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# Can growth inhibition assays (GIA) predict blood-stage malaria vaccine efficacy?

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**Abbreviations:** GIA, growth inhibition assay; IIA, invasion inhibition assay; PMR, parasite multiplication rate; BS, blood-stage; CHMI, controlled human malaria infection; ITN, insecticide treated bed nets; ACT, artemesinin-combination therapy; AMA-1, apical membrane antigen 1; MSP-1, merozoite surface protein 1; HIV, human immunodeficiency virus; WHO, World Health Organization; EBA, erythrocyte binding antigen; GLURP, glutamate-rich protein; GFP, green fluorescent protein ; ELISA, enzyme-linked immunosorbent assay; NYVAC, attenuated copenhagen strain vaccinia virus; PEV3A, virosome formulation of apical membrane antigen-1 and circumsporozoite protein; FP9, fowlpox-9; MVA, modified vaccinia virus ankara strain; AS, adjuvant system; AMA-1/C1, apical membrane antigen-1/combination 1; WRAIR, Walter Read Army Institute for Research, USA; QIMR, Queensland Institute for Medical Research, Australia; CCVTM, Centre for Clinical Vaccinology and Tropical Medicine, UK; CHMI, controlled human malaria infection; Spz, sporozoite challenge; BSP, blood-stage parasite challenge; PMR, parasite multiplication rate (in vivo); ND, not done; NP, not presented; qPCR, quantitative polymerase chain reaction; PcMSP-$1_{19}$/PyMSP-$1_{19}$, chimeric *P. falciparum* expressing *P. chabaudi* or *P. yoelii* merozoite surface protein-$1_{19}$; pLDH, parasite lactate dehydrogenase activity; $^3$H, tritium radiometric viability assay



An effective vaccine against *P. falciparum* malaria remains a global health priority. Blood-stage vaccines are an important component of this effort, with some indications of recent progress. However only a fraction of potential blood-stage antigens have been tested, highlighting a critical need for efficient down-selection strategies. Functional in vitro assays such as the growth/invasion inhibition assays (GIA) are widely used, but it is unclear whether GIA activity correlates with protection or predicts vaccine efficacy. While preliminary data in controlled human malaria infection (CHMI) studies indicate a possible association between in vitro and in vivo parasite growth rates, there have been conflicting results of immunoepidemiology studies, where associations with exposure rather than protection have been observed. In addition, GIA-interfering antibodies in vaccinated individuals from endemic regions may limit assay sensitivity in heavily malaria-exposed populations. More work is needed to establish the utility of GIA for blood-stage vaccine development.

## Introduction

*P. falciparum* malaria is the pre-eminent tropical parasitic infection, causing approximately 300 million infections and around 800,000 deaths per year (World Malaria Report, WHO, 2010). Effective control strategies such as insecticide-treated bed-nets (ITNs) and artemesinin-combination therapies (ACT) have contributed to considerable and impressive reductions in malaria incidence in some countries,[1] prompting renewed calls for malaria eradication.[2] Yet the evolution of parasite resistance to drugs[3] and vector resistance to insecticides[4] continues to challenge control efforts, and the development of an effective malaria vaccine is a global public health priority.[5,6]

While a partially effective vaccine is aiming for licensure in 2015,[7] a highly effective vaccine against *P. falciparum* malaria remains elusive. There are many challenges to overcome,[8,9] including considerable parasite genetic diversity, a lack of suitable animal models, and an incomplete understanding of the effector mechanisms that determine natural immunity in humans.[10] A variety of vaccine strategies targeting all stages of the parasite life-cycle have been pursued, including recombinant protein-in-adjuvant preparations,[11] replication-deficient viral vectors encoding malaria antigens[12] and attenuated whole parasites.[13] Fewer than 0.5% of malaria proteins have been explored as potential candidate vaccine antigens,[9] but the presence of naturally acquired immunity (in contrast to other important pathogens such as HIV), together with evidence of experimentally-induced immunity in humans,[14] offers the promise that better understanding of protective immune effector mechanisms might accelerate the vaccine development process.[9]

