Natural Protection Against Severe Plasmodium falciparum Malaria Due to Impaired Rosette Formation

By Johan Carlson, Gerard B. Nash, Vilma Gabutti, Fadwa Al-Yaman, and Mats Wahlgren

Genes for two lethal diseases, thalassemia and sickle cell anemia, are favored by evolution because, in their heterozygous form, they protect against cerebral malaria. Rosette formation, the binding of uninfected red cells (RBCs) to Plasmodium falciparum-infected RBCs (PRBCs), has previously been found to be associated with cerebral malaria, the most important severe manifestation of P falciparum malaria. We show here that thalassemic RBCs and, under certain conditions, even hemoglobin S (HbS)-containing RBCs possess an impaired ability to bind to PRBCs, forming small and weak erythrocyte rosettes compared with rosettes formed by normal RBCs. This decreased rosetting ability is associated with the small size of the thalassemic RBCs and with distortion of the mechanical properties of HbS-containing RBCs. The impairment of rosette formation may hinder the development of cerebral malaria by abatement of sequestration.

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EXCESSIVE BINDING of Plasmodium falciparum-infected erythrocytes [parasitized red blood cells (PRBCs)] to endothelial cells and to uninfected red blood cells (RBCs) seems crucial for the occurrence of microcirculatory obstruction in human cerebral malaria, but the underlying processes that lead to this state of infection are complex. One attractive approach to the understanding of the pathophysiologic mechanism is the study of the red cell defects known to protect against severe malaria. It has been shown that individuals with α- and β-thalassemia and with sickle cell trait (HbAS) can acquire P falciparum malaria, but that they experience reduced fatality and severity of the disease; eg, the protection against cerebral malaria in children with HbAS is more than 90%, but the effect on parasite densities is less pronounced. Blood group O was recently found also to be associated with significant resistance to cerebral malaria as compared with blood group A or B (Hill and Hill et al, personal communication, 1992). It has been suggested that the hampered growth and an increased tendency to sickling of HbAS-infected cells and neoantigen expression on thalassemic RBCs are the reasons for the protective effects. However, while these mechanisms may play roles in the resistance to malaria, they do not fully explain the absence of microcirculatory obstruction seen in individuals with aberrant red cells. Could it be that these RBCs are less prone to forming rosettes and to binding to endothelial cells and, therefore, hinder the development to coma and death?

Increased frequency of spontaneous erythrocyte rosetting around PRBCs has been shown to be associated with cerebral malaria. At autopsy, Hidayat and others observed "rosetting of the parasitized erythrocytes . . . within the partially occluded lumens," and in ex vivo experiments, the obstruction of the blood flow was found to be considerably more pronounced with a rosette-forming parasite than with a nonrosetting parasite that merely bound to the vascular endothelium. Moreover, anti-rosetting antibodies were frequent in sera of children with mild malaria but absent or only present at low levels in those with severe disease. Thus, rosetting has been proposed to play a key role in the excessive sequestration of PRBCs and RBCs in the microvasculature and in the pathogenesis of cerebral disease. Therefore, it could be postulated that the protective effect of certain red cell disorders against cerebral malaria is mediated via impaired rosette formation. We investigated a number of red cell disorders with respect to their impact on rosetting and report that RBCs from individuals naturally protected against cerebral malaria exhibit an impaired rosette-forming ability, with data suggesting that this is a mechanism that could mediate natural protection against the disease.

MATERIALS AND METHODS

P falciparum culture. The P falciparum R7PAI parasite, a cloned, rosetting parasite obtained from the Palo Alto Ugandan strain, was cultured according to standard procedures with 10% normal AB+ Rh+ serum added to the buffered malaria culture medium (MCM).

