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Development and Optimization of High-Throughput Methods To Measure *Plasmodium falciparum*-Specific Growth Inhibitory Antibodies

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Antibodies that inhibit replication of *Plasmodium falciparum* in erythrocytes are thought to be important both in acquired immunity to malaria and as mediators of immunity generated by candidate blood-stage vaccines. However, several constraints have limited the study of these functional antibodies in population studies and vaccine trials. We report the development and optimization of high-throughput growth inhibition assays with improved sensitivity that use minimal volumes of test serum. The major inhibitory activity of serum from exposed donors was antibody mediated, but nonspecific inhibitory factors were found in untreated serum. Culture volumes could be effectively reduced to 25 μl to limit amounts of test serum or inhibitors used in assays. Performing inhibition assays over two cycles of parasite replication gave greater sensitivity than single-cycle assays, and a simple two-cycle inhibition assay was developed that yielded highly reproducible results. Determination of parasite growth by flow cytometry was most suitable for high-throughput assays using small culture volumes and was more sensitive than parasite lactate dehydrogenase assays and less prone to error and variation than microscopy. We evaluated and optimized methods to remove antimalarials and nonspecific inhibitory factors from serum that are suitable for use with small volumes of samples that are typically obtained from clinical studies. Both microdialysis and immunoglobulin purification by ammonium sulfate precipitation were effective and practical. These methods should facilitate evaluation of vaccine trials and clinical studies of immunity and are also suitable for testing drugs and other compounds for antimalarial activity.

Plasmodium falciparum malaria is a major cause of mortality and morbidity, resulting in around 500 million clinical cases each year (25). At present, there is no effective vaccine for the prevention of malaria, and escalating drug resistance has presented an increasing barrier to effective disease control. Those who live in areas of malaria endemicity and do not die from the disease at a young age eventually develop effective immunity against malaria that limits blood-stage parasitemia and prevents severe and symptomatic malaria (4, 18). Antibodies are believed to be an important component of acquired protective immunity. Passive transfer of immunoglobulins (Ig) from immune donors to individuals with *P. falciparum* infection has been shown to reduce parasitemia and clinical symptoms (9).

Antibodies that inhibit the invasion of red blood cells by the merozoite form of the parasite are thought to be an important component of protective immunity by limiting parasite blood-stage growth in vivo (6, 8), thereby reducing total parasite biomass and organ-specific sequestration that contribute to disease pathogenesis. Monoclonal and polyclonal antibodies against several merozoite antigens generated by vaccination in animals inhibit invasion (7, 19, 26) and may confer protection in animal models (11, 23). However, very few studies have examined in detail the association between inhibitory antibodies and protective immunity in human studies due to methodological constraints on performing these assays in large studies in a reliable and reproducible manner with a limiting amount of test sera available. Although measuring antibodies to recombinant merozoite antigens by enzyme immunoassays has been widely applied in population studies, this approach has significant limitations and does not appear to be sufficiently informative when used alone. Recombinant antigens may not be in the same conformation as native proteins, and it is unclear how antibody levels relate to inhibitory function. Furthermore, such assays typically do not account for antibody affinity and fine specificity, which may be critical for inhibitory activity. Production of full-length and correctly folded recombinant malaria proteins is generally highly challenging and has only been achieved with a very limited number of candidate antigens. In the case of merozoite surface protein 1 (MSP1), for example, recent studies found a poor correlation between antibodies to recombinant MSP1-19 and MSP1-19-specific growth inhibitory antibodies (14, 20). Furthermore, acquired antibodies to MSP1 do not necessarily inhibit invasion and can block the action of inhibitory antibodies (13). Antibodies may also act by inhibiting the processing of merozoite antigens required for erythrocyte invasion (3, 12); these antibodies are not measured by conventional immunoassays using recombinant proteins. Such issues emphasize the need for functional assays to study immunity.

Reproducible high-throughput assays are essential for examining the role of inhibitory antibodies in protective immunity in

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population studies and vaccine trials and for the identification of targets of inhibitory antibodies. However, a number of factors have limited the application of growth inhibition assays (GIAs) to large population studies of malarial immunity. These include the time-consuming nature of the assays, small volumes of serum available from donors, particularly children, and the presence of antimalarial drugs in many clinical samples that hamper the measurement of inhibitory antibodies. In addition, there is a need for inhibitory assays with greater sensitivity to detect inhibitory antibodies in samples. An increasing number of transgenic parasite isolates with defined modifications to specific merozoite antigens (10) are valuable tools for identifying targets of inhibitory and/or protective antibodies. Presently, standard inhibition assays evaluate inhibitory effects during one cycle of erythrocyte invasion, and parasitemia is determined by microscopy, which is time-consuming and difficult to apply on a large scale. Here, we have addressed these constraints through the development and optimization of high-throughput inhibitory assays with improved sensitivity that generate reproducible results and use minimal volumes of serum. We have also developed and evaluated methods to remove antimalarials and nonspecific inhibitory factors from small-volume serum samples for use in GIAs that are suitable for large population studies.

MATERIALS AND METHODS

***P. falciparum* culture.** *P. falciparum*-infected erythrocytes (IEs) of the 3D7, D10, W2mef, and CS2 lines were maintained in vitro at pH 7.4 in plastic petri dishes using human group O+ erythrocytes, at 3% hematocrit, in RPMI-HEPES medium supplemented with 50 μg/ml hypoxanthine, 25 mM $NaHCO_3$, 20 μg/ml gentamicin, 5% (vol/vol) heat-inactivated pooled human sera (of various blood groups) from donors resident in Australia, and 0.25% Albumax II (Gibco, Invitrogen, Mount Waverly, Australia) maintained in an atmosphere of 1% O_2, 4% CO_2, and 95% N_2 at 37°C, as previously described (1). Cultures were synchronized two or three times per week by resuspending culture pellets in 5% D-sorbitol (Sigma, St Louis, MO) in water to lyse trophozoite- and schizont-infected erythrocytes.

Growth inhibition assays. Parasite cultures were synchronized the day before starting the assay. At the commencement of the assay, the majority of parasites were at the late-pigmented-trophozoite to schizont stage (IEs) with few ring forms present. Parasite suspensions at 0.1 to 1% parasitemia and 1% hematocrit were cultured in 96-well plates that were incubated in a sealed, humidified, gassed box. Sterile U-bottom (353077) and flat-bottom (353072) plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) were used with a culture volume of 25 to 50 μl/well, and small-volume plates (Microscreen; Robbins Scientific, Sunnyvale, CA) were used with a culture volume of 10 μl. The outer wells of each plate were not used but were filled with 150 μl of buffer for humidification purposes (resulting in 60 usable wells per plate). Parasitemia was determined using flow cytometry unless stated otherwise. In the optimized two-cycle assay, 25 μl of parasite suspension was added per well at a starting parasitemia of 0.4%, using U-bottom plates. Thereafter, 2.5 μl of test serum was gently mixed into each well and incubated at 37°C. After 48 h, 5 μl of culture medium was gently mixed into all wells and incubation continued. After 80 to 96 h, parasitemia was assessed using flow cytometry (see below). Parallel cultures were maintained and monitored for parasite development to determine the optimal time to measure parasitemia (when most parasites were late ring forms or early pigmented trophozoites). Incubation time was influenced by the stage and synchronicity of parasite cultures at commencement of the assay and by the length of the life cycle of the parasite line used. Serum and antibody samples were tested in duplicate.

