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Rosetting of *Plasmodium falciparum* Required Multiple Components of the Uninfected Erythrocytes

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SUMMARY The mechanism of rosette formation of uninfected erythrocytes with *Plasmodium falciparum*-infected erythrocytes is rarely described. In this study, rosetting of uninfected normal erythrocytes with infected erythrocytes significantly reduced after treatment of the uninfected erythrocytes with neuraminidase. In contrast, the rosetting property of the infected erythrocytes was abolished by trypsinization but not by neuraminidase. The *in vitro* rosetting model showed that uninfected thalassemic erythrocytes poorly formed rosettes with infected normal erythrocytes when compared with normal erythrocytes of the same blood group. A rosetting parasite clone showed significant reduction in rosetting with thalassemic erythrocytes of all blood groups, however, this reduction was not obvious when the wild *P. falciparum* isolates were studied. These results suggest that while parasites from a single clone can rosette with uninfected erythrocytes via carbohydrate component, there is more than one type of receptor on uninfected erythrocytes involved in rosette formation with the heterogeneous populations of the wild *P. falciparum* isolates.

The *Plasmodium* species known to produce erythrocyte rosettes are *P. falciparum*,[1-2] *P. vivax*,[3] *P. ovale*,[4] and *P. malariae*[5] in human hosts; *P. coatneyi* in the rhesus monkey,[6] and *P. fragile* in the torque monkey.[7] Although erythrocyte rosettes are found in infections by many species of plasmodia, the feature is neither malignant nor benign. The formation of rosettes caused by some plasmodium parasites may be one of the mechanisms enhancing pathogenesis of malaria leading to severity and mortality. Infection by *P. falciparum*[8] or *P. coatneyi*[9] can be fatal due to the fact that these parasites can sequester, or cytoadhere in deep vasculatures of the infected hosts. Together with the ability to sequester, rosette formation by *P. falciparum* has been described as an additional virulence factor possibly aggravating pathophysiologic conditions in malaria afflicted hosts.[10-13] However, most studies have not elaborated on the mechanism of rosetting due to a lack of specific reagents for characterization of the molecules, either of host or of parasite origin, involved in the rosetting.

In this study we approached the study of the rosetting mechanism by inducing an alteration of the uninfected erythrocyte membrane, using various enzymes to deplete a single, or multiple, components of the membrane. Another approach was to use the inherited alteration of the membrane-bound proteins in thalassemic erythrocytes. These studies were done to elucidate the receptor on uninfected erythrocytes for the rosetting ligand of the *P. falcipa-*

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rum-infected erythrocytes, This information may allow us to intervene in part of the process of malaria pathogenesis.

MATERIALS AND METHODS

Parasite cultures

An erythrocyte-rosette forming *P. falciparum* clone RPA1 from the Palo Alto strain of Uganda (maintained for several years in *in vitro* culture),[1] and three wild parasite isolates from Thailand, TM267R (maintained for 2 years),[14] TM345R and TM347R (freshly isolated and maintained for only 1 month to prepare cryopreserved aliquots for the study) were maintained in culture at 37°C in 5% CO_2-atmosphere as previously.[15] The parasites were grown in blood group O-erythrocytes in RPMI-1640 medium (Seromed, Germany), pH 7.4, supplemented with 10% heat-inactivated human serum, 2 mM glutamine, 25 mM HEPES and 20 µg/ml gentamicin. The wild isolates were cultured *in vitro* for less than 6 months during which the study was completed.

Erythrocytes

Erythrocytes from 89 healthy volunteers, 22 α-thalassemia (HbH disease) and 30 β-thalassemia/HbE volunteers were collected in citrate dextrose solution. Hemoglobin genotype of all samples was confirmed by electrophoresis on cellulose acetate at the Faculty of Medicine, Chulalongkorn University, Thailand. A standard blood group typing was performed. The erythrocytes were then washed in RPMI-1640, the buffy coat removed, and the erythrocytes resuspended to 50% hematocrit in serum free RPMI-1640, pH 7.4 and stored at 4°C before use in the study within one week.