With so many potential vaccine candidates and platforms, robust down-selection strategies are required for candidate antigens. In the case of vaccines to the asexual blood-stage, the most commonly employed strategy for candidate antigen

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down-selection has been the detection of antibody with in vitro activity in growth inhibition assays (GIA).[15,16] In primate challenge models induced antibodies with high levels of GIA activity against blood-stage antigens such as apical membrane antigen-1 (AMA-1) and merozoite surface protein-1 (MSP-1) have been associated with protection against lethal challenge.[17,18] But can the in vitro GIA activity of induced antibodies predict blood-stage vaccine efficacy in humans? Here we attempt to address this question using data from published immunoepidemiological, CHMI and field efficacy studies.

## Parasite Growth and Invasion

The malaria lifecycle is complex, involving several stages. Infected Anopheles mosquitoes inject sporozoites of *P. falciparum* present in their salivary glands when taking a blood meal. These sporozoites migrate to and invade liver cells, setting up the liver (or pre-erythrocytic) stage of infection. After around seven days each infected liver cell releases approximately 30,000 merozoites into the bloodstream. These merozoites invade and replicate asexually within red blood cells (erythrocytes), leading to an exponential increase in parasites in the blood (parasitemia). This is the blood-stage of infection—the only stage at which clinical disease occurs. Later in blood-stage replication a few parasites develop into male and female gametocytes, and these in turn may be taken up by feeding mosquitoes, leading to sexual reproduction in the mosquito that produces a new generation of sporozoites.

Invasion of erythrocytes by merozoites is rapid[19] and involves three main phases: (1) attachment, (2) apical re-orientation and (3) invasion.[20] Various merozoite antigens are involved in these processes; such as the merozoite surface proteins (MSPs, particularly MSP-1) in attachment; the apical membrane antigen 1 (AMA-1) in re-orientation; and two families termed the erythrocyte binding antigens (EBAs) and the Rh proteins in invasion.[21] Some of these proteins are leading BS vaccine targets (e.g., AMA-1 and MSP-1), although many more are untested.[15]

## Blood-Stage Immunity

Although the precise immunological mechanisms underpinning malaria immunity are unresolved, its natural history is well established.[22] Immunity develops over time with repeated exposure to the malaria parasite (providing death does not occur), first to severe disease in infants, then to clinical disease in children and young adults. Immunity is rarely "sterilizing" (i.e., asymptomatic parasitemia is often observed in adults), suggesting that naturally acquired immunity occurs mainly at the blood-stage.[10] Attempting to accelerate and improve upon this naturally acquired immunity is the major goal of blood-stage vaccines.[15,16] Malaria immunity is maintained by continued exposure to parasite antigens, and the ideal BS vaccine will be similarly boosted by, but will not require, natural exposure.[16] Studies of experimentally induced human malaria (used as a therapy for neurosyphilis in the pre-antibiotic era) have demonstrated that the immunity that develops is both strain and species specific.[23] Even in a single host, substantial variation of surface antigen expression may enable evasion of host immunity.[24]

A few BS vaccine candidates have demonstrated limited evidence of strain-specific efficacy,[25-28] however the most effective malaria vaccine to date has been the protein-adjuvant anti-sporozoite (pre-erythrocytic stage) vaccine RTS,S, which is the only candidate malaria vaccine to have progressed to phase III efficacy trials.[7] Yet RTS,S is only partially effective (30–50% efficacy against clinical malaria in phase IIb studies,[29-32] 50% in an interim analysis of the phase III trial[7]) and recent data suggest that by reducing the frequency of exposure to blood stage parasites, RTS,S may actually increase the probability of clinical malaria (vs. asymptomatic parasitemia) in those who are subsequently infected.[33] This increased probability of clinical disease is outweighed by the vaccine-induced reductions in exposure that reduce the incidence of clinical malaria overall.[33] Nevertheless, the impact of changing transmission patterns on disease severity is uncertain,[34] and the desirability of a complementary BS vaccine to mop-up leaky pre-erythrocytic immunity, and potentially deal with epidemic transmission patterns, is generally acknowledged.[8,9,15]

