Blood-stage Challenge for Malaria Vaccine Efficacy Trials: A Pilot Study with Discussion of Safety and Potential Value

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Abstract. There is increasing interest in malaria vaccines targeting the asexual blood stage of Plasmodium falciparum. Without accepted immunologic correlates of clinical protection, challenge studies are useful for assessing the efficacy of candidate vaccines in vivo in healthy volunteers. We report a pilot study of a safe and robust challenge protocol using a blood-stage inoculum. We have applied well-validated trial endpoints and twice daily real-time quantitative polymerase chain reaction monitoring of parasitemia to blood-stage challenge, which enabled direct comparison with sporozoite challenge. We found that greater accuracy in quantification of blood-stage growth rates can be achieved with blood-stage challenge. This finding may provide greater power to detect partial efficacy of many blood-stage candidate vaccines. We discuss the potential utility of blood-stage challenge studies in accelerating malaria vaccine development.

INTRODUCTION

Malaria vaccine development requires reliable means of selecting the best candidates for efficacy trials in malaria-endemic areas. In the case of pre-erythrocytic vaccines, the relative ease and wide acceptance of sporozoite challenge, in which vaccinated healthy volunteers are infected with malaria by the bites of infected mosquitoes, has enabled iterative vaccine development.1

There is currently renewed interest in vaccine candidates targeting the asexual blood stage of the parasite. Without preliminary human efficacy data to select leading candidates, substantial investment in malaria-endemic area trial capacity would be required to assess all experimental blood-stage vaccines in the field.

Information about immune mechanisms of protection against blood-stage parasites is complex and sometimes conflicting. Direct antibody effects, antibody-dependent cellular infection (ADCI), and antibody-independent cell mediated immunity may all have roles.2–4 Correlation of in vitro measures of these activities with protection in field studies is problematic. Simple assays such as an enzyme-linked immunosorbent assay may not detect quantitative differences between antibodies.5 Functional assays are heavily dependent on both assay method and the specific antigen.6,7 Natural immunity to single antigens may never reach levels induced by subunit vaccines, which prevents identification of correlates of protection in field studies. To our knowledge, no blood-stage vaccine trial has yet demonstrated a correlation between in vitro immunologic measurements and clinical protection of individual vaccinees. Thus, the use of purely immunologic measurements to select candidates for field trials is risky. Similarly, extrapolation to humans of the results of animal challenge studies is fraught with difficulty.7

Assessment of efficacy of blood-stage vaccines against parasite challenge in small groups of healthy human subjects is therefore desirable. Use of microscopic parasitemia as an endpoint, as is usual in sporozoite challenge, would not necessarily signify vaccine failure; an effective blood-stage vaccine might reduce malaria-associated morbidity and mortality without producing sterile protection. Instead, a challenge system would have to detect reduction in the blood-stage growth rate of the parasite. Sub-patent parasite growth has been quantified by quantitative real time polymerase chain reaction (PCR) and mathematically modeled after sporozoite challenge.8,9 However, the number of parasites released from the liver after sporozoite challenge is variable and sometimes close to the threshold for patent parasitemia, which hinders growth rate quantification.9

Use of blood-stage parasites to challenge vaccine study subjects could eliminate these problems. We believe that concerns about the transmission of blood-borne infection by this route can be addressed by stringent safety measures.10 We recognize that such a non-physiologic challenge would fail to detect any pre-erythrocytic immunity induced by a blood-stage vaccine and might therefore underestimate vaccine efficacy, but believe it is unlikely that such an effect would be large.

Building on the work of Cheng and others10 and the earlier studies of Whitrow,11 we report infection of five malaria-naive volunteers using blood-stage inocula. We have reproduced and extended previously published data demonstrating safety, reliability, and parasite growth rates after this challenge. Furthermore, we report the application to this challenge of the rapid, sensitive and robust quantitative real-time PCR used in sporozoite challenge studies of more than 200 volunteers to date. This application enables comparison with data obtained from sporozoite challenge studies, which demonstrates the greater accuracy of blood-stage challenge in quantifying blood-stage growth rates.

