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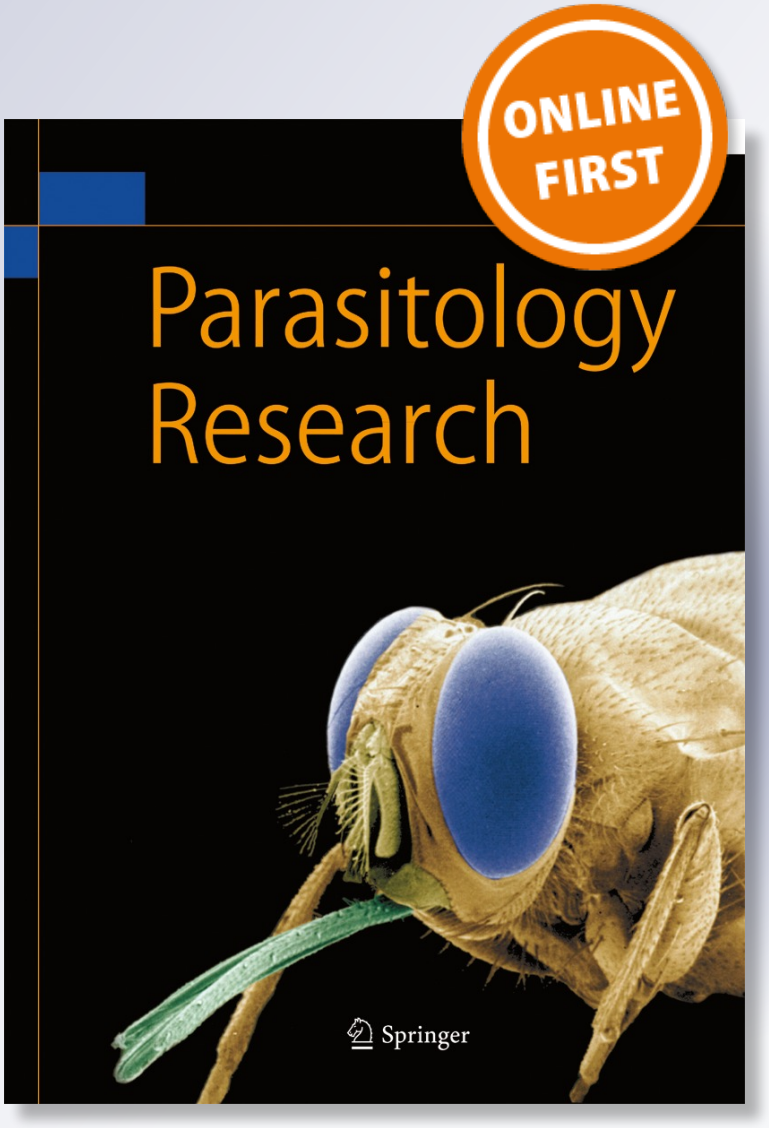


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ORIGINAL PAPER

# Maturation of *Plasmodium falciparum* in multiply infected erythrocytes and the potential role in malaria pathogenesis

**Augustine U. Orjih**

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**Abstract** Erythrocytes containing two or more parasites, referred to here as multiply infected erythrocytes (MIEs), are common in the blood of humans infected by *Plasmodium falciparum*. It is necessary to study these cells closely because the excess numbers of parasites they contain suggest that they could be overloaded with virulence factors. Here, microscopic examinations of blood smears from patients showed that up to seven merozoites can successfully invade an erythrocyte and mature to ring stage. However, in vitro culture showed that only up to three parasites can mature to late schizont stage. These observations were made by culturing the parasites in erythrocytes containing hemoglobin AA (HbAA), HbAS, and HbSS. Biochemical analysis of saponin-concentrated culture suggests that more hemozoin is produced in a MIE than in a singly infected erythrocyte (SIE). Studies have shown that ingestion of excessive hemozoin destroys monocytes and neutrophils, which could impair the immune system. Cultured parasites were also examined by transmission electron microscopy, and it was found that the quantity of knobs was dramatically increased on the membranes of erythrocytes containing multiple schizonts, compared to those containing only one schizont. Knobs contain, among other things, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) complex which mediates sequestration and promotes severe malaria. These findings suggest that *P. falciparum* increases its virulence by producing MIEs. On sexual life cycle of the parasite, microphotographs are presented in this report showing, for the first time, that two gametocytes can develop in one erythrocyte; they are referred to here as *twin* gametocytes. It is not known whether they can infect mosquitoes.

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## Introduction

*Plasmodium falciparum* is the deadliest malaria parasite for humans, killing in the order of a million people annually, although the number has gone down recently (Bousema and Drakeley 2011; Ridley 2002). Part of its lethality includes apparent non-selectivity in the erythrocytes it invades, unlike *Plasmodium vivax* and *Plasmodium ovale* that invade primarily immature erythrocytes (Kerlin and Gatton 2013; Triglia et al. 2001). It completes its intraerythrocytic life cycle in 48 h, unlike *Plasmodium malariae* that requires 72 h. It produces the highest number of merozoites per life cycle, compared to the other species. It sequesters in deep tissues during maturation in vivo, which could result in blockage of tiny blood vessels (Andrews and Lanzer 2002; Berendt et al. 1992; Newton et al. 2000). *P. falciparum* sequestration is the principal cause of cerebral malaria and coma (Ponsford et al. 2012). The sexual life cycle of *P. falciparum* is quite different from other species. Among other characteristics, it takes sexually committed *P. falciparum* ring form 7–10 days to mature from stage I to stage V gametocytes and become able to initiate the sexual life cycle in the vector *Anopheles* mosquitoes, which ends with production of sporozoites (Sinden 2009; Hawking et al. 1971; Baton and Ranford-Cartwright 2005; Rathore et al. 2003).

The general life cycle of human malaria parasites is well defined (Gerald et al. 2011). It begins with injection of the infective stage, sporozoites, by the infected female *Anopheles* mosquitoes (definitive host and vector), into the blood stream of a person from whom the fly is taking blood meal. The

sporozoites invade hepatocytes in the liver and ultimately produce merozoites that invade erythrocytes in the blood. There, the parasites transform to ring stage and develop to trophozoites with enlarged nucleus which divides and becomes a schizont (Meis et al. 1990). Mature schizonts of *P. falciparum* contain 8–20 or more merozoites, which when released, invade other erythrocytes in blood circulation. Normally, trophozoites and schizonts sequester in the vasculature of deep tissues where merozoites are released. It has been determined that merozoites have up to 6 min to circulate extracellularly in blood and invade other erythrocytes (Boyle et al. 2010). The parasite factors necessary for erythrocyte invasion are essentially common to all *Plasmodium* species that infect humans (Magowan et al. 1995). The changes that malaria parasites introduce into the host cell membrane have been studied in *P. falciparum* more extensively than any other species. They include incorporation of different parasite proteins into the host cell membrane, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and knobs that mediate sequestration as well as ring-infected erythrocyte surface antigen (RESA) (Goldberg and Cowman 2010; Naumann et al. 1992; Diez et al. 2005). Attention has recently been drawn to the findings that the RESA stabilizes and strengthens erythrocyte membrane, protecting it against mechanical damage and high febrile temperature (Diez et al. 2005; Pei et al. 2007). In the present report, differences in knob density are addressed, showing that a *P. falciparum* strain can produce different quantities of knobs on erythrocytes in the same culture. Hemozoin production by *P. falciparum* was also investigated because it is another virulence factor that targets phagocytic cells, interfering with the adaptive and innate defense system of infected persons (Martin Olivier et al. 2014; Martiney et al. 2000; Brown et al. 1990; Facer and Brown 1981; Vernes 1980).

## Materials and methods

### Source of malaria parasites

Malaria is not endemic in Kuwait, but imported cases are often detected in the hospitals. Usually, about 80 % of the cases is *P. vivax* infection and the rest are *P. falciparum*. Mixed infections of both species have been reported (Orjih 2008). Other species are rare. As service to the community, our research laboratory in Kuwait University often received blood samples that had already been identified in hospitals as malaria positive to be tested for mixed infections, using PCR methodology. Sometimes, attempts were made to culture the parasites in vitro. A blood smear prepared in Nigeria from a case of severe malaria is also presented in this study to show that MIEs can be very high in vivo.

