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Consistent Safety and Infectivity in Sporozoite Challenge Model of *Plasmodium vivax* in Malaria-Naive Human Volunteers

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Abstract. A safe and reproducible *Plasmodium vivax* infectious challenge method is required to evaluate the efficacy of malaria vaccine candidates. Seventeen healthy Duffy (+) and five Duffy (−) subjects were randomly allocated into three (A–C) groups and were exposed to the bites of 2–4 *Anopheles albimanus* mosquitoes infected with *Plasmodium vivax* derived from three donors. Duffy (−) subjects were included as controls for each group. Clinical manifestations of malaria and parasitemia were monitored beginning 7 days post-challenge. All Duffy (+) volunteers developed patent malaria infection within 16 days after challenge. Prepatent period determined by thick smear, was longer for Group A (median 14.5 d) than for Groups B and C (median 10 d/each). Infected volunteers recovered rapidly after treatment with no serious adverse events. The bite of as low as two *P. vivax*-infected mosquitoes provides safe and reliable infections in malaria-naive volunteers, suitable for assessing antimalarial and vaccine efficacy trials.

INTRODUCTION

Plasmodium vivax is the second most common *Plasmodium* species causing human malaria worldwide, and it is the most common species in most endemic areas outside Africa.[1] Limited succes of classic malaria control measures has prompted the search for vaccines and because of the epidemiological importance of *P. falciparum*, which is responsible for ~80% of the malaria cases globally, greater efforts have been invested in this parasite species than in *P. vivax.* However, progress is also being achieved in the development of *P. vivax* subunit vaccines. Two candidates, one based on the circumsporozoite (CS) protein and another based on the oocyst/ookinete *Pv*s25 protein, have been tested in phase I clinical trials.[2–4] Recent phase I clinical trials conducted using different formulations of *P. vivax* CS-derived subunit vaccines based on long synthetic peptides (LSP) have indicated that such formulations are safe, well tolerated, and immunogenic in malaria-naive volunteers. Additionally, an *Escherichia coli* recombinant chimeric full-length molecule of the *P. vivax* CS has also been recently reported. Sera from individuals naturally exposed to malaria in endemic areas and from immunized mice displayed high antibody titers to the recombinant protein. This construct is also being considered as a vaccine candidate and being proposed for further clinical trials.[5] During the last few years we have been developing a *P. vivax* challenge model because assessing the protective efficacy of *P. vivax* malaria vaccines requires a safe, reliable, and reproducible method of infecting human volunteers with sporozoites.[6] A sporozoite challenge model has been available for *P. falciparum* for several decades and has led to significant progress in vaccine development for this species, including extensive immunological analyses of volunteers exposed to *P. falciparum* irradiated-sporozoite immunizations.[6–8] A similar model for *P. vivax* is more demanding because in contrast to *P. falciparum* where continuous *in vitro* cultures allows regular production of mature, infective gametocytes,[9–11] the lack of *P. vivax* cultures imposes the need to use blood from patients with *P. vivax* infection carrying mature gametocytes capable of infecting adult mosquitoes. Besides the logistical difficulties, this model has the risk of greater variability because every batch of infected mosquitoes is derived from a new donor harboring a different parasite population.

We recently conducted a first *P. vivax* sporozoite challenge trial in Colombian malaria-naive volunteers that were exposed to the bite of 2–10 *Anopheles albimanus* mosquitoes experimentally infected by artificial membrane feeding with blood from a single infected patient.[12] Most volunteers (17/18) became infected and showed a relatively narrow range of prepatent periods (9–13 d, mean 10.6 d). Individuals were treated immediately after peripheral blood smears became positive by microscopy and all of them responded rapidly to the antimalarial treatment without developing any severe or serious adverse events (AEs). Herein, we describe the reproducibility of this infectious challenge system by using parasite isolates derived from three different infected blood donors. For these repeat challenges, we selected the "minimal doses" (2–4 bites) of infected mosquitoes for the challenge because in the previous study 6/6 volunteers challenged with this dose became infected. The volunteer who did not become infected in the first challenge trial was in the high dose (8–10 bites) group and it was suspected that this individual may have received autoprescribed anti-malarials.[12]

