CRITICAL EVALUATION OF DIFFERENT METHODS FOR MEASURING THE FUNCTIONAL ACTIVITY OF ANTIBODIES AGAINST MALARIA BLOOD STAGE ANTIGENS

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Abstract. Antibodies are thought to be the primary immune effectors in the defense against erythrocytic stage Plasmodium falciparum. Thus, malaria vaccines directed to blood stages of infection are evaluated based on their ability to induce antibodies with anti-parasite activity. Such antibodies may have different effector functions (e.g., inhibition of invasion or inhibition of parasite growth/development) depending on the target antigen. We evaluated four methods with regards to their ability to differentiate between invasion and/or growth inhibitory activities of antibodies specific for two distinct blood stage antigens: AMA1 and MSP1.42. We conclude that antibodies induced by these vaccine candidates have different modes of action that vary not only by the antigen, but also by the strain of parasite being tested. Analysis based on parasitemia and viability was essential for defining the full range of anti-parasite activities in immune sera.

INTRODUCTION

Antibodies directed against bloodstage malaria have been shown to be efficacious in the prevention of disease as shown in passive transfer experiments in humans.1-3 The mechanisms by which the antibodies neutralize parasites in vitro differ greatly depending on the target antigen. Modalities include merozoite opsonization, targeting them toward phagocytic cells of the host,4 prevention of invasion,5 inhibition of parasite development within the erythrocyte,6,7 and interference with merozoite dispersal by agglutination.8,9 Most methods for analyzing functional antibodies against bloodstage parasites in vitro are based on microscopic evaluation of blood smears or detection of DNA in erythrocytes and do not assess parasite viability.8,9,10-12 In contrast, the measurement of enzymatic activity of the parasite-derived lactate dehydrogenase (pLDH),13,14 the conversion of dihydroethidium to ethidium by NADPH oxidase and interference with merozoite dispersal by agglutination.8,9 Most methods for analyzing functional antibodies against bloodstage parasites in vitro are based on microscopic evaluation of blood smears or detection of DNA in erythrocytes and do not assess parasite viability.8,9,10-12 In contrast, the measurement of enzymatic activity of the parasite-derived lactate dehydrogenase (pLDH),13,14 the conversion of dihydroethidium to ethidium,15,16 and the quantification of 3H-hypoxanthine incorporation into newly synthesized DNA17,18 all assess parasite viability and thus can measure both invasion and growth inhibition.

The objective of this study was to compare parasite inhibition results obtained with four methods that measure either in vitro invasion inhibition or growth inhibition (viability) using a model system comprised of antigen-specific immune sera and 3D7 as well as FVO parasite cultures. Antisera were raised against the 3D7 and FVO alleles of AMA1 and MSP1.42,20,21 antigens that are candidates for bloodstage malaria vaccines. Invasion inhibition was measured by quantifying parasites in Giemsa-stained blood smears and by flow cytometric analysis of parasites whose DNA was stained with Syto16.22 Whereas staining with Giemsa can reveal antibody-induced, morphologic changes in parasite development, concluding that these changes also affect parasite viability is subjective. Syto16 readily permeates membranes of both viable and non-viable cells and is therefore not useful for measuring cell viability. Growth inhibition (viability) was measured by flow cytometric analysis of parasites whose DNA was stained by using hydroethidine (HE) and by measuring the enzymatic activity of pLDH.13,14 HE staining depends on the intracellular conversion of HE into ethidium by NADPH oxidase and has been described in various protozoan systems including malaria to be a reliable metabolic indicator of parasite viability.15,16

Comparing the four techniques in our model system allowed us to determine that these Abs function either by invasion inhibition or by growth inhibition and that the mechanism of inhibition depended on the parasite test strain. In cases where Abs acted by invasion inhibition, all four methods gave similar results. As observed previously, AMA1-specific Abs were invasion inhibitory,23,24 whereas antibodies directed against MSP1 preferentially inhibited invasion or inhibited parasite growth and development, depending on the parasite test strain. This study clearly shows that, when analyzing bloodstage malaria parasite-specific antibodies, methods that can distinguish between invasion inhibition and viability of the intraerythrocytic parasite must be used to more fully define the Ab mechanism of action.

