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Rosette Formation of *Plasmodium falciparum*-Infected Erythrocytes from Patients with Acute Malaria

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Noninfected erythrocytes form rosettes around those infected with trophozoites and schizonts of *Plasmodium falciparum* in vitro. These rosettes are thought to contribute to the microvascular obstruction which underlies the pathophysiology of severe falciparum malaria. To determine whether the percentage of infected erythrocytes forming rosettes for a parasite isolate in vitro correlates with the in vivo severity of disease, we studied the rosette formation behavior of 35 isolates of *P. falciparum* from patients with uncomplicated, severe, and cerebral malaria. There was a wide variation in the degree of rosette formation (0 to 53%). Four parasite isolates formed rosettes well (30 to 53%), and seven isolates formed rosettes poorly or not at all (0 to 5%), while the majority of the isolates formed rosettes to various degrees between these two extremes. In this relatively small sample of patients, we were unable to demonstrate a significant association between in vitro rosette formation and patients with cerebral malaria or conscious patients with significant renal (serum creatinine >200 μmol/liter) or hepatic dysfunction (serum bilirubin >50 μmol/liter and aspartate aminotransferase >50 Reitman-Frankel units). However, there was an inverse relationship between rosette formation and cytoadherence ($r = -0.575$, $P < 0.01$) which could not be explained on the basis of steric hindrance. This finding suggests that cytoadherence and rosette formation properties are intrinsic to the parasites, with isolates having a greater propensity for one or the other but not both. Further studies are required to establish the occurrence and pathophysiological role of rosette formation in vivo.

It is now generally recognized that deep vascular sequestration of parasitized erythrocytes is the central pathological event in falciparum malaria (8). As a result, considerable interest has been focused on the underlying mechanisms in the hope that new therapeutic modalities can be devised for the treatment of severe malaria. Cytoadherence of parasitized erythrocytes to the vascular endothelium of venules and capillaries appears to be the principal process which leads to sequestration (20). A relatively newly reported phenomenon, which has also been suggested as a contributing factor to microvascular obstruction, is rosette formation. This is the agglutination of nonparasitized erythrocytes around erythrocytes containing trophozoites and schizonts of *Plasmodium falciparum* (3). Rosette formation is thought to be related to cytoadherence because rosette formation occurs with species of plasmodia which also exhibit cytoadherence (4), and both phenomena are limited to parasitized erythrocytes containing mature stages of *P. falciparum* (3). In addition, rosette formation can be reversed by immune sera which also reverse cytoadherence (3), and the antigens responsible for rosette formation and cytoadherence are both protease sensitive (3, 14).

Although rosette formation has been observed in the microvasculature of the rat mesoappendix experimentally perfused with *P. falciparum*-infected erythrocytes (7), its occurrence in vivo in human malaria remains to be confirmed. Most initial studies on rosette formation have been performed with simian malarias (3) or laboratory-adapted *P. falciparum* lines and clones (2, 14). The biological characteristics of these parasites may or may not be similar to those of parasite isolates from naturally acquired infections. More recently, cryopreserved and fresh parasite isolates from patients with acute malaria have been studied in vitro (1, 5, 18). The results indicate that rosette formation is a characteristic of *P. falciparum* from diverse geographical locations (5, 18) and that it may contribute to the pathogenesis of cerebral malaria in children (1).

To determine whether in vitro rosette formation is associated with clinical markers of disease severity in vivo in adults with acute falciparum malaria, we have studied the rosette formation behavior of 35 fresh isolates of *P. falciparum* from patients with naturally acquired infections.

MATERIALS AND METHODS

Patients. We studied 35 consecutive *P. falciparum* isolates which grew to the trophozoite and/or schizont stage in vitro from patients with acute falciparum malaria at the Hospital for Tropical Diseases, Bangkok and Paholpolpayuhasena Hospital, Kanachanaburi, Thailand. These were adult patients who either had a few falciparum malaria attacks in the past or were experiencing their first infection. The diagnosis of malaria was confirmed by the detection of asexual stages of *P. falciparum* in peripheral blood smears. On admission, a complete physical examination and routine hematological and biochemical investigations were performed. Five milliliters of whole blood was drawn into heparinized tubes for parasite culture before the start of antimalarial therapy. Erythrocytes were washed three times in RPMI medium (Flow Laboratories, Ayreshire, Scotland) and either were

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put into culture immediately (15) or were resuspended in medium and kept overnight at 4°C.

