Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development

Michelle J. Boyle[a,b,1], Danny W. Wilson[a,1], Jack S. Richards[a,b,2], David T. Riglar[a,b,2], Kevin K. A. Tetteh[c], David J. Conway[c,d], Stuart A. Ralph[e], Jake Baum[a], and James G. Beeson[a,3]

[a]Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia; [b]Department of Medical Biology, University of Melbourne, Victoria 3010, Australia; [c]London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom; [d]Medical Research Council Laboratories, Banjul, Gambia; and [e]Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Victoria 3010, Australia

Communicated by Gustav J. Nossal, The University of Melbourne, Victoria, Australia, June 30, 2010 (received for review May 3, 2010)

During blood-stage infection by *Plasmodium falciparum,* merozoites invade RBCs. Currently there is limited knowledge of cellular and molecular invasion events, and no established assays are available to readily measure and quantify invasion-inhibitory antibodies or compounds for vaccine and drug studies. We report the isolation of viable merozoites that retain their invasive capacity, at high purity and yield, purified by filtration of highly synchronous populations of schizonts. We show that the half-life of merozoite invasive capacity after rupture is 5 min at 37 °C, and 15 min at room temperature. Studying the kinetics of invasion revealed that 80% of invasion events occur within 10 min of mixing merozoites and RBCs. Invasion efficiency was maximum at low merozoite-to-RBC ratios and occurred efficiently in the absence of serum and with high concentrations of dialyzed nonimmune serum. We developed and optimized an invasion assay by using purified merozoites that enabled invasion-inhibitory activity of antibodies and compounds to be measured separately from other mechanisms of growth inhibition; the assay was more sensitive for detecting inhibitory activity than established growth-inhibition assays. Furthermore, with the use of purified merozoites it was possible to capture and fix merozoites at different stages of invasion for visualization by immunofluorescence microscopy and EM. We thereby demonstrate that processing of the major merozoite antigen merozoite surface protein-1 occurs at the time of RBC invasion. These findings have important implications for defining invasion events and molecular interactions, understanding immune interactions, and identifying and evaluating inhibitors to advance vaccine and drug development.

host cell invasion | immunity | inhibitors | malaria | imaging

Malaria resulting from *Plasmodium falciparum* infection is a major cause of mortality and morbidity, particularly among young children (1, 2). There is an urgent need for an effective vaccine and new antimalarial agents to reduce the burden of malaria and combat drug resistance. The pathogenic processes of malaria occur during blood-stage infection when merozoites invade RBCs and replicate inside them. During RBC invasion *P. falciparum* merozoites use multiple receptor–ligand interactions in a series of coordinated events, but current knowledge of these interactions and mechanisms of invasion are limited (3, 4). This has impeded the development of approaches to block essential interactions with vaccine-induced antibodies or with molecules that could be used therapeutically.

Merozoite antigens are a major focus of vaccine development, in which immunization generally aims to induce antibodies that inhibit invasion and subsequent replication (5). Antibodies to merozoite antigens are also believed to be important in mediating acquired immunity (6–8). Inhibitory compounds also have significant potential for therapeutic development (9, 10). However, very few invasion-inhibitory compounds have been identified to date because of a lack of suitable methods to test potential inhibitors and a limited understanding of processes that could be targeted. Standard assays measure total growth inhibition (11–14), which could be mediated by inhibitory effects acting at different stages of parasite development. These assays are typically not able to specifically measure invasion-inhibitory activity or to dissect the timing of action of inhibitory antibodies and compounds.

Very little is known about merozoite survival after release or the kinetics of invasion, yet this knowledge is important to understanding immune effector mechanisms and advancing vaccine and drug development. It is widely thought that merozoite survival following release from schizonts is very brief, and that invasion must occur rapidly, within seconds to minutes (15, 16). Most attempts to purify merozoites that retain their invasive capacity from human malaria parasites have been unsuccessful (17) or yielded merozoites with very low invasive capacity. This has hindered the development of methods to fix and image merozoites in the process of invasion by standard microscopy, fluorescence microscopy, or EM. An improved understanding of invasion, and an ability to image events and identify and quantify inhibitors, would facilitate a more targeted approach to vaccine and drug development. Isolation of viable merozoites from a related species *Plasmodium knowlesi* has been achieved (18–20), but there are major differences between *P. knowlesi* and *P. falciparum*.

We report the isolation of viable merozoites from *P. falciparum*, which retain their invasive capacity, at high purity and high yield. Using these methods, we have advanced our understanding of merozoite invasive capacity after schizont rupture, the kinetics of invasion, and conditions for invasion. Furthermore, we have developed and optimized high-throughput invasion assays that can be used to test inhibitory compounds and antibodies as well as methods to study and visualize molecular and cellular interactions during invasion.

Results

Isolation of Merozoites That Retain Their Invasive Capacity. Previous studies report that merozoites collected from spontaneously ruptured schizonts, usually several hours after rupture, retain little or no invasive capacity (17). We explored whether mature

Author contributions: M.J.B., D.W.W., J.S.R., D.T.R., K.K.A.T., D.C., S.A.R., J.B., and J.G.B. designed research; M.J.B., D.W.W., J.S.R., D.T.R., S.A.R., J.B., and J.G.B. performed research; M.J.B., D.W.W., J.S.R., K.K.A.T., D.C., S.A.R., J.B., and J.G.B. contributed new reagents/analytic tools; M.J.B., D.W.W., J.S.R., K.K.A.T., D.C., S.A.R., J.B., and J.G.B. analyzed data; and M.J.B., D.W.W., J.S.R., D.T.R., K.K.A.T., D.C., S.A.R., J.B., and J.G.B. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]M.J.B. and D.W.W. contributed equally to this work.

