An Opsonic Phagocytosis Assay for Plasmodium falciparum Sporozoites

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ABSTRACT Plasmodium falciparum malaria remains the deadliest parasitic disease worldwide. Vaccines targeting the preerythrocytic sporozoite and liver stages have the potential to entirely prevent blood-stage infection and disease, as well as onward transmission. Sporozoite surface and secreted proteins are leading candidates for inclusion in a preerythrocytic stage-specific, antibody-based vaccine. Preclinical functional assays to identify humoral correlates of protection in vitro and to validate novel sporozoite protein targets for inclusion in multisubunit vaccines currently do not consider the interaction of sporozoite-targeting antibodies with other components of the immune system. Here, we describe the development of a simple flow cytometric assay to quantitatively assess the ability of antibodies directed against P. falciparum sporozoites to facilitate their phagocytosis. We demonstrate that this sporozoite opsonic phagocytosis assay (SOPA) is compatible with both monoclonal antibodies and human immune serum and can be performed using cryopreserved P. falciparum sporozoites. This simple, accessible assay will aid with the assessment of antibody responses to vaccination with Plasmodium antigens and their interaction with phagocytic cells of the immune system.

KEYWORDS Plasmodium falciparum, antibodies, assay development, malaria, opsonization, phagocytosis, sporozoite, vaccines

The parasite Plasmodium falciparum causes hundreds of millions of clinical malaria infections and half a million deaths annually (1). The parasite is transmitted by deposition of sporozoite stages into the skin through the bite of infected Anopheles mosquitoes. Motile sporozoites then move in the dermis to locate a capillary, gaining access to the circulation, through which they sequester in the liver. Here sporozoites select and infect hepatocytes, and the parasite then develops as a liver stage, producing tens of thousands of red blood cell-infectious merozoites that initiate blood-stage infection. Stages prior to merozoite emergence from hepatocytes are collectively known as preerythrocytic (PE) stages. The PE stages of infection are asymptomatic and are caused by a relatively small number of parasites. Elimination of PE stages prevents progression to blood stages of infection, responsible for the clinical symptoms of malaria and onward transmission of the parasite (2). These features render PE stages attractive vaccine targets.

Sporozoite surface and secreted proteins are rational antibody targets for vaccines that aim to block sporozoite infection. The most advanced PE stage-specific vaccine candidate consists of the circumsporozoite protein (CSP), which is abundantly expressed on the sporozoite surface. Immunization with a CSP-based vaccine, RTS,S/AS01, a virus-like particle expressing CSP adjuvanted with AS01, achieved modest protection in phase 3 pediatric clinical trials in areas where malaria is endemic that was in part mediated by antibodies (3, 4). An alternative approach, vaccination with live-attenuated sporozoites that are unable to progress to the blood stage, also engenders sporozoite-
neutralizing antibody responses that, together with cellular responses against the liver stages, confer high-level, sterilizing protection in controlled human malaria infection (CHMI) studies (5–7).

Antibodies against sporozoite proteins can block PE-stage infection in a number of ways, including by immobilizing sporozoites in the skin or in the blood or by directly blocking their entry into the liver parenchyma and subsequent hepatocyte infection (8–10). The effects of PE-stage-specific antibodies on these different sporozoite activities can be partially assessed by in vitro assays modeling the transition of the sporozoite from the skin to the liver. These assays include the sporozoite gliding motility and cell traversal assays that model parasite activities in the skin and liver (7, 11), sporozoite invasion assays for hepatocyte infection (12–14), and assays measuring liver-stage development within primary hepatocytes (15). Antibodies are tested for inhibitory activity in these assays, and the collective results are then used to prioritize candidate antigens for further development (16). However, none of the aforementioned assays currently analyze the downstream effects of anti-sporozoite antibodies in conjunction with the effects of cellular components of the immune system.