Antibodies are central to BS immunity. The role of antibodies was first identified by passive transfer experiments in rhesus macaques in 1937.[35] Seminal experiments by Cohen and colleagues established the protective role of antibody against clinical disease by transferring purified IgG from semi-immune adults to young children with acute malaria.[36] Additional immune responses may also be involved in BS immunity.[10] Th1 $CD4^+$ effector memory responses to BS parasites[37,38] are associated with long-term protection after repeat sporozoite exposure under drug treatment, although the precise mechanisms of this protection are unclear. A previous study had also proposed T-cell mediated protection resulted from repeated exposure to ultra-low dose BS parasite inoculation followed by drug treatment,[39] but these protection data were confounded by residual antimalarial activity at the final challenge.[40]

## In Vitro Growth Inhibition Assay (GIA).

The activity of immune sera[41] or purified immunoglobulin[42-44] in parasite growth inhibition assays (GIA) has been recognized for many years. Cohen and colleagues published the first report of the in vitro growth inhibitory activity of *P. knowlesi* antibody in 1969.[45] Antibodies with GIA activity against *P. falciparum* can be effectively induced by immunization in humans using protein-in-adjuvant and vectored subunit vaccine approaches (reviewed in refs. 15 and 16), although the magnitude of vaccine-induced GIA responses are dependent on the population group immunized[46] and the vaccine antigen.[47] For the best-studied BS antigens AMA-1 and MSP-1, significant antigen-specific variation is observed in the GIA response, with AMA-1 vaccines providing greater GIA activity than $MSP\text{-}1_{42}$ based vaccines.[47] The vaccine-induced GIA response is also highly parasite strain-dependent, particularly for AMA-1,[48] which mirrors the strain-specificity of the AMA-1 vaccine-induced protective immunity in vivo.[25] In

addition, vaccine-induced increases in GIA responses in adults in endemic regions appear less pronounced.[46]

The development of the growth inhibition assay (GIA) is indebted to the in vitro *P. falciparum* culture methods developed by Tragar and Jensen.[49] Various GIA techniques have been used to qualitatively assess antibody-mediated effects on parasite growth and/or invasion in vitro, from labor-intensive microscopy[42-44,50] and high-throughput biochemical assays of parasite proliferation and viability,[48,51,52] to the use of chimeric murine parasites[53] and GFP-expressing parasites in flow cytometric assays.[54,55] The recent development of isolation and culture methods for *P. falciparum* merozoites has also allowed the development of a highly sensitive merozoite invasion assay.[19]

It is important to recognize that these various functional assays measure distinct components of the antibody response.[52] Most microscopy and flow cytometry assays measure growth as a function of invasion capacity (in other words they are invasion inhibition assays—IIA—but confusingly this term is often used synonymously with GIA). These assays do not measure parasite viability, and some cannot distinguish dead from live parasites. On the other hand, viability assays (e.g., biochemical measurement of parasite lactate dehydrogenase (pLDH)) measure intracellular growth characteristics as well as the invasion capacity of the parasite.[52]

These different methodologies produce functional readouts that do appear to correlate,[52,56] although head-to-head comparisons of different methods are infrequently employed in clinical trials.[27,56] Standardization of the functional assay chosen is extremely important, since the read-outs can be influenced by methodological factors such as the use of whole sera or purified immunoglobulin, methods of immunoglobulin purification, the use of dialysis to remove potential contaminating antimalarial drugs, the number of growth cycles and heat-inactivation of complement.[52,55,57-61] Furthermore, GIA activity is rarely normalized to the concentration of purified immunoglobulin or the sera dilution, and this creates further heterogeneity which limits comparability between studies: for example, AMA-1 vaccine-induced GIA was quoted as 63% in one study using 10 mg/mL of purified IgG,[62] and 70–77% in another using 4 mg/mL of purified IgG.[27] Therefore headline GIA activity may be misleading without appreciating the antibody concentration tested, yet this is rarely reported prominently. Although a somewhat more labor intensive approach, titration of sera/immunoglobulin and presentation of the 50% inhibitory concentration ($IC_{50}$),[63] would greatly improve inter-study comparability. Further moves to standardize the GIA have been taken, including the establishment of the PATH-Malaria Vaccine Initiative funded GIA reference center at the NIH in 2004, yet various iterations of the GIA continue to be employed in clinical studies. For example, in a recent clinical vaccine and challenge study, there was a 2–3-fold difference in the magnitude of GIA activity when the same samples were tested by different methodologies, although the outputs were well correlated.[27] We suggest that vaccine developers reporting GIA should endeavor to report in parallel results on their samples from the GIA reference center, to improve comparability of outcomes for the field. A potential limiting factor to its wide application in field vaccine studies is that GIA often requires a prohibitive quantity of sera considering the bleed volumes permitted in most infant studies in endemic regions, and moves to down-scale assays are welcomed.[51]

