Phase I Safety and Immunogenicity Trial of *Plasmodium vivax* CS Derived Long Synthetic Peptides Adjuvanted with Montanide ISA 720 or Montanide ISA 51

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Abstract. We assessed the safety, tolerability, and immunogenicity of a mixture of three synthetic peptides derived from the *Plasmodium vivax* circumsporozoite protein formulated in Montanide ISA 720 or Montanide ISA 51. Forty healthy malaria-naive volunteers were allocated to five experimental groups (A–E); four groups (A–D) were immunized intramuscularly with 50 and 100 μg/dose injections of a mixture of N, R, and C peptides formulated in the two different adjuvants at 0, 2, and 4 months and one group was administered placebo. Vaccines were immunogenic, safe, well tolerated, and no serious adverse events related to the vaccine occurred. Seroconversion occurred in >90% of the vaccines and antibodies recognized the sporozoite protein on immunofluorescent antibody test. Vaccines in Montanide ISA 51 showed a higher sporozoite protein recognition and interferon production. Results encourage further testing of the vaccine protective efficacy.

INTRODUCTION

A vaccine against pre-erythrocytic stages of malarial infection would be an ideal weapon to avoid clinical manifestations of disease because it would block the infection during the initial asymptomatic phase. Indeed, it has been extensively proven that animals 1,2 and humans 3–4 vaccinated with irradiated sporozoites become immune to experimental infections induced by sporozoites, and do not develop patent blood-stage infections or clinical malaria symptoms. Sera and cells from these individuals recognize proteins expressed on the sporozoites and the parasite liver forms 5–7 that have been incriminated in this protection and therefore have been proposed as malaria vaccine candidates. 8,9 Among them, the circumsporozoite (CS) protein that is abundantly expressed on the sporozoite surface has been shown to be involved in the process of parasite invasion to the hepatocyte 10,11 and its immunological blockage prevents the development of malaria infection. 12,13 The RTS-S vaccine based on a construct of the *Plasmodium falciparum* CS protein and the S antigen of human hepatitis B virus has proven to be immunogenic and partially protective in phase II studies conducted with human malaria-naive volunteers 14–18 and in adults and children from malaria-endemic areas of Africa. 19–22

Regarding the *Plasmodium vivax* CS protein, three long synthetic peptides (LSP) homologous to the amino (N), central repeat (R), and carboxyl (C) regions were initially evaluated in preclinical studies and showed high immunogenicity in mice and *Aotus* monkeys. 23–26 On the basis of those studies the same LSPs were formulated in Montanide ISA 720 and assessed in phase Ia clinical trial conducted by the Malaria Vaccine and Drug Development Center (MVDC) in Cali, Colombia. Immunization with this formulation indicated to be safe, well tolerated, and immunogenic. 27 All three peptides induced production of high titers of specific antibodies that cross-reacted with the protein on the parasite and production of interferon-gamma (IFN-γ) in most vaccinated subjects. Although the N peptide induced the highest antibody titers at three different doses tested (10, 30, and 100 μg/dose), peptides R and C were also immunogenic at high doses. In the search for an optimal vaccine formulation for human use we have conducted pre-clinical studies in mice, monkeys, and clinical trials in malaria-naive volunteers, and we performed a new series of studies to assess the safety and immunogenicity of a combination of the three peptides formulated either in Montanide ISA 720 or in Montanide ISA 51. The rationale for these mixtures was to determine the possibility of immunological interference among the different peptides or their potential synergism. These adjuvants were selected because they form stable water-in-oil emulsions and induced high antibody levels that lasted for up to 1 year in mice, rabbits, and monkeys in previous studies using recombinant malaria proteins. 27–32

More recently, a recombinant *P. vivax* CS protein produced in *Escherichia coli* and formulated in Montanide ISA 720 showed to be highly immunogenic in mice. 33 Several phase I clinical trials have been conducted using different malaria vaccine antigens in which these two adjuvants have been able to stimulate both humoral and cellular immune responses. 34–37

We present here a phase I clinical trial conducted with the same *P. vivax* CS derived peptides formulated in two different adjuvants, and provide further safety and immunogenicity data as part of a clinical development plan that aims at developing vaccines to prevent malaria.