Selective enzyme treatment

Normal uninfected erythrocytes (100 µl of cell suspension in RPMI-1640) of blood group A, B or O were treated separately with various enzymes (Sigma Chemicals Co., St Louis, MO) at final concentrations as follows, neuraminidase (0.1 and 0.5 U/ml), trypsin (10 and 100 µg/ml), alpha chymotrypsin (1.8 U/ml), phospholipase A2 (5 U/ml). All samples were incubated at 37°C for 30 minutes, washed 3 times in RPMI-1640 medium containing 10% human AB serum, resuspended to 5% hematocrit in the same medium and used in the assay. Agglutination of these enzyme-treated erythrocytes was repeated using the same anti-A and anti-B antisera. The titers of agglutination were compared with those before enzyme treatment.

Staining of erythrocytes

Enzyme-treated or untreated erythrocytes from healthy and thalassemic groups were washed 3 times in serum free RPMI-1640 medium and resuspended to 5% hematocrit in the same medium. The erythrocytes were then stained with fluorescein isothiocyanate (FITC) isomer I (Sigma Chemicals Co., St Louis, MO)[16] at a final concentration of 50 µg/ml, at room temperature for 15 min. Excess dye was removed by washing 3 times in RPMI-1640 medium containing 10% human AB serum. The stained erythrocytes were resuspended to 5% hematocrit in the same medium and were stored in a dark chamber until use in the assay.

Enumeration of rosettes

A rosette was defined as an infected erythrocyte bound to two or more uninfected erythrocytes. Percentage of rosettes formed was determined by mounting one drop of the culture under a cover slip on a glass slide and examining by light microscopy.[14] The number of rosettes per 500 infected erythrocytes containing malarial pigments was counted and expressed as a percentage of rosette formation.

Assay for rosette formation

Rosette formation of *P. falciparum*-infected erythrocytes with the enzyme-treated or untreated erythrocytes was performed using the technique previously described.[17] Briefly, 200 µl of the parasite culture and of the stained erythrocytes at 5% hematocrit were mixed and pipetted vigorously for 15 seconds using a micropipette to disrupt the preformed rosettes in the culture. Immediately, a 20 µl aliquot was mounted with a cover slip on a glass slide and examined by light microscopy to confirm the complete disruption of all rosettes and determine the ratio of stained to unstained erythrocytes. The rest of the erythrocyte suspension was then centrifuged at 1,500 rpm for 5 minutes and incubated at 37°C for 15 minutes to allow re-formation of the rosettes by infected erythrocytes. The pellet was then resuspended gently and acridine orange was added at a final concentration of 0.5 µg/ml to identify parasites in the infected erythrocytes. One drop of the cell suspension was mounted with a cover slip on a glass slide and examined by UV-light microscopy using a 100 x objective. The number of stained and unstained

erythrocytes forming rosettes with infected erythrocytes was counted in 50 rosettes. The ratios of stained and unstained erythrocytes in the rosettes at the time before and after rosette re-formation were then compared and expressed as relative ratios.

Statistical analysis

Geometric means of the ratios obtained with each parasite and different erythrocyte populations were calculated. Analysis was performed using a non-parametric Mann-Whitney's U-test (Statgraphics software, STSC Inc.).

RESULTS

Rosette formation by *P. falciparum* clone and wild isolates

During observation, all parasites were grown *in vitro* in blood group O-erythrocytes to normalise the effects of blood group which might influence the rosetting process. Under these culture conditions a *P. falciparum* clone RPA1 and three wild isolates TM267R, TM345R and TM347R formed rosettes at different percentages, giving the rosetting range of 80-95%, 39-64%, 42-55% and 18-25%, respectively.