MATERIALS AND METHODS

Study participants. Twenty-two healthy, malaria-naive subjects (19–45 years of age) participated as challenge volunteers; 17 of them were Duffy positive (Fy+) and the remaining five were Duffy negative (Fy−). As the Duffy antigen is the binding site enabling the invasion of *P. vivax* merozoites into human erythrocytes, Fy− individuals are refractory to *P. vivax* infection. Thus, this group of five volunteers served as negative controls. A total of 18 *P. vivax*-infected patients served as parasite donors. All participants were recruited after the protocol was approved by the Ethics Committee of the Universidad del Valle and the Fundación Clínica Valle del Lili. The trial complied with the ICH E-6 Guidelines for Good Clinical Practices.

During recruitment, the risks of participation, including the risk of exposure to mosquito bites, the symptoms associated to

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14. ABSTRACT

A safe and reproducible Plasmodium vivax infectious challenge method is required to evaluate the efficacy of malaria vaccine candidates. Seventeen healthy Duffy (+) and five Duffy (−) subjects were randomly allocated into three (A?C) groups and were exposed to the bites of 2?4 Anopheles albimanus mosquitoes infected with Plasmodium vivax derived from three donors. Duffy (−) subjects were included as controls for each group. Clinical manifestations of malaria and parasitemia were monitored beginning 7 days post-challenge. All Duffy (+) volunteers developed patent malaria infection within 16 days after challenge. Prepatent period determined by thick smear, was longer for Group A (median 14.5 d) than for Groups B and C (median 10 d/each). Infected volunteers recovered rapidly after treatment with no serious adverse events. The bite of as low as two P. vivax -infected mosquitoes provides safe and reliable infections in malaria-naive volunteers, suitable for assessing antimalarial and vaccine efficacy trials.

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P. vivax malaria infection including the risk of relapses and the risks and discomfort of antimalarial therapy, were explained to each prospective volunteer. Study participants were provided ample opportunity to read the consent forms, to ask questions to the investigators, and were encouraged to consult with family and friends. All volunteers had to pass an oral or written exam concerning the trial and its risks before signature of the written consent. Separate consents were obtained from each volunteer for human immunodeficiency virus (HIV) screening and for enrollment. Participants were allowed to withdraw voluntarily from the study at any time. Individuals were excluded if they had abnormal laboratory test values or had any conditions that would increase the risk of an adverse outcome, as described in a previous report.[12]

Study design. This study was designed as a randomized, open-label clinical trial with the objective of determining the reproducibility of the *P. vivax* sporozoite challenge model in malaria-naive volunteers previously established by our group.[12] We aimed at specifically determining the reproducibility of the infection using a total challenge dose of 2–4 bites of *An. albimanus* mosquitoes by infecting different batches using the blood from several different *P. vivax*-infected donor patients. This study followed the same protocol used in our previous trial, which consisted of two steps: Step A was to produce mature, infective *P. vivax* sporozoites suitable for inoculation into humans from several donors;[12] and step B was to assess the safety and reproducibility of the sporozoite challenge by using these sporozoite-infected mosquitoes to challenge three groups of volunteers.

For step A, patients attending an outpatient clinic at the Immunology Institute (IDIV) in Buenaventura (Colombia) for a febrile illness, were tested microscopically for malaria diagnosis using both thick and thin blood smears (TBS) and if confirmed to harbor *P. vivax* infection, they were asked to participate by donating 30 mL of blood. From these patients (parasite donors) whole blood was screened for co-infections that could potentially represent a threat to the health of volunteers, and a blood aliquot was used to feed *Anopheles* mosquitoes using an artificial membrane feeding system.[13] For step B, three groups of malaria-naive subjects were exposed to the bites of 2–4 infected mosquitoes randomly obtained from larger batches infected from three different parasite donors. The prepatent period was measured for each group and treatment was provided to all infected individuals.