[2]J.S.R. and D.T.R. contributed equally to this work.

[3]To whom correspondence should be addressed. E-mail: beeson@wehi.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009198107/-/DCSupplemental.

schizonts could be ruptured, and merozoites purified, by using filtration. In initial experiments, highly synchronous mature-stage parasites were isolated (to approximately 95% purity), returned to culture, and monitored for rupture. When rupture had begun to occur, whole parasite preparations were passed through a 1.2-μm filter to rupture schizonts and isolate free merozoites. Culture of the merozoite preparation with fresh RBCs confirmed that a proportion retained invasive capacity, as indicated by the presence of developing intraerythrocytic parasites.

To increase the yield of merozoites that retained their invasive potential, purified mature-stage parasites were treated with the protease inhibitor *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64), which prevents merozoite release from schizonts by inhibiting rupture (21) (Fig. S1). E64 is not thought to adversely affect merozoites (22); indeed, we found that incubating merozoites with E64 did not affect their invasive capacity. This approach enabled us to obtain a parasite preparation enriched for schizonts. After the majority of parasites were fully developed in the presence of E64, parasites were pelleted and resuspended into a small volume of culture media and merozoites were purified by filtration. Analysis of the filtrate by Giemsa-stained smears and flow cytometry (which allows for populations of E64 treated schizonts, uninfected RBCs, free merozoites, RBCs with bound merozoites, and infected RBCs to be differentiated; Fig. 1*A* and Fig. S2) showed that filtration completely disrupted schizonts and excluded parasitized and nonparasitized RBCs. The resulting filtrate contained only merozoites and hemozoin crystals. When added to uninfected RBCs, purified merozoites bound to uninfected RBCs and a proportion invaded, resulting in highly synchronous parasites with normal development (Fig. 1 *B* and *C* and Fig. S1). Merozoites from E64-treated cultures had a substantially higher proportion of merozoites that invaded compared with untreated parasite cultures (mean ± SEM, 2.1 ± 0.65 times higher). We typically found that 100 mL of parasite culture (3% hematocrit, 3% parasitemia) gave a yield of 4×10^8 merozoites.

The integrity of purified merozoites was assessed by immunofluorescence microscopy (IF) and transmission EM. By IF, a high proportion of merozoites were positive with antibodies to merozoite surface proteins; more than 90% positive for AMA1 and MSP1-19 and 70% for MSP2. Anti-AMA1 labeled the whole merozoite surface, confirming that AMA1 is released from the micronemes and redistributes over the merozoite surface postrelease (Fig. 1*D* and Fig. S3). Antibodies to RAP1 showed an apical staining pattern, suggesting that rhoptry proteins involved in invasion had not yet been released (Fig. 1*E* and Fig. S3). EM further confirmed that the majority of purified merozoites were intact and that organelles and key structures were preserved (Fig. 1*F*).

These experiments were generally undertaken with a GFP-expressing D10-PfPHG parasite line (23) because it facilitated identification and tracking of merozoites and invasion events by flow cytometry and microscopy. However, we have also successfully used this protocol to isolate merozoites and obtain invasion of RBCs for the parental D10 and 3D7 lines. Of note, we found that the majority of merozoite invasion events resulted in singly infected RBCs (2% of infected RBCs were multiply infected compared with 26% in standard culture conditions using equivalent parasitemias and hematocrits; three assays in duplicate). We found that hemozoin crystals can be removed from merozoite preparations by passage over a magnet column (Fig. S4). This is important for some applications, such as use in assays of cellular immune responses. Merozoites in suspension could be pelleted by centrifugation at 2,000 × *g*; however, a large amount of agglutination of merozoites occurred. Despite this, some viability was retained (mean ± SEM, 50 ± 11% compared with noncentrifuged controls).

Invasion Efficiency Is Dependent on the Merozoite-to-RBC Ratio. The efficiency of merozoite invasion was significantly influenced by the ratio of merozoites to RBCs. As the merozoite:RBC ratio decreased, the invasion rate (proportion of merozoites that invaded) increased (Fig. 2*A* and Fig. S5*B*). Maximum invasion rates were achieved at low merozoite:RBC ratios (i.e., an excess of RBCs). However, under these conditions, the parasitemia of postinvasion cultures was low (Fig. S5*B*). Higher parasitemias were achieved with high merozoite:RBC ratios (i.e., an excess of merozoites) with a resulting reduction in invasion rate. There was a small independent effect of hematocrit on invasion rate. Keeping the merozoite:RBC ratio fixed, the invasion rate increased in relation to increasing hematocrit (e.g., invasion rates of 7.1%, 7.7%, and 8.8% at RBC concentrations of 113, 226, and 340 × 10^3/μL, respectively; one representative experiment). The observation that increasing the relative concentration of merozoites resulted in a lower proportion of merozoites invading suggests that there may be a period of competitive exclusion or interference by merozoites that is limiting invasion. Alternatively, the number of RBCs that support efficient invasion may be limited; however,