In addition to the blocking and neutralizing properties of antibodies, phagocytic cells in the skin and liver have the potential to engulf sporozoites that are decorated with antibodies, eliminate them, and initiate an adaptive immune response through antigen presentation to T cells (17). Indeed, opsonization of sporozoites by antibodies has been shown to increase their phagocytosis and elimination by Kupffer cells of the liver (18), and opsonic phagocytosis of recombinant P. falciparum CSP (PfCSP) has been reported to correlate with protection when plasma from RTS,S-immunized individuals is used (19). However, existing assays do not measure this potentially important role of antibodies, and thus, this aspect is not included in guiding the development of a PE stage-specific vaccine for P. falciparum, despite an increasing awareness of the importance of antibody-mediated phagocytosis of blood-stage parasites. In the study described here, we developed a novel sporozoite opsonic phagocytosis assay (SOPA) that can be included in candidate vaccine antigen screening. The SOPA uses the human Fc receptor-positive monocytic cell line THP-1, suitable for phagocytosis studies (20), and live or cryopreserved P. falciparum sporozoites. We demonstrate that the rapid, flow cytometry-based assay can be used with monoclonal antibodies (MAbs) against CSP, as well as blood serum and purified IgG (pIgG) from individuals immunized with a genetically attenuated P. falciparum parasite, P. falciparum GAP 3KO (PfGAP 3KO) (21).

The resulting assay will be useful in the preclinical analysis of the function of antibodies against sporozoites and analysis of humoral responses to PE stage-specific vaccination in clinical trials.

RESULTS

Development and validation of SOPA. We developed a flow cytometry-based assay to measure the capacity of antigen-specific antibodies to facilitate opsonic phagocytosis of sporozoites by the Fc receptor-positive THP-1 cell line (Fig. 1). In developing the assay, we determined that a ratio of sporozoites/cells of 1:3 with a 15-min incubation was optimal (see Fig. S1 in the supplemental material). Using the sporozoite surface protein CSP as a marker, the assay measures changes in the frequency of intracellular parasites when P. falciparum sporozoites are opsonized with either monoclonal antibodies or clinical immune serum and then phagocytosed. Incubation of P. falciparum sporozoites with a human IgG1 MAb reactive against P. falciparum CSP (3C1) (Fig. S2), but not control nonspecific human IgG (hIgG), increased the frequency of intracellular parasites at concentrations as low as 1 ng/ml (P < 0.05), with a 50% effective concentration EC50 of 7.1 ng/ml (Fig. 2A and B). THP-1 cells not incubated with sporozoites were negative for CSP and used as an additional negative control in all assays (data not shown).

Having shown that antigen-specific antibodies facilitated an increase in the number of phagocytic cells positive for sporozoites, we set out to further substantiate that this was due to opsonic phagocytosis. We generated F(ab’1)2 fragments of MAb 3C1 and
verified digestion of the Fc region by PAGE under nonreducing conditions (data not shown). When used in the SOPA, these MAb 3C1 F(ab′)2 fragments were unable to mediate the uptake of sporozoites by phagocytic cells when they were used at the same concentration as complete 3C1, instead showing low uptake levels, similar to the results for control hIgG (Fig. 2C). Phagocytosis is an active cell-mediated process requiring actin polymerization in the phagocyte, which can be inhibited by cytochalasin D. To verify that the assay in fact measures active phagocytosis of sporozoites in the presence of MAb 3C1, we pretreated phagocytic cells with cytochalasin D or its vehicle for 30 min, followed by washing the drug out to prevent inhibition of sporozoite motility. Treatment of cells with cytochalasin D but not its vehicle resulted in a significant 3.1-fold reduction in the number of sporozoite-containing phagocytic cells (Fig. 2D). Finally, we performed the SOPA on permeabilized as well as unpermeabilized cells (Fig. 2E). While hlgG-opsonized sporozoites showed similar levels of CSP-positive (CSP +) cells regardless of permeabilization (indicating that some surface-associated sporozoites were detected in the assay), an increase in CSP + events with MAb 3C1 was seen only in permeabilized cells. Together, these data show that the increase in CSP + phagocytic cells that we observed in the SOPA requires an antigen-specific antibody (Fig. 2A and B) and an antibody interaction with phagocytic cells via their Fc region (Fig. 2C) that is dependent on actin polymerization in the THP-1 cells (Fig. 2D), resulting in an increase in intracellular sporozoites (Fig. 2E). These data are consistent with the SOPA measuring the opsonic phagocytosis of \textit{P. falciparum} sporozoites by THP-1 cells.

