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Controlled Human Malaria Infections by Intradermal Injection of Cryopreserved *Plasmodium falciparum* Sporozoites

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Abstract. Controlled human malaria infection with sporozoites is a standardized and powerful tool for evaluation of malaria vaccine and drug efficacy but so far only applied by exposure to bites of *Plasmodium falciparum* (Pf)-infected mosquitoes. We assessed in an open label Phase 1 trial, infection after intradermal injection of respectively 2,500, 10,000, or 25,000 aseptic, purified, vialed, cryopreserved Pf sporozoites (PfSPZ) in three groups ($N = 6$/group) of healthy Dutch volunteers. Infection was safe and parasitemia developed in 15 of 18 volunteers (84%), 5 of 6 volunteers in each group. There were no differences between groups in time until parasitemia by microscopy or quantitative polymerase chain reaction, parasite kinetics, clinical symptoms, or laboratory values. This is the first successful infection by needle and syringe with PfSPZ manufactured in compliance with regulatory standards. After further optimization, the use of such PfSPZ may facilitate and accelerate clinical development of novel malaria drugs and vaccines.

INTRODUCTION

Malaria caused by *Plasmodium falciparum* (Pf) causes approximately one million deaths and 250 million clinical cases annually.[1,2] Implementation of insecticide-impregnated bed nets, residual insecticide spraying, and combinations of anti-malarial drugs, has reduced malaria-associated morbidity and mortality in many areas.[1] Questions related to sustainability of this effort, however, have led to a recent delineation of requirements for new tools.[3,4] A safe, long-acting anti-malarial drug and a highly effective malaria vaccine would be powerful tools for control and elimination of Pf malaria.

Progress has been facilitated by the capacity to infect volunteers under controlled conditions to test new vaccines and drugs. Infection of volunteers by exposure to laboratory-reared *Anopheles* spp. mosquitoes transmitting Pf sporozoites (SPZ)[5] was first introduced for treatment of neurosyphilis in the 1920s.[6] The development of drugs such as chloroquine,[7] primaquine,[8] and atovaquone[9] were facilitated by these controlled human malaria infections (CHMIs). The ability to culture Pf gametocytes[10–12] enhanced the capacity to produce infected mosquitoes for CHMI studies. Although potentially serious, Pf malaria can be radically cured at the earliest stages of blood infection when risks are virtually absent. CHMIs are restricted to a few specialized centers that can produce PfSPZ-infected mosquitoes, where more than 1,300 volunteers have been safely infected by the bites of PfSPZ-infected mosquitoes since 1986, primarily for clinical trials of drugs[9] and malaria vaccines,[5,13–21] but also for trials of diagnostic tests,[22] and studying human immune responses to Pf.[23]

In addition to the use of CHMIs for testing vaccines and drugs, controlled infections can also be used to immunize against malaria. For example, immunization with radiation-attenuated PfSPZ by bites of mosquitoes protects > 90% of volunteers according to the published literature,[24–26] and recently 100% protection against CHMI was achieved by immunization of volunteers taking a prophylactic regimen of chloroquine, with PfSPZ administered by mosquito bites.[27,28]

These highly protective immunization strategies could not be translated into an implementable vaccine, because they depended on inoculation of SPZ by mosquito bites. Inoculation of SPZ by injection would be a more feasible method and was performed through the early 1950s. The SPZ preparations used, however, were heavily contaminated with bacteria and mosquito material, and rates of infection with frozen and thawed SPZ were highly variable.[29–33] A contemporary approach to production of SPZ for infection or vaccination requires generating aseptic SPZ-infected mosquitoes, purifying SPZ from mosquito tissues, vialing, preserving, and administering the SPZ by needle and syringe. Sanaria has met these requirements to produce infectious aseptic, purified, vialed, cryopreserved PfSPZ (PfSPZ Challenge), and produced and tested the world's first vaccine composed of these sporozoites.[34,35] Here, we report infection of volunteers with PfSPZ Challenge administered intradermally (ID) by needle and syringe.

MATERIALS AND METHODS

Study population and study design. This open label, Phase 1 clinical trial was performed at Radboud University Nijmegen Medical Center, the Netherlands, from October 2010 to July 2011. Volunteers 18–35 years of age were screened for eligibility by medical history, physical examination, and laboratory tests of blood, serum, and urine, including standard hematological, biochemical, and pregnancy tests, and malaria, human immunodeficiency virus (HIV), hepatitis B and hepatitis C serology. The main exclusion criteria were pregnancy; residence in a malaria-endemic area within the previous 6 months; positive Pf serology; symptoms, physical signs, or laboratory test results suggestive of systemic disorders; and history of drug or alcohol abuse interfering with normal social function. All volunteers gave written informed consent.