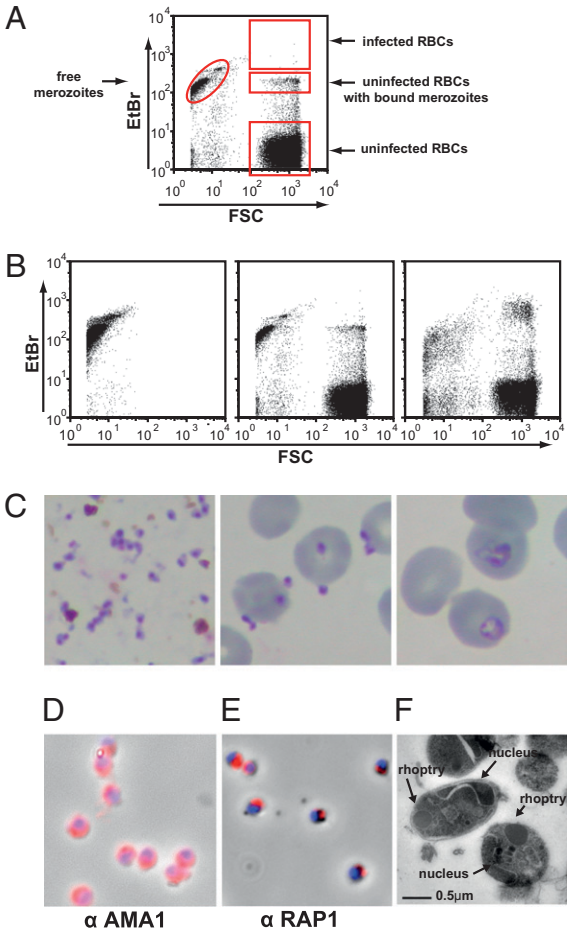

Fig. 1. Purification of merozoites and invasion of RBCs. (*A*) Representative flow cytometry plot showing different cell populations of free merozoites, uninfected RBCs, RBCs with bound merozoites and infected RBCs. Note that this plot shows no infected RBCs (see *B*, *Right* and Fig. S1 for FACS plots containing infected RBCs). (*B* and *C*) Filtration effectively purifies viable merozoites from E64-treated schizonts. FACS plots (*B*) and Giemsa-stained smears (*C*) show merozoites of high purity after filtration of E64-treated schizonts (*Left*), and that purified merozoites were able to bind and invade RBCs (*Center*), resulting in a highly synchronous population of intraerythrocytic parasites (*Right*). (*D*) Surface labeling of purified merozoites with antibodies to AMA1 [red; counter-stained with DAPI (blue) to label the merozoites' nucleus]. (*E*) Labeling of the rhoptry with antibodies to RAP1. (*F*) Transmission EM image of purified merozoites labeled to identify key structures.

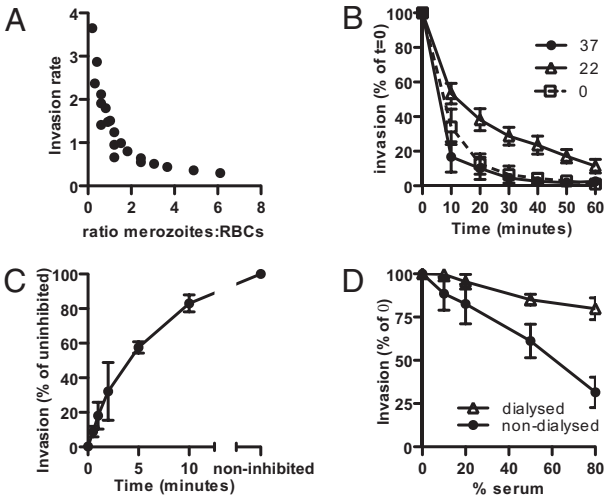

Fig. 2. Kinetics and requirements for merozoites invasion. (*A*) The proportion of merozoites that invade RBCs (invasion rate) is affected by the ratio of merozoites to RBCs. As the ratio of merozoites to RBCs increases, the invasion rate of merozoites decreases. Data are representative of four assays in duplicate. (*B*) The invasive potential of merozoites declines over time, and is affected by different temperatures. Merozoites were incubated at 37 °C, 22 °C, or on ice after purification before being mixed with RBCs to measure invasion. Data are mean ± SEM of seven assays in duplicate. (*C*) The rate of merozoite invasion over time is rapid following incubation with uninfected RBCs. The proportion of merozoites that have invaded with increasing time is shown as a percent of maximum invasion recorded in noninhibited samples. Data are means ± range of two assays in duplicate. (*D*) Merozoite invasion occurs in the presence and absence of serum components. Merozoites were tested for the ability to invade RBCs in the presence of serum at various concentrations. Serum was used with and without dialysis against RPMI-Hepes. Data are means ± SEM of three assays in duplicate and expressed as a percentage of invasion into RMPI-Hepes alone.

high parasitemias are achievable in standard culture, suggesting that RBC receptiveness is not the major factor.

For studies here, we generally balanced requirements for invasion efficiency and resulting parasitemia and performed assays between 0.5 and 2% final hematocrit and high merozoite:RBC ratios to obtain high parasitemias for FACS analysis, inhibition studies, and imaging. Invasive efficiency was also influenced by different RBC preparations, varying by ±25% compared with average (two assays in triplicate; four different RBC preparations). We obtained consistently high efficiency of merozoite invasion; the proportions of merozoites invading were 17.7%, 16.5%, and 14.9% in the three best experiments. For comparison, the invasion rate of D10-PfPHG merozoites in standard in vitro cultures was estimated at 20% to 40% based on the assumption of 16 merozoites per schizont and an observed asexual replication rate of D10-PfPHG of four- to sevenfold per cycle. Agitation of cell suspensions (400 rpm on a plate-shaker) for 10 min after mixing merozoites and RBCs increased the invasion rate and resulting parasitemia by 4.9 ± 1.3 fold (mean ± SEM; seven assays in triplicate). Of merozoites that did not invade, a proportion visibly bound the RBC surface and the remainder persisted as free merozoites (Fig. 1*B*). Similarly, free merozoites and RBC-bound merozoites could also be observed in standard in vitro culture. It was possible to isolate some RBCs with bound merozoites by flow cytometry and cell sorting (Fig. S2).