Measurement of parasitemia. Thin smears of cultures were fixed in methanol and Giemsa stained for measurement of parasitemia by microscopy. For flow cytometry, 100 μl of 10-μg/ml ethidium bromide (Bio-Rad, Hercules, CA) in phosphate-buffered saline (PBS; pH 7.3) was mixed with 25 μl of parasite culture and incubated for 1 h in darkness at room temperature. After centrifugation, the supernatant was discarded, cells were resuspended in 200 μl/well of PBS, and the samples were processed using a FACScan or a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). With the FACSCalibur, an automated 96-well plate sampler was used in high-throughput mode and each sample was mixed three times before analysis. Parasitemia was evaluated using FlowJo software (Tree Star, Inc., Ashland, OR) by first gating for intact erythrocytes by side scatter and forward scatter parameters and subsequently determining the proportion of ethidium bromide-positive cells.

Relative parasite growth rates were also determined by measuring parasite lactate dehydrogenase (pLDH) activity, as described previously (15). Parasite culture samples for testing were first frozen and thawed to lyse cells and kept on ice until used. Nitroblue tetrazolium (2 mg/ml in water) and phenazine ethosulfate (0.1 mg/ml in water) were mixed with Malstat reagent (15), in a ratio of 1:1:10, immediately before use. Fifty microliters of the reagent mix was added to 20 μl of parasite culture (1 to 2% hematocrit) in 96-well microtiter plates. We found that this ratio of regents and parasite culture gave maximum signal compared to the background. Samples were incubated for at least 45 min at room temperature to allow color development, and absorbance was measured at 655 nm. Uninfected red blood cells (RBCs) at equivalent hematocrit were included as controls. To calculate relative growth of samples, absorbance values for uninfected RBCs were deducted from all sample values, and the value for each test sample was expressed as a percentage of control serum samples. All samples were tested in duplicate or triplicate.

Treatment of serum samples. Serum samples (50 to 100 μl) were dialyzed against PBS in 50-kDa-molecular-weight-cutoff (MWCO) dialysis tubes (2051; Chemicon, Temecula, CA) or in 60-kDa-MWCO Micro dispo dialyzer tubes (Spectrum Laboratories, Inc., Rancho Dominguez, CA) and subsequently reconcentrated to the original starting volume using centrifugal concentration tubes (100-kDa MWCO; Pall Corp., Ann Arbor, MI). For purification of Ig by ammonium sulfate, 70 μl of serum was diluted with 360 μl sterile 0.15 M NaCl and kept on ice, and 300 μl of saturated ammonium sulfate was slowly added. After 30 min of incubation on ice, the precipitate was isolated by centrifugation and washed once with 50% saturated ammonium sulfate solution before the pellet was dissolved in PBS. Samples were then dialyzed against PBS and reconcentrated by centrifugation, as described above, to the starting volume of 70 μl. IgG was purified using magnetic beads coated with protein G (Dynal Biotech ASA, Oslo, Norway) or protein G-Sepharose (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's instructions. Control human IgG was obtained from Pierce Biotechnology (Rockford, IL). Serum samples used were obtained from adults and children resident in the Kilifi District, Kenya, anonymous adult blood donors in Papua New Guinea (PNG), pregnant and nonpregnant adults resident in the Blantyre area, Malawi (1), and nonexposed adult residents in Melbourne, Australia. Ethical approval was obtained from the Ethics Committee of the Kenya Medical Research Institute, Nairobi, Kenya; the Medical Research Advisory Committee, PNG; the College of Medicine Research and Ethics Committee, Blantyre, Malawi; and the Walter and Eliza Hall Institute Ethics Committee, Melbourne, Australia. Informed consent was obtained from all donors.

RESULTS

Optimization of conditions for growth inhibition assays. To generate reproducible results, we first optimized growth of asexual-stage parasites in vitro in 96-well tissue culture plates. Using the 3D7 line, maximum multiplication rates were obtained using RPMI-HEPES supplemented with 0.25% Albumax and 5% pooled human serum from nonexposed donors, with additional glutamine added (2 mM). The addition of HEPES (25 mM) to RPMI 1640 medium resulted in a 5% increase in growth. When using medium supplemented with 5% pooled serum from nonexposed donors (with or without 0.25% Albumax II), the addition of extra serum (up to a total serum concentration of 15%) did not consistently or significantly alter parasite growth. Hanks medium, in place of RPMI-HEPES, was investigated for both the 3D7 and D10 *P. falciparum* lines, but no obvious advantage was seen using this medium (the growth of 3D7 was 3% more in Hanks medium than in RPMI-HEPES, and for D10, it was 2% less). Using 3D7, growth was reduced by 35% at pH 7.0, in comparison to growth at pH 7.4, but increased by up to 30% when pH was 7.5

(all of the above experiments were performed twice, in duplicate, in 96-well microtiter plates, generating similar results).

Sera or plasma from donors, especially children, are usually obtainable only in small quantities. Therefore, there is a need to minimize the amount of test serum used in assays, especially if individual sera are to be tested in a range of assays. We established that high replication rates are obtained in 96-well plates using small incubation volumes. We evaluated replication rates in round-bottom plates (50- and 25-μl incubation volumes), flat-bottom plates (50 μl), and small-volume plates (10 μl). Using a starting parasitemia of 0.4 to 1% and incubation for one cycle of replication, no significant differences could be seen between the volumes of 50 and 25 μl using round-bottom plates (for example, when the starting parasitemia was 0.6, end parasitemia was 3.08 and 3.15%, respectively, mean of triplicate samples) or between flat-bottom and round-bottom plates. Multiplication rates achieved with D10, 3D7, and W2mef across many assays typically ranged from five- to eightfold per cycle. As a negative control, wells filled with uninfected red blood cells (incubated for 48 h and stained with ethidium bromide) gave a "parasitemia" of 0.05% using flow cytometry. Parasite growth was also achieved in 10-μl volumes in plates with small-volume wells; however, growth of parasites was reduced to 20% (mean of two experiments) compared to 50- or 25-μl culture volumes. Subjecting parasite culture plates to continuous agitation using a circulating tube roller, gel rocker, or plate shaker, did not improve the assay with regard to multiplication rates or sensitivity for detecting inhibitory antibodies (data not shown) and was associated with increased evaporation and lysis of cells in some experiments.