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Eighteen healthy malaria-naive volunteers were included in this trial. Groups of six volunteers were injected intradermally (ID) with 2,500, 10,000, or 25,000 PfSPZ Challenge. The sample size of six per group had a power of 75% to show a difference between 2 of 6 volunteers infected in the 2,500 PfSPZ group and 6 of 6 volunteers infected in the 25,000 PfSPZ group. Dose escalation was done at a minimum interval of 3.5 weeks.

The trial was performed in accordance with Good Clinical Practice and an Investigational New Drug application filed with the U.S. Food and Drug Administration, and approved by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO NL31858.091.10). Clinicaltrials.gov identifier: NCT 01086917.

Study intervention (PfSPZ Challenge). The PfSPZ Challenge contains aseptic, purified, cryopreserved PfSPZ isolated from salivary glands of aseptically reared mosquitoes.[34,35] *Anopheles stephensi* mosquitoes were raised under aseptic conditions, and then fed on cultured Stage V gametocytes of the NF54 strain of Pf.[36] Approximately 2 weeks later, mosquito salivary glands containing PfSPZ were dissected, and PfSPZ were purified, formulated, vialed (15,000 PfSPZ per vial), and cryopreserved in liquid nitrogen vapor phase at –140°C to –196°C.[34,35] The PfSPZ Challenge released for clinical use met quality control specifications including sterility (USP 71 compendial assay), purity (Supplemental Figure S1), and potency (Table 1).

Potency was assessed as previously described[34,35] by quantification of late liver stage parasites expressing Pf merozoite surface protein 1 (PfMSP-1)[37] in cultured human hepatocytes (HC-04 cells)[38] 6 days after addition of PfSPZ (Table 1, Supplemental Figure S2). For this 6-day hepatocyte potency assay, 4.0×10^4 HC-04 (1F9) cells/well in triplicate were infected with 5.0×10^4 PfSPZ and incubated for 6 days with daily media change. Late liver stage parasites expressing PfMSP-1 were counted by staining the slides with an anti-PfMSP-1 mAb and fluorescently labeled secondary antibody. As previously described[34,35] the membrane integrity of PfSPZ was used to assess cell viability (Table 1). For the sporozoite membrane integrity assay, propidium iodide and SYBR Green were added to 15,000 PfSPZ. PfSPZ were applied to a hemocytometer and incubated in a dark humidity chamber for 20 minutes, at which point the red PfSPZ (those with compromised membranes) and green PfSPZ (those with intact membranes) were counted under a fluorescent microscope. Those with intact membranes were considered viable, and viability is expressed as the percentage of total green PfSPZ over the total number of PfSPZ. Sporozoites was assessed before cryopreservation, for release of the lot, and to assess stability at defined time points after cryopreservation.

TABLE 1

Results of potency and sporozoite membrane integrity assays on the lot of PfSPZ Challenge used in this clinical trial

Time point	Potency (no. of parasites expressing PfMSP-1/well)	% Viability (sporozoite membrane integrity assay)
Fresh	27 ± 4.6	ND
Release	20 ± 1.7	83.3% ± 6.5%
6 Month	18 ± 2.1	86.6% ± 1.9%
9 Month	20 ± 2.1	83.7% ± 8.4%
12 Month	21 ± 1.5	84.8% ± 3.0%
18 Month	20 ± 0.6	83.7% ± 4.2%
24 Month	18 ± 1.0	86.0% ± 1.5%
Pre-1st clinical dose (26 Month)	17 ± 0.6	79.4% ± 6.5%
Post-last clinical dose (30 Month)	16 ± 2.6	87.4% ± 1.9%

Fresh PfSPZ used for the lot of PfSPZ Challenge used in this clinical trial produced 26% more PfMSP-1-expressing parasites in this assay than did PfSPZ that had been cryopreserved for several days (Release); at 30 months, several weeks after inoculation of the last volunteers the PfSPZ had a 40.7% reduction in potency by this assay as compared with fresh PfSPZ. There was no reduction in the results of the sporozoite membrane integrity of cryopreserved PfSPZ during 30 months of storage.

The sporozoite membrane integrity assay was not done on fresh PfSPZ for this particular lot. In our most recent three production campaigns for PfSPZ Challenge, fresh viability was 97.8%, 99.0%, and 98.2%, whereas after cryopreservation viability was reduced to 90.9%, 91.5%, and 87.4%, respectively, a mean reduction of 8.5%.