**Measurement of SOPA response with serum from whole sporozoite-immunized volunteers.** Having demonstrated the proof-of-concept for the SOPA using a \textit{Pf} CSP-specific MAb, we next sought to determine the compatibility of the assay with serum from humans immunized with whole \textit{P. falciparum} parasites (21).

Serum from two volunteers that had received a single high dose of a triple gene deletion-attenuated sporozoite (\textit{PfGAP 3KO}) (21) was tested in a dilution series in the SOPA to identify the dilution range yielding the greatest fold change between naive and immune sera (Fig. 3). Compared to the responses obtained with untreated sporozoites, we observed differences between the responses of the volunteers’ naive serum, as well as the maximum fold change between matched naive and immune serum samples in the SOPA. One volunteer showed a negligible increase in CSP + cells with naive serum at all tested dilutions (Fig. 3A), while in the other volunteer, a significant increase in CSP + cells was observed at naive serum dilutions of less than 160-fold (Fig. 3B). In both volunteers, the greatest fold change between naive and immune sera was observed at 80- to 160-fold dilutions and at the lowest dilution where naive serum had a negligible effect in the assay. We observed similar differences between volunteers when pIgG rather than whole serum was used in the SOPA (Fig. S3).
To test the reproducibility of the assay, as well as determine its capacity to measure differences in the SOPA response between individuals, we screened the serum of five PfGAP 3KO-immunized volunteers at an 80-fold dilution in the assay (Fig. 4). The maximum fold change between naive and immune sera from these five volunteers revealed significant differences between the responses of their samples (Fig. 4A and B).
These differences were reproduced in a second, independently performed assay, and a significant correlation between the two assay runs was observed, demonstrating the reproducibility of the results (Fig. 4C). Although protection data for PfGAP 3KO are not yet available, these reproducible differences demonstrate that the SOPA could be useful for identifying correlates of protection with human clinical samples. Importantly,

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**FIG 3** Determining parameters for use of human serum in the SOPA. Naive (blue line) and immune (red line) sera from two volunteers (A and B) immunized with PfGAP 3KO were tested in the SOPA across a dilution series. For one volunteer, naive serum performed comparably to untreated sporozoites (dashed line) at all tested dilutions (A), while in another volunteer, a background effect of naive serum was observed at dilutions of less than 160-fold (B). In both volunteers, the percentage of CSP$^+$ cells with immune serum peaked at an 80-fold dilution, with the greatest fold change between naive and immune sera (yellow line) being seen at dilutions of 80-fold or above. Data are means ± SDs from a representative experiment.

**FIG 4** Reproducible differences between clinical serum samples are measured by the SOPA. (A) Matched naive (day 0) and immune (day 13 postimmunization) serum samples from five volunteers immunized with PfGAP 3KO were tested in the SOPA (dashed line, untreated sporozoites). (B) The fold change between matched serum samples for each volunteer was calculated, and statistically significant differences between the responses of the volunteers were observed. (C) The experiment was performed a second time, and the mean fold change between the naive and immune sera for each of the two independent experiments was significantly correlated using Pearson’s correlation. (D) No correlation between CSP antibody titers and the fold change obtained by SOPA was seen for either of these two experiments. The data in panels A and B represent the means ± SDs from one representative experiment. The data were analyzed by one-way analysis of variance with Bonferroni’s post hoc test.* $P < 0.05$; **,$P < 0.01$; ***,$P < 0.001$. The data in panels C and D were analyzed by use of the Pearson correlation. AU, arbitrary units.
the magnitude of the response of clinical samples in the SOPA did not always correlate with the titers of antibodies against PfCSP (Fig. 4D) (21), suggesting that sporozoite antigens other than CSP might be able to facilitate opsonic phagocytosis in this assay. Alternatively, the opsonic activity of CSP antibodies could differ between immunized volunteers.