In addition to growth or invasion inhibition assays, promising assays to measure additional Fc receptor (FcR) dependent functional antibody activities have been developed. A limited number of field studies have identified that clinical protection or parasitemia correlated with antibody parasite inhibitor activity in the presence of monocytes[64,65] or neutrophils,[66] whereas antibody alone did not. Although highly promising as a functional readout,[67] such assays are technically challenging[66-68] and have not been widely replicated by other groups, therefore their utility to the broader vaccine development field has been limited to date. Another approach takes advantage of transgenic murine parasite lines expressing *P. falciparum* antigens and transgenic mice expressing human FcRs to examine FcR-mediated effector functions in detail.[69] Together these functional assays are likely to contribute significantly to future vaccine development efforts.[16] However, we focus here on the most widely used and repeatable functional assays in blood-stage vaccine development, the growth/invasion inhibition assays (GIA).



## Acquisition of Inhibitory Antibodies

Before examining the utility of GIA in BS vaccine development, it is important to understand more about the kinetics of inhibitory antibody development. Although the well-standardized controlled human malaria infection (CHMI) models in malaria-naïve volunteers provide an appropriate framework to explore such questions, no CHMI studies have reported GIA post-challenge, meaning it is unclear whether a single episode of malaria infection during CHMI results in detectable functional antibody in GIA.[26,27,38,39,62,70-74] In macaques challenged with *P. knowlesi*, substantial increases in GIA activity have been noted post-challenge.[75]

Uncertainty also surrounds the acquisition of inhibitory antibodies after naturally acquired infection. Eisen and colleagues identified that invasion inhibitory antibodies developed rapidly in non-immune travelers who had recently acquired *P. falciparum*,[76] yet in transmigrants the development of MSP-$1_{19}$ inhibitory antibodies required two or more infections.[77] Incremental boosting of the memory B cell compartment by repeated infection has been observed in individuals in malaria transmission settings,[78] but GIA activity appears to be acquired at an early age in high transmission settings,[79] does not appear to be boosted by repeated infections and in fact often decreases with age (see **Table 1**). Using sera from semi-immune Sudanese adults, inhibition of intraerythrocytic parasite growth, but not invasion activity, was temporally associated with transmission.[50] In addition, an increase in autologous parasite inhibition activity was observed over a two-week period in 57% of infected individuals in Burkina Faso.[80] A similar kinetic of increased invasion inhibition activity was observed one month after infection in Vietnam, but by contrast, MSP-$1_{19}$ specific invasion activity fell rapidly, and the end of the transmission season was also associated with