Comparison of methods for measurement of parasite growth. Different methods can be used to measure parasitemia and determine the relative inhibitory activity of samples. The use of flow cytometry to measure parasitemia using nucleic acid stains, such as ethidium bromide, has been validated and reported elsewhere (2). We confirmed this using our methods with which we found a very high correlation between parasitemia determined by flow cytometry compared to microscopy across several assays (e.g., $r = 0.940$, $n = 12$, one experiment). Using flow cytometry to measure parasitemia in single-cycle growth inhibition assays, we consistently found a high level of reproducibility in results, and repeat assays were significantly correlated (e.g., $r = 0.904$, $P < 0.0001$, correlation between two experiments using 24 dialyzed serum samples). To further evaluate results using microscopy versus flow cytometry, we performed a 48-h, 25-μl growth assay in round-bottom plates and parasites were highly synchronous at commencement of the assay. By flow cytometry, 100,000 events were counted, whereas by microscopy, 1,000 red blood cells were counted. When microscopy was used, 98% of the ring-stage parasites found at 24 h went through to pigmented trophozoites at 48 h, indicating that there was no major inhibition in the growth of parasites at these stages (data not shown). At 48 h, microscopy and flow cytometry gave results that were within the same range. When five of the slides were recounted again by the same person, the mean standard deviation was 32%, whereas by flow cytometry, it was only 4%.

An alternative high-throughput assay for evaluation growth inhibition is measurement of pLDH, which has been validated for drug inhibition assays (15). However, a modified assay (described in Materials and Methods) was required here that

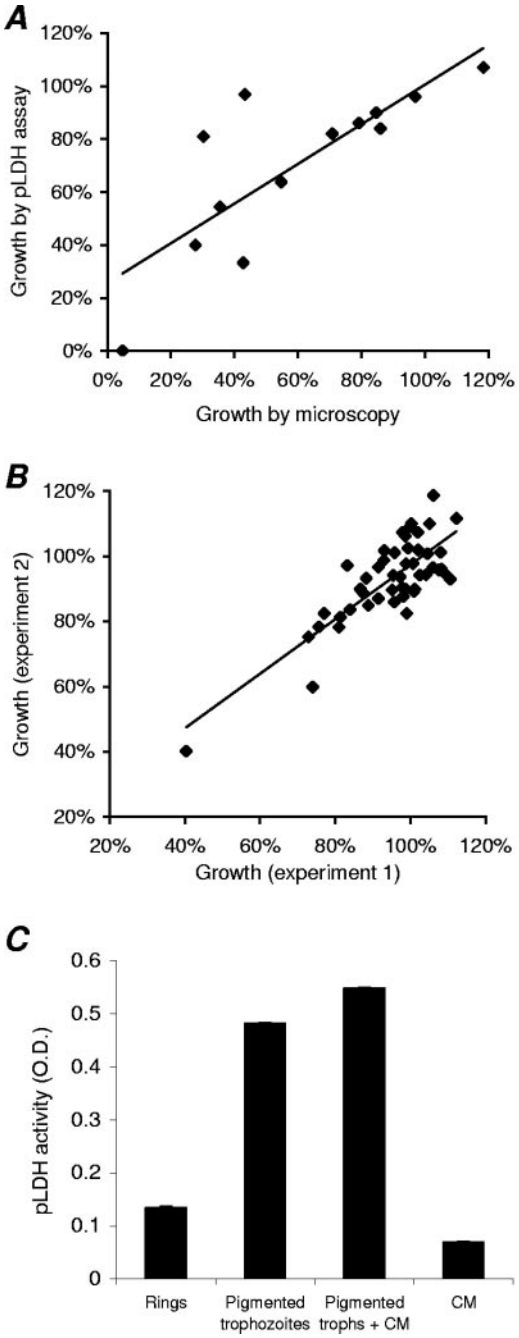

FIG. 1. Measurement of parasite LDH activity for evaluation of *P. falciparum* growth. (A) Sera from adult donors were tested in a growth inhibition assay over one cycle of parasite replication, and parasitemia was measured using microscopy or pLDH activity. Growth inhibition determined by the two methods was significantly correlated ($r = 0.794$, $P = 0.001$). Results are expressed as percentages of parasite growth among nonexposed serum controls. (B) Sera were tested in repeat growth inhibition assays over one cycle of replication, with evaluation of parasitemia by pLDH activity. Results were significantly correlated ($r = 0.804$, $P < 0.001$). Data are expressed as percentages of parasite growth among nonexposed serum controls. (C) Parasite LDH activity was measured among ring-stage parasites (rings), mature pigmented trophozoites, pigmented trophozoites (trophs) with spent culture medium (CM), or spent culture medium alone. LDH activity was severalfold higher among pigmented trophozoites compared to rings, and the majority of activity was present in parasitized erythrocytes rather than in the parasite culture medium. The parasite line CS2 was used. Results represent means + standard errors of the means (samples tested in triplicate).

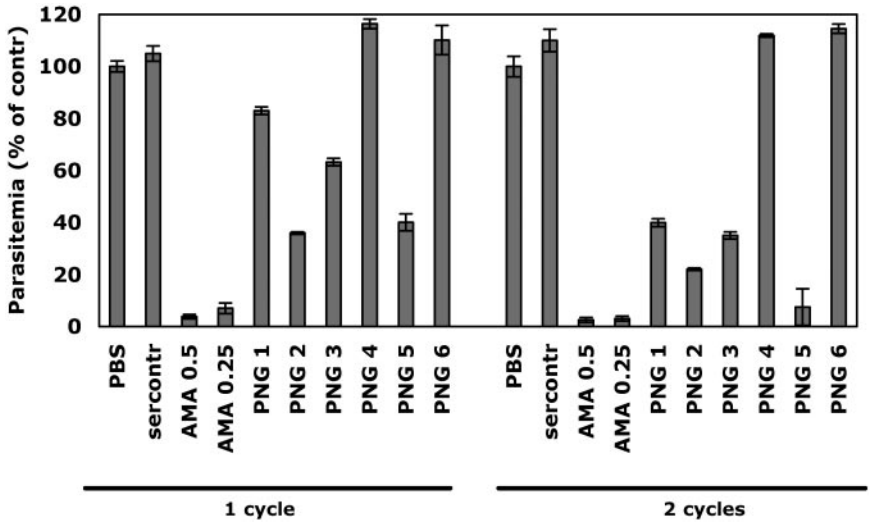

FIG. 2. Comparison of *P. falciparum* growth inhibition assays performed over one or two life cycles. The starting parasitemia (using parasite line 3D7) for the two-cycle assay was one-fourth of the parasitemia for the single-cycle assay. The degree of inhibition by sera or antibodies was greater in the two-cycle assay. Results are expressed as percentages of PBS controls (contr) for each assay. Sercontr, serum from normal Australian donor; AMA, rabbit polyclonal antibody against AMA1, used at 0.5 or 0.25 mg/ml; PNG 1 to 6, dialyzed sera from Papua New Guinean adults. All samples were used in duplicate, and error bars indicate ranges.