Blood donation and blood quality assurance. Thirty mL of whole blood were collected from patients attending the outpatient malaria clinic, presenting with parasitemia ≥ 0.1% and who signed an informed consent for the use of their blood. Blood samples were collected using Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing EDTA, heparin, or no anticoagulant and were divided into three aliquots: a 15 mL sample for mosquito feeding (Hep), 10 mL (EDTA) and a 5 mL (without anticoagulant) samples for routine screening for a panel of common infectious agents (viral, bacterial, parasitic) including confirmation of *Plasmodium* species (*P. vivax*, *P. falciparum, P. malariae*) by polymerase chain reaction (PCR).[14] Blood screening was performed at the blood bank of the Valle del Lili Clinic in Cali, following the same protocol used for blood donations to the blood bank, and mosquitoes were discarded using a biosafety procedure if found to have fed on blood determined to have any co-infection. The blood screening tests are described in detail in the previous publication.[12]

Mosquito infection. Two *An. albimanus* mosquito colonies were available for the study, one in Cali, a non-endemic area, and the other in Buenaventura, the endemic area where blood donor patients were recruited. Several lots of 4,000 adult mosquitoes (3–4 d old), reared as described before, were fed within 2–3 hours after the *P. vivax*-infected blood was collected.[13] The 15 mL blood to be used for mosquito feeding were centrifuged at 500 × *g* for 5 minutes at room temperature, plasma was removed, and cells were washed once with RPMI 1640 medium (Gibco Cell Culture Systems; Invitrogen, Grand Island, NY). The erythrocyte fraction was reconstituted to 50% hematocrit using a human AB non-immune, complement-inactivated serum pool, obtained from the Red Cross blood bank, and was provided to mosquitoes using a water-jacketed membrane feeding apparatus at 37°C.[15] Fed mosquitoes were maintained under strict biosafety conditions (locked, restricted access insectary) at 27 ± 1°C and relative humidity of 82% and were fed a sugar solution supplemented with 0.05% para-aminobenzoic-acid.[16] To ascertain mosquito infections, samples of fed mosquitoes were dissected and microscopically examined 7–8 days after the blood meal to determine the presence of oocysts in the midgut, and 14 days after the blood meal to assess the sporozoite load in salivary glands. Mosquito infections were graded as 1+ (1–10 spz), 2+ (11–100 spz), 3+ (101–1,000 spz), and 4+ (> 1,001 spz), similar to the grading system used for *P. falciparum*.[11,17]

Sporozoite challenge. On the basis of our previous trial where three different infective biting doses appeared capable of inducing malarial infection (~3, 6, or 9 bites), we designed this second study to determine the capability of the lowest infective biting dose previously tested (3 ± 1) using mosquitoes fed with different parasite isolates.[13] For this purpose, we selected three different mosquito lots fed on blood from three different parasite donors, each of which fulfilled the condition of having > 50% of mosquitoes infected with sporozoites. For the infectious challenge we used screen-meshed boxes (7 × 7 × 7 cm) filled with four mosquitoes, to have a better chance of achieving the targeted mosquito dose in a single biting round.[18] Participants who did not complete the minimal targeted dose (two bites) within the first biting round were subjected to a second one to complete the target dose. A mosquito bite was considered infectious if the mosquito was scored with at least 1+ of spz load (1–10 spz). Although 24 malaria-naive volunteers had been randomly assigned to one of three groups (N = 8) during recruitment, one of the Fy+ volunteers and one Fy– declined their participation the day before challenge (Group C). Therefore, this group (Group C) was composed of only six volunteers.

Sporozoite challenge was carried out under strict adherence to experimental protocol in a secure room in the entomology unit at the IDIV. Volunteers were asked not to use any topical chemicals (e.g., soap, deodorant, perfume) that could influence mosquito feeding. Mosquitoes were allowed to bite the flexor side of the forearm for a 5-minute period, previously determined to be sufficient for full *An. albimanus* engorgement.[18] After biting, all mosquitoes were dissected to confirm the presence of blood meal and sporozoites. Study participants were followed up at 1, 8, and 24 hr after challenge to assess their response to mosquito bites and parasite challenge.

