LACK OF SIGNIFICANT ASSOCIATION BETWEEN ROSETTE FORMATION AND PARASITIZED ERYTHROCYTE ADHERENCE TO PURIFIED CD36

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Abstract. The ability of *Plasmodium falciparum* infected erythrocytes from 162 Thai patients with uncomplicated malaria, 82 patients with severe malaria and 19 patients with cerebral malaria to form rosettes *in vitro* was studied. Of 263 isolates, 62 were evaluated for their adherence to different target molecules. We found that wide variation occurred in isolates from all groups in the level of rosette formation and adherence to CD36, intracellular adhesion molecule-1, thrombospondin and chondroitin sulfate A. No statistically significant correlation between the magnitude of rosette formation and disease severity was found ($p > 0.05$). In addition, our results from the use of purified CD36 as an adherence receptor showed no association between the degree rosetting and level of cytoadherence ($p > 0.05$, $r = -0.04$). Our data provide evidence that rosette formation and cytoadherence involve different molecular mechanisms and both phenomena can occur in all manifestations of the disease.

INTRODUCTION

Rosette formation, the binding of uninfected erythrocytes to *Plasmodium falciparum* infected erythrocytes, has been suggested to play a critical role in the induction of cerebral malaria. In some studies freshly isolated parasitized erythrocytes (PE) obtained from children with cerebral malaria were capable of rosetting, while others from patients with mild disease were not (Carlson *et al*, 1990; Ringwald *et al*, 1993; Rowe *et al*, 1995). From the studies, it has been proposed that rosettes cause impairment of microvascular blood flow and this in turn leads to organ complication in falciparum malaria (Carlson *et al*, 1990; Wahlgren *et al*, 1994; Rowe *et al*, 1995). However, no such association was found in studies in Papua New Guinea (Al-Yaman *et al*, 1995) and Pakistan (Iqbal *et al*, 1993). The definitive molecular mechanism involved in rosette formation and identity of the relevant components on uninfected erythrocytes and PE remain to be elucidated. Some evidence suggests that the capacity to form rosettes is influenced by ABO blood group saccharides (Carlson and Wahlgren, 1992; Rowe *et al*, 1995) whereas other evidence implicates CD36, which has been detected on the surface of uninfected erythrocytes (Handunetti *et al*, 1992).

In the present study, we investigated the involvement of CD36 on rosette formation. We also examined the association of rosette formation and cytoadherence to different purified adhesion molecules of PE freshly obtained from 64 patients. Rosette disruption by anti-CD36 antisera was also evaluated.

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MATERIALS AND METHODS

Parasites and patients

Plasmodium falciparum isolates were obtained from 262 patients admitted to the Bangkok Hospital for Tropical Disease, Bangkok, Thailand, between February 1995 and July 1996. These patients came from several provinces near the Thai-Myanmar border and the Thai-Cambodian border. Informed consent was obtained from the donors before collection of venous blood. Clinical assessment of severity was determined according to World Health Organization Criteria (WHO, 1990). Patients were divided into two main clinical groups, *ie* those with severe falciparum malaria and those with uncomplicated malaria. Of 100 severe patients, 19 were

classified as having cerebral malaria.

Prior to anti-malarial treatment, parasitized blood was collected in heparinized tubes for transport to the laboratory. Blood cells were washed 3 times, with buffy coat depletion. Parasites were then cultured at 4% packed cell volume in RPMI 1640 medium, buffered with 25 mM HEPES and supplemented with 10% human AB serum, 2 mM L-glutamine, 10 μg/ml of gentamicin, and 25 mM sodium bicarbonate, as previously described (Trager and Jensen, 1976). Assessment of rosetting was done during the first cycle of culture when the parasites had grown to mid-late trophozoites. Cytoadherence assays were performed when the parasitemia of trophozite-infected erythrocytes was above 1%, since field isolates varied in their growth rate and maturity.

Rosette formation

The assessment of rosette formation was made after 24 hours. A sample of resuspended parasite culture (15 μl) was mixed with 5 μl of ethidium bromide (1 mg/ml), mounted on a microscopic slide and examined using a 40x lens in incident UV light. At least 100 trophozoite-infected erythrocytes were counted for each sample. Infected erythrocytes that had bound 2 or more uninfected erythrocytes were scored as rosettes. The rosetting rate was expressed as the percentage of trophozoite-infected erythrocytes that formed rosettes.