would be suitable for small culture volumes to minimize use of serum or Ig samples. In an initial analysis, there was a strong correlation ($r = 0.97$) between parasitemia and pLDH signal using serial dilutions of mature-stage IEs from culture. In one-cycle growth inhibition assays with serum from adult donors, there was an overall correlation between the inhibitory activities of samples determined by pLDH assay versus microscopy (Fig. 1A) ($r = 0.794$, $n = 13$, $P = 0.001$), although there were substantial differences observed with some sera. We also generally observed a good correlation between flow cytometry-based assays and pLDH-based assays; however, the pLDH assays were typically less sensitive for detecting inhibitory activity of samples (addressed in further detail below). Using synchronous parasites, results from repeat testing of 50 samples in separate one-cycle GIAs were highly correlated (Fig. 1B) ($r = 0.804$, $P < 0.001$). The major component of pLDH activity arises from mature pigmented trophozoite IEs present in cultures. Using the CS2 parasite line, pLDH activity from pigmented trophozoite-stage IEs was 3.6-fold higher than ring-stage IEs collected 24 h earlier (Fig. 1C). Some pLDH was also present in the medium (13% of the total signal from a culture of mature-stage IEs). We did not detect any significant signal from serum samples from exposed donors that were used in the assays (data not shown).

Development of a high-throughput two-cycle growth inhibition assay. We aimed to develop a high-throughput assay with greater sensitivity for detecting inhibitory antibodies. For this reason, we investigated assays that measured inhibitory activity over two cycles of replication to amplify any inhibitory effects of samples, with measurement of parasitemia by flow cytometry. Performing comparisons between single- and two-cycle assays, conducted under optimized conditions, detailed above, demonstrated that the two-cycle assay was substantially and significantly more sensitive across many different experiments. A representative experiment is shown in Fig. 2. Starting parasitemia for the two-cycle assay was one-fourth of that for the one-cycle assay, with the assays performed in parallel. The effect of anti-AMA1 antibodies was substantially greater after two cycles (57% more inhibition using 0.25 mg/ml of antibody). Furthermore, sera from adults living in areas of malaria endemicity in Papua New Guinea were clearly more inhibitory than controls after two cycles than after one cycle ($P < 0.001$ for 29 sera tested in parallel in one- and two-cycle assays; Mann-Whitney U test). For reproducibility reasons, we found it important to perform careful gating of parasitized red blood cells when evaluating the results from flow cytometry. For parasite lines 3D7 and D10, ending the two-cycle assay after approximately 80 h made gating easier when analyzing flow cytometry data, since almost all of the parasites were in the late ring or early trophozoite stage at this time (Fig. 3). When gating was made at 96 h, some parasites had already reached early ring stage in a third cycle, which made it more difficult to differentiate normal and parasitized red blood cells. However, for some lines, it was preferable to end the assay at up to 96 h incubation. In all cases, a parallel culture was monitored for parasite development and growth. Selective gating of ring- and mature-stage parasites was possible and gave very similar results to gating the whole parasite population (data not shown). Low hematocrit and starting parasitemia values were required so that two-cycle assays with high replication rates could be performed without changes of culture medium. However, the addition of 5 or 10 μl of culture medium after the first cycle significantly enhanced parasite replication (30% increase when 5 μl was added and 25% when 10 μl was added). Therefore, in all two-cycle assays, we included the addition of 5 μl of culture medium after the first cycle.

When flow cytometry was compared to microscopy for the two-cycle assay, both methods gave similar results (Fig. 4A). We also evaluated the effect of intermittent agitation of culture plates on assay results. When the plates were agitated twice a

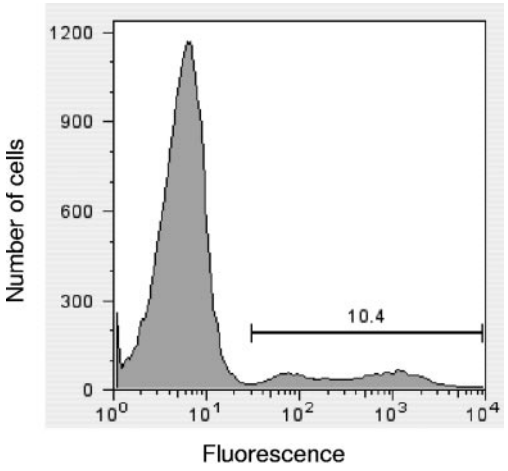

FIG. 3. Evaluation of parasitemia using flow cytometry. 3D7 parasites were used in a growth inhibition assay performed over two life cycles; the assay was stopped after approximately 80 h. Parasitized erythrocytes were labeled with ethidium bromide, and cells were processed using a FACSCalibur flow cytometer. Data were analyzed and plotted using FlowJo software. The population of intact erythrocytes was first gated and then plotted as a histogram. The *y* axis shows the number of events, and the *x* axis shows fluorescence in channel 2 (for ethidium bromide). The total parasitemia in this example was 10.4%.

day (cells were resuspended without removing them from sealed culture boxes) during the two-cycle assay, the differences between samples increased. Inhibitory samples were generally somewhat more inhibitory, whereas noninhibitory samples tended to result in increased growth compared to plates that were not agitated (Fig. 4B). Furthermore, we found the two-cycle assay to be highly reproducible and to give consistent findings. For example, results from repeat testing of dialyzed serum from exposed PNG donors were significantly correlated (Fig. 4C) ($r = 0.872$, $n = 21$, $P < 0.001$).

To further establish the validity of the assays, we examined parasite replication at different starting parasitemias and the effect of increasing concentrations of inhibitory AMA1 antibody (Fig. 5). With increasing starting parasitemia, final parasitemia after 80 h increased in a predictable manner. Multiplication rates were similar across the range of starting parasitemias (0.1 to 0.8%) but were maximal at 0.2 to 0.4% (which gave multiplication rates of 4.7 to 4.6 per cycle). The addition of AMA1 antibodies gave an approximately linear relationship between inhibition and antibody concentration. In this assay, we also compared flow cytometry with pLDH for evaluation of parasitemia. Flow cytometry was clearly more sensitive for detecting inhibitory activity of antibodies and differences in growth rates; the pLDH assay did not differentiate well between the inhibitory effects of different concentrations of AMA1 antibodies or between different total parasitemias resulting from varying the starting parasitemia.