Malaria diagnosis and patient follow-up. For malaria diagnosis, volunteers had daily follow-up visits from Day 7 post-challenge onward. During these visits symptoms

and signs of malaria were assessed and blood for TBS and *Plasmodium* PCR was collected. The TBS were stained by Giemsa staining and were read by experienced microscopists as described elsewhere.[18] The PCR was performed later for retrospective analysis. As soon as parasites became detected by TBS, participants were treated with chloroquine (1,500 mg chloroquine base provided orally in divided doses: 600 mg initially followed by 450 mg given 24 and 48 hr later) and primaquine (30 mg daily for 14 d), administered directly by the medical team. The grade of severity was scored using National Cancer Institute (NCI) common terminology criteria for AEs, 1–5 as follows: Grade 1 = mild, Grade 2 = moderate, Grade 3 = severe, Grade 4 = life-threatening or disabling AE, and Grade 5 = death. All the volunteers were followed up during a period of 1 year to look for possible relapses or AEs.

Clinical laboratory tests. A comprehensive clinical laboratory screening including G6PD status, similar to that described in our previous study, confirmed the health status of the selected naive volunteers within 1 month before challenge.[12] All individuals underwent electrocardiographic examination to help exclude individuals with pre-existing heart disease out of concern that a febrile illness might pose risks for these individuals. Tests for hemoglobin, white blood cell count, platelet count, and total bilirubin were performed again on Days 9–16 and Day 53 post-challenge.

Statistical methods. Sample size of Fy+ subjects ($N = 6$ per group) was based on the minimum number of individuals that would allow to observe the occurrence of rare events (e.g., events that occur in ~5% of individuals) with reasonable probability based on a binomial assumption. Each group was meant to include two Fy– volunteers to have a total of eight volunteers. Prepatent periods and duration of symptoms were expressed as geometric means. Probability of infection and of clinical symptoms was estimated using the Kaplan-Meier failure function. The log-rank test was used to reject null hypothesis of equality of failure functions between the groups. Furthermore, differences of median values among the groups were estimated by the Kruskal-Wallis test. All tests were two-tailed and considered statistically significant at P values less than 0.05.

RESULTS

Study population. A total of 22 of the expected 24 volunteers completed the study. Seventeen Fy+ volunteers (8 men, 9 women) with a mean age of 26.8 years received sporozoite challenge. They were randomly assigned to three groups: two groups (A and B) of 6 volunteers each and a group (C) of 5 volunteers. Five Fy– volunteers were considered negative controls an where allocated, 2 in each A and B groups and 1 in Group C (Table 2). No age differences among groups were observed (data not shown).

Mosquito infection. The 18 infected donors from whom blood was obtained had parasite densities ranging from 2,800 to 33,920 (mean 9,069) asexual-phase parasites/μL and gametocytemia ranging from 240 to 2,080 (mean 1,082) parasites/μL. From the 18 mosquito lots exposed to infected blood, 7 lots were discarded because of blood co-infection, and 4 lots because of low sporozoite infection; the other 7 lots with > 50% of the mosquitoes showing oocysts were considered useful for challenge.[18] On the day before challenge, 3 mosquito lots that were arbitrarily selected from 7 eligible mosquito lots, were examined microscopically and showed the presence of sporozoites in 94.7%, 84.2%, and 55.3% of the mosquitoes in each lot, respectively.[18]

TABLE 1
Local adverse events (AEs) related to mosquito bite

	Group			Total
Local AEs	A ($N = 8$) n*	B ($N = 8$) n	C ($N = 6$) n	($N = 22$) n
Pruritus	6	2	4	12
Swelling	0	2	0	2
Erythema	4	5	3	12

*Number of events.

Sporozoite challenge. Each group (A–C) of volunteers was exposed to mosquito biting on a different day and all three groups completed the feeding process and biting assurance within a period of 36 to 54 minutes. Most of the volunteers (18/22) completed the biting dose in a single biting cycle, 3 in 2 cycles and the last one in 3 cycles (Table 2). Local AEs are shown in Table 1. Pruritus and erythema that disappeared within 2 days were the most frequent local AEs reported by the participants.