MATERIALS AND METHODS

Volunteers and clinical method. The study was reviewed and approved by the Oxfordshire Research Ethics Committee (study reference C03.061) and an independent local safety monitor was appointed. Healthy volunteers were recruited in Oxford within the age range of 18–45 years. All subjects provided informed consent and underwent medical screening. Exclusion criteria were as previously described for sporozoite challenge studies and additionally included seronegativity for Epstein Barr virus (EBV) or cytomegalovirus (CMV).12

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Eleven prospective subjects were screened, of whom six were excluded because of seronegativity for EBV or CMV. The remaining five subjects underwent blood-stage challenge. Subjects were observed for one hour after inoculation and underwent clinical review and blood withdrawal as previously described on day one, twice daily from day two until reaching one of the study endpoints and daily for two days thereafter. Endpoints were as follows: one or more parasites visible on thick blood smear; manifestation of typical clinical symptoms or signs of malaria with a PCR result positive for malaria; reaching the evening of day 12 without symptoms or positive blood film. Upon reaching an endpoint, subjects were treated with their choice of chloroquine or artemether-lumefantrine (Riamet®; Novartis, Basel, Switzerland), using standard dosing regimens. A final follow-up clinical evaluation was conducted six weeks after inoculation.

**Inoculum.** Erythrocytes infected with chloroquine-sensitive *Plasmodium falciparum* strain 3D7 were prepared as previously described. The blood donor was seronegative or PCR negative, or both, for a panel of parenterally transmissible agents at the time of donation and one year later, but was seropositive for EBV and CMV.

Inocula were thawed and prepared for use at Oxford using a published protocol, under new microbiologic hoods in a laboratory not previously used for pathogens. No contamination of the hoods was detected by settle plates used according to a protocol supplied by the Cardiac Homograft Unit, John Radcliffe Hospital (Oxford, United Kingdom). Microscopic estimates of the donor’s parasite density prior to freezing of blood samples were used to prepare inocula, each less than $10^5$ and containing an estimated 3,000 infected erythrocytes. The time between thawing and inoculation was 40 minutes, during which time inocula were kept on ice. All volunteers were inoculated intravenously within five minutes of each other.

The viability of the thawed parasites was assayed by adding a sample of inoculum to 26 wells at a concentration estimated on the basis of the pre-freezing microscopic parasite count to be one infected erythrocyte per well. The sample used was taken from the same vial used for inoculation, immediately after the last volunteer had been inoculated. Parasites were cultured and presence or absence of parasite multiplication in each well determined by PCR as previously described. PCR was adapted to a LightCycler® 2.0 PCR machine (Roche, Burgess Hill, United Kingdom) and Quantitect SYBR Green I chemistry (Qiagen, Crawley, United Kingdom) using the instructions of the manufacturers. The number of viable parasites was estimated from the percentage of PCR-positive wells by fitting to a Poisson distribution and a 95% confidence interval (CI) for this estimate calculated using StatsDirect software (StatsDirect, Altrincham, United Kingdom).

**Parasite growth monitoring, modeling, and power calculation.** Blood was obtained twice a day for parasite growth measurements. Samples were analyzed in batches within 48 hours by quantitative real-time PCR using Plasmodipur sample filtration as described elsewhere. Growth rate modeling was carried out using a sine wave function applied to data from individual volunteers. This method was first used to model parasitemias seen after treatment with iatrogenic *P. falciparum* infection for treatment of neurosyphilis. In contrast to the situation after sporozoite challenge, the size of the parasite inoculum is known after blood stage challenge; thus the model was constrained to fit this value.