The above-described experiments were performed using freshly isolated P. falciparum sporozoites, the production of which requires specialized insectary facilities and personnel not available in many laboratories. Methods to cryopreserve P. falciparum sporozoites have been developed, making them more broadly available for research. We thus tested cryopreserved P. falciparum sporozoites in the SOPA to determine their compatibility with the assay (Fig. 5). Opsonization of cryopreserved P. falciparum sporozoites with MAb 3C1 or immune serum from PfGAP 3KO-immunized volunteers resulted in an increase in the number of sporozoite-containing phagocytic cells similar to that seen with fresh sporozoites, indicating that the assay is not restricted to use with fresh sporozoites.

**DISCUSSION**

The contribution of sporozoite-targeted antibodies to protective PE-stage immunity is increasingly recognized for both subunit and whole attenuated sporozoite vaccination (5, 22). These antibodies can act at multiple points during the journey of sporozoites from the dermis to the liver, and a number of in vitro assays are available to measure the inhibition of distinct processes at multiple steps in this journey. These include assays for the inhibition of motility in the skin as sporozoites search for a capillary, traversal of multiple cell types as sporozoites gain access to the circulation and subsequently to the liver parenchyma, as well as, ultimately, the invasion of hepatocytes by sporozoites (16). While these assays are certainly important tools in the analysis of humoral responses against subunit and whole attenuated sporozoite vaccination, they measure only the neutralization of sporozoite protein functions and do not consider the interaction of these parasite-bound antibodies with other immune components.
The significance of antibody effector functions, mediated by the interaction of antibody Fc regions with other immune components, has been recognized in functional assays against the blood stages of malaria infection. The growth inhibition assay (GIA) quantifies reductions in the merozoite invasion of red blood cells with antibodies against merozoite proteins (23). The addition of complement or phagocytic cells to the GIA increases inhibition by most antibodies in this assay and increases the capacity of the assay to predict protection (24–29). Recently, complement fixation by antibodies recognizing glycan residues on sporozoites has been shown to be protective in vivo (30), suggesting that the role of Fc-mediated functions should also be more carefully considered in PE-stage immunity and vaccine development. The SOPA that we report here is the first description of a functional assay using Plasmodium falciparum sporozoites that considers the interaction of antibodies with other immune components via their Fc-mediated effector functions.

Opsonic phagocytosis of sporozoites could be expected to augment clearance of the parasite from the host, as well as enhance the initiation of adaptive immune responses by phagocytic cells. Sporozoite traversal of the sinusoidal barrier in the liver has been shown to facilitate their evasion of phagocytosis and clearance by Kupffer cells (31), and incubation of sporozoites with specific antibodies increases parasite clearance by Kupffer cells in vitro (18). These studies indicate that antibodies bound to sporozoites could enhance their clearance at the parasites’ final barrier to the establishment of liver-stage infection. In the skin-draining lymph nodes, CD11c+ and CD8α+ dendritic cells appear to be important for the initiation of the protective CD8+ T-cell responses observed in whole sporozoite immunizations (32). Enhanced phagocytosis and enhanced antigen presentation have been described for a variety of pathogens with opsonizing antibodies (17), and antibodies recognizing sporozoites could be expected to enhance adaptive immunity during P. falciparum infection. Finally, opsonic phagocytosis has been investigated in the context of RTS,S immunization. In that study, plasma from immunized volunteers facilitated the uptake of recombinant PfCSP, and this correlated with protection (19). It is noteworthy that the previous assay used a protein corresponding to the immunogen of RTS,S immunization, whereas the SOPA that we describe uses whole P. falciparum sporozoites. The use of sporozoites in our assay constitutes a significant improvement over previous approaches, as all sporozoite antigens are present in their natural cellular context and the activity of antibodies to proteins other than CSP can be investigated.