Prepatent period and clinical follow-up. All 17 Fy+ volunteers developed malaria as confirmed by TBS and PCR. Patent parasitemia was confirmed between 9 and 16 days (median: 12 d) after the mosquito bites. For Group A median prepatent period (14 d, range: 12–16 d) was longer than for Group B (10 d, range: 9–16 d), and Group C (10 d, range: 10–12 d) (median test = 0.03). Prepatent periods are shown in Figure 1. Parasitemia at the time of microscopic diagnosis ranged from 80 to 480 parasites/μL (Table 2). As expected none of the Fy– volunteers developed signs or symptoms compatible with malaria and all of them had negative TBS and PCR during follow-up.

All 17 infected volunteers developed signs and symptoms consistent with malaria infection between Days 8 and 15 post-challenge. The most frequent symptoms reported were, in descending order, malaise, headache, chills, fever, myalgia, weakness, and arthralgia. Fever (body temperature ≥ 38°C) was documented in 15 volunteers, and the first registered day of fever was similar to that of the initiation of other symptoms (Table 3). Figure 2 indicates the relationship between time of appearance of patent parasitemia and appearance of salient symptoms (fever, malaise, and headache) and the relationship between day of TBS positivity and severity of the same symptoms.

All individuals cleared parasitemia between 24 and 48 hr after initiating antimalarial treatment, and all successfully recovered within 2–3 days without any severe or serious AE. None had to be hospitalized because of the infection, although one volunteer attended to the emergency service because she had an anxiety crisis 1 day after the initiation of antimalarials. The most frequent symptoms associated with treatment were, nausea, diarrhea, abdominal (epigastric) pain, and dizziness that remained for up to 4 days. In addition, seven patients developed blurred vision that lasted for 2 days after treatment initiation (data not shown). Although Fy– participants did not become infected, they were treated with a full course of chloroquine and primaquine, initiated 1 month after the day of challenge, in accordance with the institutional review board (IRB) approved research protocol guidelines. One of the volunteers corresponding to Group C developed malaria signs and

TABLE 2
Plasmodium vivax infected mosquito challenge, prepatent period and malaria diagnose

Group	Participant	Number of mosquito rounds	Total number of mosquitoes used	Total number of biting	Number of infective bites received per subject	Prepatent period (days)	Parasite density (mL)	Duffy phenotype
A	1	2	5	3	2	13	160	+
	2	2	5	2	2	15	160	+
	3	1	5	5	4	–	–	–
	4	2	5	4	2	16	160	+
	5	1	4	3	2	13	160	+
	6	1	4	4	4	12	480	+
	7	2	7	7	2	–	–	–
	8	1	4	4	2	15	400	+
B	1	1	3	3	3	10	160	+
	2	3	8	3	2	16	80	+
	3	1	3	2	2	10	80	+
	4	1	3	2	2	10	80	+
	5	1	3	2	2	–	–	–
	6	1	3	3	2	9	80	+
	7	1	3	3	3	10	160	+
	8	2	5	2	2	–	–	–
C	1	1	3	3	3	12	160	+
	2	1	3	3	3	10	80	+
	3	2	6	4	3	10	80	+
	4	1	3	3	2	10	80	+
	5	1	3	3	3	–	–	–
	6	1	3	2	2	12	320	+

symptoms 2 months after treatment that were diagnosed as *P. vivax* by the TBS method. This episode was treated and the patient recovered completely. Although this volunteer did not accept having visited the endemic area, microsatelite analyses indicated that this second infection was due to a different *P. vivax* parasite isolate.

Clinical laboratory follow-up. Clinical laboratory follow-up is shown in Table 4. All participants showed a decrease in lymphocyte counts, with lymphocytes < 1,200 in 15; no differences were noted among groups. None of the participants presented neutrophil count < 1,500. Platelet counts presented a decrease as compared with values recorded before challenge. None of the volunteers presented low hemoglobin compared with their initial values or increased values of reticulocytes. Concerning blood chemistry, the most frequent finding was a low level increase in transaminases values (> 40 U/L) in four of the volunteers, and a small increase in alkaline phosphatase values. No alterations in the renal function tests were registered.