Rosette formation of all four *P. falciparum* parasites with erythrocytes of different blood group from healthy donors was studied. The mean relative values of rosette formation are summarized in Table 1. Erythrocytes infected with any parasites formed rosettes with blood group A- or B-erythrocytes better than with blood group O cells. These differences were significant with the clone RPA1 and two wild isolates, TM267R and TM347R but not with TM345R. O-erythrocytes used in maintaining the parasite cultures did not show agglutination with the normal nor with the thalassemic erythrocytes.

Effect of enzymes on the ability of uninfected normal erythrocytes to form rosettes

In this study uninfected erythrocytes were treated with one of the enzymes (Table 2) to define the nature of rosetting receptor on these erythrocytes. Antibody agglutination titer with neuraminidase-treated A- or B-erythrocytes was not reduced, giving a titer of 1:100 with anti-A or anti-B antisera, respectively. Similar results were obtained with trypsin-, α-chymotrypsin-, or phospholipase A2-treated erythrocytes.

Results of this experiment are summarized in Table 2. Of all the enzymes used, neuraminidase produced the greatest reduction in the rosetting receptor of the uninfected erythrocytes. The reduction of rosetting frequency by neuraminidase was dose-dependent, and significant with 0.5 U/ml of the enzyme with all groups of erythrocytes. In contrast, treating unin-

Table 1 Rosette formation of *P. falciparum*-infected erythrocytes with normal uninfected erythrocytes

Parasite clone[1]/ wild isolates	Erythrocytes		
	O	A	B
RPA1[1]	1.23 ± 0.53[2] (27)	2.33 ± 0.70[3] (31)	2.07 ± 0.62[3] (31)
TM267R	0.89 ± 0.24 (17)	2.29 ± 1.12[3] (18)	1.81 ± 0.59[3] (15)
TM345R	1.17 ± 0.55 (11)	1.81 ± 0.55 (13)	1.57 ± 1.02 (13)
TM347R	1.30 ± 0.57 (13)	3.25 ± 0.98[3] (13)	2.44 ± 0.91[3] (14)

[2]Mean ± SD of the relative value of rosette formation of *P. falciparum*-infected erythrocytes with normal uninfected erythrocytes. All parasites were grown in blood group O-erythrocytes. Numbers of samples in individual groups are shown in parentheses.

[3]Comparison of rosette formation with uninfected group A or B erythrocytes to group O, $p < 0.05$, using a non-parametric Mann-Whitney U-test.

Table 2 Effects of enzymes on uninfected erythrocyte ability to rosette with *P. falciparum*-infected erythrocytes

Enzymes	Concentration	Blood group		
		O	A	B
None		0.89 ± 0.24[1] (17)	2.29 ± 1.12 (18)	1.81 ± 0.59 (15)
Neuraminidase	0.1 U/ml	0.68 ± 0.26	1.86 ± 0.24	1.14 ± 0.28[2]
Neuraminidase	0.5 U/ml	0.36 ± 0.17[2]	1.48 ± 0.20[2]	1.08 ± 0.13[2]
Trypsin	10 μg/ml	1.02 ± 0.08	3.10 ± 1.14	2.32 ± 0.58
Trypsin	100 μg/ml	0.90 ± 0.16	2.68 ± 0.41	1.68 ± 0.35
α-Chymotrypsin	1.8 U/ml	0.92 ± 0.11	2.44 ± 0.53	2.08 ± 0.38
Phospholipase A2	5.0 U/ml	0.98 ± 0.14	2.40 ± 0.62	1.40 ± 0.34

Enzyme-treated or non-treated uninfected erythrocytes were used to rosette with a standard *P. falciparum*-infected erythrocytes (TM267R) grown in blood group O-erythrocytes.

[1]Mean ± SD of the relative values of rosette formation. Numbers of samples in each study are 5 unless otherwise shown in parentheses.

[2]Comparison of rosette formation of the corresponding blood group of non-treated to enzyme-treated erythrocytes, $p < 0.05$, as described in Table 1.