### *P. falciparum* cultivation

The code names of wild *P. falciparum* cultures used in this study include KU1, HA, SAKU, ML21, and DOHA1. None of them was contaminated with other species. Cultures of the internationally known *P. falciparum* HB3 strain were also studied. Another strain, PB112, which was isolated in our laboratory and maintained in continuous culture in both active growth and frozen states for several months was used for the experiments with sickle cells.

The parasites were cultured in the growth medium Roswell Park Memorial Institute (RPMI) 1640, which was prepared from components purchased from Sigma (St. Louis, Missouri, USA). One sachet of RPMI 1640 powdered medium (10.4 g) was dissolved in 500 ml of double-distilled water with 5.95 g of HEPES, 2 g of D-glucose, 50 mg of hypoxanthine, and 20 mg of gentamicin. After the powder was completely dissolved, 400 ml of double-distilled water was added and the solution was mixed for 10 min, using a magnetic stirrer. Before use, 4.2 ml of 5 % sodium bicarbonate solution and 10 ml of red blood cell (RBC)-compatible human serum were added to 85.8 ml of the medium to obtain what is referred to here as complete medium. All solutions were sterilized by passage through a membrane filter with 0.2 μm pore size, except for the serum which required 0.45 μm pore size.

To culture the wild isolates for the first time, the infected blood samples were centrifuged for 10 min at 3,000 rpm and the plasma was discarded. The pellet of each sample was washed three times by suspending the cells in 10 ml of the RPMI 1640 medium described above and centrifuging each time for 10 min at 3,000 rpm. The final pellet was suspended in 7 ml of complete medium and transferred into a 50-ml sterile plastic cell culture flask, with loosely screwed cap, that was then placed in a 5-l modular incubator chamber (Billups-Rotthenberg, Del Mar, CA, USA). Air in the chamber was displaced completely with a gas mixture that contained 3 % oxygen, 4 % carbon dioxide, and 93 % nitrogen (Kuwait Oxygen & Acetylene Co., Kuwait), and the gas outlets and inlets were sealed airtight. In most cases, the cultures were incubated continuously for 10–24 days at 37 °C, replacing spent supernatant daily with equal volume of fresh medium. No fresh erythrocytes were added to the cultures of KU1, HA, SAKU, ML21, and DOHA1 during the period relevant to the present study.

### Saponin hemolysis for concentrating *P. falciparum* ring-infected erythrocytes

This method has been described in different publications (Orjih 2008). In a typical experiment, $5–8\times10^9$ RBCs, 2–7 % of which contained synchronized ring-infected erythrocytes (RIEs), were suspended in 7 ml of 0.015 % isotonic saponin in modified Krebs-Ringer phosphate (MKRP) buffer

solution, pH 7.4. The tube was placed on ice and allowed to stand for 30 min. During this incubation period, the cells were kept in suspension by hand mixing at 5-min intervals. Each mixing period involved inverting the tube about100 times upside down. The bottom of the tube was frequently tapped at to make sure that no sediment was formed. After 30 min, the tube was centrifuged for 5 min; the supernatant was discarded. The pellet was suspended again in 7 ml of saponin solution and incubated on ice for 4–7 min, during which the gentle mixing by hand continued and the suspension was watched closely until it appeared almost completely hemolyzed. The suspension was centrifuged for 5 min at 3,000 rpm, after which the supernatant was discarded. The pellet was quickly suspended in 7 ml of RPMI 1640 medium supplemented with 10 % of human O-positive serum. After mixing several times by hand, the suspension was centrifuged for 5 min at 3,000 rpm and the supernatant was discarded. Thin smears of the final pellet were prepared for staining with Giemsa stain. Where necessary, the concentrated ring forms were cultured further for 24–48 days to test for viability.

## Giemsa staining and microscopy

Thin and thick smears of the cultures were prepared and air-dried at room temperature. Thin and thick smears were usually prepared on the same slide. Stock solution of Giemsa stain was diluted to 10 % with phosphate-buffered water, pH 7.2, and used to stain the smears for 30 min at room temperature. Before staining, the thin smears were fixed with absolute methanol, while the thick smear remained not fixed. After staining and air-drying, the smears were examined for morphology under oil immersion (100×) objective. Thick smears were helpful in detecting aggregates formed by phagocytes and mature parasites.

## Quantitative measurement of hemozoin production

Synchronized or saponin-concentrated ring forms of *P. falciparum* HB3 strain were suspended in RPMI 1640 medium supplemented with 10 % O-positive human serum. Two-milliliter aliquots of the cultures were prepared in high speed Oak Ridge centrifuge tubes and covered loosely with the screw cap. The tubes were placed in the incubator chamber, and air was displaced with mixed gas containing 3 % oxygen, 4 % carbon dioxide, and 93 % nitrogen for growth at low oxygen tension described above. Aliquots for determination of hemozoin at 0 h were kept at 4 °C to be processed after 30 h when those at 37 °C were ready for hemozoin extraction.

After 30 h, one aliquot each from the sorbitol synchronized and the saponin-concentrated cultures was used to prepare smears for morphological evaluation under the microscope. The other aliquots were processed for hemozoin quantitation. The tubes were centrifuged in refrigerated centrifuge at 27,000×*g* for 30 min, after which the supernatant was carefully removed and discarded. The pellet was loosened, and 10 ml of 5 mM sodium phosphate solution, pH 7.6, was added to each tube. The tubes were shaken vigorously by hand and kept on ice for 10 min to hemolyze before being centrifuged again for 30 min at 27,000×*g*. The supernatant was carefully removed and discarded without disturbing the hemozoin pellet. It was washed once with 10 ml of 20 mM Tris buffer, pH 7.2, again centrifuging for 30 min at high speed. The washed pellet was suspended in 7 ml aqueous sodium dodecyl sulfate (SDS) solution, pH 7.8, and left overnight to purify the beta-hematin content. The quantity of this extract was determined by converting the beta-hematin to free ferriprotoporphyrin IX (FP). This was done by adding 0.2 ml of sodium hydroxide to the tube and making sure that all the hemozoin in the tube was dissolved. Then, 1.8 ml of SDS solution was added to the tube, and the solution was shaken vigorously by hand and left for about 2 h before scanning with a spectrophotometer. The absorbance at 400 nm was used with the millimolar extinction coefficient, 90.8, and total volume (ml) to calculate the hematin concentration, expressing it as pmol FP/$10^6$ IEs.

## Electron microscopy for knobs

The methods for preparation and electron microscopy of malaria parasite cultures described by Orjih et al. (Orjih et al. 1994) were used. Briefly, the growth of *P. falciparum* HB3 strain in culture was synchronized at the ring stage through treatment with sorbitol, after which the parasites were allowed approximately 44 h to mature to schizonts. The culture was then centrifuged, and the pellet was prepared for electron microscopy as follows: The cells were incubated with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, which contained 3 % sucrose and 2 mM calcium chloride. They were postfixed for 6 h with 1 % osmium tetroxide in cacodylate buffer containing 2 % sucrose. The pellet was washed twice with distilled water, each time for 5 min. All the procedures were done at 4 °C. At each step, the cell suspension was mixed on an aliquot mixer and supernatant was discarded after centrifugation. The final pellet was washed and stained with 2.5 % aqueous uranyl acetate for 16 h at 4 °C, after which it was washed twice for 10 min with distilled water at room temperature. The pellet was dehydrated twice for 5 min, using 35, 50, 70, 95, and 100 % ethanol and twice for 10 min with propylene oxide. It was then infiltrated for 3 h with equal part mixture of Polybed and propylene oxide; and then with 100 % Polybed for 3 h. Polybed was obtained from Polysciences, Inc., Warrington, Pennsylvania, USA. The final pellet was embedded in fresh Polybed and polymerized for 24 h at 70 °C. Sectioning was done from trimmed tissue block with a diamond knife