**Table 1.** GIA/IIA field studies

| Ref. | Setting | Design | N | Population | Endpoint | Method | Relationship of GIA to: | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Exposure | "Protection" | Age |
| Jensen[83] | Sudan | Cross-sectional | 177 | Adults | Clinical malaria (oral history) | GIA ($^3$H) sera (all) and IgG (n = 12) | Yes | Yes (sera, not purified IgG) | Not assessed |
| Marsh[84] | Gambia | 1. Cross-sectional 2. Prospective | 1. 280 2. 134 | 1.Children and adults 2. Children | Clinical malaria | GIA ($^3$H) sera | Yes | No | Decreased with age |
| Bolad[80] | Burkina Faso | Prospective | 24 | Children | Acquisition | IIA (fluorescence microscopy) | Yes | Not assessed | Not assessed |
| John[86] | Kenya | Prospective | 76 | Children and adults | Time to infection | PcMSP-1$_{19}$ IIA | Not assessed | Yes | No |
| Corran[85] | Gambia | Cross-sectional | 187 | Children and adults | Infection | PcMSP-1$_{19}$ IIA | Partial (parasiteamia > 5000p/uL) | No | No |
| Perraut[56] | Senegal | Prospective | 205 | Children and adults | Clinical malaria | GIA ($^3$H), PcMSP-1$_{19}$ IIA | Not assessed | No | No |
| Dent[68] | Kenya | Prospective | 98 children 99 adults | Children and adults | Time to infection | GIA (flow cytometry, pLDH) | Not assessed | Yes | Decreased with age |
| McCallum[79] | Kenya | Prospective + Cross sectional | 387 | Children and adults | Infection Acquisition | GIA (flow cytometry) | Transmission intensity | No | Stable/Decreased with age |
| Courtin[87] | Senegal | Retrospective | 305 | Children | Clinical malaria | GIA (pLDH) | No | No | Decreased with age |
| Murhandarwati[81] | Vietnam | Prospective | 134 | Children | Time to infection | PyMSP-1$_{19}$ IIA | Yes | No | No |
| Crompton[88] | Mali | Prospective | 220 | Children and young adults | Clinical malaria | GIA (pLDH) | Not assessed | Yes | Increased with age |
| Bejon[33] | Kenya | Prospective | 866 | Young children vaccinated with RTS,S | Clinical malaria | GIA (pLDH) | Yes | No (inverse correlation, lost on correcting for exposure) | Decreased with age |
| Wilson[106] | Papua New Guinea | Prospective | 206 | Children | Time to infection Clinical malaria | PcMSP-1$_{19}$ IIA | Not assessed | No | No |

GIA, Growth inhibition assay; IIA, invasion inhibition assay; PcMSP-119/PyMSP-119 (chimeric P. falciparum expressing P. chabaudi or P. yoelii merozoite surface protein-119), pLDH, parasite lactate dehydrogenase activity; 3H, Tritium radiometric viability assay



a fall in MSP-1$_{19}$ invasion inhibition activity.[81] Overall the kinetics of acquisition of GIA activity suggest an association with parasite exposure, although this does not necessarily imply a protective role.

## Immunoepidemiology Studies

Many prospective studies have examined the association between protection against malaria infection or clinical disease and antibodies to merozoite surface proteins, and this topic has been subject to a recent comprehensive systematic review in reference 82. Despite considerable heterogeneity in study design and clinical endpoints there is evidence for a positive relationship between the levels of antibodies (measured by ELISA) to several leading blood-stage malaria vaccine candidate antigens [including AMA-1, MSP-1$_{19}$, MSP-3 and glutamate-rich protein (GLURP)] and protection in malaria-exposed populations.[82]

Simply measuring the magnitude of immune responses yields no information on the qualitative nature of the induced antibodies. Unfortunately, far fewer studies have focused on functional characteristics of the antibody response and whether these correlate with protection.[33,56,68,79-81,83-88] Those studies published to date are summarized in **Table 1**.

It can be seen from **Table 1** that the conclusions of these studies in terms of the role of GIA activity in protection are highly conflicting. While some studies suggest a protective role for GIA activity,[68,83,86,88] most do not.[33,56,79,81,84,85,87] These conflicting data have several possible origins. Cross-sectional studies are less informative than prospective studies. In addition, the choice of trial endpoints is influential. A problem common to all field studies is the difficulty in differentiating between the absence of malaria exposure and "protection."[89] Whether this has a significant impact on the data are dependent on the intensity of transmission and other important confounding factors such as the use of ITNs.[90] Misclassification of unexposed individuals as "protected" will significantly bias interpretation of the immune response measured.[33,90] This is a particular problem for studies using GIA since the acquisition of growth inhibitory antibodies appears to be linked to exposure (see **Table 1**). The use of more clinically relevant endpoints such as protection against clinical disease (defined as parasitemia with clinical symptoms vs. asymptomatic parasitemia) may be more informative. However, the definition of clinical disease is also subject to inter-study variability and a lack of agreed standardization.[82]