The powers of studies using blood-stage and sporozoite challenge to detect differences in two endpoints induced by a putative effective blood-stage vaccine were calculated using STATA Release 9.2 (Stata Corporation, College Station, TX). To calculate the power to detect differences in parasite multiplication rate estimated from PCR studies, a normal distribution of log-transformed growth rates was assumed, with an equal mean 48-hour multiplication rate for blood-stage and sporozoite challenge (18-fold for controls). Standard deviation was as quoted elsewhere in this report and was as-

![PCR determined parasite growth curves](image-url)
sumed to be constant in vaccinated and control subjects. The power was estimated for a two-sample comparison of means, with the percentage efficacy applied to the linear growth rate. To calculate the power to detect vaccine induced delay to microscopic patency, bootstrap samples were selected from the actual data, with simulated delays added for vaccinated groups. The outcomes of 1,000 such simulated studies were then examined to estimate the power of the comparison.

RESULTS

Endpoints and clinical events. No serious adverse events were recorded. All five volunteers became infected and developed microscopically detected parasitemia. The median time from challenge to microscopic patency was eight days (range = 7–9 days, SD = 0.9 days). One volunteer had symptoms of malaria prior to initiating treatment; the remaining four volunteers were treated on the basis of microscopic parasitemia first detected on days seven or eight. Two subjects experienced nausea with chloroquine treatment. All volunteers had recovered completely within five days of starting antimalarial drugs. At the six-week follow-up, all volunteers were clinically well. One female subject had marginal iron-deficiency anemia that was not thought to be related to the study. No other blood test abnormalities were clinically significant in the opinion of the investigators.

Parasite viability and calculation of inocula. In the viability assay, parasite multiplication was demonstrated in 12 of 26 wells at a dilution that was estimated on the basis of the pre-freezing parasitemia to produce one infected erythrocyte per well. Fitting to a Poisson distribution suggests an actual mean of 0.62 viable infected erythrocytes per well (95% CI = 0.35–1.00). This finding implies that the inocula estimated to contain 3,000 infected erythrocytes actually contained approximately 1,800 viable infected erythrocytes (95% CI = 1,050–3,000).

Parasite growth rates. Parasite growth curves determined by quantitative real-time PCR for the five subjects are shown in Figure 1. Parasitemia first became detectable by PCR on day 3.5 post-challenge in four subjects and day 4.0 in the fifth subject. Two timepoints for subject 5 did not provide sufficient blood for PCR analysis. Four samples were PCR negative or below the quantitation threshold of 20 parasites/mL of blood after an initial PCR-positive result. This is consistent with the expected cyclic pattern of parasitemia. The mean number of PCR-positive data points per subject was eight (range = 6–11).

Forty-eight-hour parasite multiplication rates determined by modeling were as follows for each volunteer: 15.0 fold (95% CI = 10.3–21.8), 16.4 fold (95% CI = 10.8–24.8), 21.5 fold (95% CI = 14.8–31.1), 23.9 fold (95% CI = 16.2–35.1), and 31.7 fold (95% CI = 22.8–43.9). The geometric mean 48-hour multiplication rate was 20.9 fold per 48 hours (95% CI = 11.5–38.1) compared with a geometric mean of 17.8-fold multiplication (95% CI = 1.8–179.8) among five controls in a randomly chosen sporozoite challenge study and a geometric mean of 10.5-fold multiplication (95% CI = 1.4–79.3) among a pool of 19 controls from five consecutive such studies modeled by Bejon and others9 and Walther and others.15 Figure 2 compares multiplication rates after blood-stage and sporozoite challenge.

DISCUSSION

Blood from a single donor has now been used in four published trials to infect 31 volunteers with no serious adverse events.10,13,16 The risk of viral transmission by blood transfusion from Australian donors around the time of donation has been estimated to be 2.71 per million donations for hepatitis B virus, 4.27 per million for hepatitis C virus, and 0.79 per million for human immunodeficiency virus.17 The combination of long experience using this inoculum and the use of a minute volume (< 10 μL) of erythrocytes free of leukocytes and plasma argues strongly that the risk of transmission of any infectious agent through blood-stage challenge is extremely low, and far less than the already small risks pertaining to therapeutic transfusion.