Clinical assessment of severity. The classification of patients as having uncomplicated, severe, or cerebral malaria was carried out according to published criteria (21). Briefly, patients were considered to have cerebral malaria when admitted in a state of unrousable coma (19). Patients with severe malaria were those who were conscious on admission but had either elevated creatinine (>200 μmol/liter) or elevated bilirubin (>50 μmol/liter) and aspartate aminotransferase (>50 Reitman-Frankel units) levels in serum. All other patients were considered to have uncomplicated malaria.

Rosette formation assay. The rosette formation assay was performed according to the method of David et al. (3). Parasite development was examined every 8 to 10 h by light microscopy until the parasites had reached the trophozoite and/or schizont stage. The parasitemia was adjusted to between 1 and 3% by serial dilution with normal group O erythrocytes before testing. Ten microliters of a 10% suspension in RPMI, pH 7.2, was mixed with 10 μl of heat-inactivated fetal bovine serum (Flow Laboratories). Ten microliters of the mixture was put on a glass slide and covered with a coverslip (22 by 22 mm). One hundred infected erythrocytes were examined under oil immersion by phase-contrast microscopy. Infected erythrocytes were identified by the presence of a golden brown refractile malaria pigment. The percentage of infected erythrocytes with two or more uninfected erythrocytes attached was scored.

In preliminary experiments with eight parasite isolates, two or three suspensions of infected erythrocytes with between 1 and 3% parasitemia were prepared for each isolate and examined for rosettes. The coefficient of variation of duplicate or triplicate samples ranged from 2.8 to 17%, with a median of 9.4%. Because of the small interassay variations demonstrated, all subsequent assays were performed at 25°C with a single parasite suspension. Duplicate slides were prepared, and each slide was counted twice, so that the percentage of rosette formation for each isolate represented the average of four separate readings.

Rosette formation in vivo. To determine whether rosette formation occurs in vivo, erythrocytes from the peripheral blood of a patient with severe malaria which contained 4 late trophozoites in addition to 54 ring forms per 1,000 erythrocytes were prepared and examined by phase-contrast microscopy in the same manner as parasites which had been cultured in vitro.

Cytoadherence to C32 melanoma cells. The cytoadherence of 23 parasite isolates to C32 melanoma cells (MC) (ATCC CRL 1585; American Type Culture Collection, Rockville, Md.) was determined as described previously (6) at the same time as the rosette formation assay. Briefly, a 2% suspension of infected erythrocytes in RPMI 1640 medium, pH 7.2, was added to duplicate formalin-fixed monolayers each containing 10^5 MC. The monolayers were incubated at 37°C in 5% CO_2 for 1.5 h with gentle mixing every 15 min. At the end of the incubation, the monolayers were rinsed four times gently in RPMI and left to dry in air. The monolayers were fixed with methanol, stained with 10% Giemsa stain and examined microscopically. At least 1,000 MC and adherent infected erythrocytes were counted per slide, and cytoadherence was expressed as the number of infected erythrocytes per 100 MC. Because previous experiments have shown that cytoadherence of a given isolate is directly related ($r = 0.99$ to 1.0) to the trophozoite and/or schizont parasitemia at the time of the cytoadherence assay (6), the cytoadherence at 1% parasitemia was obtained for purposes of comparison among isolates by dividing the raw count by percentage parasitemia (corrected binding).

To determine the effect of rosette formation on cytoadherence in a given isolate, a 2% suspension of parasitized erythrocytes of three isolates was incubated with and without preservative-free heparin (2.5 units/ml) for 1 h at room temperature before the cytoadherence assay was performed. This dose of heparin has been shown to reduce rosette formation to <50% of the control value without affecting cytoadherence (16).