Determining Merozoite Survival After Release and Invasion Kinetics. To determine the kinetics of survival, merozoites were incubated in culture medium on ice, at 22 °C, or at 37 °C for different times after purification from schizonts. Merozoites were then mixed with RBCs and incubated at 37 °C to allow invasion to occur. After preincubation at 37 °C, the invasive potential of merozoites decreased rapidly, with a half-life of 5 min (Fig. 2*B*), and was similar after incubation on ice. At room temperature, the invasive half-life was increased to 15 min; this longer survival has important practical value, allowing sufficient time to perform treatments or manipulations of merozoites before testing their invasive capacity. At 40 °C, invasion was reduced to only 16.5 ± 3% (mean ± SEM; three experiments in duplicate) of the level of invasion observed at 37 °C, suggesting that high fevers associated with malaria may impact on parasite replication in the blood stream.

To define the kinetics of merozoite invasion, we used the invasion-inhibitor heparin (9) to block invasion so that it occurred for defined periods of time. Merozoites and RBCs were coincubated and heparin was added at different time points to stop further invasion. We found that invasion occurred at a steady rate over a period of 10 min and more than 80% of maximal invasion occurred over this time (Fig. 2*C*). Although agitation of merozoite:RBC suspensions increased the proportion of merozoites that invaded (described earlier), it had little effect on the rate of invasion over time. As the majority of purified merozoites invade within the first 10 min of mixing with RBCs, it is possible with this method to obtain cultures that have a much tighter synchronicity than achieved with commonly used methods of synchronization; the addition of heparin can be used to exclude any potential later invasion events without adversely affecting intraerythrocytic development (9).

Effect of Human Serum on Invasion. Presently it is not known whether invasion requires, or is enhanced by, serum components. E64-treated schizonts were washed and filtered in protein free RPMI-Hepes and then added to RPMI-Hepes with different concentrations of human serum (heat-inactivated, pooled from nonexposed donors); serum was used with or without dialysis against RPMI-Hepes [10,000 molecular weight cut-off (MWCO) membrane]. Merozoites were allowed to invade RBCs for 1 h, and cells were then washed and returned to normal culture conditions (Fig. 2*D*). Merozoite invasion was maximal in serum-free RPMI-Hepes, indicating that invasion does not require serum and is not enhanced by serum. Invasion rates at concentrations as high as 10% dialyzed serum or nondialyzed serum were similar to the invasion rate in serum-free conditions; 10% serum is typically used for standard in vitro culture. The invasion rate was substantially lower in the presence of high concentrations of nondialyzed serum (69% reduction with serum at 80%). However, invasion into dialyzed serum occurred efficiently even at high concentrations (in 80% serum invasion was 80% compared with no serum). Therefore, studies of invasion efficiency or inhibitors can be performed using antibodies or serum components at concentrations that are close to those in vivo, but can also be performed under conditions that require an absence of serum components or protein.

Development of an Invasion Inhibition Assay. Presently, there are no assays to specifically measure inhibition of invasion. Therefore, we developed an invasion-inhibition assay (IIA) based on these methods that would be suitable for high-throughput testing of antibodies and novel compounds using small volume microtiter plates and evaluating invasion using flow cytometry. Using this assay, known invasion inhibitory compounds (Table S1), heparin (9), AMA1-binding peptide R1 (10), and cytochalasin D (19) effectively inhibited merozoite invasion (Fig. 3*A*). EDTA also inhibited invasion as previously suggested (24), presumably by interfering with calcium flux, which is thought to be essential for invasion (25). The anti-AMA1 MAb 1F9 inhibited invasion, whereas MAb 2C5 did not, as reported (26). Invasion inhibitory compounds were also tested for activity against schizont rupture. Heparin, R1, IF9, cytochalasin D, and EDTA at tested concentrations showed no activity against schizont rupture (CSC and 2C5 not tested; Fig. 3*B*). Conversely, the cysteine protease inhibitor

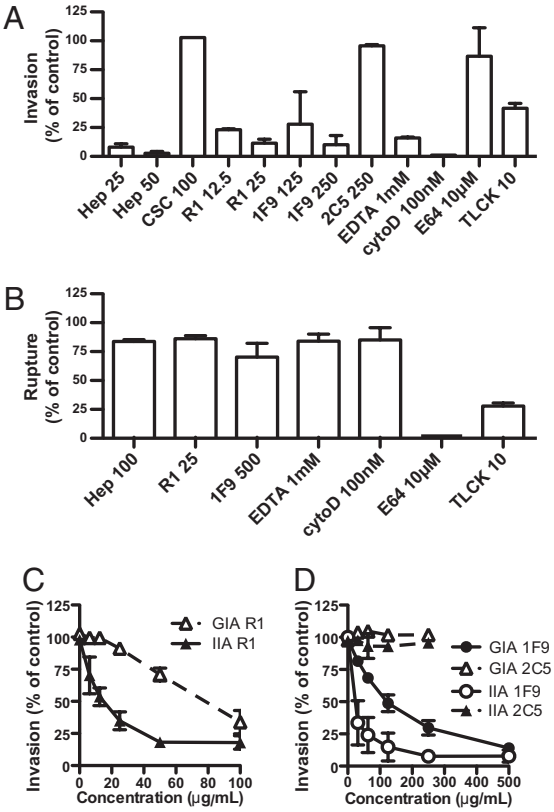

Fig. 3. Development of an invasion inhibition assay using purified merozoites. (*A*) Various inhibitory and noninhibitory compounds and antibodies were tested for their ability to inhibit invasion of purified merozoites in IIAs. Invasion is expressed as a proportion of control. The concentration of inhibitors is in μg/mL unless otherwise indicated. Data are mean ± range of two assays in duplicate. (*B*) Compounds were tested for inhibition of schizont rupture by incubating with late stage parasites and measuring parasitemia and schizont rupture by flow cytometry over time. Rupture is expressed as a proportion of control. Data are mean ± range of two assays in duplicate. (*C* and *D*) Comparison of inhibitory activities in IIA versus conventional GIA of AMA1-binding peptide R1 (*C*) and the anti-AMA1 MAbs 1F9 and 2C5 (*D*).

