Brief Definitive Report

PLASMODIUM FALCIPARUM-INFECTED ERYTHROCYTES FORM SPONTANEOUS ERYTHROCYTE ROSETTES

By RACHANEE UDOMSANGPETCH,* BIRGITTA WÅHLIN,*
JOHAN CARLSON,* KLAVS BERZINS,* MOTOMI TORII,§
MASAMICHI AIKAWA,§ PETER PERLMANN,* and MATS WAHLGREN*‡

From the *Department of Immunology, University of Stockholm, S-106 91 Stockholm, Sweden; the
‡Department of Infectious Diseases, Karolinska Institute, Roslagstull Hospital, S-114 89 Stockholm,
Sweden; and the §Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

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 erythorcytes (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 µg/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.

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 μg 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 μl of parasite culture/well. Various concentrations of IgG or the mAb OKM5 (0.1, 1, or 10 μg/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 Wåhlin 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.

Transmisson 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
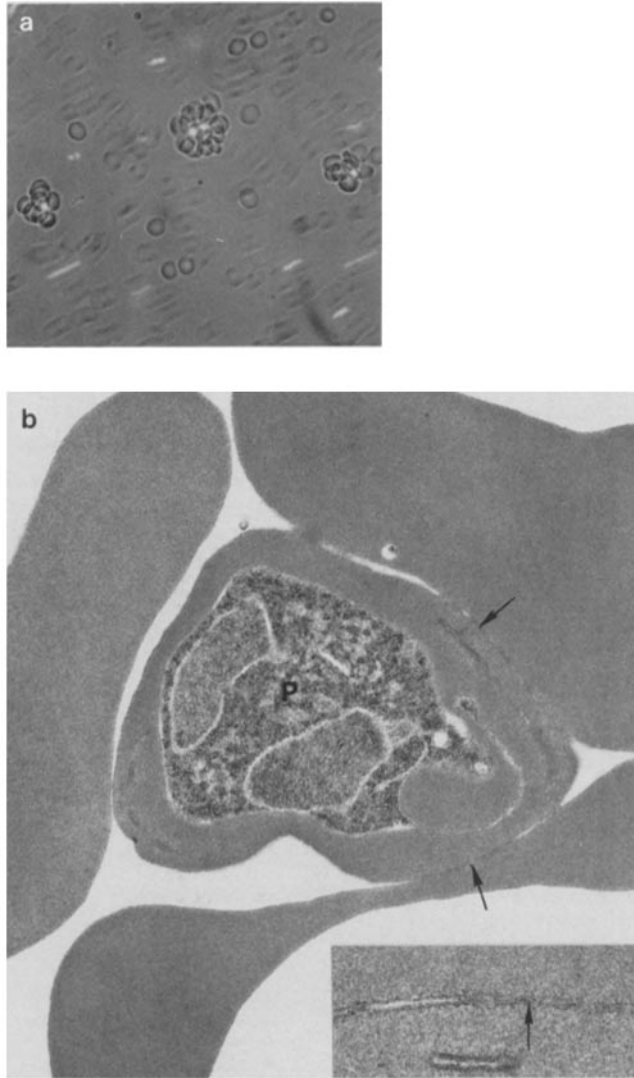
FIGURE 1.   Rosette formation of *P. falciparum* (PA) in in vitro culture. (*a*) Parasitized erythrocytes are surrounded by adhering noninfected erythrocytes. The parasites were visualized in the fluorescent microscope by staining with acridine orange. (*b*) Electronmicrograph of rosette. Membrane of *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 $\mu g/ml$ trypsin, all rosettes were disrupted. When trypsin-treated E_i were washed and returned to culture, rosettes reappeared after ~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 μg 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 ~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
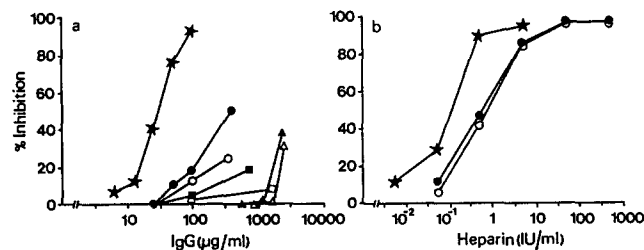

FIGURE 2.   Inhibition of rosette formation or merozoite invasion by IgG or heparin. (*a*) Inhibition of rosette formation by IgG from malaria-immune donors. 089 (□), wl83 (●), BD377 (○), Ki (■), 255 (▲), Im 8 (★) (Liberia), IS (△) (Uganda); time of assay, 20 h. The parasites were enriched for rosetting cells. Percent rosettes in control culture with normal human IgG, 82–86%. (*b*) Effect of heparin on rosette formation (★) or merozoite invasion of rosette-forming clone R^+PA (○) or R^-PA (●); 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^+ and R^- PA parasites in a similar dose-dependent fashion, with a 50% inhibition titer of $\sim$1 IU/ml (Fig. 2 b). When heparin was mixed with C^+E_i-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_i varied drastically but consistently between different *P. falciparum* isolates. Rosette formation involved adherence between the membranes of a central E_i and surrounding E_o 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_i of *P. fragile* (3) and *P. chabaudi* (1), two species that sequester in vivo. Sequestration reflects binding of late stage E_i to the endothelial lining in postcapillary venules (3, 9), a phenomenon paralleled by the cytoadherence of these E_i 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_i surface (3). Incubation of rosetting E_i 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^-) claimed to be necessary for cytoadherence, the fraction of R^+ cells steadily decreased when the parasites were enriched for cytoadherent cells (also K^-) 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^+ clone PA1 was C^- 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_i to melanoma cells and rosetting are two phenomena involving different molecular mechanisms. We think this also applies to cytoadherence of K^+ 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^+ 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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