ND = not done.

The lot of PfSPZ Challenge used in this study had been cryopreserved in liquid nitrogen vapor phase for 27 (dose of 2,500 PfSPZ) to 30 months (dose of 25,000 PfSPZ) before administration. Immediately before use, a vial of PfSPZ Challenge was thawed and diluted with phosphate buffered saline containing human serum albumin. Volunteers were injected within 30 minutes of thawing.

CHMI. Three groups of six volunteers each were injected ID with PfSPZ Challenge over the deltoid muscle, one injection in each upper arm. Each injection of 50 μL contained half the total dose. After injection, volunteers were observed for at least 60 minutes. Inoculations of volunteers were spaced 60 minutes apart. In each dose group, two volunteers were inoculated 3 days before the remaining four volunteers.

Volunteers made at least one daily outpatient clinical visit beginning 5 days after inoculation of PfSPZ Challenge. All symptoms and signs (solicited and unsolicited) were recorded and graded by the attending physician as follows: mild (easily tolerated), moderate (interferes with normal activity), or severe (prevents normal activity); fever was recorded as grade 1 (> 37.5–38.0°C), grade 2 (> 38.0–39.0°C), or grade 3 (> 39.0°C). Hematological and biochemical parameters were monitored daily. Because of a previous cardiac-related serious adverse event (SAE) following CHMI with Pf infection,[39] markers of cardiac damage and coagulation were assessed. Troponin, lactate dehydrogenase (LDH), platelets, and D-dimer were assessed daily during the period when blood stage parasitemia was expected, and for 3 days after initiating curative treatment with atovaquone/proguanil. If D-dimer or LDH were abnormal, blood samples were tested for fragmentocytes and von Willebrand cleaving protease activity, as markers for vascular endothelial cell activation.[40] Final follow-up visits were on Days 35 and 140 after infection.

As soon as parasites were detected by microscopic examination of blood smears, volunteers were treated with atovaquone/proguanil (1,000/400 mg) administered orally once daily for 3 days. Complete cure was confirmed in all volunteers by two consecutive parasite-negative blood slides after treatment, at least 4 days apart. Volunteers who did not develop parasitemia by Day 21 after challenge were presumptively treated with the same regimen.

Outcomes. The primary outcome was occurrence of Pf parasitemia detected by microscopic examination of blood smears. Sampling was done twice daily on Days 5 and 6 post-inoculation, thrice daily on Days 7–11, twice daily on Days 12–15, once daily on Days 16–21, and for 2 days after initiation of treatment. To make thick blood smears, 15 μL of EDTA-anti-coagulated blood was spread on each well of a 3-well glass slide (CEL-LINE Diagnostic Microscope Slides, 30-12A-black-CE24, Braunschweig, Germany). After drying, wells were stained with Giemsa for 45 minutes, and examined at 1,000 × magnification to assess 0.5 μL of blood. The smear was scored as positive if two unambiguous parasites were

found. Thus, volunteers could be diagnosed with as few as 4 parasites/μL of blood. The pre-patent period was defined as the period between inoculation of PfSPZ Challenge and appearance of first positive blood smear.

Retrospectively, parasitemias were determined by real-time quantitative polymerase chain reaction (qPCR), performed on all samples collected after challenge, as previously described.[41] The sensitivity of qPCR was 20 parasites/mL of blood.

Statistical analysis. Data analysis was performed using SPSS software version 16.0. The qPCR results were assessed by analysis of variance (ANOVA) on log-transformed data.

RESULTS

Parasitemia after injection of PfSPZ Challenge. Thirty-six healthy, malaria-naive volunteers were screened and 18 were included. All volunteers completed follow-up (Supplemental Figure S3). After ID injection of PfSPZ Challenge, 15 of the 18 volunteers developed a positive blood smear for Pf, five of six volunteers from each group (Table 2). The slide-negative volunteers in each group were presumptively treated with atovaquone/proguanil at 21 days post-infection.

Blood slides were first positive 11 to 14.3 days after administration of PfSPZ Challenge. The geometric mean (GM) prepatent period was similar for all groups, i.e., 13.0, 12.7, and 13.0 days for the groups receiving 2,500, 10,000, and 25,000 PfSPZ Challenge, respectively (ANOVA $P = 0.92$). The GM parasite densities by microscopy at the time of diagnosis were 12.4, 11.2, and 23.4 parasites/μL blood (ANOVA $P = 0.69$ on log-transformed data) (Table 2).