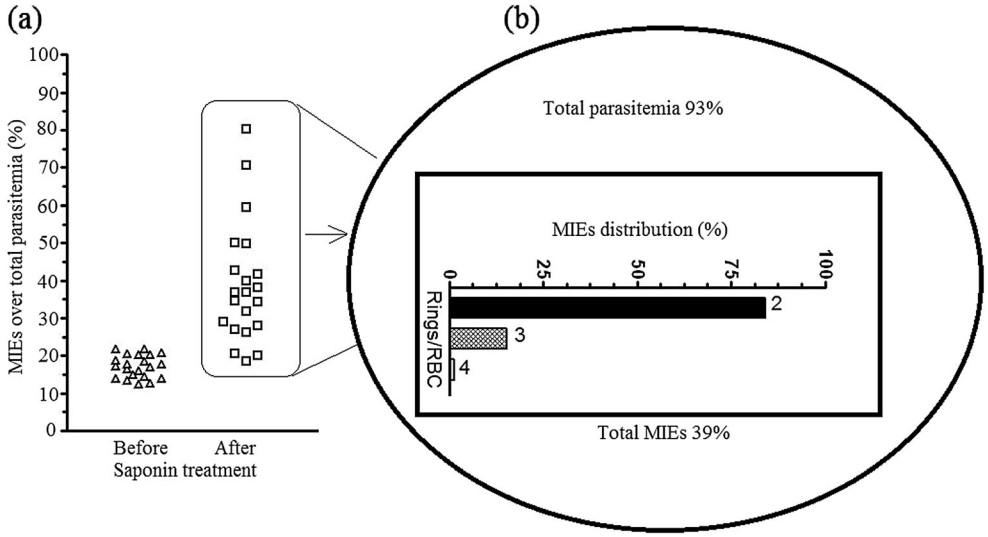


**Fig. 1** Proportion of MIEs in sorbitol synchronized and saponin-concentrated ring forms of *P. falciparum* culture. HB3 culture was treated with 5 % sornbitol solution, followed by treatment with 0.015 % solution of saponin. Thin smears of the preparations were stained with Giemsa stain and evaluated microscopically for MIEs. **a** The proportion of MIEs after sorbitol treatment (*triangle*) and then after saponin (*square*). The numbers of parasites in each MIEs were determined and the percentages of those containing two, three, or four parasites plotted as shown in the histogram enclosed in the circle representing the total parasitemia (93 %) in the saponin-concentrated culture (**b**)

on a LKB Ultracut E ultramicrotome. The sections were collected on copper grids and poststained with uranyl acetate and lead citrate. The knobs on the infected erythrocyte membrane were identified and photographed, using a JEOL 100 CX electron microscope at 60 kV.

## Results

### Production of MIEs by laboratory strain of *P. falciparum*

*P. falciparum* HB3 strain was cultured in vitro by the standard method, and parasite growth was synchronized by treatment with 5 % sorbitol solution. To increase the proportion of MIEs, synchronized cultures were immediately subjected to a two-step treatment with 0.015 % isotonic saponin solution. The first treatment lasted for 30 min after which the spent solution was replaced with fresh saponin solution and the suspension was incubated for additional 3–7 min. This procedure is described in detail here in "Materials and methods" section, as well as in previous publications (Orjih and Cherian 2013). Morphologies of the parasites were evaluated in Giemsa-stained thin smears of the cultures; usually, 1,000–2,000 infected erythrocytes (IEs) were counted under oil immersion (100×) objective.

In 21 experiments, the mean parasitemia after synchronization with sorbitol was 3.8 %, but it increased to 93 % after saponin treatment. MIE concentration was calculated relative to the absolute number of the IEs in the culture, excluding uninfected erythrocytes. MIEs were then grouped according to the number of parasites in an erythrocyte. The results are summarized in Fig. 1. It has been hypothesized that resistance to saponin is mediated by RESA and that increased resistance by MIEs was due to increased concentration of this protein in the host membrane (Orjih and Cherian 2013).

### Production of MIEs by wild *P. falciparum* isolates

The data presented in Fig. 1 were obtained with an internationally known laboratory strain, HB3. Figure 2 shows that high quantities of MIEs can also be produced in vivo. To study the maturation of MIEs that were produced in the wild, three isolates that were

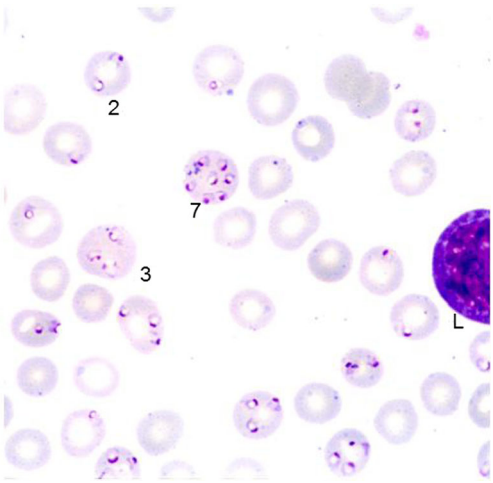


**Fig. 2** MIEs in vivo. The figure shows a blood smear collected in Nigeria from a primary school child who had severe malaria. MIEs containing two to seven *P. falciparum* ring forms were among the parasitemia. Representative images are *numbered* in the figure

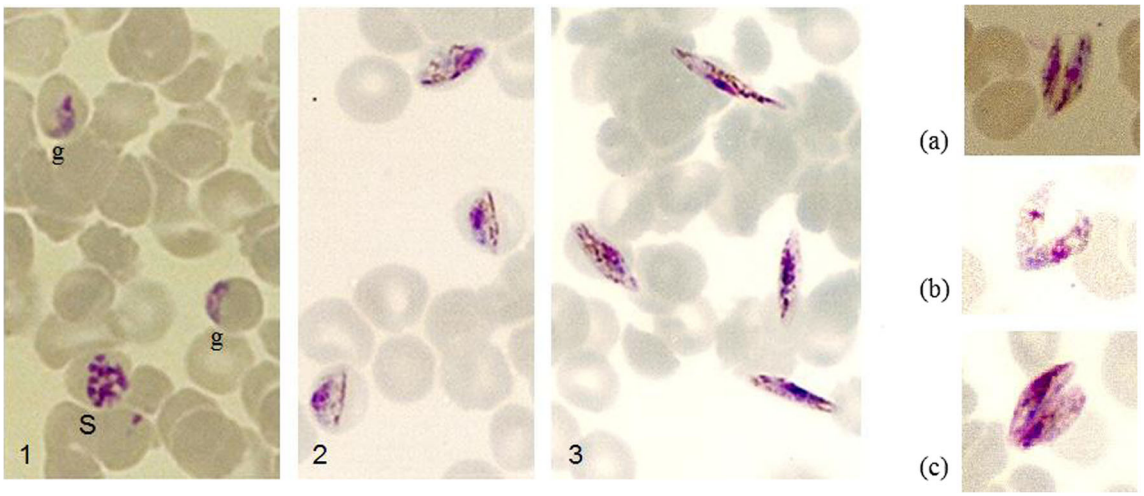


**Fig. 3** Growths of *P. falciparum* isolate KU1 in culture. The isolate was collected from a patient and put in culture on day 0. *Panels 1–3* show the culture on days 3, 4, and 7, respectively. **a**–**c** Twin gametocytes observed within days 6 and 8

collected directly from humans were cultured for up to 24 days. The three isolates were collected from adult Indian expatriates in Kuwait. One of the isolates, KU1, initially produced both asexual and sexual parasites within the first 5 days, after which only gametocytes, including *twins*, were detected (Fig. 3). Another isolate, HA, did not produce any clearly identifiable schizont throughout its 14 days in culture. Instead, the wild ring forms matured through stages I to V gametocytes within 9 days (Fig. 4). Among the gametocytes were those contained in MIEs. The third isolate, SAKU, produced both asexual and sexual parasites throughout the first 24 days, including several twin gametocytes at different stages of maturation (Fig. 5). Production of Garnham bodies by isolates HA and SAKU has been reported recently (Orjih 2012); however, these structures were not detected in twin gametocytes.