Erythrocytes. Blood was drawn into heparinized tubes or tubes containing citrate phosphate dextrose (CPD), and the RBCs were washed three times in TRIS-Hanks' solution. ABO blood typing was performed by hemagglutination with monoclonal antibodies specific for the different blood group antigens (BioCarb, Lund, Sweden). RBC morphology was evaluated by light microscopy, and the erythrocyte mean cell volume (MCV) and hemoglobin concentration were measured by a Coulter S Plus (Coulter Electronics Ltd, Luton, UK). Hemoglobin electrophoresis and electrofocusing were used to quantify the hemoglobin type of the RBCs. RBCs were obtained from four individuals with sickle cell disease (HbSS), from three that were of the sickle cell trait (HbAS), and from one exhibiting the hemoglobin SC (HbSC) phenotype. Samples from nine Papua New Guineans with Southeast (SE) Asian ovalocytosis contained 11.8% to 89.2% ovalocytic cells as counted in 500 RBCs with a 40X objective lens. Of the 19 individuals with β-thalassemia

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trait, 14 were Italians or West Africans with \( \beta^+ \), one was a Thai with the \( \beta^+/\beta^- \) thalassemia type, and three were West Africans with combined \( \beta^+ \)/HbS. All the \( \beta^- \)-thalassemic RBCs had a low MCV. Three of four individuals with \( \alpha^- \)-thalassemia trait were of the \( \alpha^- \)-thal 1 phenotype, while one (Thai) was of the Hb Constant Spring phenotype. All of these RBC samples exhibited a moderate microcytosis. RBCs were also obtained from three patients with microcytic anemia (secondary to chronic iron deficiency or severe bacterial infection), and from six samples of cord blood with high HbF contents (67% to 85% HbF).

Assessment of rosette formation and disruption of rosettes. The R'PAI strain grown in O' RBCs was subcultivated in blood from donors with normal (HbAA), \( \beta^- \)-thalassemia trait, or HbAS RBCs, and assessment for rosette formation was made as described previously. The rosetting rate was defined as the number of PRBCs in rosettes, expressed as a percentage of the total number of late-stage (trophozoite and schizont) PRBCs. The individual rosette size was defined as the mean number of uninfected RBCs bound to each PRBC. Rosettes in culture were disrupted mechanically by drawing the erythrocyte mixture through a narrow gauge injection needle six to eight times. Assessment of rosette formation was made after spontaneous reformation of rosettes, and different aliquots from the same culture were compared after different pretreatments.

Carboxy-fluorescein diacetate (C-FDA)-labeling of RBCs and assessment of rosette-forming capacity. The relative rosette-forming capacity of RBCs from patients with various red cell disorders was measured as described before, by a method where C-FDA-labeled RBCs were allowed to compete for rosetting with unlabelled RBCs. The R'PAI strain was cultured in blood group O (HbAA) RBCs, and rosettes were disrupted by addition of heparin (50 IU/mL; Kabi Pharmacia AB, Stockholm, Sweden).

Fractionation and deoxygenation of HbS-containing RBCs. HbSS or HbSC RBCs were fractionated on a Ficoll-Isopaque continuous density gradient (Pharmacia AB, Uppsala, Sweden), and the less dense cells were harvested as described. Cyclical deoxygenation-reoxygenation was performed for 15 hours with a Cyclical Gas Exchanger (Wolfson Research Laboratories, Birmingham, UK), causing changes in cell hydration and deformability that mimic the deterioration that occurs for dense cells in vivo. In separate experiments, HbAS, HbS, or HbSC RBCs were deoxygenated by adding a mixture of sodium dithionite and disodium hydrogen phosphate (2 vol 0.114 mol/L Na$_2$S$_2$O$_4$ + 3 vol 0.114 mol/L Na$_2$HPO$_4$; pH 6.8) to the erythrocyte suspension in proportion 1:3.12