Preparation of serum/plasma samples and demonstration of direct growth inhibition by acquired antibodies. The presence of antimalarials and antibiotics in serum samples collected from individuals in countries of malaria endemicity and other nonspecific factors can markedly affect results of assays to measure growth inhibitory antibodies. We found dialysis of samples to be an effective and practical way to remove anti-

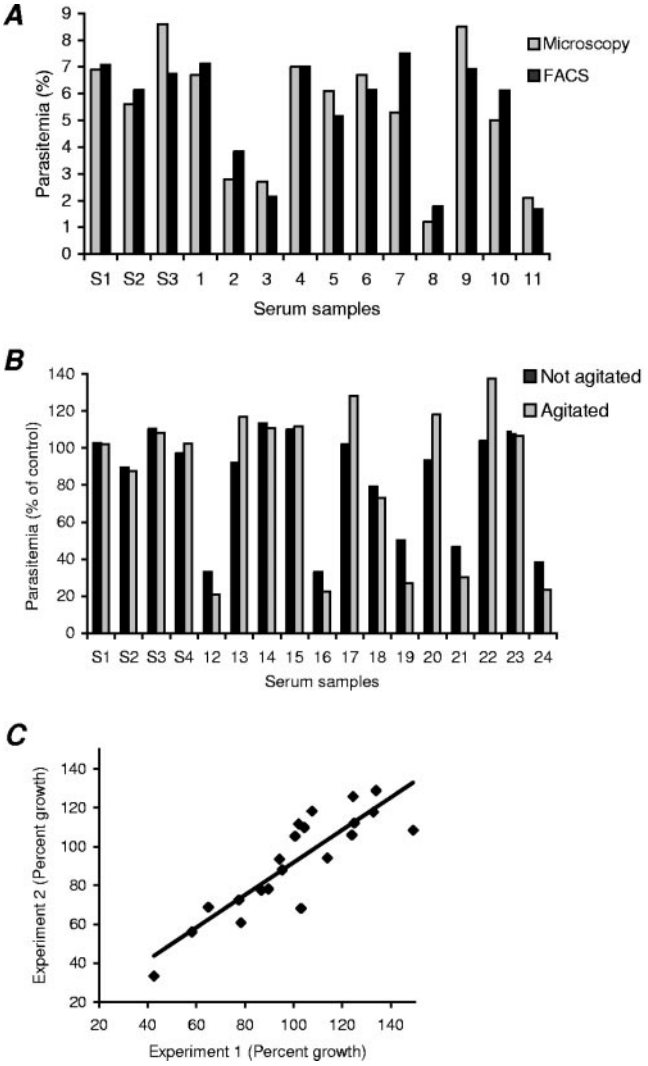

FIG. 4. Two-cycle growth inhibition assays. (A) Comparison of flow cytometry versus microscopy for evaluation of parasite growth using dialyzed serum from exposed and nonexposed donors. (B) Comparison of results from growth inhibition assays performed with or without intermittent agitation, using dialyzed serum samples. Results are expressed as a percentage of parasite growth among nonexposed serum controls. S1 to 4, sera from nonexposed Australian donors; 1 to 24, sera from adults living in Papua New Guinea. (C) Dialyzed sera were tested in repeat growth inhibition assays over two cycles of replication, with evaluation of parasitemia by flow cytometry. Results were significantly correlated ($r = 0.872$, $P < 0.001$). Data are expressed as percentages of parasite growth among nonexposed serum controls.

malarials and nonspecific inhibitory factors present in human sera using small volumes (50 to 200 μl of serum). To clearly demonstrate this, we added quinine in different concentrations to serum from an Australian donor and dialyzed 50 μl against PBS, followed by reconcentration to the original volume. This treatment resulted in successful removal of quinine. Serum with quinine added strongly inhibited parasite growth, but after dialysis, there was no inhibitory effect (Fig. 6A). We also tried treating serum using only 100-kDa-MWCO centrifugal concentration tubes (without foregoing dialysis), but this was not as effective and gave more varying results (data not shown).

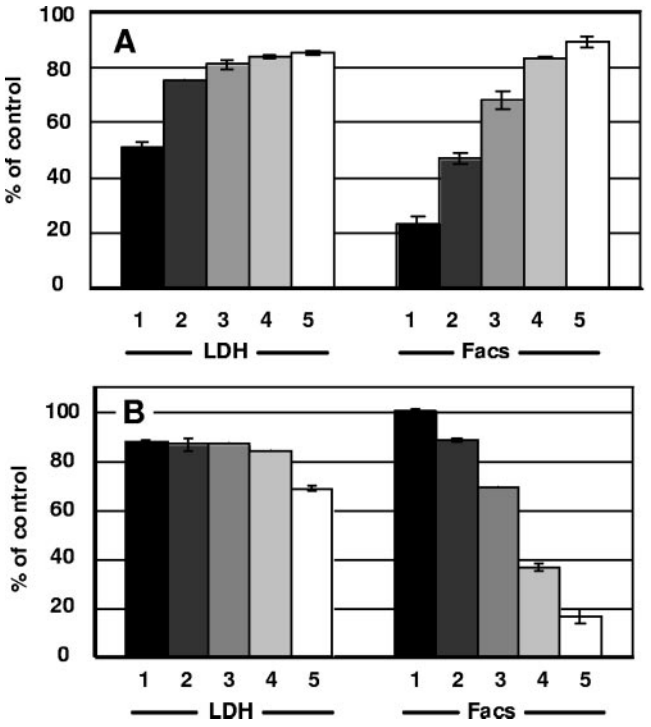

FIG. 5. Evaluation of two-cycle growth inhibition assays with measurement of parasitemia (3D7 parasites) using pLDH activity (LDH) versus flow cytometry (Facs). (A) Different concentrations of polyclonal rabbit antibody against AMA1 were tested for inhibition of parasite growth. Final concentrations (mg/ml) are as follows: 1, 0.5; 2, 0.25; 3, 0.12; 4, 0.06; 5, 0.03. (B) The effect of using different starting parasitemias on parasite replication over two cycles was evaluated. Starting parasitemias are as follows: 1, 0.8%; 2, 0.6%; 3, 0.4%; 4, 0.2%; 5, 0.1%. Results are expressed as percentages of parasite growth among nonexposed serum controls. All samples were tested in duplicate, and error bars indicate ranges.

In addition, testing dialyzed samples from a range of nonexposed donors did not demonstrate any inhibitory activity, compared to PBS as the control.

As an alternative to dialysis, and to confirm that the inhibitory activity of dialyzed serum was due primarily to antibodies, we performed small-scale Ig purification of 50- to 100-μl serum samples from adults resident in Papua New Guinea. First, magnetic beads coated with protein G were used in different amounts (according to the manufacturer's instructions). This resulted in a yield of protein so low (<0.5 mg/ml) that we did not pursue this method any further. Protein G-Sepharose was tested with 100 to 200 μl dry gel and 100 to 600 μl binding buffer in different combinations. The yield of protein from these experiments varied from 2 to 3 mg/ml. Ig was also purified by ammonium sulfate precipitation, followed by dialysis and reconcentration to the original starting volume, resulting in a harvest of 4 to 8 mg/ml protein. This method gave the best yield of protein, was also practical for processing large numbers of samples, and was lower in cost. We performed sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of untreated and dialyzed serum samples and Ig purified from sera by ammonium sulfate precipitation (Fig. 7). This showed that, after dialysis alone, most serum proteins remained, although some smaller proteins were present at a lower concentration than in untreated serum. After ammonium sulfate precipitation, the majority of protein obtained had a size that corresponded to IgG.