MATERIALS AND METHODS

Parasite cultures. Complete medium was prepared with RPMI 1640 (Invitrogen, Carlsbad, CA) containing 25 mmol/L HEPES, 7.5% wt/vol NaHCO3, and 10% human pooled serum (blood type O+). Plasmodium falciparum strains 3D7 and FVO were maintained and synchronized by the temperature cycling method.25

For the evaluation of immune sera, triplicate cultures were set up in replicate culture plates in the presence or absence of 20 vol% immune serum ~6 hours before rupture occurred (starting parasitemia, 0.3%; 1% hematocrit uninfected erythrocytes) in 96-well plates under static conditions. Replicate culture plates were set up to preclude repeated sampling at different time-points from the same plate, ruling out sampling effect on the growth of the parasites. Time-points indicated refer to time after schizont rupture in every experiment. Thus, each time-point was collected from its own plate and analyzed by the various methods in triplicate. All experiments were repeated independently at least three times.
Antisera. New Zealand White rabbits (Spring Valley Laboratories, Woodbine, MD) were immunized subcutaneously four times with either recombinant MSP1_{42} of the FVO strain (36/36/36/36 μg, six rabbits) or the 3D7 strain (200/50/50/50 μg, five rabbits) emulsified in complete/incomplete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO), and serum pools were prepared. Antisera against recombinant AMA1 of the FVO and the 3D7 strains were raised in individual rabbits after immunizing intramuscularly three times with 50 μg PpAMA1 using Montanide ISA720 (SEPPIC, Fairfield, NJ) as adjuvant. Pooled control sera were prepared from sera of four rabbits immunized three times with 50 μg of reduced/alkylated MSP1_{42} (3D7) and MSP1_{42} (FVO) in complete/incomplete Freund’s adjuvant.

Microscopic analysis. Cultures were harvested at various time-points as indicated, and blood smears were made from each well. Blood smears were fixed in methanol and stained in a 10% Giemsa solution (Sigma) for 10 minutes. Slides were washed in water and allowed to air dry before analysis. Evaluation was performed by a Nikon (Nikon, Tokyo, Japan) E400 Eclipse. Three slides per group were evaluated by counting 2,000 red blood cells (RBCs) or 100 parasitized RBCs/slide. Growth inhibition was calculated using the following formula: percent growth inhibition = (1 − [parasitemia of culture/parasitemia of control culture]) × 100.

Flow cytometry. Cells were recovered at different time-points, and a 50-μL aliquot was transferred into polystyrene tubes (Becton Dickinson, Mountain View, CA) for subsequent staining with hydroethidine (Polysciences, Warrington, PA) or Syto16 (Molecular Probes, Eugene, OR). Stock solutions of HE were prepared at 10 mg/ml dissolved in dimethylsulfoxide (DMSO) (Sigma) and stored at −30°C. Samples were stained by adding 500 μL of freshly diluted HE (diluted 1:200 in 37°C phosphate buffered saline [PBS]; BioWhittaker, Walkersville, MD) to the parasite suspensions and incubated for 20 minutes at 37°C. Syto16, purchased as a 1 mM/L solution, was diluted to 200 mM/L with PBS; a 500-μL aliquot was added to the parasite suspensions and incubated for 30 minutes at 37°C. To stop the staining for both methods, samples were transferred to ice (which allowed stabilization of the staining for up to 2 hours) and were diluted with 1 mL PBS before analysis. The data were acquired by a FACScalibur flow cytometer (Becton Dickinson) using CellQuest software for acquisition and analysis. Growth inhibition was calculated using the following formula: percent growth inhibition = (1 − [parasitemia of culture/parasitemia of control culture]) × 100.