The morphology of the RBCs was studied before and after cyclical deoxygenation-reoxygenation or treatment with dithionite, after fixation with 1% glutaraldehyde. HbAS RBCs and less dense HbSS RBCs were essentially discyctic, except that the latter contained a few distorted, boat-shaped cells (irreversibly sickled cells, ISC). Treatment with dithionite led to the formation of distorted cells and of ISC, and few discocytes remained (about 30% for HbAS cells and less for HbSS cells). Cyclical deoxygenation of the less dense HbSS RBCs also led to formation of distorted RBCs, as well as some ISCs (8% on average). A distinction should be made, however, between dithionite-treated cells, which are distorted by the formation of polymerized HbS, and cells subject to cyclical deoxygenation-reoxygenation, which dehydrate and accumulate membrane damage but do not contain polymer when reoxygenated for further study. No changes were seen in HbAA RBCs treated with either of the above-mentioned methods.

Measurement of erythrocyte binding strength by a micropipette method. Samples of malaria cultures were diluted 100-fold in MCM, and the suspension was placed in a micropipette chamber where the binding strength of individual RBCs within rosettes was studied by a dual-micropipette method (Fig 1), as described previously. The aspiration pressure (P) required to detach cells from rosettes by a micropipette (internal diameter, D) was recorded, and the force was calculated as $F = (\pi/4)(D^2)P$. In some experiments, after all the cells had been stripped from a rosetting, parasitized cell, other cells in the chamber were picked up with one of the pipettes and brought into contact with the striped parasitized cell, allowing cell-cell binding to occur. After a delay of 3 minutes, the force to detach the nonparasitized cell was measured, as described.

RESULTS

RBCs from individuals with various red cell disorders were examined for their capacity to form rosettes in competition with normal (HbAA) O, Rh+ RBCs. Whereas cord RBCs with a high HbF content or RBCs from individuals with SE Asian ovalocytosis bound equally well or only slightly less as compared with normal RBCs, thalassemic RBCs and other microcytic RBCs exhibited a reduced rosette-forming capacity, as did HbS-containing RBCs under deoxygenated conditions (Fig 2).

Thus, both \( \alpha^- \) and \( \beta^- \)-thalassemic RBCs exhibited a reduced rosette-forming capacity when competing with RBCs from normal (HbAA) individuals (Fig 2). When RBCs from four individuals with \( \beta^- \)-thalassemia trait were compared with RBCs from five normal (HbAA) individuals, a significant
reduction in mean binding strength was found for the smaller \( \beta \)-thalassemic RBCs (Fig 3). These studies also confirmed the previously reported finding of a strain-specific ABO blood group preference in rosette-forming capacity. A higher binding capacity and larger rosettes were found for the \( \beta \)-PA1 clone with blood group A/AB RBCs compared with group O/B RBCs,\(^\text{24}\) and similarly, when using the same \( P \)

\[
\begin{array}{c|c|c|c}
\text{HbAA} & \text{HbF} & \text{Ovalocytosis} & \text{HbAS} \\
\text{HbSS} & \text{HbSC} & \text{HbEF} & \text{\( \alpha \)-thal minor} \\
\text{\( \beta \)-thal minor} & \text{HbAA microcytosis} & \\
\hline
\end{array}
\]

**Fig 2.** Relative rosetting capacity of various C-FDA-labeled RBCs to HbAA infected RBCs. The tested RBCs were from nine individuals of normal hemoglobin phenotype (normo- or microcytic) or from 35 individuals with various red cell disorders (CS, Hb Constant Spring; S/\( \beta \), mixed HbS/\( \beta \)-thal trait). ■, RBCs under normal ambient \( O_2 \); □, RBCs treated by cyclical deoxygenation/reoxygenation; △, RBCs deoxygenated by sodium dithionite. The binding of C-FDA-labeled RBCs from an \( O \) Rh+ donor was used as index (100%), and the blood group A/AB preference of the strain was compensated for as described previously.\(^\text{24}\)