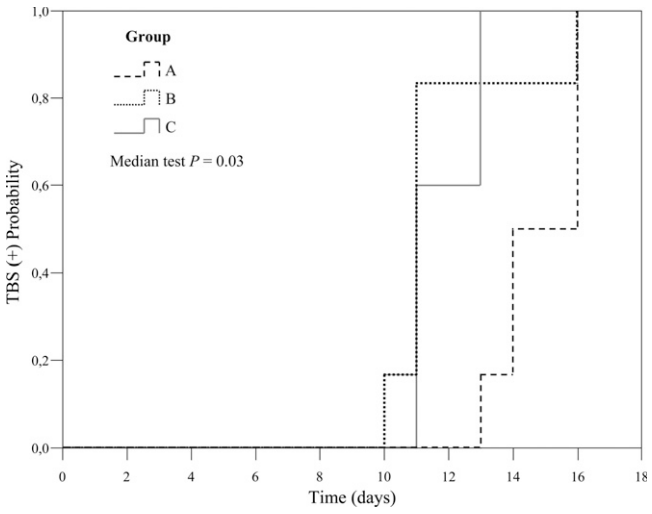

FIGURE 1. Probability of thick blood smear (TBS) positivity after *Plasmodium vivax* malarial challenge by group.

DISCUSSION

We showed that malaria-naive human volunteers can be safely and consistently infected by bites of as low as two *An. albimanus* mosquitoes carrying sporozoites from different *P. vivax* wild isolates. In a previous study conducted with different mosquito bites (2–10 bites/dose), we showed that the prepatent period (9–13 d) was independent of the mosquito biting dose.[12] Together in these two studies a total of 34 out of 35 Fy+ volunteers were successfully infected with these doses, 24 of them with low biting doses (2–4 bites). These results are in contrast with those found with *P. falciparum* where previous reports indicate an inverse relationship between the number of mosquito bites and both the prepatent period and the reproducibility of the infection. In those studies, sporozoites inoculated by < 5 mosquitoes led to an irregular infection in malaria-naive human volunteers.[19–22] This appears to be a significant difference between the two parasite species because most studies reported with *P. falciparum* sporozoites have used highly efficient and heavily infected vector mosquitoes, whereas here we have not selected any particularly high sporozoite load and *An. albimanus* is considered not to be an efficient vector.[23] Although recent reports indicate that under certain conditions, Fy– volunteers exposed to *P. vivax* infection in nature become infected, we did not expect to have Fy– developing the blood parasite cycle; the previous finding appears to be rather exceptional.[26]

Additionally, because of the establishment of the NF54 *P. falciparum* isolate in culture and the isolation of several parasite clones, human challenge trials have been performed using laboratory cultured parasites, which allow greater reproducibility and ensure high safety standards for the volunteers.[20] The lack of *P. vivax* cultures or gametocyte cryopreservation systems does not allow the possibility of using *P. vivax* master cell lines for mosquito infection and challenge; therefore

TABLE 3
Symptoms and signs related to *Plasmodium vivax* infection

Events associated with malarial infection	Total ($N = 17$)	Groups								
		A ($N = 6$)			B ($N = 6$)			C ($N = 5$)		
			Duration			Duration			Duration	
		n	First day*	Median (days)	n	First day*	Median (days)	n	First day*	Median (days)
Clinical signs and symptoms										
Malaise	17	6	12	4.0	6	9	5.0	5	10	4.0
Headache	16	5	12	3.0	6	10	2.5	5	10	3.5
Chills	15	6	13	3.0	4	11	1.7	5	10	4.0
Myalgia	15	6	12	3.0	5	10	3.0	4	10	3.0
Temperature ≥ 38°C	15	6	13	2.0	5	9	1.6	4	10	2.0
Weakness	15	6	12	3.0	5	10	3.0	4	10	3.5
Arthralgias	13	6	13	1.8	3	11	1.6	4	10	2.0
Dehydratation	9	4	13	1.5	3	9	1.3	2	10	2.0
Nausea	5	5	12	2.0	0	–	–	0	–	–
Diaphoresis	3	1	14	2.0	1	9	3.0	1	11	1.0
Ocular pain	1	0	–	–	0	–	–	1	10	2.0
Pallor	1	1	13	2.0	0	–	–	0	–	–