LDH assay. Cultures for growth inhibition assays were set up at the schizont stage in 96-well plates and cultured for one cycle (i.e., 40 hours for 3D7, 48 hours for FVO). Cells were harvested, washed, and frozen at −30°C until analysis. pLDH was detected and measured as described elsewhere. Growth inhibition was calculated using the following formula: % growth inhibition = (1 − [(OD_{sample} − OD_{RBC})/(OD_{control serum} − OD_{RBC})]) × 100.

Statistical methods. Results from detection of growth/invasion inhibition obtained using Syto16 and HE in three independent experiments were compared with the Student t test (two-tailed). For comparing the results of all four assays, the Box-Cox power transformation was used to stabilize variance in the data. Differences in growth inhibitory activity among these factors were evaluated by using an analysis of variance technique for a 3 × 4 factorial experiment of a randomized block design and adjusted for multiple comparisons with Tukey’s simultaneous test (family error rate = 5%). Results from the two parasite test strains were evaluated separately.

RESULTS

Microscopic analysis of invasion/growth inhibitory effects of bloodstage-specific antisera. Parasite cultures were established at the early schizont stage in the presence of immune or control sera. The AMA1-specific sera were only tested against the homologous strain, whereas anti-MSP1_{42} was tested against both homologous and heterologous strains. Figure 1 summarizes the microscopic evaluation of blood smears stained with Giemsa. The graphs show the mean percentage of reduction in parasitemia and the 95% CI of 3D7 (Figure 1A) and FVO (Figure 1B) cultures at various time-points during the bloodstage cycle. For this analysis, every parasitized cell was counted as one invasion event including cells infected with multiple parasites. We counted all parasites associated with RBCs, i.e., rings as well as residual schizonts. We did not exclude these residual schizonts from the parasitemia analysis because of the possibility that they may rupture at a later time-point and produce a delayed new burst of young rings. In three independent experiments conducted with 3D7 parasite cultures, treatment with MSP1_{42} (3D7)-specific, MSP1_{42} (FVO)-specific, and AMA1 (3D7)-specific antisera caused an overall reduction of parasitemia by 13%, 25%, and 46%, respectively. Similarly, with FVO parasite cultures, treatment with MSP1_{42} (3D7)-specific, MSP1_{42} (FVO)-specific, and AMA1 (FVO)-specific antisera caused an overall reduction of parasitemia by 10%, 52%, and 42%, respectively. To determine if any of the antibodies merely delay development rather than inhibit growth, we extended the analysis by 6 hours after the second round of invasion was completed. Typically for the in vitro culture conditions, the 3D7 and FVO strains, have cycle lengths of 40 and 48 hours, respectively. Thus, for the extended analysis, 3D7 and FVO cultures were collected 48 and 58 hours, respectively, after the initial invasion event. No delays were observed (data not shown).

Comparison of DNA-binding dyes Syto16 and HE for their ability to measure the effect of bloodstage-specific antisera on parasite viability. We next explored flow cytometric analysis as a means for automated measurement of the anti-parasitic effects of immune sera, because analysis by Giemsa-stained blood typically is time consuming and high throughput screening is difficult. Syto16 staining is useful for detecting the presence of DNA but is not able to distinguish between live and dead cells, whereas HE has been described in various protozoan systems including malaria as a vital stain for viable parasites, owing to the requirement that parasite-derived enzymes convert hydroethidine to ethidium.

Results from Syto16 and HE staining (Figure 2), in a single cycle assay, for homologous strain pRBC cultured with AMA1-specific Ab (FVO strain P. falciparum cultured with anti-AMA1 [FVO] or 3D7 strain-cultured with AMA1 [3D7]) were essentially identical, indicating that all inhibition by AMA1-specific Abs is by invasion inhibition.