**Fig 3.** Force required to detach individual uninfected RBCs bound in a spontaneous rosette around \( P \) falciparum-infected RBCs. Values given are mean values ± SD. The binding strength was compared by measuring 50 to 68 RBCs from every patient. The \( \beta \)-thalassemia trait was \( \beta \)-type 1. ABO blood group of donors: △, A; ○, B; □, O. Statistical analysis was performed between the mean detachment force of the two groups of patients using the nonparametric Mann-Whitney \( U \) test \((P = .012)\).
with microcytic anemia secondary to iron deficiency/chronic bacterial infection were compared with ABO-matched controls (binding force: mean, $1.4 \times 10^{-10}$ and $1.6 \times 10^{-10}$ N, respectively, for the microcytic RBC populations; $2.2 \times 10^{-10}$ and $2.5 \times 10^{-10}$ N for the normocytic).

HbSS or HbAS RBCs bound normally to P falciparum-infected RBCs when competing with HbAA RBCs under normal oxygen pressure, but after exposure of HbSS cells to cyclical deoxygenation-reoxygenation or when deoxygenated with dithionite before the assay, the binding capacity was reduced (Fig 2). When P falciparum (clone R+PA1) was cultured in RBCs from two individuals with sickle cell trait (HbAS) and one with sickle cell disease (HbSS), no significant difference was seen in the rosetting rate, the mean individual rosette size, or the parasitemia compared with cultures with HbAA RBCs of corresponding ABO blood group (data not shown). However, chemical deoxygenation of the blood, followed by mechanical disruption and spontaneous reformation of rosettes, induced a significant drop in mean individual rosette size in the HbAS and HbSS cultures compared with untreated HbAS/SS controls (Table 1). By using the micropipette technique, it could also be shown that HbSS discocytes had a mean binding force not differing from that of discocytes from a normal (HbAA) individual (Fig 5). After cyclical deoxygenation-reoxygenation of the HbSS RBCs, significantly impaired binding capacity was found in the ISCs and also in the moderately distorted, dehydrated RBC population (Fig 5).

### DISCUSSION

Using three independent assays we report that certain aberrant RBCs form small and weak erythrocyte rosettes and hypothesize that this reduced ability to form erythrocyte rosettes may protect against severe P falciparum malaria, i.e., by preventing the formation of large stable red cell aggregates and obstructing the cerebral microvasculature otherwise seen in cerebral malaria.

Low cell volume, a common denominator of the α- and β-thalassemias, is associated with a reduced rosette-forming capacity. The statistical analysis was performed with simple regression analysis: $r = 0.95; r^2 = 0.91; P < .0001$. (B) MCV versus mean individual rosette size. Statistical analysis was performed with multiple regression analysis, taking into account that, when using the R+PA1 parasite, RBCs of the A/AB phenotypes form larger rosettes than O/B phenotypes, independent of other parameters: $r = .90; r^2 = .81; P < .001$.

### Table 1. Mean Individual Rosette Size Before Disruption and After Spontaneous Reformation of Rosettes in P falciparum Cultures Grown in HbAS and HbSS RBCs

<table>
<thead>
<tr>
<th>Donor Initials/Phenotype</th>
<th>Before Disruption</th>
<th>After Reformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA/HbAS Deoxy</td>
<td>3.3 ± 0.8</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>3.6 ± 0.7</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>HB/HbAS Deoxy</td>
<td>4.2 ± 1.1</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>Control</td>
<td>4.5 ± 1.2</td>
<td>4.2 ± 1.1</td>
</tr>
<tr>
<td>NS/HbSS Deoxy</td>
<td>4.2 ± 1.2</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>Control</td>
<td>4.2 ± 1.2</td>
<td>4.0 ± 0.9</td>
</tr>
</tbody>
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The P falciparum clone R+PA1 was cultured in untreated RBCs and RBCs deoxygenated by dithionite (see Materials and Methods) from three different donors. The cultures were assessed for mean individual rosette size (the number of uninfected RBCs bound to parasitized RBCs ± SD) by counting 50 rosettes, and each determination was performed twice. Statistical analysis was performed between the individual rosette sizes before and after reformation using the non-parametric Mann-Whitney U test.