E64 inhibited schizont rupture, with no activity on invasion. The serine and cysteine protease inhibitor N-α-Tosyl-L-lysine chloromethyl ketone (TLCK) was inhibitory against both merozoite invasion and schizont rupture. These findings indicate the specificity of the assay and its value in distinguishing invasion inhibition from other mechanisms of growth inhibition. Furthermore, the IIA was more sensitive than standard growth inhibition assays (GIAs), in which inhibitors are added to a culture of mature-stage parasites and incubated for 24 h or longer (11–14). This was demonstrated using R1 peptide and MAb 1F9 (e.g., the IC_{50} of 1F9 in GIA was 125 μg/mL compared with 28 μg/mL in merozoite IIAs; Fig. 3 *C* and *D*). Agitation of merozoite:RBC suspensions did not appear to alter the activity of inhibitors. To the best of our knowledge, this is the first demonstration of an assay that can clearly distinguish inhibitors of *P. falciparum* merozoite invasion from other growth inhibitory activity.

Imaging Invasion Events. Presently, there are no reliable methods to fix *P. falciparum* merozoites in the process of invasion to enable visualization and identification of invasion interactions or the processing of merozoite proteins. Using our methods, invading merozoites and parasites immediately after invasion could be captured for imaging by IF and EM (Fig. 4). This was achieved by mixing purified merozoites with RBCs and fixing cells after 2 min. All stages in merozoite invasion could be observed from initial

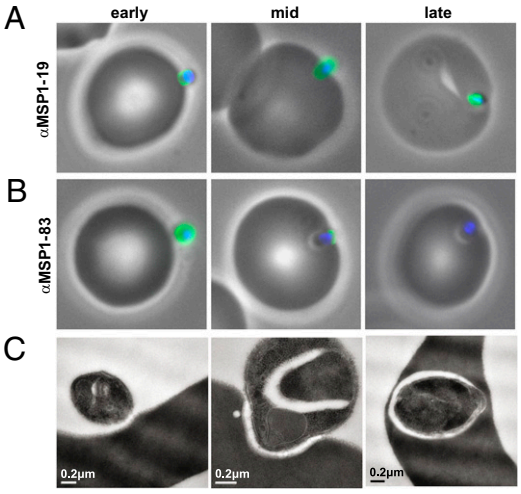

Fig. 4. Imaging of merozoite invasion in fixed cells. Purified merozoites were fixed in the process of invasion to visualize invasion events. Merozoites were examined at the point of initial binding to the RBC surface (*Left*), midway through RBC invasion (*Center*), and after invasion was complete (*Right*). Merozoites in the process of invasion were labeled with antibodies to MSP1-19 (*A*) or MSP1 block 2 (*B*) (green). Nucleus is stained with DAPI (blue). (*C*) EM images of fixed invading merozoites showed all stages of merozoite invasion.

contact through to complete invasion. Conditions were also developed to image merozoites at different stages of invasion by transmission EM (Fig. 4*C*). The high invasion rate and ability to control the timing of invasion using these methods allowed the efficient and reproducible capture of invading merozoites.

We were able to demonstrate the processing of merozoites surface protein 1 (MSP1) during invasion. MSP1 is a major merozoite protein that is cleaved from the merozoite surface by SUB2, leaving only the short GPI-anchored MSP1-19 fragment on the surface (27). This event is thought to occur around the time of invasion; however, this has not been shown directly and its timing is unclear. Antibodies to the N-terminal block 2 region of MSP1 and to MSP1-19 were used to examine the processing of MSP1 during invasion (Fig. 4 *A* and *B*). We found that MSP1 block 2 antibodies labeled free merozoites and merozoites that were bound to the RBC surface, but had not commenced invasion. Among merozoites in the process of invading, the extracellular pole of the merozoites was labeled by antibodies, but the intracellular apical end of merozoites was not labeled, suggesting that processing of MSP1 was occurring at the point of invasion. Merozoites immediately after invasion were not labeled by antibodies to the N-terminal of MSP1, indicating that processing was complete by this time. In contrast, we found that antibodies to MSP1-19–labeled merozoites at all stages of invasion from initial attachment through to complete invasion (Fig. 4*A*). These findings provide an important insight into processing events during invasion and may facilitate the identification of specific inhibitors.

Discussion

We have developed and applied approaches to obtain important insights into the kinetics of invasion and merozoite survival and merozoite surface antigen processing. It was previously thought that merozoite survival after release was very brief and that merozoites must invade RBCs within seconds or no later than 1 to 2 min. We found the half-life of merozoite invasive potential was 5 to 6 min at 37 °C, and most invasion events occurred over a period of 10 min. This survival period is considerably longer than expected and may be important physiologically. Parasitized RBCs sequester in vascular beds and are thought to develop through to schizonts while bound to endothelial cells. Presumably, merozoite

viability would need to be maintained for several minutes to allow sufficient time for merozoites to enter the circulation and invade RBCs after release by rupture of sequestered schizonts. The persistence of viable extracellular merozoites for several minutes would allow sufficient time for interactions to occur between merozoites and antibodies or circulating immune cells, or for cellular interactions in the spleen, as approximately 5% of circulating blood volume passes through the spleen per minute.

