

**PLASMODIUM FALCIPARUM-INFECTED ERYTHROCYTES  
FORM SPONTANEOUS ERYTHROCYTE ROSETTES**

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Erythrocytes infected with late asexual stages of *Plasmodium falciparum* and *P. fragile* may form spontaneous rosettes with uninfected erythrocytes (1-3). Here we present a detailed study of rosette formation in *P. falciparum* malaria.

**Materials and Methods**

*Assessment of Rosetting.* *P. falciparum* parasites were cultured in vitro under standard conditions. Infected erythrocytes ( $E_i$ ) were mixed with acridine orange, and  $E_i$  that had bound two or more noninfected erythrocytes ( $E_o$ ) were scored as rosetting. Blood samples drawn from patients with a blood smear positive for *P. falciparum* were washed and cultured in vitro. The number of rosettes was counted after 35 h and expressed as a percentage of the total number of trophozoite- or schizont-containing erythrocytes.

*Enrichment of Rosetting and Nonrosetting Parasites.* Cultures containing mature parasite-infected erythrocytes were layered on Ficoll-Isopaque (FIP) and centrifuged for 5 s at room temperature (RT). The cells that passed through the FIP layer were collected in a pellet, washed twice in RPMI 1640, and cultured as described. For enrichment of nonrosetting  $E_i$ , the cultures were layered over 60% Percoll and centrifuged (500 g) at RT for 20 min. The layer at the interface was collected and washed twice in RPMI 1640 and cultured as above. Rosette-forming parasites were cloned by limiting dilution and screened for rosetting on day 10-14.

*EM.* Erythrocytes from *P. falciparum* cultures were fixed with 2% glutaraldehyde in 0.05 M phosphate buffer containing 4% sucrose (pH 7.4) for 1 h, followed by embedding and cutting procedures as described by Aikawa et al. (4).

*Effect of Enzymes on Rosetting Parasites.* Cultures containing rosetted  $E_i$  were washed three times in RPMI 1640, treated with 10 or 100  $\mu\text{g}/\text{ml}$  of trypsin for 15 min at 37°C, and subsequently washed. In some experiments, trypsinized  $E_i$  were cultured in complete medium for observation of reappearance of rosettes. Experiments with neuraminidase were performed as above using 0.2, 1, and 5 U/ml, respectively.

*Cytoadherent Parasites.* The assay for cytoadherence of  $E_i$  to melanoma cells (ATCC 1585, C32r; American Type Culture Collection, Rockville, MD) was performed as described elsewhere (5). To enrich for cytoadherent parasites, *P. falciparum* cultures were incubated with melanoma cells grown in a tissue culture flask at RT for 1-2 h with continuous rotation. The bound erythrocytes were retrieved by flushing the melanoma cells with culture medium.

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The inhibitory effect on cytoadherence of mAb OKM5 (lot M506; Ortho Diagnostic Systems Inc., Westwood, MA) was studied by incubating the melanoma cells with 0.25–10  $\mu\text{g}$  antibody/ml at RT for 30 min. After washing in PBS the assay was performed as described. Inhibition by heparin was studied by incubating the parasite cultures with 5–50 IU of heparin/ml at RT for 15 min before addition to the melanoma cells. Enzyme treatments (trypsin or neuraminidase) of cytoadherent parasites were done as described above.

*Inhibition or Reversal of Rosette Formation by Antibodies or Heparin.* Studies were performed in duplicates in 96-well flat-bottomed tissue culture plates supplemented with 100  $\mu\text{l}$  of parasite culture/well. Various concentrations of IgG or the mAb OKM5 (0.1, 1, or 10  $\mu\text{g}/\text{ml}$ ) (6) were added to cultures of rosetting parasites (3–5% parasitemia at ring stage and 2% haematocrit), which then were incubated overnight. For inhibition or reversal with heparin, 10-fold dilutions (0.005–5 IU/ml) of purified heparin without preservative were added to *P. falciparum* cultures and treated as described above or kept for 1 h at RT before examination (reversal).

*Merozoite Invasion Inhibition Assay.* Merozoite invasion inhibition in vitro was performed as described by Wählin et al. (7).

## Results

*Binding of Uninfected Erythrocytes to Trophozoite/Schizont-infected Erythrocytes.* When cultured *P. falciparum* parasites of different geographical origins were monitored by light microscopy, spontaneous erythrocyte rosettes appeared when the ring stage parasites developed to trophozoites. The rosettes consisted of a central trophozoite or schizont  $E_i$ , binding up to 13  $E_o$  (Fig. 1 a). Similar rosettes were found when  $E_i$  obtained from patients with acute *P. falciparum* malaria were grown for a single cycle in vitro. Rosettes containing two or more central late stage  $E_i$  surrounded by large numbers of  $E_o$  were seen on occasions when parasitemia reached high levels (10–15%) in vitro. Agglutination of  $E_o$  in the absence of  $E_i$  was never observed.