Abbreviation: NS, not significant.
IMPAIRED ROSETTE FORMATION IN MALARIA

Fig 5. Force required to remove individual uninfected RBCs attached to a rosetting P falciparum-parasitized cell for 3 minutes. Values given are mean attachment force (± SD) as measured by micromanipulation of 28 to 36 uninfected RBCs per assessed cell population. Cylindrical deoxygenation/reoxygenation was performed with a Cylindrical Gas Exchanger (see Materials and Methods). Donors (initials and hemoglobin phenotype): □, BC/HbAA; ■, MS/HbAA; ○, BT/HbSS; ●, NS/HbSS; ●, CS/HbSS. Statistical analysis was performed with Student's t test for each HbSS donor between the discocytes and the distorted RBC population and between the discocyte and the ISC populations, respectively. All differences in mean detachment forces between the various RBC populations were statistically significant (all P values < .001).

Rosettes. Other investigators have studied features of microcytic cells that might protect against malaria, such as restriction of parasite growth or impairment of cytoadherence of infected cells, but have not found convincing correlations.16,39 Restricted rosette formation may be a common protective mechanism for microcytic RBCs, thus conferring significant clinical protection against cerebral malaria in disorders with profound microcytosis like the thalassemias. This hypothesis is supported also by the recent report by other investigators of impaired rosette formation of thalassemic RBCs.31 The possible protective effect of impaired rosette formation in other disorders might be more difficult to assess (e.g., iron deficiency, with a mixed pattern of microcytosis) although malnutrition—and especially iron deficiency—has in fact been claimed to confer protection against malaria. However, this issue is highly controversial, and polar views have been adopted.32,34

A diminished rosette-forming capacity was evident already for deoxygenated, slightly distorted HbAS, HbSC, or HbSS cells. In addition, after repeated sickling of HbSc or HbSS cells, rosetting was inhibited even when the cells were oxygenated. Thus, already moderate changes in cellular mechanical properties, known to occur not only with HbSS RBCs35 but also with both infected and uninfected HbAS RBCs under unfavorable conditions,36 cause a significant loss of rosette-binding capacity. The obstruction of the microvasculature and the concomitant lower oxygen tension seen in cerebral malaria seems likely to be such an unfavorable or even extreme condition capable of inducing changes in the rheologic properties of a significant number of HbAS cells. Acknowledging the well-known difficulties in extrapolating in vitro data to in vivo conditions, we nevertheless suggest that the impaired rosette-forming ability expressed even in moderately changed HbAS RBCs, in conjunction with other potential protective phenomena,13,13 may contribute to the protection against cerebral malaria. Paradoxically, this potential mechanism of resistance, as well as others previously described,14,37 seems to involve all HbS-containing RBCs, despite the fact that individuals with homozygous sickle cell disease often suffer more severely from malarial disease with high mortality. However, this fact may most likely be attributed to the sickle cell disease per se despite an innate resistance to malarial infection existing also in homozygotes.38,39

Rosette formation is mediated by protein ligands, rosettes, on the infected RBCs that bind to carbohydrate receptors on the uninfected RBCs.27,24,60 Whether the decreased rosette binding with microcytotic RBCs is due to a lower availability and/or a lower expression of the carbohydrate structures remains to be investigated. For HbSS and HbAS RBCs, on the other hand, it is not lack of receptors but the accessibility of them, influenced by the distortion or rigidification of the cells, that abates binding, as the rosetting capacity is dependent on the ambient oxygen pressure.

Some studies also give support for a modifying role of SE Asian and Melanesian ovalocytosis and HbF in P falciparum malaria.41,42 However, the protection conferred by SE Asian ovalocytosis or a high HbF content does not seem to involve erythrocyte rosetting.

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