This blood-stage challenge trial consistently applied prospectively defined treatment initiation points of either microscopic parasitemia or malarial symptoms. These points have been shown by extensive experience to be safe and tolerable by volunteers undergoing sporozoite challenge, with microscopy being the current gold standard method of malaria diagnosis. Furthermore, they enable use of delay to microscopic parasitemia as a trial outcome measure in addition to calculated growth rates.
Although there has been some variability in blood-stage parasite viability from study to study, there have been no failures of infection in any trials using this inoculum preparation protocol. Storage conditions and time between thawing and inoculation are the major factors that affect parasite viability (Cheng Q, unpublished data). There does not appear to be any downward trend in parasite viability despite nine years of frozen storage prior to this study, and parasites remained viable when tested after an additional three years of storage (Watkins KR and others, unpublished data).

Results of this study generally correlate well with relevant previously published data. The mean rate of parasite multiplication observed in this study (20.9 fold per 48 hours, 95% CI = 11.5–38.1) was not significantly different from mean multiplication rates seen after sporozoite challenge (10.5 fold, 95% CI = 1.4–79.3) or previously published blood-stage challenge multiplication rates calculated using an estimated starting parasitemia (11.9-fold per 48 hours). We discuss below some discrepancies between the PCR-determined parasite density seen in this study and those reported by Cheng and others.

The quantitative real-time PCR protocol used in this study has significant advantages relative to the two-step nested PCR method of quantification used in previous blood-stage challenge studies. The nested PCR protocol developed by Cheng and others has been used to monitor volunteers in one-phase IIa vaccine trial but the relative speed and simplicity of the quantitative real-time PCR make it more suited in this context. We report twice daily parasite density data after blood-stage challenge, which improves the quality of data available for growth modeling. This technique could make results available before the next clinical visit in future studies.

Although a sensitivity of 10 parasites/mL has been claimed for nested PCR, the detection of approximately 30 parasites/mL in some of the volunteers studied by Cheng and others would require implausibly rapid multiplication (50-fold in 24 hours). The quantitative real-time PCR has a reported sensitivity of 20 parasites/mL, and, as a closed PCR system, has a greatly reduced risk of false-positive results because of contamination than the nested PCR.

The level of PCR-determined parasitemia at days 7.0–8.0 in the current study was approximately 10-fold higher than that published by Cheng and others. The upper limit of the standard curve used for quantification by Cheng and others was 3,333 parasites/mL, and parasite densities exceeding this value on days seven and eight post-challenge in that study may be underestimates. Densities from 20 to \(2 	imes 10^6\) parasites/mL were used to construct the standard curve used for the quantitative real-time PCR, and only one data point in the present study was recorded above this range. We therefore believe that the data presented here represent parasite density after blood-stage challenge more accurately than data in previous publications.

We have shown above that blood-stage challenge reduces inter-individual variability and standard error of calculation in multiplication rates compared with sporozoite challenge (Figure 2). This finding probably largely reflects elimination of variability of liver-to-blood inoculum size after sporozoite challenge. Although five infectious bites is the standard inoculum in sporozoite challenge studies, there is considerable variation in observed salivary gland parasite densities between infectious mosquitoes and further possible variability caused by different amounts of probing by mosquitoes prior to taking a blood meal.

The median period in this study from challenge to microscopic patency was 8.0 days compared with a median of 4.5 days between parasite release from the liver and microscopic patency after sporozoite challenge of 31 volunteers in five consecutive studies in our unit. Blood-stage challenge thus provides a prolonged period of blood-stage multiplication during which blood-stage immunity induced by a partially effective vaccine could act to increase the delay to microscopic patency. Figure 3 shows that a vaccine reducing parasite growth by 30% would be expected to produce approxi-
mately a 3.0-day delay to patency under blood-stage challenge versus a 1.5-day delay under sporozoite challenge.