The frequency of rosetting  $E_i$  varied greatly and consistently between different in vitro strains, as well as ex vivo grown parasites. Thus, while two in vitro grown strains contained few rosette-forming parasites (IMTM22 clone 7G8, Brazil; F32, Tanzania), two others contained 20–40% rosette-forming late stage  $E_i$  (K1, Thailand; Palo Alto [PA], Uganda). Parasites obtained directly from patients showed a similar range of variation in the number of rosette-forming  $E_i$  after culture for 30–40 h.

*Transmission Electron Microscopy (TEM) of Rosettes.* Analysis of rosettes in TEM showed that the membranes of  $E_i$  and surrounding  $E_o$  were closely adhered (Fig. 1 b). In addition, merozoites with what appear to be membrane fragments between the erythrocytes were sometimes seen (not shown). The  $E_i$  lacked electron-dense knob protrusions at the  $E_i$  surface (4).

*Enrichment and Cloning of Rosetting Parasites.* The Palo Alto strain of *P. falciparum*, containing 40% rosetting  $E_i$ , was used for enrichment and cloning of rosette-forming  $E_i$ . When the parasite culture was centrifuged on an FIP gradient, the number of rosetting late stage  $E_i$  increased from 40% to 60% after the first centrifugation and reached 80–85% after repeating this procedure four to five times. Higher frequencies were not obtained with this technique. The rosetting phenotype was stable as 80% of the late stage  $E_i$  rosetted after 4 mo of continuous culture. Conversely, the  $E_i$  fraction depleted of rosetting erythrocytes by Percoll gradient centrifugation showed almost no rosetting (3–5%  $R^+$ ), and the  $R^-$  phenotype was also sustained during at least 5 mo of culture. When cultures of  $R^+$ -enriched parasites

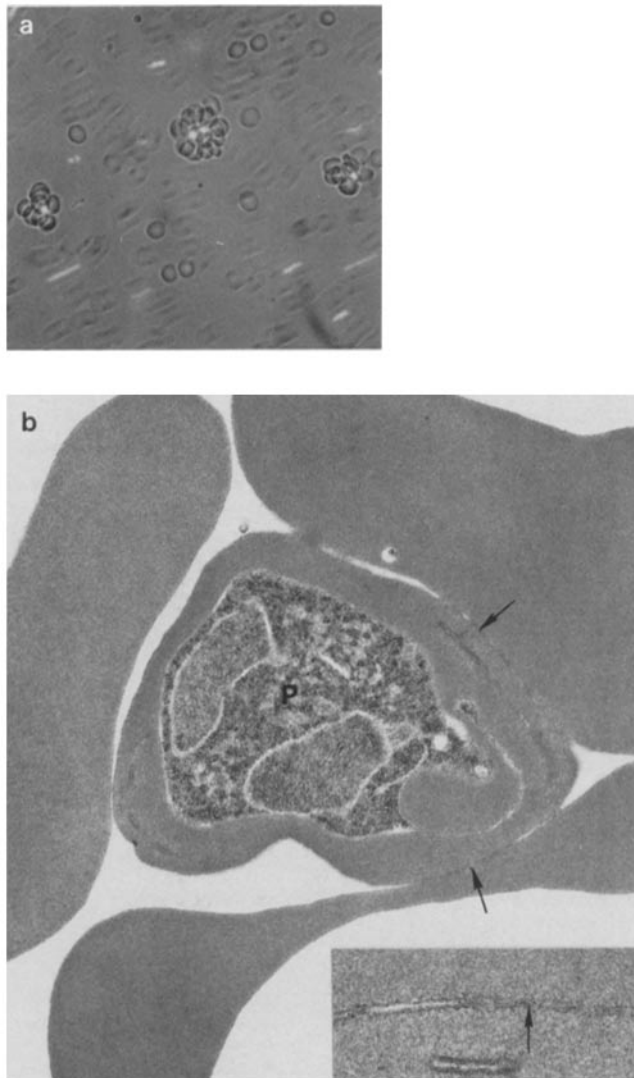


FIGURE 1. Rosette formation of *P. falciparum* (PA) in in vitro culture. (a) Parasitized erythrocytes are surrounded by adhering noninfected erythrocytes. The parasites were visualized in the fluorescent microscope by staining with acridine orange. (b) Electronmicrograph of rosette. Membrane of *P. falciparum* (*P*)-infected erythrocyte forming adherence (arrow) with proximal membranes of noninfected erythrocytes ( $\times 14,000$ ). (Inset) A higher magnification micrograph of membrane adherence (arrow) ( $\times 55,000$ ).

were cloned by limiting dilution, clones of either  $R^+$  or  $R^-$  phenotype were obtained. One  $R^+$  and one  $R^-$  clone were propagated further over a 2-mo period and showed a stable frequency of  $>95\%$   $R^+E_i$  or  $R^-E_i$ , respectively.