In the study described here, we developed a simple, medium-throughput in vitro assay able to reproducibly measure the antibody-mediated opsonic phagocytosis of whole P. falciparum sporozoites. This SOPA is efficient with both clinical samples (1 to 4 μl of serum is required per sample per experiment) and sporozoites (1.5 × 10⁶ sporozoites are sufficient to test 14 clinical samples with matched naive and immune serum samples) and can be completed in less than 1 day. Using the SOPA, we observed significant differences in opsonic phagocytosis between five volunteers immunized with a PfGAP 3KO vaccine that did not correlate with anti-PfCSP titers. While the small sample size in this study is a limitation, these data suggest that antigens other than CSP may be able to contribute to the opsonic phagocytosis of sporozoites. It is also possible that the difference in the SOPA response observed between these volunteers is related to the specific IgG subclasses of the opsonizing antibodies present in the samples, but this was not further investigated. It will be important to explore this possibility in future studies. Nevertheless, the SOPA appears to be sensitive enough to enable correlative analyses to be performed in the future using samples from subjects with known protection outcomes.

We performed our analysis using the fold change between naive and immune sera, although it is important to note that alternative methods of analysis are possible. For example, the percentage of CSP+ cells observed at a given dilution of immune serum could be employed. Indeed, where fold change was reported, the worst performer among the analyzed volunteers also exhibited the highest percentage of CSP+ cells using immune serum at an 80-fold dilution (Fig. 4). As protection was not assessed in this PfGAP 3KO phase Ia study, we are unable to resolve which method best reflects the
contribution of opsonic phagocytosis to protection in vivo, and the predictive power of the assay remains to be determined.

MATERIALS AND METHODS

THP-1 cell culture. THP-1 cells were maintained in RPMI 1640 complete medium containing 10% fetal bovine serum (Thermo Fisher Scientific), 200 U/ml penicillin-streptomycin (Thermo), and 2.5 µg/ml amphotericin B (Thermo Fisher Scientific) at a cell density of between 0.1 × 10^6 and 1.0 × 10^6 cells per ml. Pretreatment of THP-1 cells with cytochalasin D (Sigma) or its vehicle (dimethyl sulfoxide) to inhibit actin polymerization in some experiments was performed for 30 min at 37°C, followed by two washes in complete medium to remove residual compound before the addition of cells to sporozoites.

Mosquito rearing, sporozoite production, and isolation. Mosquito rearing and P. falciparum sporozoite production were performed as previously described (33). Briefly, P. falciparum NF54 gametoctye cultures were fed to adult female Anopheles stephensi mosquitoes 3 to 7 days after their emergence. Blood-fed mosquitoes were then maintained according to standard protocols for 14 days, after which their salivary glands were dissected and infectious sporozoites were isolated by grinding and centrifugation. In some experiments, cryopreserved sporozoites were used. Following their dissection, sporozoites to be cryopreserved were first purified using a discontinuous density gradient (34) and then resuspended in Schneider’s insect medium (Lonza) containing 300 mM trehalose and 200 µg/ml 1,2-dioleoyl-sn-glycero-3-phosphocholine. Resuspended sporozoites were incubated at room temperature for 10 min before addition of hydroxyethyl starch to a final concentration of 1.7% (vol/vol), followed by rapid freezing by immersion in liquid nitrogen. Prior to their use in the SOPA, cryopreserved sporozoites were thawed at room temperature and pelleted by centrifugation, and the cryopreservation medium was removed.