The relatively short viability of merozoites probably explains why prior attempts to isolate merozoites from naturally ruptured schizonts have generally been unsuccessful; harvesting merozoites is commonly done several hours after rupture and involves significant handling and washing steps (17). The success of our method is achieved through forced rupture of schizonts by filtration, and optimizing the protocol to minimize handling. Generating a highly synchronous starting culture and using E64, which inhibits schizont rupture (28), enabled us to obtain a parasite preparation enriched for schizonts and therefore a high yield of purified merozoites. IF and EM confirmed that purified merozoites were intact. Surprisingly, we found that merozoite invasion occurred efficiently in serum-free and protein-free conditions. In vitro culture of asexual parasites is known to require serum components, but it had not been known whether this was also a requirement for invasion (29). The ratio of merozoites to RBCs had a significant effect on invasion rate, being highest with a low ratio. This is reflective of conditions in vivo, in which a low parasitemia is typically observed in human malaria. Invasion rates were increased by agitating merozoite:RBC suspensions during the period in which invasion occurs. An objective of this work was to develop methods that were technically straightforward and low in cost that could be used in diverse settings, including laboratories in malaria-endemic countries. Our protocol requires no more than cell culture facilities and basic laboratory equipment.

By using purified merozoites, we developed an invasion inhibition assay that has significant potential for vaccine and drug development. As a proof of principle, we demonstrated inhibition of invasion by antibodies (e.g., 1F9) and various compounds (e.g., heparin, cytochalasin D, and R1 peptide), whereas schizont rupture inhibitor E64 did not inhibit and the serine/cysteine protease inhibitor TLCK was inhibitory against both schizont rupture and merozoite invasion. We also demonstrated the invasion-inhibitory activity of EDTA, confirming the expected importance of calcium for invasion. Importantly, our IIA was substantially more sensitive for detecting invasion inhibitory activity than standard GIAs, but specificity was retained; the IC_{50} was much lower for 1F9 and R1 in IIA compared with GIA, but MAb 2C5 and E64 remained noninhibitory in IIA. Established assays typically measure inhibition of blood-stage growth and cannot specifically measure invasion inhibition by antibodies or compounds. The ability to specifically measure invasion-inhibitory activity, separately from total growth inhibition, is important because some antibodies to merozoite antigens are known to inhibit intraerythrocytic development of parasites as well as invasion (12, 30). A further advantage is that our IIAs can be performed with the use of serum or serum components at near-physiological concentrations (up to 80% concentration), which is important for testing human antibodies and understanding their role in vivo.

To extend these findings, we developed conditions to fix and preserve merozoites at different stages of erythrocyte invasion for imaging by IF and EM. This provides a valuable approach to define invasion events and interactions and the inability to image invasion in this way has limited progress toward a better understanding of RBC invasion by *P. falciparum*. We were able to clearly visualize the processing of MSP1 during invasion and showed that the N-terminal region of MSP1 was cleaved from the merozoite surface at the point of invasion, with the MSP1-19 fragment being carried into the RBC. Although MSP1 processing has been described (31), it has not been possible to precisely define the timing of this event. This establishes an important principle and approach that can be extended to other antigens, and it may be possible to adapt these approaches to isolate protein complexes to identify key interactions during invasion. We also showed that it is possible to label and image parasites immediately after invasion, which will facilitate studies of early postinvasion events. Methods to purify merozoites that retain invasive capacity, and their use in invasion assays, also have applications in proteomics, metabolomics, and transcriptional analyses. It may be possible to use purified merozoites for transfection, as is done with *Plasmodium berghei*, to obtain higher transfection efficiencies.

In conclusion, these findings significantly advance our understanding of host–parasite interactions during the blood-stage replication of *P. falciparum* and parasite biology. The ability to isolate viable merozoites, identify and quantify invasion-inhibitory activity of antibodies and compounds, and image erythrocyte invasion events and interactions have significant potential to advance vaccine and drug development.

Methods

Parasite Culture and Synchronization. *P. falciparum* isolates were cultured as described, in RPMI-Hepes culture medium containing 10% pooled human serum (11, 32). The GFP-labeled parasite line D10-PfPHG (23) was used in most experiments because of its 48-h life cycle, which facilitated obtaining synchronous cultures, and expression of GFP enhanced detection by flow cytometry and fluorescence microscopy. Parasites were synchronized using sorbitol treatment (33) and by using the invasion-inhibitory properties of heparin (9). Parasites were cultured in the presence of 30 IU of medical-grade heparin (porcine mucous; approximately 230 μg/mL; Pfizer) until the majority of parasites were at the schizont stage. Heparin was then removed from cultures for 4 to 6 h to allow schizont rupture and merozoite invasion to occur. After the invasion period, heparin was added to cultures, resulting in the blocking of any further invasion events.

Merozoite Invasion Assay. Late-stage parasites (40-46 h after invasion) were isolated (>95% purity) from uninfected RBCs with a MAC magnet separation column (Macs; Miltenyi Biotec). Parasites were incubated with 10 μM of E64 (Sigma) for 6 to 8 h. Schizonts were pelleted at 1,900 × *g* for 5 min. Parasites were resuspended in a small volume of culture medium or incomplete culture media (containing no protein) and filtered through a 1.2-μm Acrodisc 32-mm syringe filter (Pall). Filtered merozoites were added to uninfected RBCs with or without inhibitors (Table S1) and cultured according to standard methods in 96-well plates. To test the effect of serum on invasion (Fig. 2*D*), pooled serum from malaria-unexposed donors in Melbourne, Australia, was dialyzed (10,000 MWCO) against RPMI-Hepes, or untreated, and then filter-sterilized. Merozoites were incubated with RBCs and 0% to 80% serum for 1 h and then washed twice with culture media before culturing in standard conditions for 27 to 40 h. To investigate the invasive half-life of merozoites (Fig. 2*B*), merozoite preparations were filtered into culture medium at room temperature (23 °C). Merozoites were aliquoted into 1.5-mL tubes and incubated on ice, at room temperature, or at 37 °C. At regular intervals, an aliquot of parasites from each treatment was mixed with uninfected RBCs (final hematocrit, 1%) in a 96-well plate and incubated per normal culture.