MATERIALS AND METHODS

Study design and population. This was a phase I double-blind, controlled vaccine trial, evaluating safety, tolerability, and immunogenicity of mixtures of N, R and C LSP derived from the *P. vivax* CS protein formulated in two adjuvants Montanide ISA 720 and Montanide ISA 51. The primary objective was to assess in malaria-naive adults, the safety and reactogenicity of these peptides formulated in the two adjuvants. Study protocol was approved by the Institutional Review Boards (IRB) of the Universidad del Valle and Centro Médico Imbanaco (CMI), and the study complied with Declaration of Helsinki principles, International Conference
The primary endpoints were the occurrence of solicited signs and symptoms during an 8-day period after each vaccination. Volunteers were encouraged to maintain contact with the research team by phone and whenever needed personally, at any time of the day, to inform the occurrence of any symptom during the 1-year study period. Volunteers were observed for 60 minutes after injection and evaluated by the study team at 8 and 24 hrs after injection and again on Day 7 after vaccination. Safety was assessed by a complete physical examination and clinical laboratory tests on all study participants during monthly visits up to Month 5 post immunization and followed up by phone between months 5 and 12. Blood samples were collected for complete blood count, sedimentation rate, prothrombin time, partial thromboplastin time, blood urea nitrogen, and serum glucose, total protein, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and bilirubin. Immediately before each

### Table 1

<table>
<thead>
<tr>
<th>Recruitment process</th>
<th>Volunteers asked to participate</th>
<th>Refused to participate</th>
<th>Not meet inclusion criteria</th>
<th>Reserved if needed</th>
<th>Volunteers briefed</th>
</tr>
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<tr>
<td>Number of volunteers (n)</td>
<td>100</td>
<td>51</td>
<td>7</td>
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<td>40</td>
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#### Consecutive allocation

<table>
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<tr>
<th>Montanide adjuvant</th>
<th>Immunogen</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st dose</td>
<td>N + C</td>
<td>100* (N = 8)</td>
<td>100 (N = 8)</td>
<td>200 (N = 8)</td>
<td>200 (N = 8)</td>
<td>SS† (N = 8)</td>
</tr>
<tr>
<td>2nd dose</td>
<td>N + R + C</td>
<td>150 (N = 8)</td>
<td>150 (N = 7)</td>
<td>300 (N = 7)</td>
<td>300 (N = 8)</td>
<td>SS (N = 8)</td>
</tr>
<tr>
<td>3rd dose</td>
<td>N + R + C</td>
<td>150 (N = 8)</td>
<td>150 (N = 7)</td>
<td>300 (N = 6)</td>
<td>300 (N = 8)</td>
<td>SS (N = 8)</td>
</tr>
</tbody>
</table>

*μg/dose.
†Saline solution.
vaccination, we tested female volunteers for pregnancy using a beta human chorionic gonadotropin test. Solicited symptoms were grouped by local and systemic. Local symptoms included pain, swelling, and erythema, whereas systemic symptoms were fever, flu-like symptoms, headache, dizziness, nausea, emesis, and abdominal pain. Signs and symptoms were graded from mild to serious with a four level score. The AE were recorded throughout the study and were defined as any new or worsening sign or symptom of illness or an abnormal laboratory test during the protocol specified follow-up. Each AE was evaluated by the study clinician for its severity, and relatedness to vaccination and SAE were reported as required by the World Health Organization (WHO). The data safety monitoring board was constituted by an external clinical monitor and an internal clinical monitor who met three times during the study.

