Levine M, Nussenzweig RS, Clyde D, Edelman R, 1991. Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. *Am J Trop Med Hyg* 45: 539–547.
- Hoffman S, Miller L, 1996. Perspectives on malaria vaccine development. Hoffman S, ed. *Malaria Vaccine Development*. Washington, DC: ASM Press, 1–13.
- Nussenzweig RS, Vanderberg J, Most H, Orton C, 1967. Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. *Nature* 216: 160–162.
- Herrera S, Corradin G, Arevalo-Herrera M, 2007. An update on the search for a *Plasmodium vivax* vaccine. *Trends Parasitol* 23: 122–128.
- Herrera S, Bonelo A, Perlaza BL, Valencia AZ, Cifuentes C, Hurtado S, Quintero G, Lopez JA, Corradin G, Arevalo-Herrera M, 2004. Use of long synthetic peptides to study the antigenicity and immunogenicity of the *Plasmodium vivax* circumsporozoite protein. *Int J Parasitol* 34: 1535–1546.

21. Dicko A, Sagara I, Ellis RD, Miura K, Guindo O, Kamate B, Sogoba M, Niambele MB, Sissoko M, Baby M, Dolo A, Mullen GE, Fay MP, Pierce M, Diallo DA, Saul A, Miller LH, Doumbo OK, 2008. Phase 1 study of a combination AMA1 blood stage malaria vaccine in Malian children. *PLoS One* 3: e1563.
22. Elliott SL, Suhrbier A, Miles JJ, Lawrence G, Pye SJ, Le TT, Rosenstengel A, Nguyen T, Allworth A, Burrows SR, Cox J, Pye D, Moss DJ, Bharadwaj M, 2008. Phase I trial of a CD8+ T-cell peptide epitope-based vaccine for infectious mononucleosis. *J Virol* 82: 1448–1457.
23. Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, Fay MP, Narum D, Rausch K, Miles AP, Aebig J, Orcutt A, Muratova O, Song G, Lambert L, Zhu D, Miura K, Long C, Saul A, Miller LH, Durbin AP, 2008. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS ONE* 3: e2636.
24. Karbach J, Gnjatich S, Bender A, Neumann E, Weidmann E, Yuan J, Ferrara CA, Hoffmann E, Old LJ, Altorki NK, Jager E, 2010. Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. *Int J Cancer* 126: 909–918.
25. Valmori D, Pessi A, Bianchi E, Corradin G, 1992. Use of human universally antigenic tetanus toxin T cell epitopes as carriers for human vaccination. *J Immunol* 149: 717–721.
26. Verdini A, Terenzi S, Brossard V, Roggero M, Corradin G, 2008. Oxidative folding of synthetic polypeptides S-protected as tert-butylthio derivatives. *J Pept Sci* 14: 1271–1282.
27. Atherton E, Logan JC, Sheppard RC, 1981. Peptide synthesis procedures for solid phase synthesis using N-fluorenylmethoxycarbonyl-amino-acid on polyamide supports: synthesis of substance P and of acyl carrier protein 65–74 decapeptide. *J Chem Soc Lond Perkin Trans 1*: 538–548.
28. Arevalo-Herrera M, Roggero MA, Gonzalez JM, Vergara J, Corradin G, Lopez JA, Herrera S, 1998. Mapping and comparison of the B-cell epitopes recognized on the *Plasmodium vivax* circumsporozoite protein by immune Colombians and immunized *Aotus* monkeys. *Ann Trop Med Parasitol* 92: 539–551.
29. Herrera S, Fernandez O, Manzano MR, Murrain B, Vergara J, Blanco P, Palacios R, Velez JD, Epstein JE, Chen-Mok M, Reed ZH, Arevalo-Herrera M, 2009. Successful sporozoite challenge model in human volunteers with *Plasmodium vivax* strain derived from human donors. *Am J Trop Med Hyg* 81: 740–746.
30. Solarte Y, Manzano Mdel R, Rocha L, Castillo Z, James MA, Herrera S, Arevalo-Herrera M, 2007. Effects of anticoagulants on *Plasmodium vivax* oocyst development in *Anopheles albimanus* mosquitoes. *Am J Trop Med Hyg* 77: 242–245.
31. Herrera S, Fernández OL, Vera O, Cardenas W, Ramirez O, Palacios R, Chen-Mok M, Corradin G, Arevalo-Herrera M, 2011. Phase I safety and immunogenicity trial of *Plasmodium vivax* CS-derived long synthetic peptides adjuvanted with Montanide ISA 720 or ISA 51. *Am J Trop Med Hyg* 84 (Suppl 2): 12–20.
32. Frevert U, Sinnis P, Cerami C, Shreffler W, Takacs B, Nussenzweig V, 1993. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J Exp Med* 177: 1287–1298.