## Asynchronous maturation of parasites in MIEs

Most of the two to three parasites in MIEs appeared to have matured at the same rate, but as shown in Fig. 6, a few of the parasites were at different stages of development. The erythrocyte shown in a, for example, contains one mature schizont

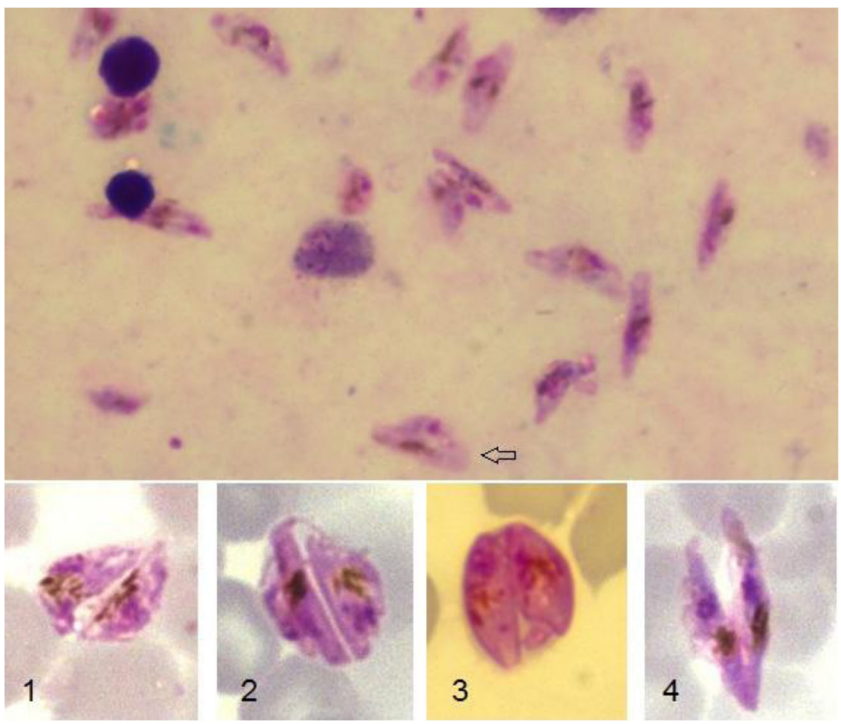


**Fig. 4** Development of in vivo committed gametocytogenesis in vitro. The wild isolate HA was collected from the patient and introduced into culture on day 0. The culture was maintained for 12 days. *Top panel* shows a thick smear of the culture on day 5. Notice that there is no asexual parasite in the field. *Arrow* points at a twin gametocyte in the thick smear, whereas *images 1–4* show twin gametocytes identified in thin smears

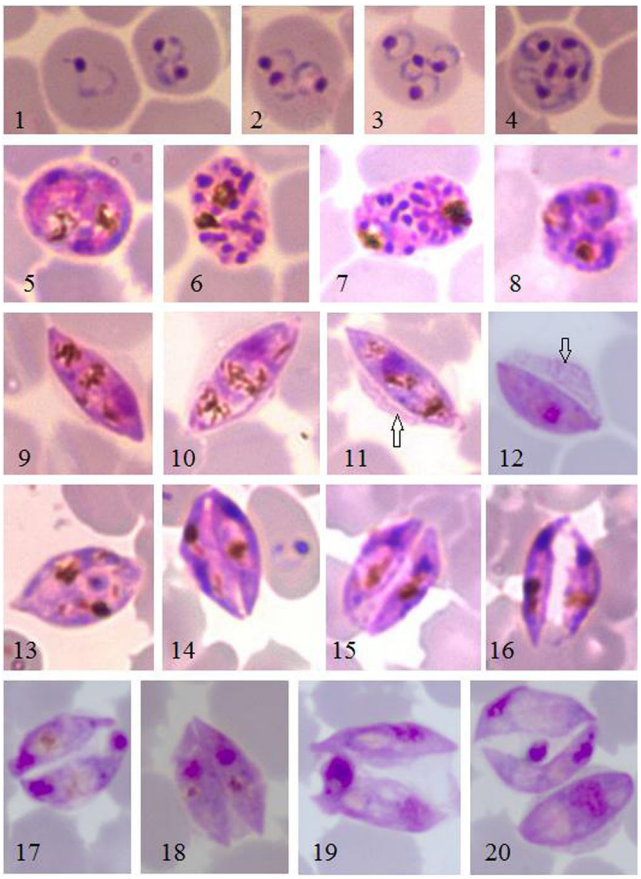


**Fig. 5** In vitro growth of *P. falciparum* SAKU isolate. *Images 1*, *2*, *3* and *4* show erythrocytes containing two, three, four, and five parasites, respectively. *Images 5–8* show asexual maturation in MIEs. *Rows 9–12* show the shapes of normal gametocytes on days 5–7. *Images 11* and *12* contain Garnham bodies (*arrow*). *Images 13–20* show some of the twin gametocytes identified in the culture. *Scale bar* represents 6.3 μm. The isolate was placed in culture within 24 h after collection from the patient (day 0), and all the parasites shown here were detected in the culture between days 3 and 24

and two ring forms; the three parasites appear morphologically healthy, suggesting that the difference in stages might not be merely due to growth retardation of the ring forms. Indeed, all the other parasites in panels b–j appear morphologically healthy. The gametocyte in j was probably invaded about 2–3 days later by a new merozoite.

## Production of MIEs in sickle cells

It is widely believed that *P. falciparum* maturation is retarded in sickle cells (Bunn 2013; Luzzatto et al. 1970; Gong et al. 2012). Here, PB112 strain (Orjih 2008) was cultured in HbAS and HbSS erythrocytes. The growth in HbAS was normal at low oxygen tension (3 % oxygen, 4 % carbon dioxide, and 93 % nitrogen), and the parasites in MIEs were able to mature to late stage schizonts. MIEs were also produced in HbSS, although the maturation appeared somewhat retarded (Fig. 7).

## Multiple infections increase hemozoin production

In 12 experiments with HB3 strain, parasite cultures were synchronized with sorbitol only or treated thereafter with saponin. The cultures that were treated with sorbitol only contained, on the average, 17 % MIEs, whereas the saponin-concentrated culture contained 40 % MIEs. The cultures were diluted with complete growth medium to contain approximately $2\times10^7$ IEs/ml. They were allowed to grow for 30 h before processing for hemozoin measurement. Hemozoin concentration was determined by the standard method (Orjih et al. 1994) and expressed as pmol FP/$10^6$ IEs. To account for contaminating hemozoin released during treatment with sorbitol and saponin, the quantity at 0 h was subtracted from that at 30 h of growth period. The results show that significantly more hemozoin was produced in cultures that contained increased proportions of MIEs, namely the saponin-concentrated parasites (Fig. 8).

## Hemozoin destroys phagocytic cells

Observations by light microscopy suggest that accumulation of hemozoin is destructive to monocytes and neutrophils. In severe malaria cases, phagocytosis of hemozoin can be detected directly in peripheral blood smears (Mundwiler-Pachlatko and Beck, 2013; Cooke et al. 2001). To simulate what could be happening in deep tissues, where mature parasites sequester, peripheral blood obtained directly from a falciparum malaria patient was cultured in vitro for 2 days, allowing ring forms to mature to late stage schizonts. Giemsa-stained smears of the culture showed extensive hemozion phagocytosis by monocytes and neutrophils (Fig. 9a–d). In many cases, the hemozoin was part of ingested schizonts (Fig. 9c, d). Mature schizonts usually contain 10–20 merozoites (Fig. 9e–k). Potential effect of such phagocytosis on innate and acquired immunity has been established by other investigators (Martiney et al. 2000; Brown et al. 1990; Facer and Brown 1981; Vernes 1980).

