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, vialled, cryopreserved Pf sporozoites (PISPZ) 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 PISPZ manufactured in compliance with regulatory standards. After further optimization, the use of such PISPZ 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. 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. Questions related to sustainability of this effort, however, have led to a recent delineation of requirements for new tools. 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) was first introduced for treatment of neurosyphilis in the 1920s. The development of drugs such as chloroquine, primaquine, and atovaquone were facilitated by these controlled human malaria infections (CHMIs). The ability to culture Pf gametocytes 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 PISPZ-infected mosquitoes, where more than 1,300 volunteers have been safely infected by the bites of PISPZ-infected mosquitoes since 1986, primarily for clinical trials of drugs and malaria vaccines, but also for trials of diagnostic tests, and for trials of diagnostic tests, and studying human immune responses to Pf.

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 PISPZ by bites of mosquitoes protects > 90% of volunteers according to the published literature and recently 100% protection against CHMI was achieved by immunization of volunteers taking a prophylactic regimen of chloroquine, with PISPZ administered by mosquito bites.

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. 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, vialled, cryopreserved PISPZ (PISPZ Challenge), and produced and tested the world’s first vaccine composed of these sporozoites. Here, we report infection of volunteers with PISPZ Challenge administered intradermally 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.
Eighteen healthy malaria-naïve volunteers were included in this trial. Groups of six volunteers were injected intradermally (ID) with 2,500, 10,000, or 25,000 PISPZ 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 PISPZ group and 6 of 6 volunteers infected in the 25,000 PISPZ 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 (PISPZ Challenge)**. The PISPZ Challenge contains aseptic, purified, cryopreserved PISPZ isolated from salivary glands of aseptically reared mosquitoes. Anopheles stephensi mosquitoes were raised under aseptic conditions, and then fed on cultured Stage V gametocytes of the NF54 strain of P.falciparum. Approximately 2 weeks later, mosquito salivary glands containing PISPZ were dissected, and PISPZ were purified, formulated, vialled (15,000 PISPZ per vial), and cryopreserved in liquid nitrogen vapor phase at −140°C to −196°C. The PISPZ 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 by quantification of late liver stage parasites expressing Pf merozoite surface protein 1 (PfMSP-1) in cultured human hepatocytes (HC-04 cells) 6 days after addition of PISPZ (Table 1, Suppemental 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 PISPZ 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, the membrane integrity of PISPZ was used to assess cell viability (Table 1). For the sporozoite membrane integrity assay, propidium iodide and SYBR green were added to 15,000 PISPZ. PISPZ were applied to a hemocytometer and incubated in a dark humidity chamber for 20 minutes, at which point the red PI(PISPZ (those with compromised membranes) and green PISPZ (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 PISPZ over the total number of PISPZ. Sporozoites was assessed before cryopreservation, for release of the lot, and to assess stability at defined time points after cryopreservation.

The lot of PISPZ Challenge used in this study had been cryopreserved in liquid nitrogen vapor phase for 27 (dose of 2,500 PISPZ) to 30 months (dose of 25,000 PISPZ) before administration. Immediately before use, a vial of PISPZ 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 PISPZ 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 PISPZ 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, 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. 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 x 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) pre-patent 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 = 0.24), respectively. The GM parasite densities by PCR at the time of thick smear diagnosis were 35, 5, and 132 parasites/μL blood (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.

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.

Table 2

| 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 thick smear diagnosis (Pf/μL) |
|----------------|-------------------------|--------------------------------------|---------------------|-----------------------------------------------|---------------------------------------------------------------|
| 606–18         | 12.3                    | 4                                    | 9.6                 | 0.08                                          | 5                                                             |
| 711–08         | 14.0                    | 16                                   | 12.8                | 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                    |                                       |                     |                                               |                                                               |
| 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                    |                                       |                     |                                               |                                                               |
| 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                    |                                       |                     |                                               |                                                               |

N/A = not applicable; thick-smear negative volunteers were presumptively treated on day 21 after infection.
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 PISPZ Challenge, with mean duration of events*<sup>a</sup>

| Any adverse event | 2,500 PISPZ (N = 6) | 10,000 PISPZ (N = 6) | 25,000 PISPZ (N = 6) |
|-------------------|---------------------|---------------------|---------------------|
|                   | 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 PISPZ Challenge was well tolerated. The AEs were expected and attributed to malaria.