Quantitative PCRs were first positive 9.0 to 12.0 days after challenge (Table 2). Volunteers in the 2,500, 10,000, and 25,000 PfSPZ Challenge groups had similar GM times to first detection of parasites by qPCR of 10.6, 10.3, and 9.9 days (ANOVA $P = 0.486$) at a GM parasite density of 0.07, 0.2, and 0.2 parasites/μL blood (ANOVA $P = 0.24$), respectively. The GM parasite densities by PCR at the time of thick smear diagnosis were 35, 5, and 132 parasites/μL (ANOVA $P = 0.23$). qPCR was negative throughout the 21-day follow-up for the three slide-negative volunteers. Parasite growth was cyclical, and was similar in all dose groups (Figure 1), and the parasite replication rate in the bloodstream was comparable to that seen after CHMI by exposure to the bite of PfSPZ-infected mosquitoes, ~11.5-fold every 48 hours.[42]

Safety. Local reactogenicity was not observed after ID administration of PfSPZ in any of the volunteers. All volunteers, including the three volunteers who did not develop parasitemia, reported solicited adverse events (AEs) considered possibly, probably, or definitely related to the trial procedures (clinical malaria) (Table 3). Headache was the most frequently reported AE, and occurred in all volunteers including the three who did not develop parasitemia. There were no significant differences among the groups in solicited AEs, which were most frequently reported between Days 12 and 18 post-injection. The percentage of volunteers with related grade 3 AEs was comparable to historical data from subjects subjected to CHMIs by mosquito-bites (44% versus 49%, respectively).[42] The total number of solicited and unsolicited AEs reported over time is shown in Figure 2. There were few AEs before Day 7; PfSPZ Challenge inoculations were well tolerated.

TABLE 2
Parasitemia data by thick blood smear and quantitative polymerase chain reaction (qPCR)

	Thick smear		qPCR		
Volunteer code	Pre-patent period (day)	Parasite density at diagnosis (Pf/μL)	qPCR positive (day)	Parasite density at first day positive (Pf/μL)	Parasite density by qPCR at time of diagnosis by thick smear (Pf/μL)
		Group 1: 2,500 PfSPZ			
696–18	12.3	4	9.6	0.08	5
711–08	14.0	16	12	0.16	71
795–06	N/A	N/A	N/A	N/A	N/A
935–01	14.0	124	10.6	0.03	89
937–20	12.3	6	10.6	0.12	43
940–14	12.3	6	10.3	0.06	35
Geom. mean	13.0	12	10.59	0.1	35
No. of positives	5/6		5/6		
		Group 2: 10,000 PfSPZ			
119–03	12.6	24	9.6	0.68	6
603–11	13.0	8	11	0.17	2
736–04	11.0	6	9.6	0.04	3
783–25	13.3	6	10.6	0.03	15
788–21	14.0	26	11	1.12	6
925–26	N/A	N/A	N/A	N/A	N/A
Geom. mean	12.7	11	10.34	0.2	5
No. of positives	5/6		5/6		
		Group 3: 25,000 PfSPZ			
647–30	14.0	512	9.3	0.32	759
720–13	12.3	6	10.3	0.32	162
789–15	N/A	N/A	N/A	N/A	N/A
806–09	12.3	8	9	0.25	48
909–29	14.3	48	11.3	0.13	102
926–24	12.3	6	10	0.19	68
Geom. mean	13.0	23	9.95	0.2	132
No. of positives	5/6		5/6		

N/A = not applicable; thick-smear negative volunteers were presumptively treated on day 21 after infection.