*Day in which the event was first registered.

we have to face the risk of trial-to-trial variability. Although safety of *P. vivax* isolates directly derived from a human donor might generate some concern, there is as yet no other pathogen from human origin different *Plasmodium* known to be transmitted by *Anopheles* mosquitoes in Colombia; additionally, the comprehensive blood bank screening would ensure at least a similar standard as that of blood transfusion.[12]

Because the main interest in developing a *P. vivax* infection model based on sporozoite inoculation is to use it for assessing the protective efficacy of malaria vaccines or antimalarial drugs, we have tried to develop a mosquito challenge that mimics natural transmission as closely as possible. Most endemic regions have inoculation rates ranging from 0.6 to 814 bites/year, which would indicate that the maximal theoretical biting dose per day would be 0.002–2.23 bites every day.[26] Here, we have induced reproducible infections with two mosquito bites/hr, which is therefore close to the maximal biting intensity in nature. Additionally, because *An. albimanus* mosquitoes usually develop weaker infections than other Anopheline species, as determined by lower oocysts counts in midguts and lower sporozoite loads, it is likely that this species inoculates fewer sporozoites during a single blood meal than mosquitoes from species such as *An. gambiae, An. stephensi* and *An. dirus* that are more robust vectors;[15,18,27–29] however the number of sporozoites inoculated by a single mosquito bite appears to be relatively standard (1–50 spz/bite) independently of the mosquito sporozoite load.[30–32] On the basis of the range of mosquito bites estimated in endemic areas, we believe that the two mosquito bites that have shown to be reproducible sufficient for infection of naive volunteers in studies[18] is the maximal amount desirable, and therefore the five mosquito bites required to infect naive volunteers with *P. falciparum* might correspond to an overwhelming simultaneous sporozoite dose to assess the efficacy of *P. vivax* malaria vaccines.

Another striking feature of this study was the reproducibility in the development of prepatent periods. In every experimental group, individuals developed prepatent periods determined by TBS, within a range of 9–16 days. Together this study and our previous one indicate that four groups ($N = 23$) developed a similar prepatent period (mean of 4 groups 11.8 d), whereas only one group ($N = 6$) developed a significantly longer prepatent period (median 14.2 d).[12] This could be explained by as yet undetermined biological differences in wild parasite isolates,[33–35] because we were not able to identify any other correlates, including characteristics of the donor infection (density of asexual or sexual forms), the number of mosquito bites, or the oocyst or sporozoite burden of the mosquitoes. Recently, a significant difference in the total messenger RNA (mRNA) levels was found between different *P. vivax* isolates by transcriptome analysis,[36] and this might possibly reflect biological differences that could impact a prepatent period. Additionally, as every single *P. vivax* isolate may contain multiple genetically diverse clones,[34,37] there is a possibility to use microsatellite analyses to determine the genetic polymorphism.[38] We

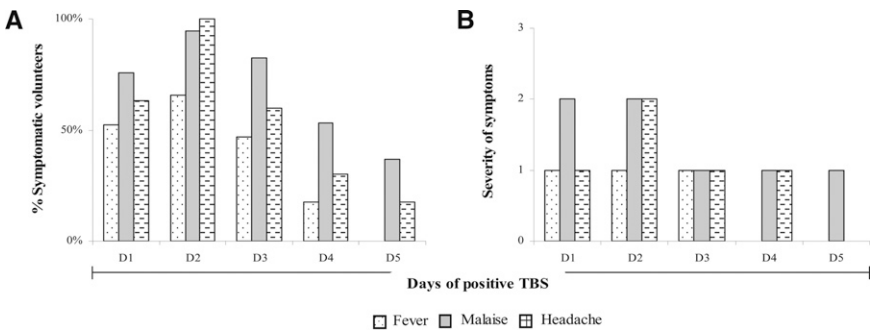

FIGURE 2. (**A**) Relationship between times of patent blood stage infection as determined by thick blood smear (TBS) and salient symptoms. (**B**) Relationship between day of TBS positivity and severity of symptoms.