Cytoadherence assay

Duplicate concentration spots of the purified adhesion molecules CD36, thrombospondin, and ICAM-1 were adsorbed to petri dishes at 1, 50, and 10 μg/ml, respectively. The dishes were subsequently blocked with 1% bovine serum albumin prior to assays. In another assay, C32 melanoma cells were seeded at a concentration of 1×10^5 cells/ml. After 24 hours, the cells were washed twice with RPMI-HEPES before addition of infected erythrocytes.

The cytoadherence assay was carried out essentially as described by Biggs *et al* (1992). Parasite cultures at the mid-late trophozoite stage were harvested and washed in RPMI 1640-HEPES. The harvested erythrocytes were then incubated with various preparations of cell line or purified adhesion molecules for 1 hour at 37°C with gentle rocking at 15-minute intervals. Unbound cells were then washed off with 10 ml of RPMI 1640-HEPES. Bound cells were fixed with 2% glutaraldehyde, and stained with 10% Giemsa. Cytoadherence assays were performed at least twice with each field isolate, depending on the amount of material available. The numbers of bound infected erythrocytes were assessed by microscopic examination. Results were expressed as the number of parasitized erythrocytes bound per 100 melanoma cells or per mm^2.

Disruption of rosette formation

A 25 μl of aliquot of culture was incubated at 37°C for 60 minutes with 25 μl of monoclonal anti-human CD36 (Sigma Immuno Chemicals, St Louis, Mo) at a final dilution of 1:10. Ethidium bromide (5 μl) was then added to the mixture (15 μl) and the rosetting rate was determined. Autologous human sera were included as controls.

Statistical analysis

Rosetting rates of the whole study population were examined against a set of variables including parasite density, blood group, severity, and CD36 adherence. Comparisons according to blood group and severity were assessed by Kruskal-Wallis one-way analysis of variance. Correlations between rosette formation, parasite density and CD36 adherence were examined by Spearman's rank correlation coefficient. P-values < 0.05 were considered to indicate significant differences.

RESULTS

During the study period, 162 patients with uncomplicated malaria (UM) (mean age 24.7 years, range 17 years to 33 years), 81 patients with severe malaria (SM) (mean age 25.9 years, range 15 years to 37 years), and 19 patients with cerebral malaria (CM) (mean age 25.1 years, range 17 years to 33 years) were recruited. Clinical data of the patients are shown in Table 1. The parasites obtained from these donors were grown *in vitro* and later scored for their ability to form rosettes. Not all isolates formed rosettes. Eighteen of the 81 isolates from

Table 1

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

Parameter	Value for patients with malaria type:		
	Uncomplicated (n = 162)	Severe (n = 81)	Cerebral (n = 19)
Age (yr)	24.7 ± 8.2*	25.9 ± 10.7	25.1 ± 8.1
Sex (M:F)	90:72	55:26	11:8
Parasite count (%)	1.3 ± 1.1	7.1 ± 5.7	7.3 ± 6.7
Hematocrit (%)	34 ± 6.7	34.6 ± 8.8	28.7 ± 6.1
Blood urea nitrogen (mg/dl)	19.1 ± 11.0	38.97 ± 29.2	69.3 ± 38.1
Creatinine (mg/dl)	1.0 ± 0.4	1.7 ± 1.2	2.2 ± 1.5
Total bilirubin (mg/dl)	2.0 ± 1.9	5.9 ± 6.6	5.9 ± 4.9
Aspartate aminotransferase (Reitman-Frankel units)	51.8 ± 40.2	98.0 ± 79.1	190.9 ± 180.5
Adhesion to CD36 (n = 64)	332.4 ± 475.7 (n = 37)	333.5 ± 364.7 (n = 25)	138.3 ± 167.2 (n = 2)
%Rosette formation	4.1 ± 6.0	5.1 ± 6.9	11.1 ± 13.6
Blood groups : A (6.5 ± 9.7, n = 72)	5.4 ± 8.2 (n = 41)	6.3 ± 10.1 (n = 19)	10.9 ± 13.1 (n = 12)
B (5.3 ± 7.3, n = 71)	4.1 ± 5.4 (n = 42)	5.5 ± 6.1 (n = 26)	21.3 ± 20.5 (n = 3)
AB (3.5 ± 5.6, n = 20)	4.2 + 2.8 (n = 14)	5.6 ± 10.8 (n = 5)	1 (n = 1)
O (3.7 ± 4.9, n = 99)	3.6 ± 5.2 (n = 65)	4.0 ± 4.3 (n = 31)	5.0 ± 6.2 (n = 3)

*Mean ± standard deviation.