## Multiple infections increase knob density

*P. falciparum* HB3 culture was synchronized with sorbitol and then allowed to grow to mature schizont stage, after which it was prepared for ultrastructural examination by transmission electron microscopy. It was found that SIEs (Fig. 10a, b) contained fewer numbers of knobs than erythrocytes containing multiple schizonts (Fig. 10c), and some of the knobs on SIEs appeared more protruded than others. There were also

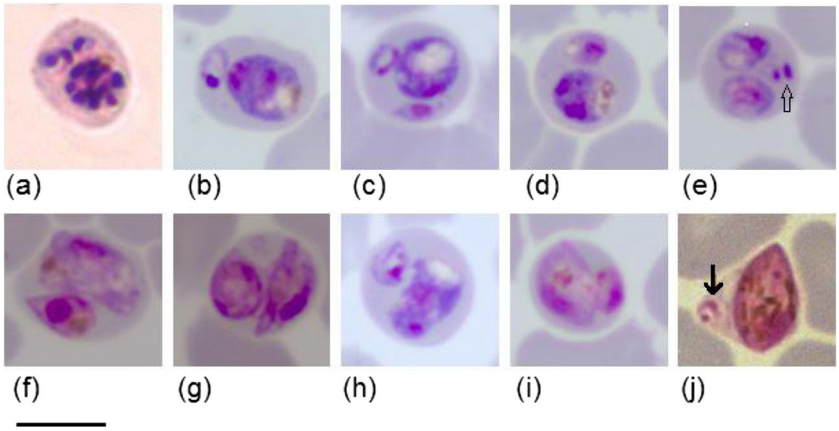


**Fig. 6** Asynchronous maturation of parasites in MIEs. The images shown here were detected in the SAKU culture described in Fig. 5. Each erythrocyte contains two or three parasites; the erythrocyte in **e** appears to contain two trophozoites and two ring forms (*open arrow*) **a**–**e** show asexual maturation, whereas **f**–**j** show that at least one of the parasites in each erythrocyte is a gametocyte. The ring form in **j** (*thick arrow*) could be the result of a merozoite invading a stage III gametocyte. *Scale bar* represents 6.3 μm

some singly infected schizonts that did not contain any detectable knob (Fig. 10d) On the whole, the figure shows that the same *P. falciparum* strain could produce knobs on some but not all infected erythrocytes in the same culture; the morphology of the knobs could also vary.

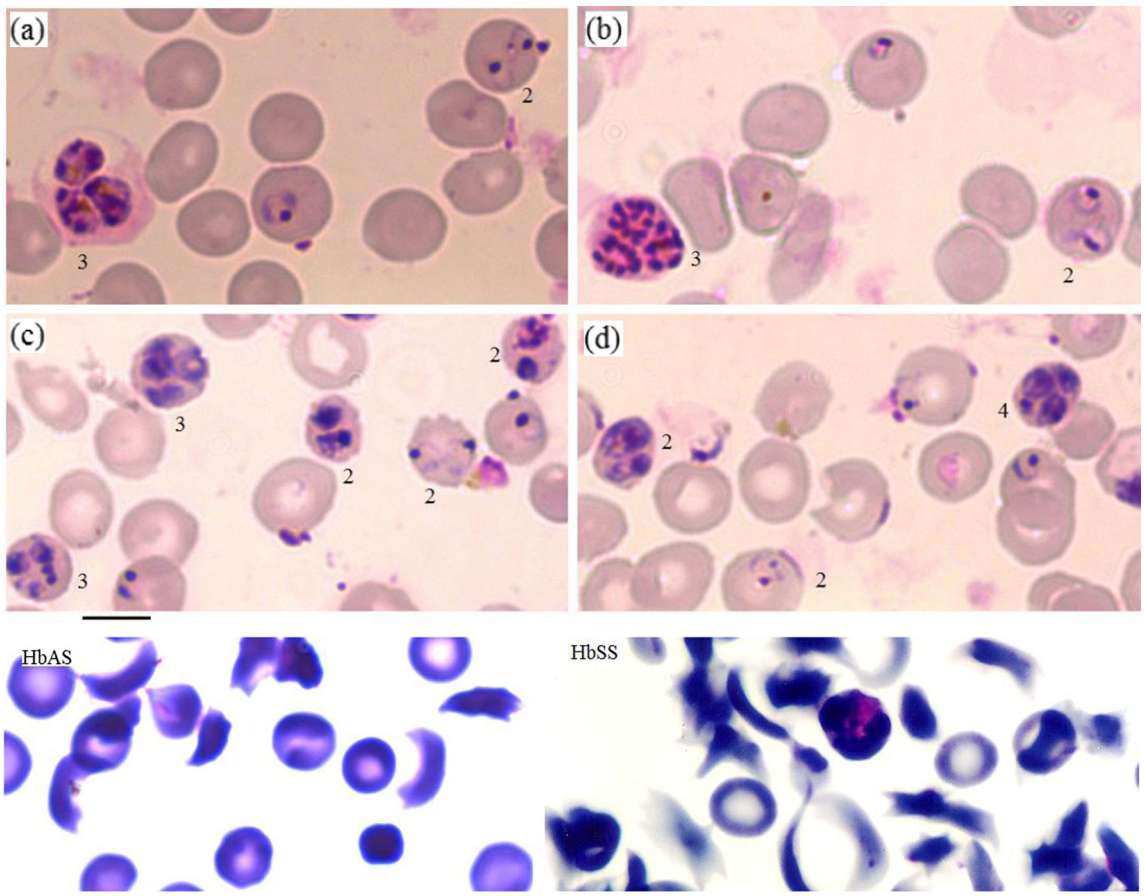


**Fig. 7** MIEs in sickle cells. *P. falciparum* PB112 strain, was maintained in HbAS (**a**, **b**) for several weeks before it was used to infect HbSS (**c**, **d**). HbAS culture was maintained in 3 % oxygen environment throughout, whereas those in sickle HbSS were cultured in candle jar. Numbers of parasites (*2*–*4*) in MIEs are written in the photographs. The effect of 3 % oxygen environment is also shown with HbAS and HbSS in the figure. Sickled shapes were retained by fixing the culture with glutaraldhyde before exposure to air. *Scale bar* represents 6.3 μm. The last row shows the sickling effect of low oxygen tension on HbAS and HbSS when incubated for 24 h and fixed with glutaraldehyde before exposure to air

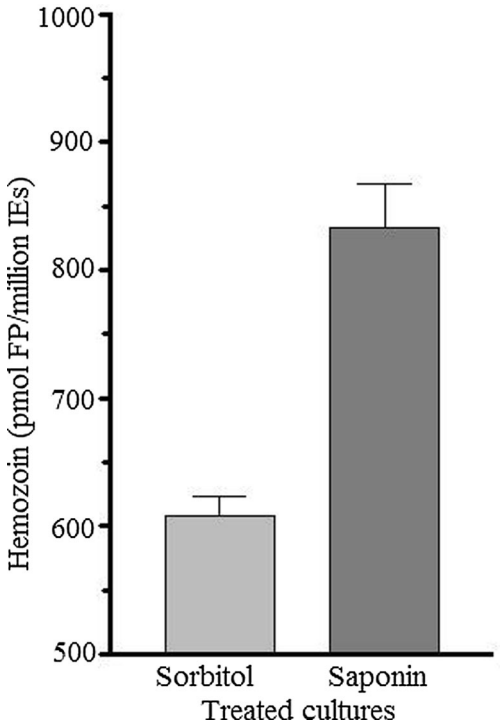


**Fig. 8** Increased hemozoin production due to MIEs. *P. falciparum* HB3 was cultured in HbAA erythrocytes for 5 days at the end of which they were synchronized at the ring stage with sorbitol, followed immediately by concentration of viable intraerythrocytic ring forms using 0.015 % saponin solution. Both the sorbitol and the saponin-treated cultures were then maintained under standard growth conditions for 30 h during which the parasites grew to mostly schizonts. Hemozoin concentrations were then determined and expressed as pmol FP/$10^6$ IEs. The histogram here represents the mean±SEM of five experiments. They are 608±16 before sorbitol and 832±35 after saponin

## Discussions

In microscopic examination of blood for malaria infection, the presence of MIEs is one of the useful morphological characteristics for differentiating *P. falciparum* from other species. Although some MIEs can be observed in most species that infect humans, the highest proportion is produced by *P. falciparum*. An additional unique characteristic of *P. falciparum* is the shape of the gametocytes; only *P. falciparum* has crescent-, sausage-, or banana-shaped gametocytes. With the exception of *P. falciparum*, all malaria parasites that infect humans reveal all maturation stages in peripheral blood, including rings, trophozoites, schizonts, and gametocytes. As shown in Fig. 2, *P. falciparum* circulates in peripheral blood mainly at the ring stage of growth, although trophozoites and schizonts could be detected in rare cases. On the other hand, *P. falciparum* gametocytes normally do not circulate in peripheral blood until they are at stage V of maturation.