Statistical analysis. The percentage of rosette formation in samples from the three patient groups was compared by using the Kruskal-Wallis one-way analysis of variance (14). Correlation between rosette formation and cytoadherence was assessed by Spearman's rank correlation coefficient.

RESULTS

Clinical. The admission clinical and laboratory features of the 35 patients are summarized in Table 1. Ten patients were classified as having uncomplicated malaria, while 16 had evidence of renal and/or hepatic dysfunction. Nine patients had cerebral malaria, of whom six also had evidence of other vital organ dysfunction.

Rosette formation. Rosette formation was observed in 31 of 35 isolates (89%). The percentage of rosette formation was readily reproducible between duplicate slides and double counts; the coefficient of variation of the four readings for each subject was always less than 10%. There was, however, wide variation in the percentage of rosette formation of different isolates, with a range of 0 to 53% (Fig. 1). Occasionally rosettes were seen which contained 2 to 3 parasitized erythrocytes, but higher numbers of such erythrocytes in a single rosette were not observed as previously reported (1, 2).

The median percentage rosette formation of the 10 patients with uncomplicated malaria was 10.7 (range, 0 to 47.3) (Fig. 1). In the 16 patients who were conscious but had clinical markers indicative of significant hepatic and renal impairment, the median percentage rosette formation was 9.0% (0 to 34%). The nine patients with cerebral malaria had the highest median percentage rosette formation, 18.3% (0 to 51.6%). When rosette formation of the three patient groups were analyzed by using the Kruskal-Wallis test, the differences were not statistically significant ($\chi^2 = 2.649$, df = 2, $P > 0.20$).

Rosette formation in vivo. An occasional rosette (3 in 50 high-power fields) similar to those seen after in vitro culture of the early stages of *P. falciparum* was observed. No attempt was made to quantitate the degree of rosette formation because of the low-trophozoite parasitemia.

Correlation between rosette formation and cytoadherence to C32 melanoma cells. When the percentage of rosette formation was plotted against corrected binding, a significant inverse correlation between the two processes was obtained ($r = -0.575$, $P < 0.01$) (Fig. 2). To determine whether this inverse relationship was the result of physical interference with cytoadherence by rosettes of erythrocytes, infected erythrocytes of three isolates were preincubated with heparin, which is known to reverse rosette formation. There was no increase in the degree of cytoadherence of heparin-treated erythrocytes compared with controls, although the degree of rosette formation was markedly reduced (Table 2).

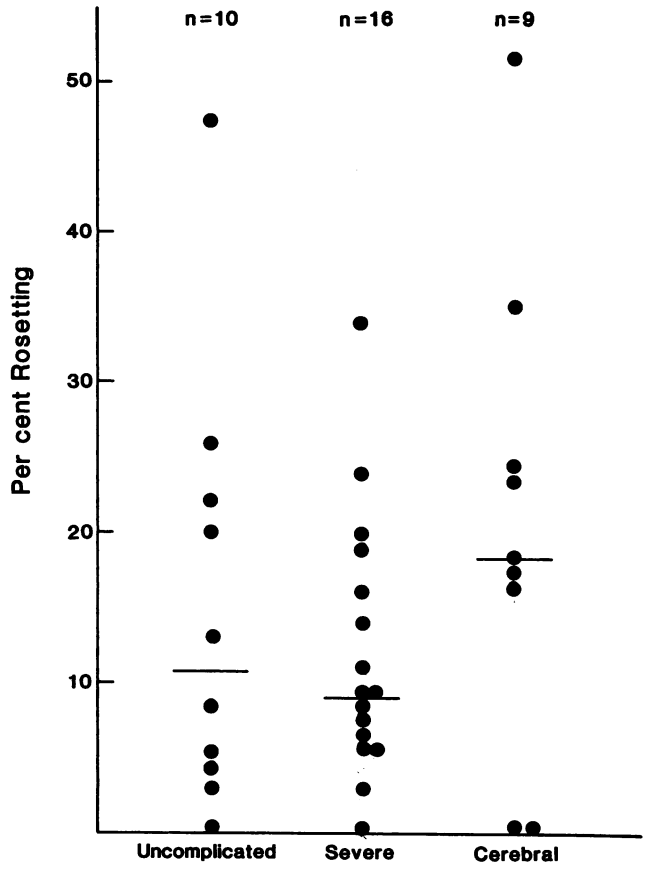

FIG. 1. Comparison of percentage rosette formation in patients with uncomplicated, severe, and cerebral malaria. The bars indicate median percentages.