Measurement of humoral responses. The immunological assays were performed using samples collected during the monthly visits. Antibody response was measured by enzyme-linked immunosorbent assay (ELISA) using as antigen the N, R, or C peptides (1 μg/mL), as described previously. The final reaction was read at 405 nm in a microplate reader (MRX; Dynex Technologies, Inc., Chantilly, VA). Cut-off points were calculated as three SD above the mean absorbance value at 405 nm of sera from healthy volunteers who had never been exposed to malaria. Controls were selected from a pool of sera from semi-immune blood donors (positive controls) and a pool of sera from malaria-naive donors (negative controls). Peptide-specific IgG isotypes were determined by modified ELISA using sera collected at Months 0 and 5 from immunized volunteers. Briefly, 1 μg/mL of peptide was used as antigen to coat microwells. Wells were blocked with phosphate buffer saline (PBS)-Tween 20 plus fat free milk and then incubated with the serum sample dilutions for 1 hr. Plates were washed with PBS-Tween 20 and then incubated with mouse anti-human IgG isotype antisera. The ELISA titers of < 1:200 were considered negative.

Anti-IgG1, IgG3, and IgG4 (Skibio, Bedfordshire, England) and anti-IgG2 (Sigma, St. Louis, MO) isotypes were used and a peroxidase conjugated goat anti-mouse IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and the reaction read at 630 nm (OD630). Data were reported as ratio (OD mean of the sample, positive or negative controls divided by the cut-off point).

Parasite recognition by anti-peptide antibodies was determined by indirect immunofluorescent antibody test (IFAT) at 0, 3, and 5 months after enrollment using P. vivax sporozoites produced in Anopheles albimanus mosquitoes. Antibody titers were determined as the reciprocal of the end-point dilution that showed positive fluorescence as described previously. In addition, 50 sera from individuals naturally exposed to malaria were also evaluated for the presence of anti-P. vivax sporozoite and comparison with antibodies induced by vaccinations.

Enzyme-linked immunosorbent spot (ELIspot) assay for single cell release of IFN-γ. Peripheral blood mononuclear cells (PBMC) collected after last immunization (Month 5) were separated from whole blood using Ficoll Histopaque (Sigma-Aldrich, St. Louis, MO) density gradients, and were resuspended in RPMI medium 1640 (Gibco, Grand Island, NY). The IFN-γ-producing cells were identified using a commercial IFN-γ ELIspot human kit (Mabtech AB, Stockholm, Sweden). Fresh PBMCs (4 × 10^6/well were then mixed with 10 μg/mL of each synthetic peptide and plates were incubated for 40 hrs at 37°C in an atmosphere of 5% CO₂. Spots were counted with a spot counting system (Scanalytics, Fairfax, VA), and the results were expressed as the mean number of IFN-γ spot-forming cells (sfc) per 10^7 PBMC. Volunteers were considered responders if the number of sfc in their samples had increased from their own baseline level (before immunization on Day 0), any increase ≥ 5 sfc were considered positive.

Statistical methods. The main outcomes we evaluated were AE and SAE related to vaccination during the study period. An AE was considered related if it was determined to be possibly, probably, or most probably related to vaccination. Rates of related and other common AE were compared among experimental groups using Fisher’s exact tests. Main comparisons of interest were between adjuvants and between vaccine doses. Antibody titers and IFN-γ production were compared among peptides, vaccine doses, and adjuvants at several points in the studies using Wilcoxon signed-rank and Kruskal-Wallis tests. Two-tailed, non-parametric P values ≤ 0.05 were considered significant.