N/A = not applicable.
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 $\gamma$GT (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 PISPZ manufactured in compliance with regulatory standards. Five of six volunteers became infected when 2,500, 10,000, or 25,000 PISPZ 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 PISPZ Challenge.

The capacity to infect volunteers with PISPZ Challenge is dependent on the efficiency of administration and the infectiousness/fitness of the cryopreserved PISPZ. 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 PISPZ-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 PISPZ 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 PISPZ group was longer than was observed after 1–2 bites of PISPZ (NF54)-infected mosquitoes at RUNMC43 but shorter than after 1–2 bites of PISPZ (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 PISPZ-infected mosquitoes. Alternatively, replication in the liver stage could have been of lower magnitude or slower with the aseptic, purified, cryopreserved PISPZ as compared with the fresh PISPZ 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 PISPZ Challenge.44

The asexual erythrocytic stage parasites in our study replicated ~11.5-fold every ~48 hours. Thus, with a 10-fold increase in PISPZ, the theoretical time until parasitemia by microscopic examination (pre-patent period) should have been 2 days less in the 25,000 PISPZ group as compared with the 2,500 PISPZ 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 PISPZ and 25,000 PISPZ

![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 PISPZ Challenge dose (green straight) groups.](Image)
groups, respectively. Thus, increasing the dose of PfSPZ Challenge 10-fold from 2,500 PISPZ to 25,000 PISPZ 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 PISPZ 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 PISPZ Challenge. A possible explanation for this lack of dose response may be trapping of PISPZ 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 PISPZ in the PISPZ Vaccine. Although not as profound, there was a lack of a linear dose response in the first trial of the PISPZ Vaccine in which irradiated PISPZ were administered in 120 μL ID or SC.35

To determine the minimal numbers of PISPZ required to achieving 100% infection rates, and a pre-patent period similar to five PISPZ-infected mosquitoes, it would be most useful to assess intravenous (IV) administration of PISPZ 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 PISPZ 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 PISPZ 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 PISPZ 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

| Date    | Status of PISPZ | Viability (SMIA) | Number of PISPZ inoculated (IV) | ID50 (number of PISPZ) |
|---------|-----------------|-----------------|------------------------------|------------------------|
| 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 PISPZ | 26.1% | 7.4-fold |

*Freshly dissected, purified P. yoelii sporozoites (PISPZ) 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 PISPZ from the same lot were cryopreserved, thawed at six different time points, assessed for viability by SMIA, and administered to mice to provide data for calculation of the number of PISPZ that infected 50% of mice (ID50, calculated using an exponential association model y = a(1-e^-y)) (CurveExpert version 1.4) with fresh and cryopreserved PISPZ, groups of five mice each received PISPZ in de-capsulating 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 PISPZ was reduced 26.1% as compared with fresh, purified PISPZ. The cryopreserved PISPZ were 7.4-fold less infective than fresh PISPZ as it took 7.4 times more cryopreserved PISPZ to achieve 50% infection of mice.

Table 4
Infectivity in mice of purified, cryopreserved PySPZ administered IV or ID*

| Date Status of PySPZ | No. of PySPZ Injected | Number of mice | Proportion infected |
|----------------------|-----------------------|----------------|--------------------|
| Oct 2009 fresh       | 33                    | 2              | 5                  | 40%                |
| Dec 2009 cryopreserved| 100                   | 1              | 5                  | 20%                |
| Jan 2010 cryopreserved| 300                   | 5              | 5                  | 100%               |
| Feb 2010 cryopreserved| 900                   | 80% infectious dose = 257 PySPZ | 100% infectious dose = 528 PySPZ |

| Date Status of PySPZ | No. of PySPZ injected | Number of mice | Proportion infected |
|----------------------|-----------------------|----------------|--------------------|
| Oct 2009 fresh       | 200                   | 2              | 5                  | 40%                |
| Dec 2009 cryopreserved| 600                   | 3              | 5                  | 60%                |
| Jan 2010 cryopreserved| 1800                  | 3              | 5                  | 60%                |
| Feb 2010 cryopreserved| 5400                  | 5              | 5                  | 80%                |

*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.

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*
CHMI by PISPZ Challenge may be a better-defined number of injected PISPZ 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 PISPZ 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 PISPZ is critical for potential development of whole PISPZ vaccines where parasite development is arrested by radiation, anti-malarial drugs, or genetic modification.

In summary, we show that aseptic, purified, vialed, cryopreserved PISPZ (PISPZ 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 PISPZ 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 PISPZ.

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