Despite the abundance of erythrocytes containing multiple ring forms in falciparum malaria patients, there has been little attempt to study the maturation of these parasites and the potential contribution to pathology. The present study has examined *P. falciparum* parasitemia in blood samples directly from infected humans, as well as those grown in vitro, with particular attention to MIEs. Data are presented here showing that whether the infection happens in vivo or in vitro, the numbers of parasites in MIEs usually range between 2 and 6; in the clinical case shown in Fig. 2, a few erythrocytes contain seven parasites. Inclusion of sickle cells in the present study established that invasions by multiple *P. falciparum* merozoites and maturation of the parasites are not prevented by variant hemoglobin types.

It has been repeatedly reported that erythrocytes containing the ring stage of *P. falciparum* are more resistant than uninfected erythrocytes to destruction by saponin, and it has been determined that MIEs are more resistant than SIEs (Orjih 2008). A recent publication has hypothesized that RESA (Orjih and Cherian 2013) could be involved in induction of saponin resistance. RESA is a protein produced in merozoites before these invasive organisms are released from schizonts. Soon after invasion of an erythrocyte, a merozoite releases RESA and the protein is transported, possibly through Maurer's clefts, to the host cell membrane (Mundwiler-Pachlatko and Beck 2013). Experiments with genetically engineered RESA protein have shown that it stabilizes the spectrin component of erythrocyte membrane, making the cell resistant to mechanical damage (Diez et al. 2005; Pei et al. 2007). As can be predicted from published experiments with genetically engineered RESA protein (Pei et al. 2007), the increased resistance of MIEs to saponin could be because each ring form introduces its RESA to the shared host erythrocyte membrane.

The reason for excessive MIEs in falciparum malaria is unclear. In suggesting that RESA serves the purpose of preventing free merozoites from invading already infected erythrocytes, Pei et al. have stated that an erythrocyte almost never harbors two parasites in different developmental stages (Pei et al. 2007). To investigate this claim, the present study has examined smears of different *P. falciparum* isolates at different developmental stages. It was found that two asexual parasites in the same erythrocyte tend to mature synchronously, but occasionally, one parasite may be different from the other in size (Fig. 6). Perhaps, it is the absence of RESA that makes it less possible for other human *Plasmodium* species to sustain multiple invasions. Although MIEs are also found in *P. vivax* infections, the number of parasites in one erythrocyte is rarely more than three, and the maximum proportion of MIEs in vivo is usually not as high as those in falciparum malaria. It is possible that, in falciparum malaria, the window to invade already infected erythrocyte closes as soon as the first ring form in an erythrocyte enters trophozoite stage; from this point, RESA is probably replaced or masked by mature parasite proteins on infected erythrocyte surface (Cooke et al. 2001; Glenister et al. 2009).

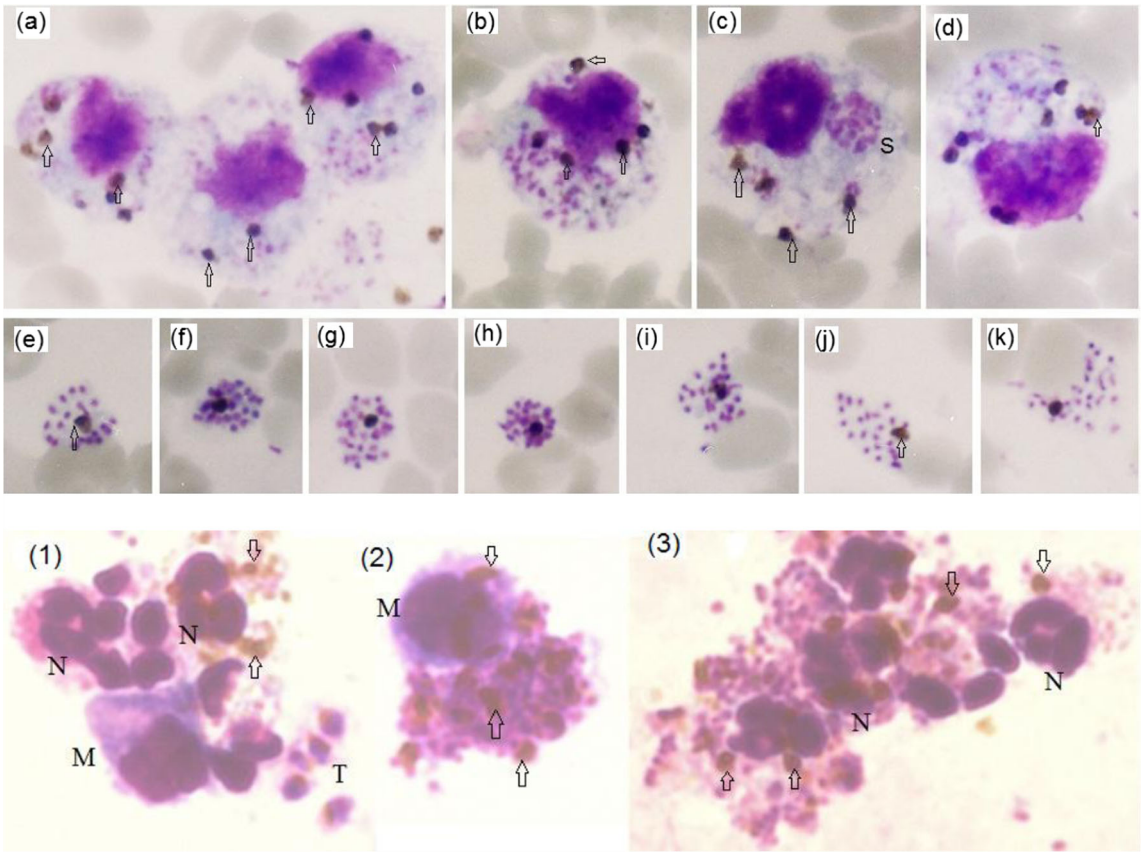


**Fig. 9** Hemozoin phagocytosis destroys monocytes and neutrophils. This figure shows phagocytosis of *P. falciparum* schizonts and hemozoin in the blood (ML21) collected from a man and cultured for 2 days. Most of the hemozoin were probably still in schizonts when they were phagocytized by monocytes (**a**–**d**). The morphology of some of the free schizonts, in the same culture is shown in **e**–**k**. Each of the schizoints contains 16–20 merozites. It is unclear whether the phagocytes formed aggregates (shown in **a**) before or after ingestion of schizonts/hemozoin. *Images 1–3* were from a 48 h culture of *P. falciparum* from another patient, DOHA1. In this case, aggregates of monocytes (M) and neutrophils (N), together with parasite schizonts and hemozoin were detected. *Arrows* point at some of the hemozoin

Therefore, it can be reasoned that RESA gene makes possible the multiple invasions of an erythrocyte by *P. falciparum* merozoites, rather than preventing the phenomenon.