RESULTS

Study population. A total of 100 people were asked to participate; of these 49 consented to screening. Finally, 40 participants willing to continue in the study (18 males and 22 females) were consecutively allocated to one of the five treatment groups, as shown in Table 1. Allocation started in the low-dose groups and once they were completed, and no SAEs were reported in response to the first vaccination dose, the study continued with the high dose. Groups were comparable in baseline demography, and clinical laboratory values did not show statistically significant differences (data not shown). One volunteer received a dose of tetanus vaccine 1 week after the first immunization and another one was immunized against rubella and smallpox at Day 68 of the study. These events were evaluated by the clinical monitor and the IRBs, and they both agreed that these two volunteers could continue in the study. Two other participants abandoned the study voluntarily, both after the first immunization, and a third participant abandoned after the second immunization.

Safety. All three immunization doses were well tolerated by all participants from the low and high vaccine doses. Low grade pain (mild or moderate) at the injection site up to 48 hrs after injection was the most frequent AE related to immunization, followed by local swelling and local erythema (Table 2). We found no statistically significant differences of AEs between doses. When comparing between adjuvants, pain at injection site was the only difference significantly higher at the third dose of the injection with Montanide ISA 720. No significant differences were observed in the occurrence of the remainder AEs. When both adjuvants were taken together and compared with placebo, local pain was more frequent in the malaria immunized group in the first and third injections (P = 0.03). Although local swelling seemed to be more frequent in the immunized group compared with placebo, the difference did not reach statistical significance. Headache was the most common systemic AE with both adjuvants, but again its difference with the placebo group did not reach statistical significance.
None of the participants experienced SAE related to the vaccine, whereas two volunteers showed SAE that were considered not related to immunization. In the placebo group, 7 days after first immunization one volunteer was diagnosed with chronic cholecystitis and another in the high dose group formulated in Montanide ISA 51 presented an acute episode of urolithiasis on Day 82 of the study. All laboratory tests for the remaining participants yielded normal values during the study period.

**Humoral response.** Figure 1 shows antibody responses to each peptide. After the first immunization with mixtures containing the N and C peptides, 74% of participants seroconverted for peptide N, but none for peptide C. With the second immunization, which was performed with the mixture of the three LSP, 100% of the volunteers became positive to N peptide and 71% of volunteers seroconverted to C peptide. Few of the volunteers seroconverted to R peptide after the second immunization that corresponded to a priming dose for R, but 93% of the volunteers responded to this peptide after first boosting (third immunization). Antibody titers to R peptide reached comparable levels as those obtained with the three doses of C peptide in most study groups, except for the group immunized with the 150 μg/dose in Montanide ISA 51 adjuvant, which showed titers four times higher to C peptide than to R peptide. In general, 96% of volunteers produced specific IgG antibodies to all peptides after the third immunization with the three peptide mixtures. Volunteers responded earlier to N peptide, and all volunteers required only two immunizations to seroconvert. The N peptide also elicited the highest median titer (1:9,600) when compared with titers after the third dose either. Concordant with all this, when the ratio of IgG1 plus IgG3 versus IgG2 plus IgG4 was evaluated, C peptide showed a higher ratio, followed by N peptide, but not a significant difference was observed between these two peptides (Figure 2).

**Sporozoite recognition.** Forty-three percent of the participants were (13/30) IFAT positive (titers ≥ 1:20) after the second immunization and 73% (21/29) after the third dose, without taking into account the type of adjuvant used. When responses to both adjuvants were compared after the third dose, 93% of participants receiving Montanide ISA 51 were IFAT positive as compared with 56% of those receiving Montanide ISA 720 (Table 4). Similarly, Montanide ISA 51 adjuvant resulted in higher sporozoite recognition antibody titers than Montanide ISA 720, independently of the vaccine dose (P = 0.01; two-tailed Kruskal-Wallis non-parametric P value, data not shown). In general, the two-dose immunization schedule using 150 μg/dose resulted in a lower level of anti-sporozoite antibodies when compared with titers after the third dose (P = 0.02). However, levels of anti-sporozoite antibodies in the groups vaccinated with both peptide doses formulated in Montanide ISA 51 were similar after the third immunization. At the higher dose, anti-sporozoite antibody levels were similar after two and three immunizations. Specificity of the antibody response was confirmed by negative results obtained by ELISA and IFAT, using sera from volunteers in the control group and from all other volunteers before immunization. Half of the individuals naturally exposed to malaria presented anti-P. vivax sporozoite antibodies titers ranging from 1:80 to 1:640. Most of them (10 individuals) had low antibody titers (1:80) similar to those observed in immunized volunteers.