Additional heterogeneity is introduced by the use of different assays, which as discussed above, measure distinct aspects of the functional antibody response; however Dent and colleagues used three different GIA methodologies and found similar associations.[68] Moreover, even with very similar assays, there can be conflicting data from different populations,[81,86] suggesting the presence of additional confounding effects of transmission intensity and/or host genetic variation.

There is also significant correlation between age and protection against malaria,[91] and to account for this prospective studies should be performed in well-defined age groups and analysis corrected for age.[68,88] Interestingly, it appears from most prospective studies that GIA activity is present in young children and then reduces with increasing age,[33,68,79,84,87] in contrast to the age-related progression of clinical immunity. This could suggest that GIA activity is unrelated to clinical disease immunity, although some authors have suggested a possible contribution to early protection against severe disease.[79] This seems unlikely, since severe disease immunity is generally acquired after one or two infections

and asymptomatic high-density parasitemia is very common in young children in endemic areas. On the other hand, malaria antibodies that interfere with vaccine-induced GIA activity are found in adults[58] and children in endemic areas,[92] and are not found in malaria-naïve individuals.[58] This suggests that GIA "interfering" antibodies may also develop with malaria exposure. The acquisition of these interfering antibodies could partially explain the lack of a positive association between GIA and age or protection. The implications of such interfering antibodies are not yet clear, but these data imply that the sensitivity of GIA may be reduced significantly in certain groups,[46] and raise concerns about the use of GIA as a marker of BS vaccine responses in malaria-experienced populations.

These observations highlight the importance of the fine specificity of the antibody response. For the leading vaccine candidates (MSP-1 and AMA-1), evidence suggests that antibodies to conformational epitopes are critical in mediating GIA activity (reviewed in refs. 11 and 93). Monoclonal antibodies (mAb) with GIA activity against AMA-1 and MSP-1 have been shown in structural studies to inhibit proteolytic processing of the precursor proteins (e.g., 88-kDa AMA1/MSP$1_{42}$), a step required for merozoite invasion, through binding to discontinuous (conformational) epitopes.[11,93] Individuals with MSP$1_{19}$ antibodies displaying cross-competition against the inhibitory monoclonal antibody (mAb) 12.10 had significantly lower parasite densities in cross-sectional studies.[85,94] However, the "interfering" antibodies described above can, through steric inhibition, block the binding of growth/invasion inhibitory antibodies and therefore interfere with this functional activity. Binding affinity, an additional aspect of the fine specificity of the antibody response, may influence the functional phenotype of the antibody, with recent suggestions that higher affinity antibodies may be associated with inhibitory activity, while lower affinity antibodies generate ADCI.[11] Finally, as mentioned briefly before, the specificity of protective antibody can be influenced by the degree of parasite polymorphism. In pre-clinical studies significant diversity of the target epitopes in AMA-1 and MSP-1 attenuates the response against heterologous (non-vaccine) strains.[95] The clinical importance of this target antigen polymorphism was recently illustrated in a phase IIb study of an AMA-1 vaccine based on recombinant 3D7 strain AMA-1, where 3D7 strain-specific protective effects were observed in immunized individuals, but no protection was conferred against heterologous (non-vaccine) strains.[25] A similar pattern was observed in the phase IIb field study of the Combination B vaccine.[28] Therefore parasite antigen polymorphism may significantly influence the association (or lack thereof) between GIA activity and clinical protection in observational studies. Moreover, it should be remembered that GIA is often tested against well-characterized laboratory strains in vitro that may not reflect the parasite strains circulating in the community, further reducing the likelihood of a relationship (if present) being observed.