**Rosetting and Cytoadherence.** Only low numbers of the original Palo Alto late stage  $E_i$  bound to the melanoma cell line ATCC (C32r). To enrich for cytoadherent  $E_i$  the culture was incubated with melanoma cells. The binding cell population, flushed loose with medium, was added back to erythrocytes for further in vitro growth. After three such adherence steps, a population of binding  $E_i$  was obtained ( $>10 E_i$ /melanoma cell) (8). However, while the cytoadherent population increased during each binding procedure to include up to  $\sim 70\%$  of the parasitized erythrocytes, the number

of rosetting  $E_i$  decreased from 40% to 10%. In general,  $R^+E_i$ -enriched preparations or clone  $R^+PA1$  did not bind to melanoma cells.

A mouse mAb, OKM5, has earlier been shown to inhibit cytoadherence (6). With  $C^+E_i$ , a dose-dependent inhibition of cytoadherence was obtained by the addition of OKM5 to the melanoma cells before incubation with the parasites (8). However, OKM5 had no inhibitory effect on the formation of rosettes when added at similar concentrations to ring stage  $E_i$ , which were allowed to mature in vitro.

*Effect of Enzymes on Rosetting and Cytoadherence.* When rosetting  $E_i$  were incubated with 10–100  $\mu\text{g/ml}$  trypsin, all rosettes were disrupted. When trypsin-treated  $E_i$  were washed and returned to culture, rosettes reappeared after  $\sim 10$  h. Trypsin treatment also abolished cytoadherence. Neuraminidase (0.2–5 U/ml) had no effect on either rosetting or cytoadherence.

*Inhibition of Rosette Formation and Merozoite Invasion with Human Serum IgG.* Fig. 2 *a* shows an inhibition experiment with IgG preparations from the sera of seven malaria-immune donors. The IgGs inhibited rosette formation with 50% inhibition titers varying between 30  $\mu\text{g}$  and  $>2$  mg IgG/ml culture medium. When the rosette-inhibitory capacity of the different IgG preparations was compared with their capacity to inhibit in vitro merozoite invasion of either  $R^+PA1$  or the nonrosetting strain F32, no correlation was seen. Similar results were obtained when invasion inhibition was studied in parallel with the  $R^+PA1$  clone and an  $R^-$  culture of the PA parasites (not shown).

*The Effect of Heparin on Rosette Formation, Cytoadherence, or Merozoite Invasion.* When clone  $R^+PA1$  ring stage parasites were incubated with 10-fold dilutions (0.005–50 IU) of sodium heparin and allowed to mature overnight in vitro, a strong dose-dependent inhibition of rosette formation was found (Fig. 2 *b*). The 50% inhibition titer was 0.1 IU/ml and maximum inhibition (90–100%) was reached at 0.5–5 IU/ml. It also effectively and rapidly reversed existing rosettes (50% reversal at  $\sim 1$  IU/ml). In some experiments in which heparin was removed by washing the cultures with PBS, immediate reappearance of rosettes was observed. Heparin also inhibited

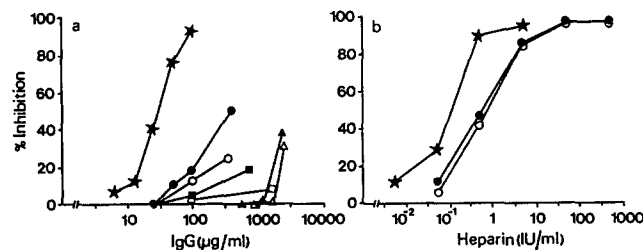


FIGURE 2. Inhibition of rosette formation or merozoite invasion by IgG or heparin. (*a*) Inhibition of rosette formation by IgG from malaria-immune donors. 089 ( $\square$ ), w183 ( $\bullet$ ), BD377 ( $\circ$ ), Ki ( $\blacksquare$ ), 255 ( $\blacktriangle$ ), Im 8 ( $\star$ ) (Liberia), IS ( $\triangle$ ) (Uganda); time of assay, 20 h. The parasites were enriched for rosetting cells. Percent rosettes in control culture with normal human IgG, 82–86%. (*b*) Effect of heparin on rosette formation ( $\star$ ) or merozoite invasion of rosette-forming clone  $R^+PA$  ( $\circ$ ) or  $R^-PA$  ( $\bullet$ ); time of assay, 20 h. Percent rosettes or parasitaemia in control cultures without heparin, 81% or 4.2%, respectively. See also reference 6.