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**Table 2**

Number of volunteers reporting vaccine-related adverse events per dose and adjuvant group

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>ISA 51</th>
<th>ISA 720</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Injection site pain</td>
<td>14</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Swelling</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Erythema</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fever (&gt; 37.5°C)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Headache</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Dizziness</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Nausea/Emesis</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenopathy (Axilar/cervical)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Montanide at 150 μg or 300 μg dose.*
† Two-tailed *P* values < 0.05 by Fisher exact test, for comparison among adverse events (AE) in adjuvant and placebo groups.
Single-cell IFN-γ production. Production of IFN-γ by PBMC varied among volunteers and immunization doses (Figure 3). The response to peptide N (median 140 sfc/10^6 PBMC) was significantly higher than to peptide R (median 80 sfc/10^6 PBMC) and peptide C (median 100 sfc/10^6 PBMC) (P = 0.001). Response induced by the 300 μg dose was higher than 150 μg dose (P = 0.001). When comparing groups by adjuvant, we found that the response to Montanide ISA 51

<table>
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<tr>
<th>Isotype</th>
<th>IgG1 Median</th>
<th>IQR</th>
<th>P</th>
<th>IgG2 Median</th>
<th>IQR</th>
<th>P</th>
<th>IgG3 Median</th>
<th>IQR</th>
<th>P</th>
<th>IgG4 Median</th>
<th>IQR</th>
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<tr>
<td>Peptide</td>
<td>N</td>
<td>10.6</td>
<td>10.9</td>
<td>10.4</td>
<td>25.1</td>
<td>25.6</td>
<td>12.0</td>
<td>15.1</td>
<td>6.0</td>
<td>7.5</td>
<td>24.2</td>
<td>2.0</td>
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<tr>
<td>R</td>
<td>0.9</td>
<td>0.6</td>
<td>1.4</td>
<td>&lt;0.001</td>
<td>0.7</td>
<td>0.5</td>
<td>0.9</td>
<td>&lt;0.001</td>
<td>0.9</td>
<td>0.8</td>
<td>1.5</td>
<td>&lt;0.001</td>
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<tr>
<td>C</td>
<td>10.1</td>
<td>6.7</td>
<td>17.7</td>
<td>0.8</td>
<td>0.7</td>
<td>1.1</td>
<td>2.0</td>
<td>1.4</td>
<td>3.3</td>
<td>1.2</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>ISA 51</td>
<td>10.3</td>
<td>1.8</td>
<td>10.9</td>
<td>0.06</td>
<td>1.0</td>
<td>0.8</td>
<td>2.6</td>
<td>0.53</td>
<td>2.6</td>
<td>1.4</td>
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<tr>
<td>ISA 720</td>
<td>6.2</td>
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<td>22.8</td>
<td>1.1</td>
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<tr>
<td>Doses μg</td>
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<td>0.9</td>
<td>10.9</td>
<td>0.70</td>
<td>0.9</td>
<td>0.7</td>
<td>3.4</td>
<td>0.79</td>
<td>2.5</td>
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<tr>
<td>300</td>
<td>10.1</td>
<td>2.4</td>
<td>10.9</td>
<td>0.9</td>
<td>0.7</td>
<td>2.6</td>
<td>1.9</td>
<td>1.1</td>
<td>24.6</td>
<td>1.3</td>
<td>0.9</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*IQR = interquartile range.
†Two-tailed, Kruskal-Wallis non-parametric P value.
CLINICAL TRIAL WITH \( P. \) VIVAX CS DERIVED LSP VACCINE