SM patients lacked parasites that formed rosettes and no rosette was detected in the parasites isolated from 2 CM patients (Fig 1). Interestingly, a high percentage (73%) of the isolates collected from UM patients formed rosettes. A wide range of variation in the rosetting rate was observed in all groups (0% - 32% in UM, 0% - 33% in SM and 0%-42% in CM) (Fig 1). There were differences in the geometric mean rosetting rates among the three clinical groups (4.1% in UM, 5.1% in SM, and 11.1% in CM) (Table 1). However, statistical analysis using the Kruskal-Wallis one way analysis of variance found no significance difference between the magnitude of rosette formation and severity of infection ($p > 0.05$). In addition, there was no difference in the number of large or giant rosettes seen in parasitized cells from CM, SM, or UM patients.

The mean of parasite density was higher in the groups with cerebral malaria (7.3% and severe malaria (7.1%) than in the group with uncomplicated malaria (1.3%) (Table 1). We found a statistically significant correlation between *in vivo* parasitemia and rosette formation rate among isolates tested ($p < 0.05$, $r = 0.168$).

When rosette formation capacity of infected erythrocytes of different ABO blood groups was analyzed, the parasite isolates from patients with blood groups A and B had higher geometric mean rosetting rates (6.6% for A, 5.3% for B, 3.5% for AB, and 3.7% for O). However, these values were not significantly different after statistically analyzed ($p > 0.05$) (Fig 2).

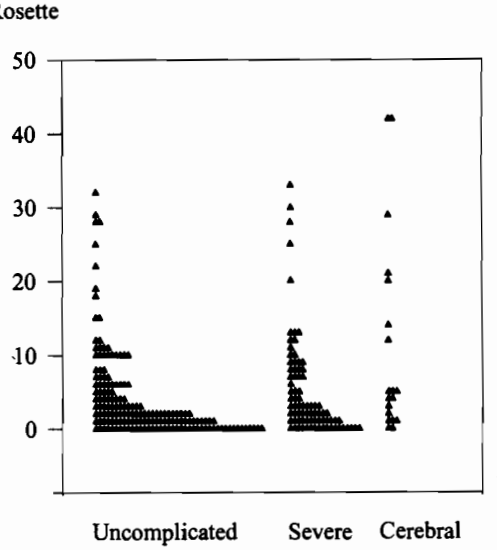

Fig 1–Degree of rosette formation of infected erythrocytes from fresh malarial isolates obtained in Thailand.

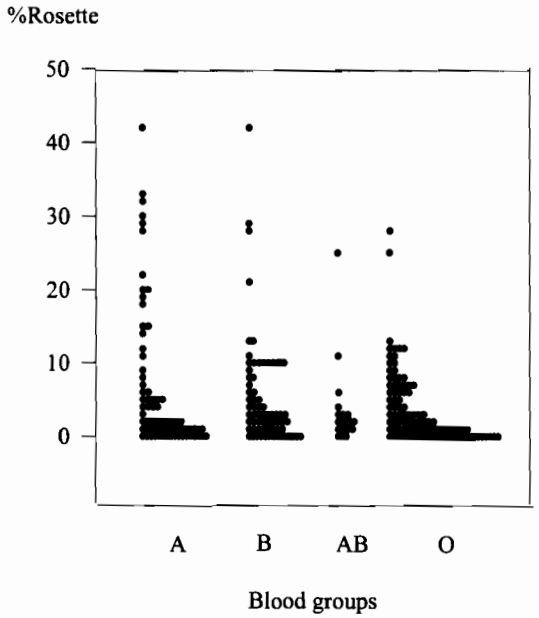

Fig 2–Rosetting capacity of infected erythrocytes from donors belonging to different ABO blood group.