TABLE 1. Clinical and laboratory features of patients with uncomplicated, severe, and cerebral malaria

Malaria classification	Age[a] (yrs)	No. of parasites (geometric mean/μl)	Hematocrit[a] (%)	Blood urea nitrogen[a]		Serum creatinine[a]		Serum bilirubin[a]		Aspartate aminotransferase[a]	
				mg/dl	mmol/liter	mg/dl	μmol/liter	mg/dl	μmol/liter	U[b]	μmol/liter
Uncomplicated (n = 10)	26.1 ± 11.6	107, 741	33.0 ± 7.0	23.0 ± 7.5	8.2 ± 2.7	1.3 ± 0	115.1 ± 26.6	3.6 ± 1.4	61.2 ± 23.8	35.8 ± 11.0	0.60 ± 0.18
Severe (n = 16)	22.7 ± 6.4	270, 422	34.0 ± 9.3	39.9 ± 29.4	14.2 ± 10.5	2.4 ± 0.9	212.4 ± 79.7	9.9 ± 8.5	168.3 ± 144.5	124.5 ± 62.3	2.08 ± 1.04
Cerebral (n = 9)	33.0 ± 18.8	127, 669	33.4 ± 7.7	53.6 ± 46.5	19.1 ± 16.5	2.9 ± 1.7	256.7 ± 150.5	11.7 ± 20.6	198.9 ± 350.2	79.6 ± 32.3	1.33 ± 0.54

[a] Values represent the mean ± the standard deviation.
[b] Reitman-Frankel units. Normal range, 0 to 40 units.

DISCUSSION

Since its first description 3 years ago, rosette formation has been thought to contribute to the pathogenesis of severe malaria (2, 3, 17). However, although many investigations have been directed towards the influence of various laboratory conditions such as temperature, pH, and different anticoagulants on the process (2, 16), there was relatively little information until recently on the occurrence and pathophysiological significance of rosette formation in humans with *P. falciparum* infections. In this study, rosette formation was observed in the peripheral blood of a patient who had severe malaria and who had late trophozoites in the circulation. Although this observation was one step closer to in vivo events in the patient, the possibility that the rosettes had resulted from in vitro manipulation of the infected erythrocytes (similar to the formation of platelet aggregates during venipuncture [13]) could not be ruled out.

Cytoadherence of *P. falciparum*-infected erythrocytes to C32 MC (6) and to purified CD36 protein adsorbed on plastic (11) are both positively associated with objective markers of the clinical severity of infection of the patients from whom the parasite isolates were obtained. If rosette formation is an important pathological process in vivo, there should be a similar correlation. Our results do not support such an association.

In 35 parasite isolates obtained directly from patients infected with *P. falciparum* and tested during the first cycle