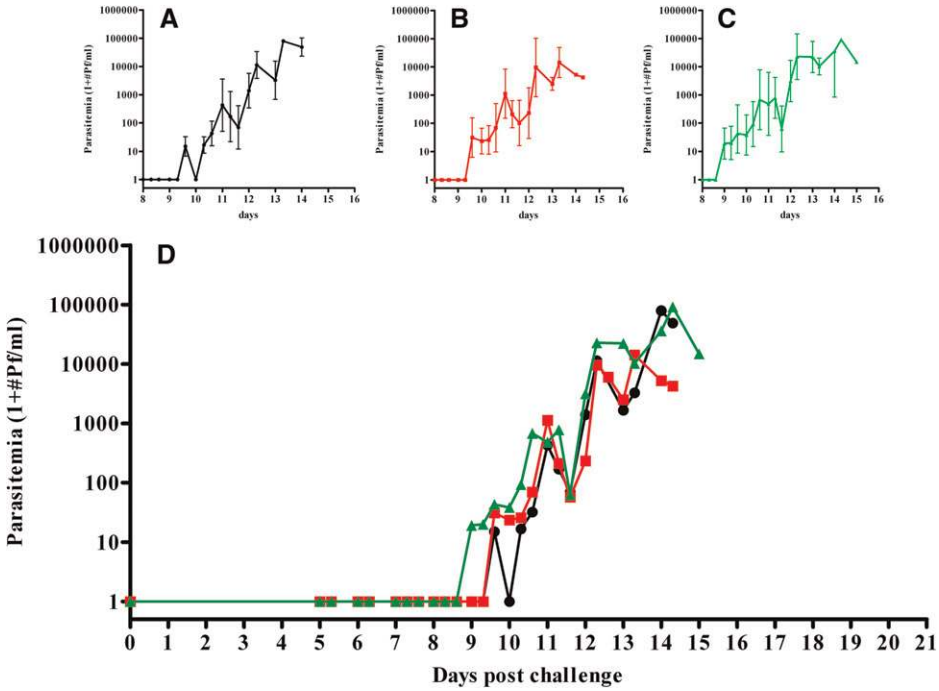

FIGURE 1. Parasite density as measured by quantitative polymerase chain reaction (qPCR) in the 2,500 (**A**), 10,000 (**B**), and 25,000 (**C**) PfSPZ Challenge dose groups. Panels A, B, and C show geometric mean parasite density of positive volunteers per group with confidence intervals ($N = 5$ for all groups) from day of inoculation through last day of positivity after initiation of treatment. Panel D shows an overlay of geometric mean parasite densities of positive volunteers in each group.

Routine daily laboratory tests showed no clinically significant abnormalities before initiation of anti-malarial treatment. Three or 4 days after receiving the first dose of atovaquone/proguanil, four volunteers had thrombocyte levels in the range 78–95 $\times$ 10^9/L, which was below the lower limit of normal (120 $\times$ 10^9/L). Leukocyte counts decreased after initiation of treatment in all thick smear positive volunteers (minimum 2.89 $\times$ 10^9/L compared with 5.46 $\times$ 10^9/L at baseline). In 13 volunteers, D-dimers were > 500 ng/mL, the upper limit of normal (ULN), at 1 or 2 days after initiation of anti-malarial

TABLE 3

Numbers of volunteers reporting solicited adverse events possibly, probably, or definitely related to administration of PfSPZ Challenge, with mean duration of events*

	2,500 PfSPZ ($N = 6$)		10,000 PfSPZ ($N = 6$)		25,000 PfSPZ ($N = 6$)	
Any adverse event	Number of volunteers	Mean duration ± SD (days)	Number of volunteers	Mean duration ± SD (days)	Number of volunteers	Mean duration ± SD (days)
Abdominal pain	1	2.9	1	0.04	2	0.3 ± 0.1
Arthralgia	0	N/A	0	N/A	0	N/A
Chest pain	1	0.04	0	N/A	0	N/A
Chills	1	2.0	2	0.3 ± 0.2	2	0.9 ± 0.6
Diarrhea	0	N/A	0	N/A	1	0.8
Fatigue	5	2.9 ± 3.3	3	2.5 ± 1.7	5	3.0 ± 3.9
Fever	3	1.6 ± 1.5	2	1.8 ± 0.6	4	0.8 ± 0.4
Headache	6	1.1 ± 1.1	6	1.5 ± 1.6	6	1.4 ± 2.6
Malaise	2	2.2 ± 2.4	5	1.8 ± 1.4	1	0.7
Myalgia	2	3.7 ± 3.2	2	1.3 ± 0.5	2	0.8 ± 0.1
Nausea	3	1.7 ± 1.3	5	0.9 ± 0.9	3	1.0 ± 0.9
Vomiting	0	N/A	2	0.01 ± 0.0	0	N/A
Any	6	2.0 ± 1.4	6	1.1 ± 0.8	6	1.1 ± 1.0
Grade 3 adverse event						
Fatigue	0	N/A	0	N/A	1	2.2
Fever	0	N/A	1	1.2	0	N/A
Headache	2	3.0 ± 0.4	0	N/A	0	N/A
Malaise	1	4.8	0	N/A	1	0.1
Vomiting	0	N/A	2	0.01 ± 0.0	0	N/A
Any	2	3.9 ± 0.2	3	0.6 ± 0.0	2	1.2 ± 0.0

*There were few AEs before Day 7 (Figure 2). Thus, administration of PfSPZ Challenge was well tolerated. The AEs were expected and attributed to malaria.
N/A = not applicable.