If multiple *P. falciparum* parasites invade an erythrocyte what do they do? Apparently, each of them is independent of the others. Each carries out its own metabolic activities, ingesting and digesting hemoglobin and producing its own hemozoin, among other things. Each of the parasites is demarcated from the others by its own parasitophorous vacuole membrane (pvm). Among the limiting factors on growth could include fixed quantity of hemoglobin and space in the host erythrocyte. These could explain why not more than three parasites can successfully complete the intraerythrocytic life cycle. The effect of multiple mature *P. falciparum* parasites on host erythrocyte membrane is clearly demonstrated by transmission electron microscopy showing more knobs on an erythrocyte containing multiple schizonts than those on an erythrocyte containing only one parasite (Fig. 9). Each transports its proteins, such as RESA and PfEMP1, to the common host cell membrane, presumably unaffected by the other parasites. Thus, the number of knobs, where PfEMP1 is stored, is higher on MIEs than those on SIEs.

A good deal of malaria pathology is initiated by the infected erythrocytes binding to receptors on vascular endothelium, forming rosettes of infected and uninfected erythrocytes in deep tissues (Ho et al. 1991; Doumbo et al. 2009). The effector molecules for sequestration and cell aggregation are located on the surface of the infected erythrocytes. Malaria pathology can also be initiated when phagocytic cells, including monocytes and neutrophils, ingest infected erythrocytes. Trophozoites, schizonts, and gametocytes of *P. falciparum* contain hemozoin particles which have been shown to interfere with innate and actively acquired immunity (Mujuzi et al. 2006; Schwarzer et al. 1998). Taken together, it is reasonable to suggest that an infected erythrocyte is loaded with malarial virulence factors, more so in MIEs.

It was also found in the present study that two ring forms in an erythrocyte could be sexually committed. In 1935, Thomson and Robertson published drawings of two *P. falciparum* gametocytes in one erythrocyte, but the authors did not suggest how such morphology could be formed

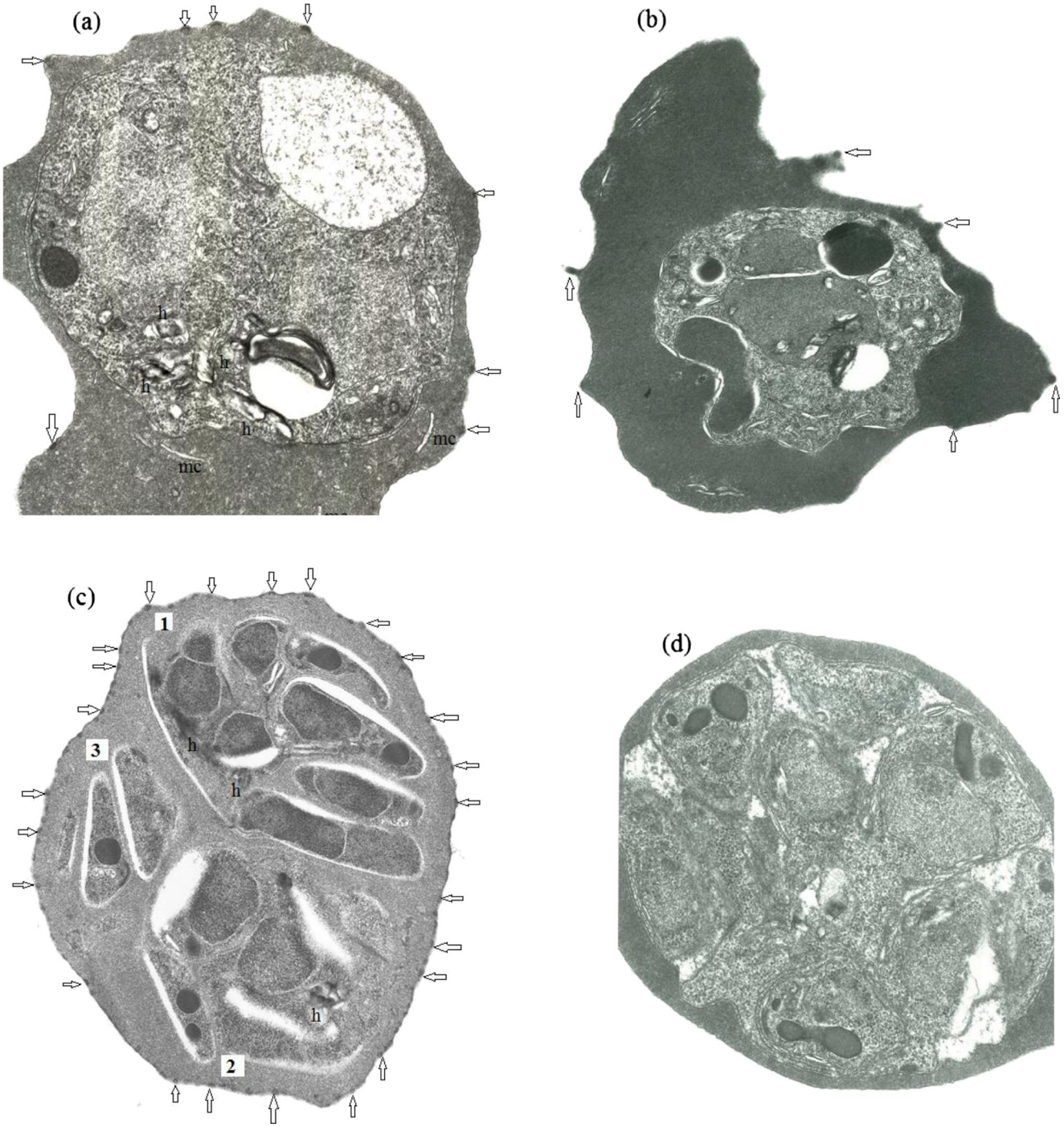


**Fig. 10** Schizonts in MIEs produce excess knobs. The images in this figure were from the same culture of HB3 strain. The erythrocytes in **a** and **b** contain one schizont each, whereas **c** contains three schizonts numbered *1*, *2*, and *3*. *Arrows* point at some knobs. Hemozoin crystals (*h*) are indicated in the parasites. Maurer's clefts, *mc*, were observed in some of the schizonts. No knob was detected on the schizont shown in **d**

(Thomson and Robertson 1935). The present study has established that twin gametocytes result when two sexually committed merozoites simultaneously invade the same erythrocyte and become attached to each other, making them appear as if they were surrounded by the same pvm. Twins could also develop if two merozoites are not fully separated before invading an erythrocyte; the invasion process could be initiated by one, or if properly oriented, by both merozoites; in that case, the two parasites could have a common pvm. Usually, the parasites develop synchronously to stage II gametocytes with their flat sides facing each other. As the parasites grow through stages III and IV, the pvm breaks, but the two could still be surrounded by the host erythrocyte membrane. Toward the end of maturation, they tend to assume

a V-shape (Fig. 6a) and could separate (Fig. 6b). Early in the maturation phase, twin gametocytes are difficult to differentiate from one another, but each accumulates its own hemozoin, indicating independent transport and catabolism of hemoglobin in separate food vacuoles. Morphological appearance of the hemozoin suggests that the parasites are metabolizing at about the same rate. It is unclear whether twin gametocytes could eventually initiate the sexual life cycle and produce infective sporozoites in mosquitoes. Furthermore, it would be interesting to investigate whether a male and a female gametocyte could develop to full maturity in the same erythrocyte.

In conclusion, this study considers *P. falciparum*-infected erythrocytes as cytological time bombs. The question is which infected erythrocytes are more dangerous, MIEs or SIEs? Because each MIE is densely studded with knobs at schizont stage, it is more likely than SIE to effectively bind to vasculature and form rosettes that could interfere with blood flow. MIEs could also contain increased quantities of hemozoin which, when phagocytized by neutrophils and macrophages, could interfere with protective activities of these cells.