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DISCUSSION

Our results provide evidence that vaccination of malaria-naive volunteers with mixtures of LSP derived from the \( P. \) vivax CS protein formulated in Montanide ISA 720 and in Montanide ISA 51 is safe, well tolerated, and immunogenic. This study also confirms data previously reported by our group on the vaccination with similar \( P. \) vivax CS derived LSP individually formulated in Montanide ISA 720. In this regard AEs related to vaccination occurred with similar frequency in all groups and transient pain at the site of injection, which was described as similar to that caused by commercially available vaccines, was the most frequent complaint. Moreover, the trial indicated that although the peptide mixture as formulated did not appear to produce any synergism on immune responses, we expect an additive effect of cell-mediated immunity and antibodies, especially on their blocking activity on sporozoite invasion, which may be more effective than vaccination with individual peptides.

The peptide mixtures induced antibody titers to each peptide that were similar to those from our previous trial, in which volunteers were immunized with single peptides. This suggests that there is no detectable interference in the peptide mixture. The strategy of initially giving a priming vaccine containing N and C peptides only was used to avoid the potential imbalance that might produce the immunodominant R fragment. It has been shown that after continued exposure to malaria, sera of individuals from endemic areas recognize the R region better than the N- and C-terminal regions, which may be caused by its repetitive structure, where the same B- and T-cell epitopes are repeated numerous times, whereas any individual epitope present in the N and C flanking regions is present only once in each CS molecule. This immuno-dominance may represent a “smoke screen” that diverts the response toward it, preventing the development of stronger responses to the flanking regions that contain functional cell-binding domains (regions I and II) required for parasite invasion.

Although the use of the full-length protein may be considered desirable, besides its immunodominance, the \( P. \) vivax central repeat domain is highly polymorphic. Use of the LSP approach allows an easy, inexpensive, and accurate methodology to down-select protein fragments containing epitopes relevant in protection. \( Plasmodium falciparum \) RTS-S, the most advanced malaria vaccine candidate is based on a recombinant construct containing protein fragments similar to the \( P. \) vivax R and C fragments studied here. Although that construct might be the best selection for \( P. \) falciparum, results here indicate that other combinations, i.e., including the N fragment with other CS fragments or other antigens may be more effective in \( P. \) vivax. In this and in our previous vaccine trial, the C fragment was less immunogenic than N and R.

Although the choice of protective protein fragment is critical, adjuvants are also fundamental. Montanide ISA 720 and Montanide ISA 51 represent a new generation of promising adjuvants, with an overall acceptable safety profile and strong B cell and Th stimulation capacity. Montanide ISA 51 is a mineral

![Figure 2. Ratio of immunoglobulin isotypes by time and peptide.](image)

![Figure 3. IFN-γ production in response vaccination with a mixture of N, R, and C peptides. Median of sfc to each peptide independent of doses and adjuvant.](image)

| Table 4 | Recognition of \( Plasmodium vivax \) sporozoites by immunofluorescence assay |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Montanide adjuvant | Dose ug | Immunization | Second | Third | Natural infection* |
| | | | | | |
| ISA 51 | 150 | n | Median† | IQR† | n | Median† | IQR† | 10 | 80 |
| | | | 7 | 0 | 0–10 | 40 | 40–320 | 0.02 |
| | 300 | 7 | 20 | 20–80 | 7 | 20 | 20–80 | 0.25 |
| ISA 720 | 150 | 8 | 10 | 10–20 | 8 | 10 | 0–60 | 0.22 |
| | 300 | 8 | 20 | 10–20 | 7 | 20 | 10–20 | 0.91 |
| | 6 | 320 |
| | | 4 | 640 |
| | | 25 | Negatives |

*Anti-\( P. \) vivax sporozoite antibodies titers of 50 adults from malaria endemic regions.
†Values are reciprocal dilution titers.
‡IQR = interquartile ratio. Two-tailed, Wilcoxon signed-rank test non-parametric \( P \) value, \( H_\text{2nd} \) 2nd = 3rd.
and moderate local reactogenicity; however, a trial conducted with a malaria vaccine candidate formulated in this adjuvant had to be suspended because of the presence of SAEs.