Generation of PGAP-immunized clinical samples. Naive and immune sera from human volunteers immunized with a P. falciparum genetically attenuated parasite (PGAP 3KO) in a phase la clinical trial were collected as described elsewhere (21). Briefly, volunteers received a single exposure of 150 to 200 bites from PGAP 3KO-infected A. stephensi mosquitoes. Serum was collected from volunteers before infection (naive serum) and 13 days after infection (immune serum), and purified IgG (pIgG) was isolated from these serum samples using protein G columns (catalog number 28-9852-55; GE Healthcare). Immunization of volunteers with PGAP 3KO was approved by the Western Institutional Review Board (WIRB) and the United States Army Research and Material Command, Office of Research Protections, Human Research Protection Office.

Generation and human chimerization of monoclonal antibody 3C1 against P. falciparum CSP. The coding sequence of PCSP (3D7 strain) was cloned into the pET20b vector, expressed in the BL21(DE3) Escherichia coli strain, and protein purified by Ni-affinity chromatography. Subsequent immunization of mice and hybridoma fusion were performed by Green Mountain Antibodies, Inc. (Burlington, VT). Briefly, mice were primed with 50 µg of PCSP emulsified with complete Freund’s adjuvant (CFA), followed by weekly immunization of 50 µg of PCSP emulsified with TiterMax adjuvant (Sigma) or Sigma Adjuvant System (Sigma). One week following the seventh immunization, B cells were isolated from lymph nodes and fused with a mouse myeloma cell line, and positive clones secreting anti-PCSP IgG were selected by enzyme-linked immunosorbent assay. The leading clone was selected for expression as a chimeric antibody with human constant regions. The DNA sequences encoding the heavy- and light-chain variable region genes for the leading clone were amplified by PCR and cloned into expression vectors containing the human κ light-chain constant region and human γ1 heavy-chain constant region. The expression vectors were transfected into HEK293 cells for transient production of chimeric MAb 3C1. The cells were grown in chemically defined medium, and full-length 3C1 was purified from the cell culture supernatant by protein A-affinity chromatography (protein A resin; GE Healthcare).

SOPA. An overview of the SOPA workflow is shown in Fig. 1. Salivary gland P. falciparum sporozoites were first prepared in complete medium at a concentration of 8 × 10^6/ml, and then sporozoites were opsonized with 3C1-hIgG1, nonspecific human IgG (hIgG), or serum or pIgG derived from patients immunized with PGAP 3KO (21). Sporozoites were then incubated at 37°C for 15 min, before 1.5 × 10^4 opsonized sporozoites were plated into triplicate wells of a 96-well V-bottomed plate. THP-1 cells, prepared at 1 × 10^6/ml in complete medium, were added to each well at a 1:1 volume with sporozoites to achieve a ratio of sporozoites/cells of 1:3. The plate was then centrifuged at 500 × g for 3 min and incubated at 37°C for 15 min. Cells were then immediately fixed without washing in Perm/Fix buffer (BD Biosciences), blocked, permeabilized in Perm/Wash buffer (BD Biosciences), and then stained with a monoclonal antibody directed against PCSP conjugated to Alexa Fluor 647 (clone 2A10). THP-1 cells containing sporozoites were identified by flow cytometry using a BD LSR II flow cytometer (BD Biosciences), and data analysis was performed using FlowJo software (TreeStar). In some experiments, an F(ab)’2 fragment of 3C1 generated by pepsin digestion of the Fc region (catalog number 44688; Thermo Fisher Scientific) was used in place of full-length 3C1.

Statistical analysis. Data were analyzed using GraphPad Prism software (version 6) for Windows, and differences were considered statistically significant when P was <0.05.

SUPPLEMENTAL MATERIAL

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TEXT S1, PDF file, 0.5 MB.
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