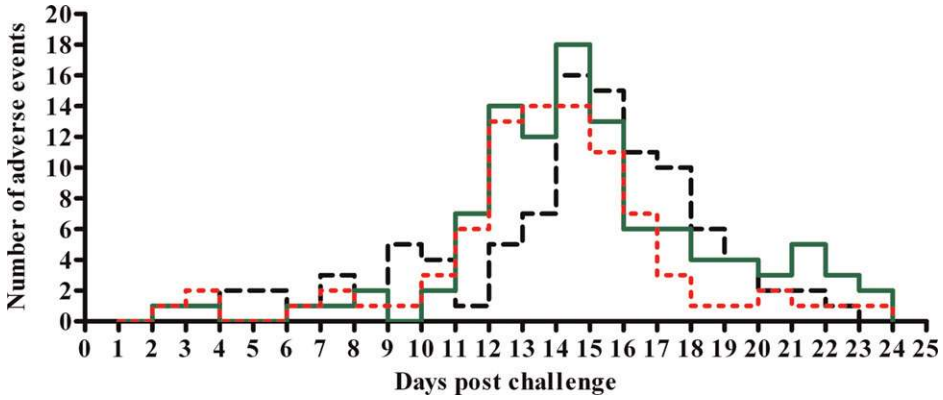

FIGURE 2. Number of possibly, probably, or definitely related solicited and unsolicited adverse events reported over time in the 2,500 (black dashed), 10,000 (red dotted), and 25,000 PfSPZ Challenge dose (green straight) groups.

treatment (range of peaks: 540–10,200 ng/mL). D-dimer increases most likely reflect non-specific inflammatory responses to parasite-derived material released after initiation of treatment. In all volunteers, D-dimer concentrations normalized without complications. One volunteer had abnormal liver function tests at Day 2 post atovaquone/proguanil initiation. Maximum values were 526 U/L ASAT (ULN 40 U/L), 745 U/L ALAT (ULN 45 U/L), 777 U/L LDH (ULN 450 U/L), and 74 U/L γGT (ULN 50 U/L). Bilirubin and alkaline phosphatase were normal. Abnormal values had returned to baseline levels at Day 100 after infection.

One SAE occurred in a volunteer who reported chest pain 1 day after the first dose of atovaquone/proguanil. Based on medical history, the chest pain was initially considered possibly consistent with angina pectoris. Pain resolved within 1 hour without treatment. The volunteer was admitted to the cardiac care unit for monitoring for 6.5 hours. The first electrocardiogram (ECG) had a negative T-wave in V2, which was absent at the time of study initiation. All subsequent ECGs, beginning 2.5 hours after the first ECG, were comparable to baseline, with a negative T in V1 only. Troponin T levels were normal at the time of chest pain, 6 and 17 hours later, daily for the next 3 days and at trial Days 28 and 35. As per protocol, the trial was put on hold, and the event was reported to the Safety Monitoring Committee (SMC) and regulatory authorities. The SMC concurred with the principal investigator's attribution of the chest pain as "possibly related" to participation in the trial. The SMC concluded that although the cause of chest pain was not clear, the clinical data suggested that the SAE was not a serious cardiac event, and recommended resumption of the trial within 3 days of the event. The regulatory authorities concurred.

DISCUSSION

We report for the first time that healthy, malaria-naive volunteers can be infected with *P. falciparum* malaria by injection of aseptic, purified, cryopreserved PfSPZ manufactured in compliance with regulatory standards. Five of six volunteers became infected when 2,500, 10,000, or 25,000 PfSPZ were inoculated ID. The AEs were comparable with those in mosquito bite challenge trials.[17,19,42,43] Virtually all related AEs were attributed to malaria, not to the inoculations with PfSPZ Challenge.

The capacity to infect volunteers with PfSPZ Challenge is dependent on the efficiency of administration and the infectiousness/fitness of the cryopreserved PfSPZ. It can be expressed by the success rate of infection in the exposed individuals and/or the pre-patent period, i.e., the time from inoculation until first detected parasitemia. Since 1986 CHMIs have been performed by exposing volunteers to bites of laboratory-reared mosquitoes infected by feeding on Pf gametocyte-infected erythrocytes grown in culture.[12] Essentially, all volunteers challenged by bites of five PfSPZ-infected mosquitoes develop Pf parasitemia.[5,12,17,19] When numbers are reduced to one or two mosquitoes, success rates drop to 50% or less.[43–45] The ID inoculation of the lowest dose of 2,500 cryopreserved PfSPZ Challenge, which resulted in infection of 5 of 6 volunteers in the current study, was thus at least as effective in achieving blood stage infection as the bites of 1–2 infected mosquitoes.