merozoite invasion of both R<sup>+</sup> and R<sup>-</sup> PA parasites in a similar dose-dependent fashion, with a 50% inhibition titer of ~1 IU/ml (Fig. 2 *b*). When heparin was mixed with C<sup>+</sup>E<sub>i</sub>-enriched cultures before incubation with melanoma cells, no inhibition was seen with doses up to 5 IU/ml. A slight inhibition was observed at the highest concentration of heparin used (20% inhibition at 50 IU/ml).

### Discussion

The binding of uninfected erythrocytes to certain *P. falciparum* trophozoite/schizont-infected erythrocytes results in the formation of rosettes. The number of rosetting E<sub>i</sub> varied drastically but consistently between different *P. falciparum* isolates. Rosette formation involved adherence between the membranes of a central E<sub>i</sub> and surrounding E<sub>o</sub> but the molecular events responsible for rosetting are unknown.

Rosette formation was inhibited with IgG preparations from different *P. falciparum*-immune donors. However, the 50% inhibition titers varied widely, indicating either weak or transient immune responses in different donors or antigenic diversity of the epitopes seen by the antibodies. A dose-dependent inhibition of rosette formation as well as reversal of already formed rosettes was also obtained with heparin. Heparin similarly inhibits reinvasion of erythrocytes by merozoites but the possible relationship between rosetting and invasion remains to be established.

Rosetting has also been shown for late stage E<sub>i</sub> of *P. fragile* (3) and *P. chabaudi* (1), two species that sequester in vivo. Sequestration reflects binding of late stage E<sub>i</sub> to the endothelial lining in postcapillary venules (3, 9), a phenomenon paralleled by the cytoadherence of these E<sub>i</sub> to endothelial cells and certain melanoma cells in vitro (5). With cloned *P. fragile*-infected erythrocytes, both rosetting and sequestration occur in parallel and are related to the presence of parasite-derived antigens on the E<sub>i</sub> surface (3). Incubation of rosetting E<sub>i</sub> of both *P. fragile* and *P. falciparum* with trypsin at concentrations that abolish or reduce cytoadherence to melanoma cells also disrupts the rosettes (2, 3). Moreover, both rosetting and cytoadherence were regained after a few hours of in vitro culture. Finally, neither rosetting nor binding to melanoma cells were susceptible to neuraminidase treatment. Taken together, this data would seem to suggest a relationship at the molecular level between rosetting and endothelial binding. However, in our experiments performed with a Palo Alto strain that lacked the knobby protrusions (K<sup>-</sup>) claimed to be necessary for cytoadherence, the fraction of R<sup>+</sup> cells steadily decreased when the parasites were enriched for cytoadherent cells (also K<sup>-</sup>) by repeated adsorption to melanoma cells (8). Conversely, when the parasites were enriched for rosetting cells by repeated gradient centrifugation, they did not cytoadhere. Heparin that efficiently inhibited rosette formation had only a slight inhibitory effect on cytoadherence when added at high concentration (>50 IU/ml). Furthermore, the R<sup>+</sup> clone PA1 was C<sup>-</sup> also when incubated in the presence of heparin at a concentration (5 IU/ml) that disrupts the rosettes but does not affect cytoadherence. When cultures were incubated with an mAb (OKM 5), previously shown to block cytoadherence by binding to a receptor protein on melanoma cells (6), cytoadherence was inhibited (8), while rosetting was not.

From these results we conclude that the cytoadherence of knobless E<sub>i</sub> to melanoma cells and rosetting are two phenomena involving different molecular mechanisms. We think this also applies to cytoadherence of K<sup>+</sup> parasites but this remains to be established. In any event, as both cytoadherence and rosetting are apparently

associated with parasites that are sequestered in the infected host (2, 3, 9), it is possible that both phenomena are also involved in sequestration. Parasites of the R<sup>+</sup> phenotype might primarily be responsible for the formation of aggregates between parasitized and nonparasitized erythrocytes obstructing capillaries or post-capillary venules in patients with cerebral malaria.

### Summary

Erythrocytes infected with trophozoites or schizonts of *Plasmodium falciparum* bind uninfected erythrocytes, leading to rosette formation. Both established laboratory strains and fresh isolates from patients form such rosettes, but at widely different frequencies. IgG preparations from the serum of some *P. falciparum*-immune donors and heparin inhibited rosette formation. The results indicate that cytoadherence of infected erythrocytes to endothelial cells and rosetting represent distinct genetic traits.

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