In this study, all formulations were immunogenic inducing both, specific anti-peptide antibodies and IFN-γ production. Importantly, even low LSP vaccine doses were enough to induce sero-conversion in 95% of the volunteers and sera from 86% of the participants recognized the native protein in sporozoites. Although IFAT titers may appear low, the high seroconversion is encouraging because in field conditions only a limited number of the individuals continuously exposed to P. vivax recognize sporozoites, even if they are continuously exposed to the complete mass of parasite proteins. Interestingly, similar antibody titers were indicated in a previous trial to induce significant in vitro sporozoite invasion inhibition.

Throughout our study, antibody responses to the N peptide developed a more homogeneous profile and were consistently stronger than those to the other peptides. As expected most volunteers produced anti-R-peptide antibodies only after a boosting dose on Month 4, thereafter they followed a trend comparable to that produced by peptide N. Similarly to our previous study, volunteers required one or two boosting doses to respond to the C-terminal peptide. Therefore, it seems that higher doses are necessary for responses to peptides R and C to reach a similar degree of maturity than induces peptide N. This may indicate the importance of attempting an even better balance among the different specificities that may be important for the protective capacity of the vaccine.

In general, antibody responses were similar in their pattern and titers to those obtained in volunteers immunized previously with individual peptides, and in preclinical trials in Aotus monkeys vaccinated with the same vaccine formulations. Moreover, titers were higher than those observed in sera of most people from P. vivax endemic areas of Colombia.

As we expected, IgG1 isotype profile response was different for each peptide. The N peptide showed increased titers for all IgG isotypes, compared with C peptide that showed more defined IgG1 and IgG3 responses. This is certainly of interest, because of reports indicating that these are the main isotypes involved in protection against malaria infection, that IgG4 is non-protective and that IgG2 is not only non-protective but could even compete and interfere with the protective isotypes.

Recognition of sporozoites by anti-peptide antibodies is encouraging because of the potential boosting effect of sporozoites on the humoral response upon natural exposure in endemic areas and the greater possibilities of a functional role of antibodies in blocking parasite invasion to hepatocytes. Although anti-sporozoite antibody titers were low, it has not yet been defined what antibody levels are needed for protection. Indeed, similar antibody titers were able to block in vitro parasite invasion. Furthermore, the fact that Montanide ISA 51 elicited a more vigorous anti-sporozoite response than Montanide ISA 720 formulations should be considered when moving forward in designing further clinical trials with these peptides.

Specific induction of IFN-γ production by T cells by all peptides but mainly by peptide N, particularly when formulated in Montanide ISA 51, is consistent with the higher antibody response; peptide N may contain stronger T-cell epitopes than in R and C peptides. Recently, the N-terminal region of the P. falciparum CS in combination with the C-terminal region had a better ability to inhibit sporozoite invasion to hepatocytes and recognition of short N-terminal peptide (PfCS65-110) by sera from children living in a malaria-endemic region was associated with protection from disease.

In conclusion, vaccination of human volunteers with a combination of LSP derived from the P. vivax CS protein formulated Montanide ISA 720 or Montanide ISA 51 is safe, well tolerated, and immunogenic. Peptides formulated in Montanide ISA 51 produced better anti-sporozoite recognition antibody response, suggesting that the choice of adjuvant for the formulation of these vaccines is an important variable to be considered in future research. This study confirms LSP as a safe and immunogenic antigen. The recent development of a P. vivax challenge model for humans creates ideal conditions to assess these antigens in phase II studies (Herrera and others, 2011, in press).

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