In regard to the pre-patent period the results were not straightforward. The pre-patent period in the 2,500 PfSPZ group was longer than was observed after 1–2 bites of PfSPZ (NF54)-infected mosquitoes at RUNMC[43] but shorter than after 1–2 bites of PfSPZ (3D7)-infected mosquitoes at the Naval Medical Research Center.[44] The longer pre-patent period in our study compared with the pre-patent period after exposure to NF54-infected mosquitoes may have been caused by fewer developing liver stage schizonts after inoculation than after exposure to the bites of 1–2 PfSPZ-infected mosquitoes. Alternatively, replication in the liver stage could have been of lower magnitude or slower with the aseptic, purified, cryopreserved PfSPZ as compared with the fresh PfSPZ delivered by the mosquito bite. Finally, the findings may just reflect expected biologic variability, because the study with 1–2 3D7 infected mosquitoes showed a longer pre-patent period than after PfSPZ Challenge.[44]

The asexual erythrocytic stage parasites in our study replicated ~11.5-fold every ~48 hours. Thus, with a 10-fold increase in PfSPZ, the theoretical time until parasitemia by microscopic examination (pre-patent period) should have been 2 days less in the 25,000 PfSPZ group as compared with the 2,500 PfSPZ group. However, this was not the case as pre-patent periods of 13.0 and 13.0 days by microscopy and 10.59 and 9.95 days by qPCR were obtained in the 2,500 PfSPZ and 25,000 PfSPZ

TABLE 4

Infectivity in mice of purified, cryopreserved PySPZ administered IV or ID*

Intravenous (IV)				Intradermal (ID)			
	Number of mice				Number of mice		
No. of PySPZ Injected	Infected	Injected	Proportion infected	No. of PySPZ injected	Infected	Injected	Proportion infected
33	2	5	40%	200	2	5	40%
100	1	5	20%	600	3	5	60%
300	5	5	100%	1800	3	5	60%
900	5	5	100%	5400	4	5	80%
80% infectious dose = 257 PySPZ				80% infectious dose = 5871 PySPZ			
100% infectious dose = 528 PySPZ				100% infectious dose = N/A			

*Purified, cryopreserved PySPZ were injected IV in the tail vein or ID at the base of the tail of 6–8 week old BALB/c. Infection was determined by examination of blood smears on Days 7 and 14 after inoculation. The 80% and 100% infectious doses were calculated using CurveExpert version 1.4.

groups, respectively. Thus, increasing the dose of PfSPZ Challenge 10-fold from 2,500 PfSPZ to 25,000 PfSPZ administered ID did not increase the percentage of infected volunteers or reduce the pre-patent period. Apparently, increasing the dose administered in two 50 μL injections did not result in higher numbers of PfSPZ getting from the skin to the circulation, invading and maturing in hepatocytes, eventually resulting in merozoites that invaded and multiplied in erythrocytes. Understanding this lack of dose response will be important for optimization of administration of PfSPZ Challenge. A possible explanation for this lack of dose response may be trapping of PfSPZ at the inoculation site. The use of five mosquitoes that probe in multiple sites must result in distribution of PfSPZ in the dermis and subcutaneous tissue in at least five different sites, and probably considerably more. Therefore, increase in the number of inoculation sites and injection of much smaller volumes (< 0.5 μL) may result in better infections. Such strategies may also be useful for improving the efficiency of administration of the irradiated PfSPZ in the PfSPZ Vaccine. Although not as profound, there was a lack of a linear dose response in the first trial of the PfSPZ Vaccine in which irradiated PfSPZ were administered in 120 μL ID or SC.[35]

To determine the minimal numbers of PfSPZ required to achieving 100% infection rates, and a pre-patent period similar to five PfSPZ-infected mosquitoes, it would be most useful to assess intravenous (IV) administration of PfSPZ Challenge. Data from studies in mice show that administration of purified cryopreserved *Plasmodium yoelii* (Py) SPZ required ~23 times more PySPZ administered ID than IV to achieve 80% infection rates (ID80) (Table 4). Similar differences in liver load *in vivo* between IV and ID routes of administration were demonstrated using luciferase-labeled, bioluminescent fresh *Plasmodium berghei* (Pb) SPZ (Nganou-Makamdop and others, *Parasite Immunol*, published online ahead of print, doi:10.1111/j.1365-3024.2012.12000.x). Thus, we will conduct studies to investigate the minimal IV-dose and to optimize non-IV administration by modifying the route of administration (e.g., ID, subcutaneous, intramuscular), inoculation volume, numbers of inoculations, and sites of injection.