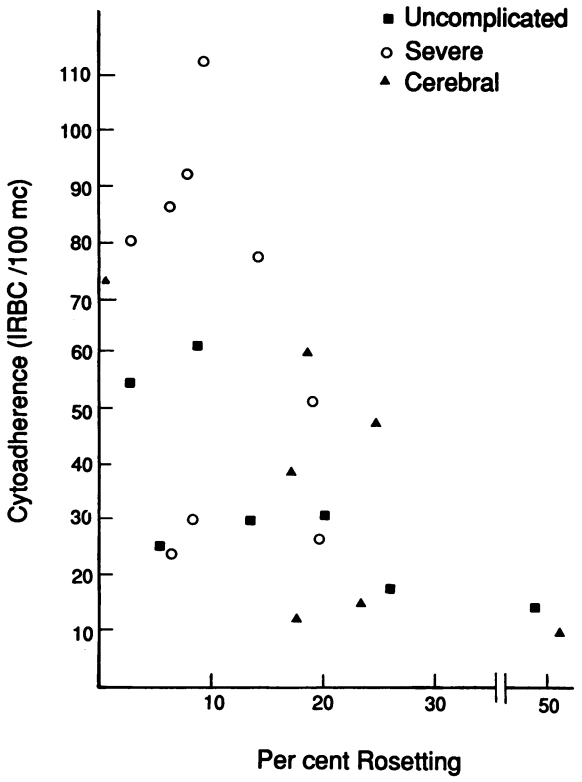

FIG. 2. Corrected binding versus percentage rosette formation.

of culture in vitro, the range of the percentages of rosette formation observed in this study was similar to that of most other studies involving *P. falciparum* from patients with natural infections (1, 5, 18) but was considerably lower than that observed in six patients from Sri Lanka (37 to 68%) (4). There appeared to be a few parasite isolates which formed rosettes well (30 to 53%), and some which formed rosettes poorly or not at all (0 to 5%), while the majority of the parasite isolates formed rosettes to various degrees between these two extremes. However, we were unable to demonstrate a significant correlation between in vitro rosette formation and cerebral complications or renal and hepatic dysfunction. This is a relatively small series, with limited power to detect small differences or weak associations, but large effects can be excluded with confidence.

The inverse relationship demonstrated between rosette formation and cytoadherence is intriguing. Rosettes may physically hinder the interaction between parasitized erythrocytes that have not formed rosettes and the C32 melanoma cell monolayer. However, this was shown not to be the case in this study, since cytoadherence did not increase when rosette formation was reduced by 60 to 70% in three isolates. This finding suggests that cytoadherence and rosette formation properties are intrinsic to the parasites, with parasite isolates having a greater propensity for one or the other but not both. This observation is consistent with the finding that isolates from patients with cerebral malaria adhere poorly to C32 MC (6, 9) and human umbilical vein endothelial cells (10) in vitro but do appear to form rosettes to a greater degree both in this study and as reported by Carlson et al. (1). Whether the inverse relationship between cytoadherence and rosette formation holds true in vivo remains to be determined.

TABLE 2. Effect of inhibition of rosette formation by heparin on cytoadherence

Isolate	Rosette formation (%)		Cytoadherence (infected erythrocytes/100 MC)	
	Heparin	No heparin	Heparin	No heparin
1	10	33	165	188
2	8	22	109	106
3	4	10	282	302

The evidence implicating rosette formation in the pathogenesis of cerebral malaria remains hypothetical. Unlike cytoadherence, for which there is unequivocal histological evidence that it parallels the degree of cerebral involvement (12), rosettes have never been observed in any histological studies of clinical or postmortem tissues. The site of rosette formation in vivo is unknown. Presumably, the lower pH and shear forces on the venous side would tend to favor rosette formation, but if complete vascular obstruction occurs, one would expect the tail of blood into the capillary to reflect the distribution of parasitized erythrocytes in the circulation. In fact, more than 90% of the erythrocytes sequestered in the cerebral vessels in cerebral malaria are parasitized (8). Rosette formation on the arterial side of the microcirculation appears unlikely as arteriolar obstruction is not a pathological feature of severe malaria. It may be that the binding forces which lead to rosette formation may contribute to a reduction in forward blood flow, which would then encourage cytoadherence.

The determination of the relative contribution of cytoadherence and rosette formation to the pathogenesis of severe malaria is obviously of great importance both in the understanding of the disease process and in designing new therapeutic approaches for the treatment of severe malaria. It has been shown that rosette formation can be inhibited by pharmacological doses of heparin and calcium chelators as well as by a monoclonal antibody against the histidine-rich protein PfHRP1 which is known to form the core of the electron-dense knobs at the point of contact between parasitized erythrocytes and the endothelium (2). However, the data of the present study emphasize that further studies to establish the pathophysiological role of rosette formation in vivo are necessary in order to determine whether such a therapeutic strategy will have a beneficial clinical effect.

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