Table 3 Rosette formation of *P. falciparum*-infected erythrocytes with thalassemic erythrocytes

Parasite clone[1]/wild isolates	Blood group	Thalassemic erythrocyte	
		Beta/HbE	Alpha
RPA1[1]	O	0.68 ± 0.22[1,2] (12)	0.77 ± 0.19[2] (10)
	A	1.25 ± 0.07[2] (2)	1.67 ± 0.54[3] (6)
	B	1.07 ± 0.47[2] (16)	1.25 ± 0.65[3] (6)
TM267R	O	0.73 ± 0.46[3] (12)	0.60 ± 0.28[3] (10)
	A	1.00 ± 0[3] (2)	2.33 ± 2.01 (6)
	B	1.11 ± 0.55[2] (16)	0.98 ± 0.48[3] (6)
TM345R	O	1.56± 1.06 (12)	0.86 ± 0.62 (10)
	A	1.15 ± 1.49 (2)	1.84 ± 0.76 (6)
	B	2.62 ± 1.65 (16)	1.84 ± 1.94 (6)
TM347R	O	1.02 ± 0.50 (12)	0.79 ± 0.26[3] (10)
	A	3.10 ± 0.14 (2)	2.85 ± 0.98 (6)
	B	1.95 ± 0.52 (16)	1.28 ± 0.28[3] (6)

[1]Mean ± SD of the relative values of rosette formation of *P. falciparum*-infected erythrocytes with thalassemic erythrocytes. All parasites were grown in blood group O erythrocytes. Numbers of samples in each group are shown in parentheses. Comparison of rosette formation of the corresponding blood group of thalassemic to normal erythrocytes as described in Table 1, [2]$p < 0.001$, [3]$p < 0.05$.

fected erythrocytes with 100 μg/ml trypsin could not deplete the rosetting receptor of the erythrocytes. Other enzymes, α-chymotrypsin, and phospholipase A2 did not reduce the rosetting receptor of uninfected erythrocytes of any blood group. Treating infected erythrocytes with trypsin at concentration as low as 10 μg/ml, completely abolished the rosetting ability for all four *P. falciparum* isolates/clones studied (data not shown).

Rosette formation of thalassemic erythrocytes

The blood group of thalassemic erythrocytes was determined. Results of rosetting of these thalassemic erythrocytes with infected erythrocytes are summarized in Table 3. These rosetting frequencies were compared with the results for the non-thalassemic cells of the corresponding blood group in Table 1. Rosetting frequencies with *P. falciparum* clone RPA1 were significantly lower with all thalassemic erythrocytes studied compared to non-thalassemic cells. The reduction was greater ($p < 0.001$) in all blood groups of the β-thalassemia/HbE compared with the α-thalassemia. The different in rosetting of α-thalassemic versus normal erythrocytes was greater for blood group O ($p <0.001$) than for group A or B cells ($p <0.05$). On the other hand, rosetting frequency with the three wild parasite isolates with thalassemic erythrocytes was different. TM267R showed significant reduction in rosetting ($p <$ 0.05 or < 0.001) with almost all thalassemic erythrocytes except with blood group A. TM345R showed little or no rosetting reduction with all thalassemic erythrocytes. TM347R showed significant rosetting reduction only with blood group O or B of α-thalassemic erythrocytes.

DISCUSSION

Rosette formation of *P. falciparum*-infected erythrocytes was compared using clone parasite and three wild isolates. Levels of rosetting caused by these parasites were different during *in vitro* culture due to an intrinsic factor of each isolate as described previously.[14] A high rosette inducing property of the clone RPA1 parasite has been obtained by a repetitive selection technique.[1] This clone parasite, therefore, provides a useful model for studying the mechanism of rosette formation and comparing it with that of the wild parasite isolates. The rosette formation assay in this study determined the ability of erythrocytes of any blood group (A or B or O) to form rosettes with infected blood group O erythrocytes. The erythrocytes used in these assays were stained with fluorescein dye in order to provide differential enumeration of each erythrocyte bound in the rosettes.