With such a complex pathogen as *P. falciparum* it seems unlikely that a single immune mediator will ever account entirely for immunological protection.[9] Assessment of a greater breadth of functional immune responses, combined with integrative systems biology approaches, will likely be required to achieve more substantial insights into the major components of protective immunity. This also applies to CHMI studies.

## Controlled Human Malaria Infections (CHMI)

CHMI by either sporozoite or blood-stage inoculation of healthy malaria-naïve volunteers can accelerate candidate vaccine development by providing rapid and robust efficacy readouts.[5,96] These studies are performed in a small number of centers worldwide.[97] Whether efficacy in CHMI predicts field efficacy in target populations remains an open question, since so few candidates have demonstrated convincing efficacy in CHMIs, but the indications are that CHMI can accurately down-select potential candidates, since very few phase IIb studies have detected efficacy in the absence of an efficacy signal in a phase IIa CHMI[5] (the only possible exception is the Combination B vaccine[28,71]). Pre-erythrocytic vaccine efficacy readouts are unambiguous (i.e., sterilizing protection or delays to patency), although mathematical modeling of parasitemia (measured by highly-sensitive quantitative polymerase chain reaction assays) also detects important reductions in liver-to-blood inocula.[98] Blood-stage vaccine CHMI efficacy readouts include delays to microscopic patency by blood-film microscopy, and/or reductions of the in vivo parasite multiplication rate.[70,99,100] Relatively few blood-stage vaccine candidates have been tested by CHMI[96] (see **Table 2**), although more studies have been performed recently in reference 27 and 62, (and clinicaltrials.gov/NCT01142765) as the utility of the CHMI model is increasingly recognized.[97] Until recently the BS vaccine goal-posts in CHMI had not been experimentally defined. PMR in semi-immune individuals appears to be considerably lower than in malaria-naïve individuals,[101] and is also considerably lower than has been achieved in any vaccine CMHI trial to date[26,27,62,71] implying that the first generation of BS vaccines was not sufficiently immunogenic to impact on in vivo PMR. PMR by CHMI is a more clinically relevant study endpoint than in vitro GIA[70] and is most appropriate to determine which candidates to take to the field,[97] but CHMI studies may be impractical in some settings due to logistics, regulatory hurdles and prohibitive costs.[15] In addition, by CHMI no consistent association has emerged between significant reductions in PMR and clinical indicators of BS protection such as delays in pre-patent period.[26,27,62,71] Robust CHMI models (e.g., those involving twice-daily qPCR monitoring of blood-stage parasitemia) should be better capable of predicting field efficacy for BS vaccines as more immunogenic BS vaccine candidates are developed.

If GIA activity is an important mechanism through which antibodies mediate BS protection in vivo, one might expect an association between in vitro GIA and in vivo PMR. This hypothesis has only been tested in a single CHMI study,[62] in which a significant positive correlation was observed in a small group of AMA-1 vaccinated malaria-naïve volunteers despite no effects on pre-patent period or overall PMR in the vaccine group. This finding needs to be replicated in larger cohorts, but does indicate that in vitro GIA may be a useful surrogate for in vivo PMR in

**Table 2.** Published blood-stage vaccine controlled human malaria infection (CHMI) studies

| Reference (year) | N | Vaccine | Location | CHMI | GIA (median) | Antibody conc. (mg/mL) | PMR (median) | Relationship to GIA | Efficacy |
|---|---|---|---|---|---|---|---|---|---|
| Ockenhouse 1998[107] | 35 | NYVAC -Pf7 (multi-stage) | WRAIR | Spz | ND | - | ND | - | Delay to patency |
| Lawrence, 2000[71] | 17 | Combination B | QIMR | BSP | ND | - | NP | - | No |
| Thompson, 2008[26] | 29 | 1. PEV3A (multi-stage) 2. PEV3A + FP9/MVA ME-TRAP | CCVTM | Spz | None | 30.0 | 1: 5.7 2: 6.3 C: 8.7 | - | Reduced PMR Trend to reduced liver-emerging parasites |
| Spring, 2009[27] | 22 | 1. AMA-1 + AS01B 2. AMA-1 + AS02A | WRAIR | Spz | 1. 70% 2. 77% | 4.0 | 1. 14.5 2. 13.9 C. 16.8 | ND | Trend to reduced PMR Significant reduction in liver-emerging parasites and decreased qPCR densities; no delay to patency |
| Duncan, 2011[62] | 8 | AMA-1/C1 + CPG 7909 | CCVTM | BSP | 63% | 10.0 | V: 17.5 C: 17.6 | Yes | No |