To validate and compare these methods, 21 serum samples from adults living in Papua New Guinea (plus 5 samples from nonexposed Australian donors) were dialyzed, Ig purified by ammonium sulfate precipitation, or used untreated in a growth inhibition assay. A selection of representative results is shown in Fig. 6B. The untreated samples were sometimes seen to be strong inhibitors of parasite growth, but this inhibitory effect sometimes disappeared after dialysis or ammonium sulfate precipitation, suggesting nonspecific inhibitory activity in serum. A number of samples remained inhibitory delete after dialysis or using purified Ig. The effects of dialysis or ammonium sulfate precipitation on parasite growth were very similar for all samples tested. Therefore, we concluded that the inhibitory effect of dialyzed samples is mainly due to Ig rather than other factors.

We generally tested serum or dialyzed serum at a 1/10 dilution. Increasing the serum concentration to 1/5 in an attempt to increase the sensitivity to detect inhibitory activity was generally not advantageous, as nonspecific inhibitory effects were sometimes observed with nonexposed serum (Fig. 6C). We consistently found that a 1/10 dilution gave greater inhibition than a 1/20 dilution, both when inhibitory sera were tested and when anti-AMA1 was added to the sera. We also observed that a small number of freeze-thaw cycles of exposed sera or purified antibodies had no significant effect on inhibitory activity. When four samples from nonexposed donors were frozen and thawed (each time left for half an hour in room temperature) 25 times, and stored at −20°C, there was a mean increase in growth of 3D7 parasites in a two-cycle assay of 34%. After only three freeze-thaw cycles, no significant difference could be seen. This suggests that serum samples should be frozen and thawed as few times as possible.

DISCUSSION

The ability to perform reliable in vitro inhibition assays is a prerequisite for evaluation of blood-stage vaccine candidates and for identifying targets of protective antibodies against malaria. Here we have developed and validated a sensitive high-throughput assay of growth inhibitory antibodies that uses small amounts of serum together with high-throughput methods for preparation of serum when using small sample volumes. Greater sensitivity and reproducibility was achieved over conventional assays by testing inhibitory activity over two cycles of replication and evaluating parasitemia by flow cytometry. We have also minimized sample volumes required for testing. Since sera from studies in regions of malaria endemicity are usually from children, the total volumes available are typically very small and it is preferable to reduce the amounts needed for each assay, particularly if multiple assays are to be performed. In our assays, we used 2.5 μl of patient serum per well, which is significantly less than what has been used in other assays (6, 8, 17, 20). Since the variation between duplicate samples in our optimized assay is very small, there is a reduced need for replicate samples and retesting, which further reduces the amount of sample required.

We validated our optimized two-cycle assay by demonstrat-

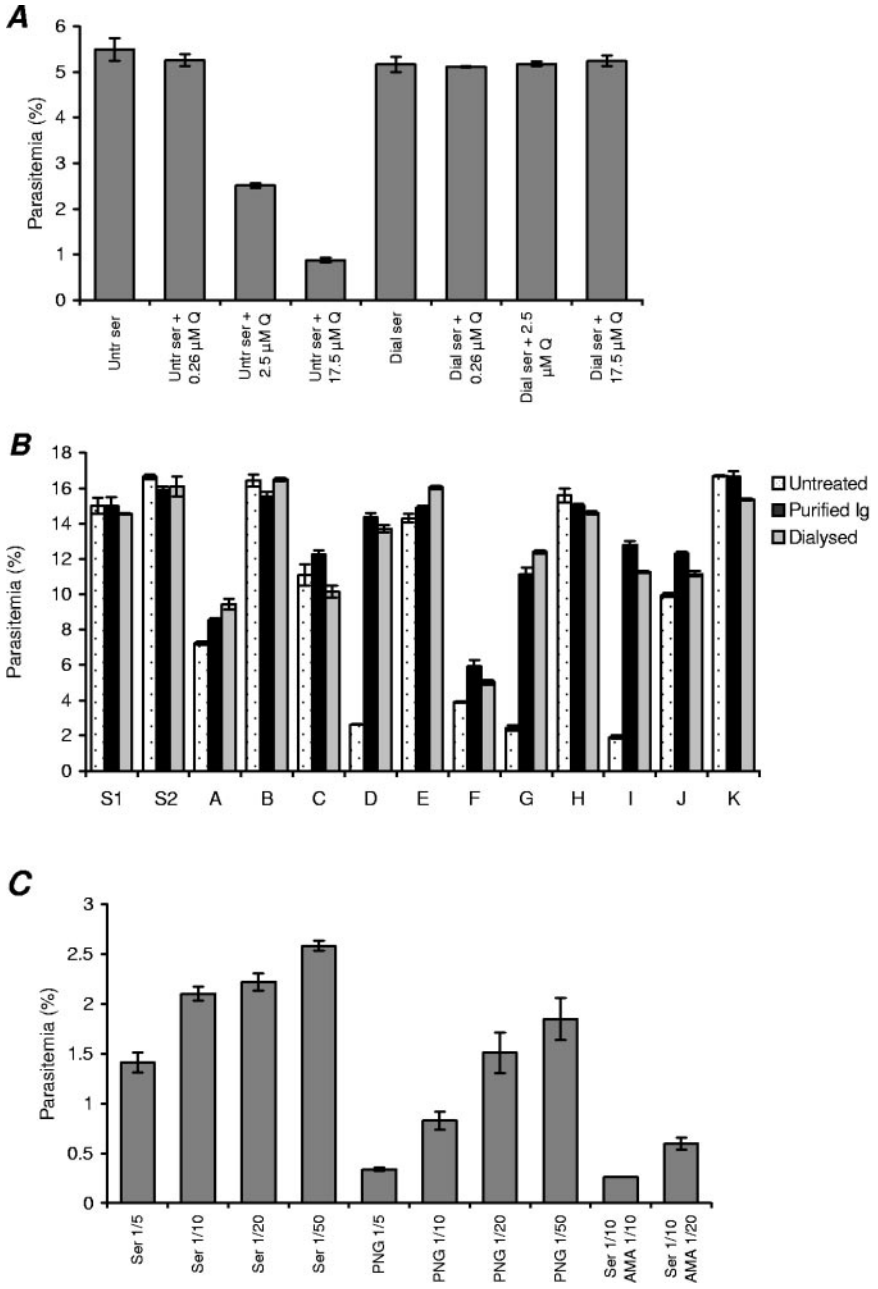

FIG. 6. (A) Dialysis of serum samples effectively removes antimalarial drugs added to serum. Quinine in different concentrations was added to serum from a nonexposed Australian donor. The serum was dialyzed and used in a growth inhibition assay performed over one cycle of replication with measurement of parasitemia by flow cytometry. Untr ser, untreated serum; Q, quinine; Dial, dialyzed. (B) Comparison of dialyzed serum samples, Ig purified from the same sera, and untreated serum tested for growth inhibitory activity using an assay performed over two cycles of parasite replication. S1 and S2, sera from nonexposed Australian donors; A to K, sera from adult residents of PNG. (C) Different dilutions of serum or plasma were tested in a growth inhibition assay performed over two life cycles. AMA, rabbit polyclonal antibody against AMA1; PNG, plasma from an adult from PNG; Ser, serum from a nonimmune Swedish donor. All samples were used in duplicate with the 3D7 parasite line, and error bars indicate ranges.

ing that results from repeat assays were highly correlated and that variation in starting parasitemia and the addition of different concentrations of inhibitory AMA1 antibody gave entirely predictable results. There was a near-linear relationship between end parasitemia and start parasitemia and between degree of inhibition and antibody concentration. Optimal performance of small-volume two-cycle assays involved using culture medium supplemented with Albumax, hypoxanthine, serum from nonexposed donors, and glutamine, cultured in volumes of 25 μl per well with the addition of 5 μl of culture medium after 48 h. Serum samples are used at a 1/10 dilution, and parasitemia is measured by flow cytometry.