33. Calvo-Calle JM, Oliveira GA, Watta CO, Soverow J, Parra-Lopez C, Nardin EH, 2006. A linear peptide containing minimal T- and B-cell epitopes of *Plasmodium falciparum* circumsporozoite protein elicits protection against transgenic sporozoite challenge. *Infect Immun* 74: 6929–6939.
34. Arevalo-Herrera M, Herrera S, 2001. *Plasmodium vivax* malaria vaccine development. *Mol Immunol* 38: 443–455.
35. Hurtado S, Salas ML, Romero JF, Zapata JC, Ortiz H, Arevalo-Herrera M, Herrera S, 1997. Regular production of infective sporozoites of *Plasmodium falciparum* and *P. vivax* in laboratory-bred *Anopheles albimanus*. *Ann Trop Med Parasitol* 91: 49–60.
36. Bongfen SE, Ntsama PM, Offner S, Smith T, Felger I, Tanner M, Alonso P, Nebie I, Romero JF, Silvie O, Torgler R, Corradin G, 2009. The N-terminal domain of *Plasmodium falciparum* circumsporozoite protein represents a target of protective immunity. *Vaccine* 27: 328–335.
37. Coppi A, Pinzon-Ortiz C, Hutter C, Sinnis P, 2005. The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. *J Exp Med* 201: 27–33.
38. Prato S, Maxwell T, Pinzon-Charry A, Schmidt CW, Corradin G, Lopez JA, 2005. MHC class I-restricted exogenous presentation of a synthetic 102-mer malaria vaccine polypeptide. *Eur J Immunol* 35: 681–689.
39. Stevenson MM, Urban BC, 2006. Antigen presentation and dendritic cell biology in malaria. *Parasite Immunol* 28: 5–14.
40. Nardin EH, Nussenzweig V, Nussenzweig RS, Collins WE, Harinasuta KT, Tapchaisri P, Chomcharn Y, 1982. Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. *J Exp Med* 156: 20–30.
41. Hollingdale MR, Nardin EH, Tharavanij S, Schwartz AL, Nussenzweig RS, 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells: an *in vitro* assay of protective antibodies. *J Immunol* 132: 909–913.
42. Stewart MJ, Nawrot RJ, Schulman S, Vanderberg JP, 1986. *Plasmodium berghei* sporozoite invasion is blocked *in vitro* by sporozoite-immobilizing antibodies. *Infect Immun* 51: 859–864.
43. Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M, 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science* 207: 71–73.
44. Potocnjak P, Yoshida N, Nussenzweig RS, Nussenzweig V, 1980. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J Exp Med* 151: 1504–1513.
45. Moorthy VS, Ballou WR, 2009. Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. *Malar J* 8: 312.
46. Sacarlal J, Aide P, Aponte JJ, Renom M, Leach A, Mandomando I, Lievens M, Bassat Q, Lafuente S, Macete E, Vekemans J, Guinovart C, Sigauque B, Sillman M, Milman J, Dubois MC, Demoitie MA, Thonnard J, Menendez C, Ballou WR, Cohen J, Alonso PL, 2009. Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children. *J Infect Dis* 200: 329–336.
47. Hoffman SL, Wistar R Jr, Ballou WR, Hollingdale MR, Wirtz RA, Schneider I, Marwoto HA, Hockmeyer WT, 1986. Immunity to malaria and naturally acquired antibodies to the circumsporozoite protein of *Plasmodium falciparum*. *N Engl J Med* 315: 601–606.
48. Nardin EH, Nussenzweig RS, McGregor IA, Bryan JH, 1979. Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria. *Science* 206: 597–599.
49. Crompton PD, Kayala MA, Traore B, Kayentao K, Onoiba A, Weiss GE, Molina DM, Burk CR, Waisberg M, Jasinskas A, Tan X, Doumbo S, Doumbo D, Kone Y, Narum DL, Liang X, Doumbo OK, Miller LH, Doolan DL, Baldi P, Felgner PL, Pierce SK, 2010. A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. *Proc Natl Acad Sci USA* 107: 6958–6963.
50. Daubersies P, Thomas AW, Millet P, Brahimi K, Langermans JA, Ollomo B, BenMohamed L, Slierendregt B, Eling W, Van Belkum A, Dubreuil G, Meis JF, Guerin-Marchand C, Cayphas S, Cohen J, Gras-Masse H, Druilhe P, 2000. Protection against *Plasmodium falciparum* malaria in chimpanzees by immunization with the conserved pre-erythrocytic liver-stage antigen 3. *Nat Med* 6: 1258–1263.
51. Galinski MR, Barnwell JW, 2008. *Plasmodium vivax*: who cares? *Malar J* 7 (Suppl 1): S9.
52. Herrera S, Perlaza BL, Bonelo A, Arevalo-Herrera M, 2002. *Aotus* monkeys: their great value for anti-malaria vaccines and drug testing. *Int J Parasitol* 32: 1625–1635.