The concentration of merozoites and RBCs was determined using CountBright Absolute Counting Beads as per manufacturer's protocol (Invitrogen). Analysis was performed using FlowJo software (Tree Star) with beads gated in FL1/FL2, merozoites gated by size in side scatter channel (SSC)/forward scatter channel (FSC) followed by fluorescence in FL1/FL2 and uninfected RBCs in gates FL2/FSC. Ethidium bromide–stained (10 μg/mL, Bio-Rad) infected RBCs were counted 27 to 40 h after invasion (11). Invasion rate was calculated as percentage of RBCs invaded × [(RBCSs per μL)/(merozoites per μL)] (20). GIA was performed as described (11). For merozoite IIA, merozoites were incubated with RBCs and inhibitors for 1 h, then cultures were washed twice to remove inhibitors and incubated as for normal culture. For further details on the methods for isolation of merozoites and invasion assays, see *SI Methods*.

Immunofluorescence Microscopy and Electron Microscopy. Cells were fixed with 4% formaldehyde/0.0075% glutaraldehyde coated onto glass slides and labeled as described (34). Cells were incubated with rabbit or mouse antibodies to MSP1-block 2 (generated in the present study), MSP1-19 (35), AMA1 (36), MSP2 (37), or RAP1 (38), followed by an Alexa 594/488–conjugated secondary antibody (Molecular Probes). Slides were mounted in VectaShield (Vector Laboratories) with 0.1 ng/mL DAPI (Invitrogen) to label the parasite

nucleus. Images were obtained using a Plan-Apochromat (100×/1.40) oil immersion phase-contrast lens (Carl Zeiss) on an AxioVert 200M microscope (Carl Zeiss) equipped with an AxioCam Mrm camera (Carl Zeiss). Images were processed using Photoshop CS4 (Adobe). To capture invasion events, purified merozoites were mixed with RBCs, allowed to incubate for approximately 2 min, and then fixed and labeled as described earlier. To generate antibodies to MSP1-block 2, a GST-fusion protein corresponding to aa 72 to 98 of the MAD20 allele of MSP1-block 2 was expressed and purified using standard methods (39). Rabbits were immunized intramuscularly with 200 μg of purified recombinant protein on days 0, 14, 28, and 49, and serum was collected on d 58. Specificity of the antibodies was confirmed by labeling of merozoites of a MAD20 parasite isolate (sequence confirmed), but lack reactivity with merozoites of 3D7 (which has a different block 2 allele).

For EM, free or invading merozoites were fixed in 1% glutaraldehyde in RPMI-Hepes on ice for 30 min. Samples were pelleted in low-melt agarose before being transferred into water, dehydrated in ethanol, and embedded in LR Gold Resin (ProSciTech). Following polymerization by benzoyl peroxide (SPI-Chem), 100-nm sections were prepared by using an Ultracut R ultramicrotome (Leica). Sections were poststained with saturated aqueous uranyl-acetate, then 5% triple lead, and observed at 120 kV on a CM120 BioTWIN transmission electron microscope (Philips).

ACKNOWLEDGMENTS. We thank Alan Cowman (Walter and Eliza Hall Institute, Australia), Paul Gilson and Brendan Crabb (Burnet Institute, Australia), Tony Hodder (Walter and Eliza Hall Institute, Australia), and Robin Anders (Latrobe University, Australia) for antibodies to RAP1, MSP1-19, AMA1, and MSP2, respectively, and helpful advice on the study; Julia Cutts for important experimental ideas; and Salenna Elliott for critical reading of the manuscript and the WEHI flow-cytometry services. RBCs and serum for parasite culture were provided by the Red Cross Blood Bank (Melbourne, Australia). Funding for the study was provided by a National Health and Medical Research Council of Australia Project grant and Career Development awards (J.G.B., J.B., and S.A.R.); a Postgraduate Research Scholarship (J.S.R.) and Independent Research Institutes Infrastructure Support Scheme grant; an Australian Research Council Future Fellowship (J.G.B.) and Grant FT0990350 (to S.A.R.); an Australian Postgraduate Award (M.J.B. and D.R.); an OIS Grant from the Victorian State Government; a Pratt Foundation Postgraduate Scholarship (D.T.R.); and a Top-Up Postgraduate Award from the University of Melbourne, School of Medicine, Dentistry and Health Sciences (M.J.B.).