We confirmed that inhibition of parasite growth was mediated by antibodies by using Ig purified from serum. Dialysis

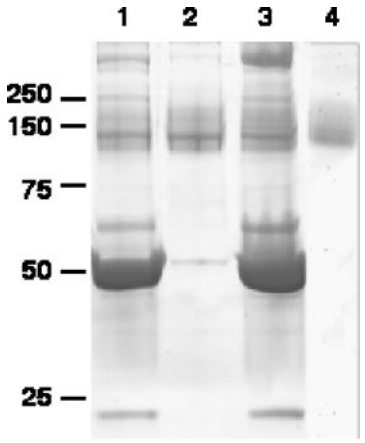

FIG. 7. SDS-PAGE of serum and immunoglobulins stained with Coomassie. Lanes 1 to 3, serum from the same nonimmune Australian donor; lane 1, dialyzed serum; lane 2, Ig purified from serum by ammonium sulfate precipitation; lane 3, untreated serum; lane 4, purified human IgG (from Pierce).

was shown to be an effective method of removing antimalarials and, possibly, other nonantibody inhibitory factors from sera and can be readily used with small sample volumes. For purification of Ig, ammonium sulfate precipitation had several advantages over protein G beads and gave a greater yield. Ammonium sulfate precipitation copurifies IgM, which may be an important component of the inhibitory activity. When the ammonium sulfate-precipitated samples were examined by SDS-PAGE, the majority of protein seen was composed of IgG. When these samples were compared with dialyzed samples, the results were very similar. However, dialysis is a more straightforward and reproducible way of preparing samples because there are fewer steps in this procedure, less protein is lost, and a smaller volume of samples can be processed. This is particularly important when volumes are scarce, which they often are in clinical studies. Removal of nonspecific inhibitory effects improves the validity and usefulness of the assay. It may be possible to modify this assay to include the addition of monocytes to measure antibody-dependent cellular inhibition (5).

We found that growth inhibition assays run over two cycles of erythrocyte invasion were more sensitive than single-cycle assays, and the degree of inhibition by test serum or antibodies increased significantly. Although parasites were typically less synchronous after two cycles than after one cycle of invasion, this problem was overcome by stopping the assay at an appropriate stage, which was typically after 80 h, and by gating all parasites when analyzing flow cytometry data. Others have successfully used multicycle assays for phenotyping the invasion characteristics of isolates (21). One factor that is probably important for reproducibility is optimization of growth conditions. Hence, for each parasite line, factors such as different culture media, starting parasitemias, and pH should be evaluated. When a large number of samples are being tested, we usually employ two people to set up the assay to minimize the setup time. To increase reproducibility, repeated freezing and thawing of patient samples should also be avoided. In developing a simple two-cycle invasion assay that did not involve changes of media, which is cumbersome and may introduce error, it was necessary to reduce the hematocrit and starting parasitemia to avoid overgrowth of parasites and exhaustion of nutrients in culture medium. We established that, under our conditions, the total parasite numbers remained below the critical support capacity of the medium. Replication rates were similar across a range of starting parasitemias (Fig. 5) and were similar between the first and second cycles of replication.

Using flow cytometry considerably facilitated measurement of parasitemia. The sensitivity in differentiating parasitized from nonparasitized red blood cells appears similar whether microscopy or flow cytometry is used. Flow cytometry is much more rapid than microscopy, and the number of red blood cells that can be counted in a short time is radically higher, leading to decreased variation between samples. When a 96-well automated plate sampler is used with the flow cytometer, even more samples can be read in the same time and the risk of error when transferring samples from plates to tubes is eliminated. Interobserver and within-observer error is a potential major problem with microcopy. Reading of 60 samples takes around 15 min with a plate sampler by flow cytometry, but with microscopy it takes 3 to 5 h. Additionally, it is difficult to prepare good quality blood smears for microscopy when performing small volume assays. Several different nucleic acid dyes are suitable for labeling parasitized RBCs. The advantage of ethidium bromide is that it can be used on standard flow cytometers, whereas dyes such as 4′,6′-diamidino-2-phenylindole (DAPI) and Hoechst require UV excitation, which is not available on standard flow cytometers.

Other high-throughput methods, as alternatives to microscopy, have been reported for evaluation of parasite growth in drug assays. These include pLDH-based assays, radiolabeled-hypoxanthine incorporation, and staining of parasite nucleic acids with fluorescent dyes (15, 22, 24). A potential limitation of these assays is that larger culture volumes and greater amounts of parasite material are generally used and they have not been widely adapted for assays with human serum. A recent study reported the use of a pLDH-based single-cycle assay to measure vaccine-induced antibodies in human sera using larger culture volumes than in our assays (16). In our studies, we found that pLDH assays generally gave reproducible results, when compared to microscopy or flow cytometry, for measurement of inhibition by serum or antibodies in small-volume single-cycle assays; however, this method was generally less sensitive than microscopy or flow cytometry. When pLDH determination was used in two-cycle assays, the sensitivity was considerably lower than for flow cytometry. The pLDH assay might have worked better if larger volumes and higher hematocrit had been employed (to give greater parasite material), but our aim was to perform assays that used volumes that were as small as possible. One potential reason for the reduced sensitivity of the pLDH-based assay for differentiating inhibitory activities or parasitemia may arise from asynchrony in parasite cultures, particularly when using a two-cycle assay. We demonstrated here substantial differences in pLDH activity among ring-stage and mature pigmented trophozoites; ring-stage and late-stage parasites can be separately analyzed among data from flow cytometry. A further limitation of indirect methods for determining parasitemia, such as pLDH assays (15), fluorimetry (24), or radioisotope incorporation (22), is that it is not possible to easily check for potential nonspecific

inhibitory effects (e.g., cell lysis) or examine differences in parasite stages between test samples. This can be readily done with flow cytometry by reviewing data plots for each sample by using the appropriate software.