Next to route of administration, our manufacturing/cryopreservation process may also be responsible for reduced infectivity. *In vitro* assays of potency and viability estimate a maximum difference of 25–30% between fresh and cryopreserved PfSPZ Challenge (Table 1). Rodent model *in vivo* data, however, suggest that a ~7-fold loss in infectivity caused by cryopreservation is more likely (Table 5). Therefore, we will continue to concentrate our efforts on improvement of infectivity of PfSPZ Challenge. Interestingly, once the merozoites are released from the liver into the bloodstream they are as fit as non-cryopreserved, mosquito-administered parasites, as their replication rates are similar.

Successful development and application of PfSPZ Challenge will increase the global capacity to conduct CHMIs, including in Africa where a CHMI consortium has been established with representative institutes from seven countries. This expansion of clinical sites conducting CHMIs will facilitate the clinical development of malaria vaccine candidates and anti-malarial drugs.[3,46] Another advantage of

TABLE 5

Effect of cryopreservation on sporozoite membrane integrity and infectivity in mice inoculated intravenously with the same lot of *P. yoelii* sporozoites (PySPZ). Infectivity was the number of PySPZ required to infect 50% of BALB/c mice*

Date	Status of PySPZ	Viability (SMIA)	Number of PySPZ inoculated (IV)	ID_{50} (number of PySPZ)
Oct 2009	fresh	96.3%	24-12-6-3	8.9
Dec 2009	cryopreserved	72.7%	200-100-50-25	33.1
Dec 2009	cryopreserved	68.3%	200-100-50-25	62.1
Jan 2010	cryopreserved	67.7%	400-200-100-50-25	103.8
Feb 2010	cryopreserved	67.1%	400-200-100-50-25	55.2
Feb 2010	cryopreserved	71.6%	400-200-100-50-25	107
Feb 2010	cryopreserved	73.9%	400-200-100-50-25	34.5
Mean	cryopreserved	70.2%		66.0
Difference between fresh and cryopreserved PySPZ		26.1%		7.4-fold

*Freshly dissected, purified *P. yoelii* sporozoites (PySPZ) were assessed by the sporozoite membrane integrity assay (SMIA) as a measure of viability, and administered to BALB/c mice by intravenous (IV) injection. The remaining PySPZ from the same lot were cryopreserved, thawed at six different time points, assessed for viability by SMIA, and administered IV to mice. To provide data for calculation of the number of PySPZ that infected 50% of mice (ID_{50} calculated using an exponential association model $y = a(1\text{-}e^{-bx})$) (CurveExpert version 1.4) with fresh and cryopreserved PySPZ, groups of five mice each received PySPZ in de-escalating doses as indicated, and their infection status was determined by assessing Giemsa-stained blood smears 7–14 days after inoculation. The viability by SMIA of purified, cryopreserved PySPZ was reduced 26.1% as compared with fresh, purified PySPZ. The cryopreserved PySPZ were 7.4-fold less infective than fresh PySPZ as it took 7.4 times more cryopreserved PySPZ to achieve 50% infection of mice.

CHMI by PfSPZ Challenge may be a better-defined number of injected PfSPZ compared with the numbers administered by mosquito bites. This may decrease the large inter-individual variation in the estimated number of infected hepatocytes.[47] Furthermore, using needle administration of defined quantities of PfSPZ Challenge from the same lot, will allow for comparisons of parallel and sequential clinical trials at multiple sites, including malaria-endemic areas. Finally, needle and syringe administration of cryopreserved PfSPZ is critical for potential development of whole PfSPZ vaccines where parasite development is arrested by radiation, anti-malarial drugs, or genetic modification.

In summary, we show that aseptic, purified, vialed, cryopreserved PfSPZ (PfSPZ Challenge) are infectious to humans for at least 2.5 years after cryopreservation. These data provide the rationale and foundation for a clinical trials program aimed at establishing a dose and route of PfSPZ that consistently achieves 100% infection rates. This will allow for the global expansion of sites that can conduct CHMIs for assessment of malaria vaccines and new drugs, and the potential to develop whole parasite vaccines based on cryopreserved PfSPZ.

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