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 434:214–217.
2. Elliott SR, Beeson JG (2008) Estimating the burden of global mortality in children aged <5 years by pathogen-specific causes. *Clin Infect Dis* 46:1794–1795.
3. Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. *Cell* 124: 755–766.
4. Dowse TJ, Koussis K, Blackman MJ, Soldati-Favre D (2008) Roles of proteases during invasion and egress by Plasmodium and Toxoplasma. *Subcell Biochem* 47:121–139.
5. Richards JS, Beeson JG (2009) The future for blood-stage vaccines against malaria. *Immunol Cell Biol* 87:377–390.
6. Doolan DL, Dobaño C, Baird JK (2009) Acquired immunity to malaria. *Clin Microbiol Rev* 22:13–36.
7. Fowkes FJ, Richards JS, Simpson JA, Beeson JG (2010) The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: A systematic review and meta-analysis. *PLoS Med* 7:e1000218.
8. Osier FH, et al. (2008) Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infect Immun* 76:2240–2248.
9. Boyle MJ, Richards JS, Gilson PR, Chai W, Beeson JG (2010) Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. *Blood* 115:4559–4568.
10. Harris KS, et al. (2005) Binding hot spot for invasion inhibitory molecules on *Plasmodium falciparum* apical membrane antigen 1. *Infect Immun* 73:6981–6989.
11. Persson KE, Lee CT, Marsh K, Beeson JG (2006) Development and optimization of high-throughput methods to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. *J Clin Microbiol* 44:1665–1673.
12. Bergmann-Leitner ES, et al. (2006) Critical evaluation of different methods for measuring the functional activity of antibodies against malaria blood stage antigens. *Am J Trop Med Hyg* 75:437–442.
13. McCallum FJ, et al. (2008) Acquisition of growth-inhibitory antibodies against blood-stage *Plasmodium falciparum*. *PLoS ONE* 3:e3571.
14. Singh S, et al. (2006) Immunity to recombinant *plasmodium falciparum* merozoite surface protein 1 (MSP1): Protection in Aotus nancymai monkeys strongly correlates with anti-MSP1 antibody titer and in vitro parasite-inhibitory activity. *Infect Immun* 74:4573–4580.
15. Gilson PR, Crabb BS (2009) Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *Int J Parasitol* 39:91–96.
16. O'Donnell RA, Blackman MJ (2005) The role of malaria merozoite proteases in red blood cell invasion. *Curr Opin Microbiol* 8:422–427.
17. Blackman MJ (1994) Purification of *Plasmodium falciparum* merozoites for analysis of the processing of merozoite surface protein-1. *Methods Cell Biol* 45:213–220.
18. Dennis ED, Mitchell GH, Butcher GA, Cohen S (1975) In vitro isolation of Plasmodium knowlesi merozoites using polycarbonate sieves. *Parasitology* 71:475–481.
19. Hadley T, Aikawa M, Miller LH (1983) Plasmodium knowlesi: Studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. *Exp Parasitol* 55:306–311.
20. Johnson JG, Epstein N, Shiroishi T, Miller LH (1980) Factors affecting the ability of isolated Plasmodium knowlesi merozoites to attach to and invade erythrocytes. *Parasitology* 80:539–550.
21. Blackman MJ (2008) Malarial proteases and host cell egress: An 'emerging' cascade. *Cell Microbiol* 10:1925–1934.
22. Glushakova S, Mazar J, Hohmann-Marriott MF, Hama E, Zimmerberg J (2009) Irreversible effect of cysteine protease inhibitors on the release of malaria parasites from infected erythrocytes. *Cell Microbiol* 11:95–105.
23. Wilson DW, Crabb BS, Beeson JG (2010) Development of fluorescent *Plasmodium falciparum* for in vitro growth inhibition assays. *Malar J* 9:152.
24. Dutta S, Haynes JD, Moch JK, Barbosa A, Lanar DE (2003) Invasion-inhibitory antibodies inhibit proteolytic processing of apical membrane antigen 1 of *Plasmodium falciparum* merozoites. *Proc Natl Acad Sci USA* 100:12295–12300.
25. Singh S, Alam MM, Pal-Bhowmick I, Brzostowski JA, Chitnis CE (2010) Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog* 6:e1000746.
26. Coley AM, et al. (2006) The most polymorphic residue on *Plasmodium falciparum* apical membrane antigen 1 determines binding of an invasion-inhibitory antibody. *Infect Immun* 74:2628–2636.
27. Harris PK, et al. (2005) Molecular identification of a malaria merozoite surface sheddase. *PLoS Pathog* 1:241–251.
28. Salmon BL, Oksman A, Goldberg DE (2001) Malaria parasite exit from the host erythrocyte: A two-step process requiring extraerythrocytic proteolysis. *Proc Natl Acad Sci USA* 98:271–276.
29. LeRoux M, Lakshmanan V, Daily JP (2009) *Plasmodium falciparum* biology: Analysis of in vitro versus in vivo growth conditions. *Trends Parasitol* 25:474–481.
30. Woehlbier U, et al. (2006) Analysis of antibodies directed against merozoite surface protein 1 of the human malaria parasite *Plasmodium falciparum*. *Infect Immun* 74: 1313–1322.
31. Holder AA (2009) The carboxy-terminus of merozoite surface protein 1: Structure, specific antibodies and immunity to malaria. *Parasitology* 136:1445–1456.
32. Beeson JG, et al. (1999) *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. *J Infect Dis* 180:464–472.
33. Lambros C, Vanderberg JP (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* 65:418–420.
34. Tonkin CJ, et al. (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol* 137:13–21.
35. O'Donnell RA, et al. (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.
36. Hodder AN, Crewther PE, Anders RF (2001) Specificity of the protective antibody response to apical membrane antigen 1. *Infect Immun* 69:3286–3294.
37. Ramasamy R (1987) Studies on glycoproteins in the human malaria parasite *Plasmodium falciparum*. Identification of a myristilated 45kDa merozoite membrane glycoprotein. *Immunol Cell Biol* 65:419–424.
38. Schofield L, et al. (1986) A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. *Mol Biochem Parasitol* 18:183–195.
39. Polley SD, et al. (2003) Repeat sequences in block 2 of *Plasmodium falciparum* merozoite surface protein 1 are targets of antibodies associated with protection from malaria. *Infect Immun* 71:1833–1842.