In conclusion, we have developed reliable high-throughput assays with greater sensitivity for measuring growth inhibitory antibodies in serum and demonstrated that the major inhibitory activity in serum is due to antibodies. Application of these assays, specifically adapted for use in population studies and clinical trials, should greatly facilitate studies of potential molecules for malaria vaccines and for the identification and measurement of mediators of protective immunity. Additionally, these assays would also be suitable for testing of other inhibitors, such as antimalarial drugs, peptides, or other molecules, particularly where greater sensitivity is needed and when the amounts of test inhibitors are limited.

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REFERENCES

1. **Beeson, J. G., G. V. Brown, M. E. Molyneux, C. Mhango, F. Dzinjalamala, and S. J. Rogerson.** 1999. *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. J. Infect. Dis. **180:**464–472.
2. **Bianco, A. E., F. L. Battye, and G. V. Brown.** 1986. *Plasmodium falciparum*: rapid quantification of parasitemia in fixed malaria cultures by flow cytometry. Exp. Parasitol. **62:**275–282.
3. **Blackman, M. J., T. J. Scott-Finnigan, S. Shai, and A. A. Holder.** 1994. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J. Exp. Med. **180:**389–393.
4. **Bloland, P. B., D. A. Boriga, T. K. Ruebush, J. B. McCormick, J. M. Roberts, A. J. Oloo, W. Hawley, A. Lal, B. Nahlen, and C. C. Campbell.** 1999. Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission. II. Descriptive epidemiology of malaria infection and disease among children. Am. J. Trop. Med. Hyg. **60:**641–648.
5. **Bouharoun-Tayoun, H., P. Attanath, A. Sabchareon, T. Chongsuphajaisiddhi, and P. Druilhe.** 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes. J. Exp. Med. **172:** 1633–1641.
6. **Brown, G. V., R. F. Anders, G. F. Mitchell, and P. F. Heywood.** 1982. Target antigens of purified human immunoglobulins which inhibit growth of *Plasmodium falciparum in vitro*. Nature **297:**591–593.
7. **Chappel, J. A., and A. A. Holder.** 1993. Monoclonal antibodies that inhibit *Plasmodium falciparum* invasion *in vitro* recognise the first growth factor-like domain of merozoite surface protein-1. Mol. Biochem. Parasitol. **60:**303–312.
8. **Cohen, S., G. A. Butcher, and R. B. Crandall.** 1969. Action of malarial antibody in vitro. Nature **223:**368–371.
9. **Cohen, S., I. A. McGregor, and S. C. Carrington.** 1961. Gamma-globulin and acquired immunity to human malaria. Nature **192:**733–737.
10. **Cowman, A. F., D. L. Baldi, J. Healer, K. E. Mills, R. A. O'Donnell, M. B. Reed, T. Triglia, M. E. Wickham, and B. S. Crabb.** 2000. Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells. FEBS Lett. **476:**84–88.
11. **de Koning-Ward, T. F., R. A. O'Donnell, D. R. Drew, R. Thomson, T. P. Speed, and B. S. Crabb.** 2003. A new rodent model to assess blood stage immunity to the Plasmodium falciparum antigen merozoite surface protein 119 reveals a protective role for invasion inhibitory antibodies. J. Exp. Med. **198:**869–875.
12. **Dutta, S., J. D. Haynes, J. K. Moch, A. Barbosa, and D. E. Lanar.** 2003. Invasion-inhibitory antibodies inhibit proteolytic processing of apical membrane antigen 1 of Plasmodium falciparum merozoites. Proc. Natl. Acad. Sci. USA **100:**12295–12300.
13. **Guevara-Patino, J. A., A. A. Holder, J. S. McBride, and M. J. Blackman.** 1997. Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. J. Exp. Med. **186:**1689–1699.
14. **John, C. C., R. A. O'Donnell, P. O. Sumba, A. M. Moormann, T. F. de Koning-Ward, C. L. King, J. W. Kazura, and B. S. Crabb.** 2004. Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage *Plasmodium falciparum* infection in individuals in a malaria endemic area of Africa. J. Immunol. **173:**666–672.
15. **Makler, M. T., J. M. Ries, J. A. Williams, J. E. Bancroft, R. C. Piper, B. L. Gibbins, and D. J. Hinrichs.** 1993. Parasite lactate dehydrogenase as an assay for Plasmodium falciparum drug sensitivity. Am. J. Trop. Med. Hyg. **48:**739–741.
16. **Malkin, E. M., D. J. Diemert, J. H. McArthur, J. R. Perreault, A. P. Miles, B. K. Giersing, G. E. Mullen, A. Orcutt, O. Muratova, M. Awkal, H. Zhou, J. Wang, A. Stowers, C. A. Long, S. Mahanty, L. H. Miller, A. Saul, and A. P. Durbin.** 2005. Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. Infect. Immun. **76:** 3677–3685.
17. **Marsh, K., L. Otoo, R. J. Hayes, D. C. Carson, and B. M. Greenwood.** 1989. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans. R. Soc. Trop. Med. Hyg. **83:**293–303.
18. **Mwangi, T. W., A. Ross, R. W. Snow, and K. Marsh.** 2005. Case definitions of clinical malaria under different transmission conditions in Kilifi District, Kenya. J. Infect. Dis. **191:**1932–1939.
19. **Narum, D. L., J. D. Haynes, S. Fuhrmann, K. Moch, H. Liang, S. L. Hoffman, and B. K. Sim.** 2000. Antibodies against the Plasmodium falciparum receptor binding domain of EBA-175 block invasion pathways that do not involve sialic acids. Infect. Immun. **68:**1964–1966.
20. **O'Donnell, R. A., T. F. de Koning-Ward, R. A. Burt, M. Bockarie, J. C. Reeder, A. F. Cowman, and B. S. Crabb.** 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J. Exp. Med. **193:**1403–1412.
21. **Okoyeh, J. N., C. R. Pillai, and C. E. Chitnis.** 1999. *Plasmodium falciparum* field isolates commonly use erythrocyte invasion pathways that are independent of sialic acid residues of glycophorin A. Infect. Immun. **67:**5784–5791.
22. **O'Neill, M. J., D. H. Bray, P. Boardman, J. D. Phillipson, and D. C. Warhurst.** 1985. Plants as sources of antimalarial drugs. Part 1. In vitro test method for the evaluation of crude extracts from plants. Planta Med. **Oct:**394–398.
23. **Rotman, H. L., T. M. Daly, R. Clynes, and C. A. Long.** 1998. Fc receptors are not required for antibody-mediated protection against lethal malaria challenge in a mouse model. J. Immunol. **161:**1908–1912.
24. **Smilkstein, M., N. Sriwilaijaroen, J. X. Kelly, P. Wilairat, and M. Riscoe.** 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob. Agents Chemother. **48:**1803–1806.
25. **Snow, R. W., C. A. Guerra, A. M. Noor, H. Y. Myint, and S. I. Hay.** 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature **434:**214–217.
26. **Thomas, A. W., J. A. Deans, G. H. Mitchell, T. Alderson, and S. Cohen.** 1984. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. Mol. Biochem. Parasitol. **13:** 187–199.