In the case of MSP1_{42}-specific Abs, the situation was more
complex. By 6 hours after invasion, detection with Syto16 or HE showed that anti-MSP1$_{42}$ (3D7)-specific antiserum suppressed homologous 3D7 strain parasite growth only marginally compared with control serum (11% inhibition). By the end of the experiment, there was no change in the amount of inhibition observed by staining with Syto16, but the amount of inhibition measured by staining with HE increased significantly to 22.6% (two-sample $t$ test, $P = 0.02$, two-tailed). This was also the case when 3D7 parasites were treated with heterologous anti-MSP1$_{42}$ (FVO) antiserum, although the overall levels of inhibition were higher (Syto16, 25% inhibition; HE, 44% inhibition, two-sample $t$ test, $P = 0.038$, two-tailed). These results indicate that the primary effect of these antisera on 3D7 strain parasites was inhibition of development rather than inhibition of invasion.

In contrast, when FVO strain parasites were treated with either homologous anti-MSP1$_{42}$ (FVO) or heterologous anti-MSP1$_{42}$ (3D7) antiserum, Syto16 and HE staining detected similar levels of inhibition throughout the experiment. By the end of the experiment, the inhibition by anti-MSP1$_{42}$ (FVO) antiserum, as detected by Syto16 and HE, was 51% and 53%, respectively ($P = 0.87$ and 0.53), and was substantially greater than the inhibition with the anti-MSP1$_{42}$ (3D7) antiserum (5% and 9%, respectively, $P = 0.31$). These results indicate that the primary effect of antiserum against the two alleles of MSP1$_{42}$ when tested against FVO strain $P$. falciparum is invasion inhibition. Thus, we were able to discern the different effects of the antiserum on the various test strains by comparing results from DNA staining using Syto16 and the vital stain HE.

**Measurement of parasite metabolic activity.** Last, we sought to confirm the parasite growth inhibition detected with HE staining by quantifying pLDH levels. Figure 3 is representative of three experiments and shows that the parasites must reach early trophozoite stage to produce enough pLDH to meet the threshold of detection. It also shows that the measurement of pLDH activity is optimal at the end of schizogony because the OD values are maximal at this time. pLDH levels were not different in 3D7 strain cultures that were either not treated or treated with pooled control sera, whereas pLDH levels in FVO strain cultures that were treated with pooled control sera were slightly higher than in untreated cultures. Both the anti-MSP1$_{42}$ (3D7) and (FVO)-specific sera caused growth inhibition when tested against the homologous parasite strain (21% and 60%, respectively) as well as the heterologous strain (20% and 41%, respectively). Considering the kinetics of the pLDH measurement and the trends observed for the various treatments, we conclude that measuring pLDH is a sensitive method for detecting viable parasites that were able to invade and continue to develop into trophozoites and schizonts in the presence of immune serum.

**Comparison of the methods for measuring the growth inhibitory activity of immune sera.** Figure 4 summarizes the mean levels of growth inhibition induced by three specific antiserum (anti-MSP1$_{42}$ (3D7), anti-MSP1$_{42}$ (FVO), and anti-AMA1), each evaluated using four different methods (Giemsa, Syto16, HE, and pLDH) against the two parasite strains. In each test strain, the antiserum produced similar patterns of inhibition among the four methods. Testing for the interaction between factors, antiserum and method, was not significant. In addition, analysis of variance revealed significant main effects among methods and among antiserum. The differences in growth inhibition among the four methods were stronger for the 3D7 test strain ($F_{3,18} = 13.9$, $P < 0.001$) but still reached significance in the FVO strain ($F_{3,18} = 3.2$, $P = 0.048$). To further evaluate differences among the four methods, Tukey’s post hoc test procedure was used. For the 3D7 test strain, there was no difference in detection of inhibition between the pair Giemsa and Syto16 or between the pair HE and pLDH, but detection of inhibition by either
Giems or Syto16 was significantly less sensitive than detection by HE or pLDH. For the FVO test strain, pLDH consistently showed the strongest detection of inhibition across all three antisera; however, the Tukey procedure failed to reject any pairwise differences between methods.