Our observations were consistent with those reported previously,[17-18] namely, that the likelyhood of erythrocytes in forming rosettes with an infected erythrocyte *in vitro* was in the order of bloodgroup A > B > O. Blood group determinants of erythrocytes are thought to be involved in the mechanism of rosetting between uninfected and infected erythrocytes. The mechanism of rosetting was elucidated further using various enzymes known to act specifically at different sites of the outer membrane components of the erythrocytes. All three blood groups were used in the enzyme treatment experiment. Neuraminidase and trypsin did not affect the agglutination titer of blood group A or B erythrocytes assayed by the conventional polyclonal antisera to blood group antigens. This indicated that phenotypic determinant of the erythrocytes was not abolished by these enzymes. Our observations showed that a parasite-derived rosetting ligand on the infected erythrocytes was trypsin sensitive but the rosetting receptor on uninfected erythrocytes was sensitive to neuraminidase. The decrease in rosette formation was significant in all blood groups of normal erythrocytes pretreated with 0.5 U/ml neuraminidase but not with other enzymes. These results suggest that although the blood group A or B determinant involved in rosetting of infected erythrocytes, it is not the essential receptor of parasite rosetting. Blood group antigens may play an accessory role in strengthening the rosette formation of infected erythrocytes. Furthermore, it is interesting that trypsin abolished rosetting ability of infected erythrocytes but failed to do so to uninfected erythrocytes. That the significant decrease in rosetting was obtained only with neuraminidase, suggests that the rosetting receptor on uninfected erythrocytes is a sialylated protein on which sialic acid may interact with the parasite rosetting ligand. A recent study[19] has shown that complement-receptor 1 can interact with the *P. falciparum* erythrocyte membrane protein-1 to induce rosette formation. However, ~ 0.5 mg/ml of soluble complement-receptor 1 inhibits only 75% of rosette formation with several

rosetting parasites but does not show any effect with a rosetting parasite clone. This recent study supports our finding that the rosetting mechanism of the parasite requires multiple interactions between the putative ligand on infected erythrocytes and multiple receptors on uninfected erythrocytes, one of which is neuraminidase sensitive.

Further investigation using erythrocytes of α- or β-thalassemia to elaborate the mechanism of rosette formation showed a significant reduction in rosetting of RPA1 clone and TM267R isolate with erythrocytes in both α- and β-thalassemia populations. However, the reduction in rosetting with thalassemic erythrocytes was rarely observed when another two wild parasite isolates, TM345R and TM347R, were used in the rosette formation assay. This may be due to the difference in the type and quantity of rosetting ligand expressed on the infected erythrocytes, owing to the difference in duration of the culture *in vitro*. A previous study has shown that the level of sialic acid on the surface membrane of β-thalassemic erythrocytes is decreased by 25% compared with that in normal erythrocytes.[20] This characteristic of the thalassemic erythrocytes and the observations of rosetting ability of these erythrocytes fit with our results for neuraminidase-treated uninfected erythrocytes, and thus strengthen the evidence for involvement of sialic acid in the formation of rosettes. Whether the level of sialic acid differs between the α- and β-thalassemic erythrocytes is not known. Our previous study using hemoglobin AS- and SS-sickle erythrocytes in the rosette formation assay showed no difference in rosetting ability of these sickle cells compared with that of normal hemoglobin erythrocytes.[17] Whether or not this is due to similar sialic acid contents in both erythrocyte membranes has not been established.

These observations suggest that the mechanism of rosette formation of *P. falciparum*-infected erythrocytes involves multi receptors on uninfected erythrocytes, one of which may be sialic acid. In addition, the differences in rosetting potency of these parasite isolates and a parasite clone with thalassemic erythrocytes may be due to differences in the nature and type of the parasite rosetting ligands, as well as the quality of the receptor on the uninfected erythrocytes. Differences between the wild isolates in the expression of parasite-derived rosetting ligand on infected erythrocytes can not be excluded. The heterogeneity of the rosetting mechanism exhibited by the wild parasite isolates creates an obstacle to the prevention of rosetting by a single receptor-ligand blockade.

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