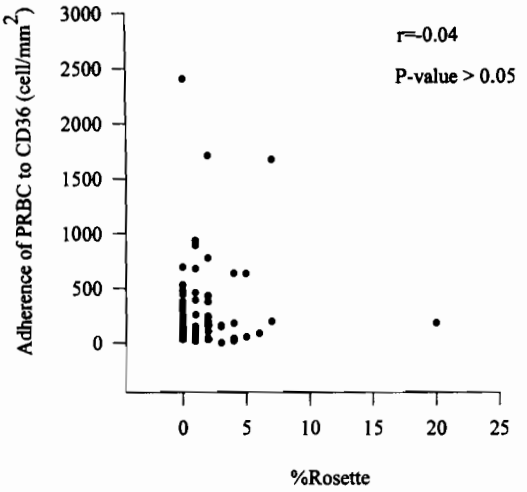

Fig 3–CD36 adherence and degree of rosette formation of 64 clinical isolates. Results show the mean number of parasitized cells bound per mm² (Y-axis) and mean percent rosetting rate (X-axis).

Of 262 isolates tested for rosetting, 64 were examined for adherence to purified CD36 and to C32 melanoma cells. Most isolates demonstrated relatively high binding to CD36 (mean adherence 326.4, 2-2408), and to C32 melanoma cells (mean adherence 522, 105-1178). Rosette formation of these isolates was compared to mean CD36 adherence. There was no correlation between rosette formation and adherence of infected erythrocytes to either CD36 (Fig 3) ($p > 0.05$, $r = -0.04$) or C32 melanoma cells ($p > 0.05$, $r = -0.05$). In addition, statistical analysis by Mann-Whitney one-way analysis of variance found no correlation between adherence of parasitized cells to CD36 and severity of infection ($p > 0.05$).

DISCUSSION

In the present study, we were unable to demonstrate any statistically significant correlation between the magnitude of rosette formation and disease severity, even though a higher geometric mean rosetting rate was associated with cerebral malaria. Our data are in agreement with those reported from Papua New Guinea (Al-Yaman *et al*, 1995), Pakistan (Iqbal *et al*, 1993) and Thailand (Ho *et al*, 1991). However, a high geometric mean rosetting rate has also been observed in CM cases in the Gambian study (Carlson *et al*, 1990; Treutiger *et al*, 1992). The mean rosette formation rates of our UM and SM isolates were much lower than those in the Gambian study but similar to those in the Papua New Guinean (PNG) study. The inability of some of our isolates to form rosettes was in agreement with most studies (Carlson *et al*, 1990; Ho *et al*, 1991; Treutiger *et al*, 1992; Iqbal *et al*, 1993) but differed from the data obtained in PNG where every isolate tested was able to form rosettes (Al-Yaman *et al*, 1995). Of note, in our study, was the fact that a high rosetting rate was associated with a high % parasitemia.

We failed to obtain a statistically significant association between rosetting and any particular donor blood group, although high geometric means tended to be associated with A and B blood group antigens. Our results differ from those of Carlson and Walgren (1992) and Udomsangpetch *et al* (1993) who proposed that parasitized red cells formed rosettes more readily with A or B donor blood groups. They also differ from those in the PNG study (Al-Yaman *et al*, 1995) which reported an association with the AB blood group antigen. On the other hand, no correlation between rosette formation and ABO blood group was reported from a study in Punjab, Pakistan (Iqbal *et al*, 1993). Hence, the significance of ABO blood group anti-

gens in rosetting remains controversial.