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## References


Andrews KT, Lanzer M (2002) Maternal malaria: *Plasmodium falciparum* sequestration in the placenta. Parasitol Res 88:715–723

Baton LA, Ranford-Cartwright LC (2005) Spreading the seeds of million-murdering death: metamorphoses of malaria in the mosquito. Trends Parasitol 21:573–580

Berendt AR, McDowall A, Craig AG, Bates PA, Sternberg MJE, Marsh K, Newbold CI, Hogg N (1992) The binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. Cell 68:71–81

Bousema T, Drakeley C (2011) Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. Clin Microbiol Rev 24: 377–410

Boyle MJ, Wilson DW, Richards JS, Riglar DT, Tetteh KKA, Conway DJ, Ralph SA, Baum J, Beeson JG (2010) Isolation of viable *Plasmodium* falciparum merozoites to define erythrocyte invasion events and advance vaccine and drug development. Proc Natl Acad Sci U S A 107:14378–14383

Brown AE, Webster HK, Teja-Isavadharm P, Keeratithakul D (1990) Macrophage activation in falciparum malaria as measured by neopterin and interferon-gamma. Clin Exp Immunol 82:97–101

Bunn HF (2013) The triumph of good over evil: protection by the sickle gene against malaria. Blood 121:20–25

Cooke BM, Mohandas N, Coppel RL (2001) The malaria-infected red blood cell: structural and functional changes. Adv Parasitol 50:1–86

Diez M, Guillotte M, LeScanf D, Contamin H, David P, Cooke B, Mercerau-Puijalon O, Bonnefoy S (2005) Revisiting putative functional properties of the *Plasmodium falciparum* Pf155/RESA protein using genetically engineered parasites. J Eukaryot Microbiol 52: 28S–34S

Doumbo OK, Thera MA, Kone AK, Rasa A, Tempest LJ, Lyke KE, Plowe CV, Rowe JA (2009) High levels of *Plasmodium falciparum* rosetting in all clinical forms of severe malaria in African children. Am J Trop Med Hyg 81:987–993

Facer CA, Brown J (1981) Lancet 1:897–898

Gerald N, Mahajan B, Kumar S (2011) Mitosis in the human malaria parasite *Plasmodium falciparum*. Eukaryot Cell 10:474–482

Glenister F, Fernandez KM, Kats LM, Hanssen E, Mohandas N, Coppel RL, Cooke BM (2009) Functional alteration of red blood cells by a megadalton protein of *Plasmodium falciparum*. Blood 113:919–928

Goldberg DE, Cowman AF (2010) Moving in and renovating: exporting proteins from *Plasmodium* into host erythrocytes. Nat Rev Microbiol 8:617–21

Gong L, Maiteki-Sebuguzi C, Rosenthal PJ, Hubbard AE, Drakeley CJ, Dorsey G, Greenhouse B (2012) Evidence for both innate and acquired mechanisms of protection from *Plasmodium falciparum* in children with sickle cell trait. Blood 119:3808–3814

Hawking F, Wilson ME, Gammage K (1971) Evidence for cyclic development and short-lived maturity in the gametocytes of *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 65:549–555

Ho M, Cavis TME, Silamut K, Bunnag D, White NJ (1991) Rosette formation of *Plasmodium falciparum*-infected erythrocytes from patients with acute malaria. Infect Immun 59:2135–2139

Kerlin DH, Gatton ML (2013) Preferential invasion by *Plasmodium* merozoites and the self-regulation of parasite burden. PLoS ONE 8(2):e57434. doi:10.1371/journal.pone.0057434

Luzzatto L, Nwachuku-Jarrett ES, Reddy S (1970) Increased sickling of parasitised erythrocytes as mechanism of resistance against malaria in sickle-cell trait. Lancet 1:319–321

Magowan C, Coppel RL, Lau AOT, Moronne MM, Tchernia G, Mohandas N (1995) Role of the *Plasmodium falciparum* mature-parasite-infected erythrocyte surface antigen (MESA/PfEMP-2) in malarial infection of erythrocytes. Blood 86:3196–3204

Martin Olivier M, Van Den Ham K, Shio MT, Kassa FA, Fougeray S (2014) Malarial pigment hemozoin and the inflammatory response. Front Immunol 05 February 2014 | doi: 10.3389/fimmu.2014.00025

Martiney JA, Sherry B, Metz CN, Espinoza M, Ferrer AS, Calandra T, Broxmeyer HE, Bucala R (2000) Macrophage migration inhibitory factor release by macrophages after ingestion of *Plasmodium chabaudi*-infected erythrocytes: possible role in the pathogenesis of malarial anemia. Infect Immun 68:2259–2267

Meis JF, Ponnudurai T, Mons B, van Belkum A, van Eerd PM, Druilhe P, Schellekens H (1990) *Plasmodium falciparum*: studies on mature exoerythrocytic forms in the liver of the chimpanzee, Pan troglodytes. Exp Parasitol 70:1–11

Mujuzi G, Magambo B, Okech B, Egwang TG (2006) Pigmented monocytes are negative correlates of protection against severe and complicated malaria in Ugandan children. Am J Trop Med Hyg 74:724–729

Mundwiler-Pachlatko E, Beck H-P (2013) Maurer's clefts, the enigma of *Plasmodium falciparum*. Proc Natl Acad Sci, USA 110:19987-19994

Naumann KM, Jones GL, Saul A, Smth R (1992) Parasite-induced changes to localized erythrocyte membrane deformability in *Plasmodium falciparum* cultures. Immunol Cell Biol 70:267–275

Newton CRJC, Hien TT, White N (2000) Neurological aspects of tropical disease cerebral malaria. J Neurol Neurosurg Psychiatry 69:433–441

Orjih AU (2008) Requirements for maximal enrichment of viable intraerythrocytic *Plasmodium falciparum* rings by saponin hemolysis. Exp Biol Med 233:1359–1367

Orjih AU (2012) Hemozoin accumulation in Garnham bodies of *Plasmodium falciparum* gametocytes. Parasitol Res 111:2353–2359

Orjih AU, Cherian PT (2013) Possible relationship between *Plasmodium falciparum* ring-infected erythrocyte surface antigen (RESA) and host cell resistance to destruction by chemicals. Parasitol Res 112: 4043–4051

Orjih AU, Ryerse JS, Fitch CD (1994) Hemoglobin catabolism and the killing of intraerythrocytic *Plasmodium falciparum* by chloroquine. Experientia 50:34–39

Orjih AU, Cherian P, AlFadhli S (2008) Microscopic detection of mixed malarial infections: improvement by saponin hemolysis. Med Princ Pract 17:458–463

Pei X, Guo X, Coppel R, Hbattacherjee S, Haldar K, Gratzer W, Mohandas N, An X (2007) The ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* stabilizes spectrin tetramers and suppresses further invasion. Blood 110:1036–1042

Ponsford MJ, Medana IM, Prapansilp P, Hien TT, Lee SJ, Dondorp AM, Esiri MM, Day NPJ, White NJ, Turner GDH (2012) Sequestration and microvascular congestion are associated with coma in human cerebral malaria. J Infect Dis 205:663–671

Rathore D, Hrstka SCL, Sacci JB, Vega PD, Linhardt RJ, Kumar S, McCutchan TF (2003) Molecular mechanism of host specificity in *Plasmodium falciparum* infection : role of circumsporozoite protein. J Biol Chem 278:40905–40910

Ridley RG (2002) Chemotherapeutic hope on the horizon for *Plasmodium vivax* malaria? Proc Natl Acad Sci U S A 99: 13362–13364

Schwarzer E, Alessio M, Ulliers D, Arese P (1998) Phagocytosis of the malarial pigment, hemozoin, impairs expression of major histocompatibility complex class II antigen, CD54, and CD11c in human monocytes. Infect Immun 66:1601–1606

Sinden RE (2009) Malaria, sexual development and transmission: retrospect and prospect. Parasitol 36:1427–1434

Thomson JG, Robertson A (1935) The structure and development of *Plasmodium falciparum* gametocytes in the internal organs and peripheral circulation. Trans Roy Soc Trop Med Hyg 29:31–40

Triglia T, Thompson J, Caruana SR, Delorenzi M, Speed T, Cowman AF (2001) Identification of proteins from *Plasmodium falciparum* that are homologous to reticulocyte binding proteins in *Plasmodium vivax*. Infect Immun 69:1084–1092

Vernes A (1980) Phagocytosis of *P. falciparum* parasitized erythrocytes by peripheral monocytes. Lancet 2:1297–1298