vaccinated malaria-naïve individuals. However the association does not imply causality. Additional immune effector mechanisms have been associated with PMR in CHMI including cytokines and regulatory T cells.[73] Unfortunately no other BS vaccine CHMI studies have directly examined the relationship between GIA and PMR. Spring and colleagues modeled PMR by a group method that does not yield values for individual volunteers, meaning correlations could not be assessed, although there was a trend to reduced PMR in vaccine groups with GIA activity.[27] By contrast, in another CHMI study of an AMA-1-containing multistage virosomal vaccine, there was no measurable activity by GIA, despite indications of blood-stage efficacy in one immunised individual,[26] suggesting that either the blood-stage efficacy observed was mediated by a separate immune mechanisms than antibody inhibitory activity, or that the GIA failed to predict potential efficacy. In a separate BS vaccine study, GIA was not performed, and the vaccine had no observed impact on PMR,[71] despite later indications of possible limited strain-specific effects in a phase IIb study.[28] Overall, there is currently insufficient data from CHMI to conclusively determine the relationship between in vitro GIA and in vivo PMR, or the predictive power of GIA, although a promising indication warrants further investigation.

Going forward, CHMI may have significant potential in proof of concept studies to validate GIA and other functional assays, in contrast to field studies where problems of unexposed individuals and interfering antibodies (discussed above) confound the assessment of protection and its relationship to immune markers. Moreover, CHMIs in well-defined semi-immune adult populations[97] may also shed light on the various contributions of functional antibodies (and other immune effector mechanisms) to BS immunity.[96]

## Field Efficacy Studies

In part due to prohibitive blood-draw limits during phase IIb studies in young children and infants, GIA has not generally been performed in field efficacy studies of BS vaccine candidates to date,[25,28,102,103] meaning that prospective correlations with efficacy cannot be assessed. In one pediatric phase IIb trial of an AMA-1 vaccine with Alhydrogel,[103] a modest vaccine-induced increase in GIA activity was observed in children without pre-existing GIA activity (< 20%),[46] but GIA did not correlate with parasitemia,[46] and there was no field efficacy with this vaccine.[103] In order to understand more about the value of functional antibody assays in predicting efficacy (or a lack thereof), approaches to facilitate GIA in field efficacy studies should be explored.[51,54] Feedback of such data will inform future vaccine design.[97]

## Closing Remarks

Although BS vaccine development has been largely disappointing to date, there are several reasons for cautious optimism. Immunogenicity of some vaccine platforms, particularly viral-vectored vaccines and prime-boost regimens, is improving significantly,[12,104] and many potential candidate antigens remain to be tested.[9] For example, a recent promising study of vectored vaccines against the conserved candidate antigen Rh5,[105] suggests that many untested antigens could be effective vaccine targets. Indications of strain-specific efficacy with some monovalent AMA-1 vaccines are also promising,[25] but more work is required to understand how best to down-select the many potential BS vaccine candidates in the pipeline. In vitro functional antibody assays may be important to this effort due to their scalable nature, but it is critical to establish whether the widely used assays such

as GIA are of value in predicting vaccine efficacy. To this end, GIA should be prospectively validated in standardized CHMI models to follow up on indications of a correlation with in vivo parasite multiplication rate.[62] The kinetics of GIA activity could be better defined by prospective studies in endemic settings by controlling for exposure, and by studying kinetics in CHMI. Finally, field vaccine efficacy studies should endeavor to report GIA data to improve our understanding of the important relationship between efficacy and in vitro GIA.

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No potential conflicts of interest were disclosed.


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