DISCUSSION

The evaluation of vaccines directed against the bloodstages of *P. falciparum* often consists of measuring vaccine induced antibody titers (e.g., by ELISA) in preclinical models and correlating these titers with some biologically relevant functional activity. It is widely considered that measuring the growth inhibitory capacity of an immune serum or antibody preparation will be one of the prime components for such an immune correlate. When selecting a technique for the evaluation of parasite inhibitory activities within immune sera, several aspects should be considered: 1) what does the selected technique actually measure, i.e., parasitemia or metabolic activity, 2) how sensitive and reproducible is the assay, and 3) how feasible is the sample preparation for large scale screening of sera. This study focuses on the first two points and compares the results obtained from methods that are based on either measurement of parasitemia and/or viability/metabolic activity of parasites. Figure 4 summarizes the inhibitory effect of the anti-MSP1\(_{42}\) and anti-AMA1 antisera, as measured by microscopic analysis of blood smears, and flow cytometric analysis using Syto16, both of which measure parasitemia, as well as by flow cytometric analysis with HE and measuring pLDH, both of which measure parasite viability. Comparison of the results from the four methods by use of a general linear model shows that 3D7 strain parasites treated with anti-MSP1\(_{42}\) antisera were more susceptible to killing by mechanisms that affect parasite viability than by inhibition of
invasion, whereas FVO strain parasites treated with the same antisera seemed to be most susceptible to invasion inhibition. When treated with homologous antisera specific for AMA1, both strains were neutralized by invasion inhibition.

The idea that parasite viability can be affected by anti-MSP1 Ab after invasion is plausible owing to the fact that MSP1-specific Abs coat merozoites and can be found on the surface of ring stage parasites after invasion. Our observation that MSP1-specific antisera can affect intracellular parasite viability is consistent with the results of another recent study, in which MSP1-specific antisera affected the progression of intracellular parasite development. In that study, Giemsa-stained parasites were evaluated for their morphologies, and flow cytometry was used to differentiate and quantify parasite populations based on DNA content by staining with propidium iodide. We prefer to measure viability by either of the methods presented above over measurement of DNA content with propidium iodide because gate settings are subjective and vary between experiments, and it is not possible to distinguish between retarded trophozoite stage and newly developing ring stage parasites.

Our data showed that invasion inhibitory activities of Ab can be measured by any of the methods used in this study, whereas growth inhibitory activities are best measured by using methods that determine viability. We also show that some Ab work by more than one modality, which can vary with the test strain. We propose that the evaluation of bloodstage-specific antisera for preclinical or clinical evaluations include techniques that are based on vital stains such as HE and/or measurement of parasite metabolic activity such as the pLDH assay. These methods measure both invasion and growth inhibition and therefore reveal a greater portion of the spec-

**Figure 3.** pLDH activity is diminished in parasite cultures incubated with immune serum. Parallel cultures of 3D7 parasites (A) and FVO parasites (B) were set up at the schizont stage (~6 hours before rupture begins) and incubated for various lengths of time with 20% of control serum, anti-MSP142 (3D7), anti-MSP142 (FVO), or anti-AMA1–specific serum (tested against the homologous strain only). Experiment is representative of three separate experiments; data shown are mean OD ± SD of triplicate cultures.

**Figure 4.** Methods evaluating the viability or metabolic activity of the parasite to measure a greater range of anti-parasite activity in immune sera. Growth inhibition of 3D7 parasites (left) and FVO parasites (right) induced by homologous or heterologous anti-MSP142 or homologous anti-AMA1 as measured by the various methods. Data are expressed as mean percentage inhibition ± SEM of three independent experiments.
trum of anti-parasite activities associated with the Ab being tested. We further propose that other unidentified parasite phenotypes might also be affected by Ab and that learning to measure these may also be useful for developing immune correlates.

Received March 31, 2006. Accepted for publication May 9, 2006.

Acknowledgments: The authors thank Kathleen Moch and Jeffrey Snavely for culturing and providing blood stage parasites.

Financial support: This work was supported by the United States Agency for International Development, Project Number 936-6001, Award Number AAG-P-00-98-00006, Award Number AAG-P-00-98-00005, and by the United States Army Medical Research and Materiel Command.

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