TABLE 4
Clinical laboratory values before and after challenge

Laboratory values	Basal values (Day –30)			After challenge values (Day 10–16)		
	Mean	95% CI		Mean	95% CI	
Hematological values						
Hemoglobin (g/dL)	15.1	14.4	15.1	15.3	14.6	16.0
Reticulocytes (%)	0.7	0.6	0.8	0.8	0.6	0.9
WBC counts ($\times 10^3/mm^3$)	7.0	6.3	7.7	5.3	4.4	6.1
Lymphocyte counts ($\times 10^3/mm^3$)	2.0	1.8	2.1	0.9	0.7	1.0
Granulocyte counts ($\times 10^3/mm^3$)	4.4	3.8	5.0	4.0	3.2	4.8
Platelet counts ($\times 10^3/mm^3$)	227.0	203.8	250.2	181.2	163.4	198.9
PT* (sec)	10.9	10.6	11.1	11.2	11.0	11.4
PTT (sec)†	27.4	26.4	28.5	28.3	27.5	29.1
Biochemical values						
Total bilirrubins (mg/dL)	0.7	0.6	0.8	0.7	0.6	0.8
ALT‡ (U/L)	16.1	13.0	19.1	28.9	20.3	37.5
AST§ (U/L)	22.1	19.4	24.9	31.8	23.8	39.8
Alkaline phosphatase (U/L)	183.1	160.0	206.2	220.4	200.6	240.1
Creatinine (mg/dL)	1.1	1.0	1.2	1.2	1.1	1.3
BUN¶ (mg/dL)	11.9	9.6	14.3	11.6	10.4	12.8
Glycemia (mg/dL)	93.2	88.3	98.2	94.1	86.4	101.8

* Prothrombin time.
† Activated patial thromboplastin time.
‡ Alanine aminotransferase.
§ Aspartate aminotransferase.
¶ Blood urea nitrogen

are currently genotyping the *P. vivax* isolates used in the two trials conducted so far, to determine the potential association between genetic compositions and prepatent periods.

Although prepatent periods in the 17 infected Fy+ volunteers in this trial varied between 9 and 16 days, all developed malaria symptoms between Days 8–15, post-challenge. Similar to our previous study, fever was not as frequently recorded as had been expected and when it occurred, it was not an early symptom.[12] Over half of the individuals presented with their first febrile episode after Day 10. Malaise, headache, chills, myalgia, and weakness occurred more frequently and appeared starting on Day 9 post-challenge.

Because of prompt diagnosis, there were no significant differences in parasite density (75–420 parasites/μL) among the three groups, and no relationship between parasitemia and malaria symptoms was observed. Parasitemia was cleared in most cases (13/17) within the first 24 hours. Decrease in lymphocyte and platelet counts at diagnosis were consistent findings among participants. Although lymphopenia is a well-established feature of *P. falciparum* malaria in humans, it is not well described in *P. vivax* infection. No alterations were seen in hemoglobin levels as expected taking into account the short duration of parasitemia.

In conclusion, this infection model is safe and reliable and therefore suitable for vaccine testing. The narrow prepatent window should allow the use of relatively small experimental groups to determine differences between controls and immunized volunteers who develop partial protection (prolonged prepatent periods). Our model can allow immediate testing of both pre-erythrocytic and asexual blood-stage antigens in phase II trials and human vaccination with *P. vivax* irradiated sporozoites. Taking advantage of our proximity to malaria endemic areas, we are currently planning controlled, small size phase IIb vaccine studies where pre-immune individuals presently living in a non-endemic city (Cali) would be enrolled for vaccination and experimental challenge. Similarly, a trial to establish a model for *P. vivax* irradiated-sporozoite vaccination is being developed as a means to search for immune correlates of protection. Finally, the model would also be suitable to better characterize the infection susceptibility of both Fy– homozygotes and heterozygotes.[24,39]

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