Both improved accuracy of multiplication rate calculation and a possible prolonged vaccine-induced delay to patency would give blood-stage challenge studies more power than sporozoite challenge to detect partially effective vaccines. To illustrate this point, the estimated power of studies using 6 controls and 12 vaccinated subjects to detect the effects of vaccines reducing blood-stage growth by 30% and 50% is shown in Table 1.

The real strength of blood-stage challenge relative to sporozoite challenge is likely to lie in detection of partial vaccine efficacy against parasite growth; it might be viewed by some, therefore, as simply a less stringent test. However, we believe there are two strong arguments for sensitive detection of partial blood-stage vaccine efficacy: as well as guiding future vaccine development, a vaccine with a relatively weak effect against parasite growth in blood-stage challenge may have a powerful clinical effect by prolonging the period over which a secondary immune response can develop. This view is supported by evidence from rodent studies that demonstrate the importance of an active immune response in mice passively immunized against the merozoite surface protein 1, blood-stage antigen.18

Certain observations raise concern that blood-stage challenge could fail to detect a clinically significant vaccine effect. The “combination B” blood-stage vaccine showed no efficacy under blood-stage challenge, but was reported to reduce density of parasitemia of the target strain in a field trial.13,19 However, this result should be interpreted with caution. There were significant differences in baseline parasitemias between vaccine and placebo groups; the vaccine was not effective in reducing parasite density in vaccinated children pre-treated with antimalarial drugs; and the trial was underpowered to detect an effect on clinical episodes, with a non-significant trend toward increased clinical episodes in vaccinated children, albeit attributed to infection with a parasite strain heterologous to that included in the vaccine. We do not therefore consider this experience particularly discouraging to the use of blood-stage challenge in vaccine evaluation.

In malaria-endemic areas, immunity resulting in asymptomatic parasitemia is common, and it remains unknown whether such immunity would reduce sub-patent parasite growth rates. Such evidence would strengthen the case for blood-stage challenge. Encouragingly, comparison of rates of PCR-detected parasitemia with blood microscopy has shown that blood-stage immunity in malaria-endemic areas controls or clears many infections at a sub-patent level, which suggests that natural immunity would be detectable by blood-stage challenge.20

Some P. falciparum antigens are expressed at more than one stage of the parasite life cycle. Clearly, blood-stage challenge would fail to detect an effect upon pre-erythrocytic stages and thus may underestimate the efficacy of vaccines inducing responses against such antigens.

There is in vitro evidence that ADCI is most effective at very high parasite densities, and it has been hypothesized that a similar phenomenon may occur in vivo.21 If ADCI were dependent upon parasite densities exceeding the microscopic detection threshold, it could not be ethically detected in experimental infection because of the onset of clinical malaria, and assessment of its induction by candidate vaccines in healthy volunteers would be limited to in vitro assays. We note that although it has been suggested that ADCI might become most effective at parasite densities around the level of microscopic patency, this suggestion is not firmly based on empirical evidence.21

Although ADCI is one example of an effect detectable in vitro that might not be detected by blood-stage challenge, we consider it likely that the reverse might occur. Blood-stage challenge may demonstrate parasite growth inhibition in vivo by immune mechanisms undetected by current in vitro assays. In view of the complexity of known immune effector mechanisms, blood-stage challenge may help clarify and quantify the additive effects of the vaccine-induced immune response in a more biologically relevant manner than any in vitro assay. Moreover, its results may allow some comparison of the effects of different vaccines, which is presently impossible because different in vitro assays are often used for immunologic assessment of each candidate vaccine.

In summary, we believe blood-stage challenge to be safe, robust, straightforward, and more physiologically relevant than in vitro or animal assays of blood-stage immunity, and likely to be more sensitive than sporozoite challenge. It should complement existing approaches to allow well-informed selection of candidate vaccines before expensive and time-consuming field trials.

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