Unlike a previous, smaller study in Thailand (Ho *et al*, 1991), we did not find an inverse correlation between rosette formation and cytoadherence of infected erythrocytes. In that study, conclusions were based on the characteristics of infected erythrocyte adherence to C32 melanoma cells. It was subsequently shown that adherence to C32 melanoma cells can involve binding not only to CD36 but also to thrombospondin and chondroitin sulfate A (Chaiyaroj *et al*, 1994). Thus, adherence to melanoma cells cannot be interpreted as indicating adhesion to CD36. Indeed, our results from the use of purified adhesion molecules showed no association between the degree rosetting and level of infected erythrocyte binding to purified CD36. A similar lack of correlation has been reported in the other studies (Reeder *et al*, 1994; Iqbal *et al*, 1993). To confirm this, we attempted to disrupt rosette formation with the monoclonal antibody anti-CD36 (Howard and Gilladoga, 1989) and no disruption was observed (data not shown). Furthermore, a few trials on addition of 1 μg/ml of purified CD36 could not competitively disrupt rosette (Data not shown). Our results indicated that rosette formation and cytoadherence involve different molecular mechanisms. Obviously, other factors including cytokines, parasite toxins, nitric oxide production and host genetics are involved in disease pathogenesis and could markedly affect rosetting ability.

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REFERENCES

Al-Yaman F, Geuton B, Mokela D, *et al.* Human cerebral malaria: lack of significant association between erythrocyte rosetting and disease severity. *Trans R Soc Trop Med Hyg* 1995; 89 : 55-8.

Biggs B, Anders RF, Dillon HE, *et al.* Adherence of infected erythrocytes to venular endothelium selects for antigenic variants of *Plasmodium falciparum*. *J Immunol* 1992; 142 : 2047-54.

Carlson J, Helmby H, Hill AV, Brewster D, Greenwood BM, Wahlgren M. Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet* 1990; 336 : 1457-60.

Carlson J, Wahlgren M. *Plasmodium* erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J Exper Med* 1992; 176 : 1311-7.

Chaiyaroj SC, Coppel RL, Novakovic S, Brown GV. Multiple ligands for cytoadherence can be present simultaneously on the surface of *Plasmodium falciparum*-infected erythrocytes. *Proc Natl Acad Sci USA* 1994; 91 : 10805-8.

Handunnetti SM, Van Schrarendijk SM, Hasler T, Barnwell JW, Greenwalt DE, Howard RJ. Involvement of CD36 on erythrocytes as a rosetting receptor for *Plasmodium falciparum*-infected erythrocytes. *Blood* 1992; 80 : 2097-104.

Ho M, Davis TM, Silamut K, Bunnag D, White NJ. Rosette formation of *Plasmodium falciparum*-infected erythrocytes from patients with acute malaria. *Infect Immun* 1991; 59 : 2135-9.

Howard RJ, Gilladoga AD. Molecular studies related to the pathogenesis of cerebral malaria. *Blood* 1989; 74 : 2603-18.

Iqbal J, Perlmann P, Berzins K. Serological diversity of antigens expressed on the surface of erythrocytes infected with *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg* 1993; 87 : 583-8.

Reeder JC, Rogerson SJ, Al-Yaman F, *et al.* Diversity of agglutinating phenotype, cytoadherence, and rosette-forming characteristics of *Plasmodium falciparum* isolates from Papua New Guinean children. *Am J Trop Med Hyg* 1994; 51 : 45-55.

Ringwald P, Peyron F, Lepers JP, *et al.* Parasite virulence factors during falciparum malaria: rosetting, cytoadherence and modulation of cytoadherence by cytokines. *Infect Immun* 1993; 61 : 5198-204.

Rowe A, Obeiro J, Newbold CI, Marsh K. *Plasmodium falciparum* rosetting is associated with malaria severity in Kenya. *Infect Immun* 1995; 63 : 2323-6.

Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; 193 : 973-5.

Treutiger CJ, Hedlund T, Helmby H, *et al.* Rosette formation in *Plasmodium falciparum* and anti-rosette activity of sera from Gambians with cerebral or uncomplicated malaria. *Am J Trop Med Hyg* 1992; 46 : 503-10.

Udomsangpetch R, Todd J, Carlson J, Greenwood BM. The effects of hemoglobin genotype and ABO blood group on the formation of rosettes by *Plasmodium falciparum*-infected red blood cells. *Am J Trop Med Hyg* 1993; 48 : 149-53.

Wahlgren M, Fernandez V, Scholander C, Carlson J. Rosetting. *Parasitol Today* 1994; 10 : 73-9.

WHO. Severe and complicated malaria. *Trans R Soc Trop Med Hyg* 1990; 84 (suppl 2): 1-65.