Alteration in Cytoadherence and Rosetting of *Plasmodium falciparum*-Infected Thalassemic Red Blood Cells

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Hemoglobinopathies have a protective role in malaria that appears to be related to alterations in red blood cell (RBC) properties. Thalassemic RBCs infected with *Plasmodium falciparum* showed greatly reduced cytoadherence and rosetting properties as well as impaired growth and multiplication. A significant decrease in the levels of falciparum antigens associated with the membrane of infected β-thalassemic RBCs was observed at trophozoite/schizont stage, but not young ring stage. This reduction was shown when a cytoadherence inhibitory monoclonal antibody, but not a noninhibitory pooled immune serum, was used. These observations suggest that protection against malaria in thalassemia is caused by both reduced parasitemia and altered adherence properties of the infected thalassemic RBCs that promote enhanced clearance of the parasite from the circulation.

*P falciparum* is the most pathogenic of human malaria parasites. After the bite of an infected anopheline mosquito, the parasite first develops in the liver and then invades the red blood cells (RBCs) where it undergoes both asexual (schizogony) and sexual (gametogony) multiplication.1 During schizogony the intraerythrocytic parasite has three characteristic stages of maturation: ring, trophozoite, and schizont. Clinical manifestations in malaria are associated with the blood phase of infection during which RBCs are progressively destroyed as the parasite develops through successive schizogonic cycles.

Genetic defects in human populations are associated with RBC variants that have different phenotypic characteristics. These genetic defects often involve altered membrane or hemoglobin (Hb) of RBCs that have been shown to interfere with invasion, maturation, and multiplication of *P. falciparum*.2,3 There are several types of abnormal Hbs that appear to interfere with the development of *P. falciparum*.3 In vitro studies have provided evidence for the impairment of *P. falciparum* multiplication in RBCs containing HbCC4 and HbEE.5 Decreased rates of parasite invasion and retardation of maturation have been shown in HbS and sickled RBCs under in vitro culture conditions with low oxygen atmosphere.6,7 Culture of *P. falciparum* in RBCs from β-thalassemia with various levels of Hbf (up to almost 100% in RBCs from β*/β*-thalassemia heterozygotes8 and in α-thalassemia with HbH disease9 showed that maturation and multiplication of *P. falciparum* were retarded. These in vitro data are consistent with epidemiological studies in different geographical endemic areas which show that thalassemia can confer protection against falciparum malaria.10,11 In northern Liberia, prevalence of falciparum malaria in individuals with β-thalassemia trait is similar to that found in normal individuals, but parasite densities and gametocyte rates are lower in the β-thalassemia group.12 Studies in Gambia have shown an absence of α-thalassemia cases in groups of children with falciparum malaria. In addition, only a small number of adults with α-thalassemia had histories of clinical malaria.13 Taken together, these observations suggest that the impaired maturation and multiplication of *P. falciparum* in the thalassemic host may be associated with alterations in other biologic properties of the infected thalassemic RBCs involved in the pathogenesis of severe disease.

In this study we show what appears to be happening in thalassemic RBCs; *P. falciparum* invades, but then undergoes an impaired development as evidenced by poor cytoplasmic characteristics, and in some cases poor DNA synthesis. This probably results in the decreased expression of specific surface protein, which is important for cytoadherence and rosetting of the infected RBC.

**MATERIALS AND METHODS**

*P. falciparum* culture. A *P. falciparum* strain TM267R from Thailand was maintained in normal group 0 RBCs. This parasite has consistently formed rosettes with uninfected RBCs and cytoadhered to live, unfixed human umbilical-vein endothelial cells since it was first isolated without being selected for rosetting or cytoadherence.14 TM267R has been maintained as previously described15 for less than 1 year at 37°C, 5% CO2-atmosphere in RPMI-1640 medium supplemented with 10% heat-inactivated human AB blood, 2 mmol/L glutamine, 25 mmol/L HEPES, and 20 μg/mL gentamicin. Parasites synchronised by sorbitol treatment were used for initiating cultures with thalassemic RBCs. Antigens. Pooled human immune serum from malaria endemic areas in Thailand was absorbed twice at 37°C for 30 minutes with human group AB RBCs at 50% cell suspension. After absorption, the pooled serum did not agglutinate RBCs of any blood group. A human monoclonal IgM antibody (MoAb 33G2) to *P. falciparum* antigen was obtained from culture supernatant of a
MoAb has been shown to bind to \textit{P. falciparum}-infected RBCs and block cytoadherence of the infected RBCs to C32 melanoma cells.\textsuperscript{16} Thalassemic RBC. Two milliliters of blood was collected after informed consent from thalassemia volunteers followed at the Haematology unit, Chulalongkorn Hospital, Bangkok, and pack cell volume of the samples was determined. Twenty heterozygous \(\alpha\) and 24 heterozygous \(\beta\)-thalassemic RBC preparations were obtained and maintained in citrate dextrose solution and used within 1 week. Hb genotype of RBCs was determined by electrophoresis on cellulose acetate.

Multiplication and intraerythrocytic development of the parasites. A synchronous culture of TM267R at 20\% to 25\% trophozoite/schizont (mature) stages in normal group O RBCs at 3\% hematocrit was used for initiating cultures in thalassemic RBC. Twenty-five microliters of TM267R culture was added to 1.5 mL of normal group O or thalassemic RBC suspensions at 3\% hematocrit in plastic Petri dishes. Paired smears were made from each culture at the initiation and 24 hours later. Smears from cultures were done again in the second schizogonic cycle. Parasitemias were determined for each pair of smears. The relative increase in parasitemia in the cultures with thalassemic RBCs was expressed as a percentage of multiplication in the culture with normal group O RBC. Parasite morphology, number of nuclei in segmented schizonts, and number of new ring-infected RBCs in subsequent cycles were also determined from blood smears.

Development of parasites from ring to mature stages was assessed by flow cytometry (FACSCAN; Becton Dickinson, Sunnyvale, CA).\textsuperscript{18} Cultures at ring stage and at mature stages in thalassemic or normal group O RBCs were fixed with 0.025\% glutaraldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature, washed twice in PBS, and resuspended in 1\% bovine serum albumin (BSA) in PBS. The fixed cultures were stained with 10 \(\mu\)g/mL propidium iodide for 1 hour at room temperature and examined by flow cytometry.

In vitro cytoadherence assay. \textit{P. falciparum} cultured in thalassemic or normal group O RBCs at 7\% to 10\% parasitemia and 1\% hematocrit were used in cytoadherence assays with live, unfixed human umbilical-vein endothelial cells isolated as described previously.\textsuperscript{18} Suspensions of endothelial cells at 10\(^5\) cells/mL were grown on gelatin precoated plastic Petri dishes for 16 to 18 hours, and then cultured in 1\% saponin solution (to solubilize phospholipid in the RBC membrane) for 15 minutes at room temperature, washed, and resuspended in PBS containing 0.1\% saponin as described previously.\textsuperscript{22} Each fraction of fixed cells was incubated with pooled immune serum (final dilution 1:4) or with MoAb 33G2 (final dilution 1:2) for 30 minutes at room temperature. The cells were then washed and stained for 30 minutes with goat antihuman Ig conjugated with FITC. After washing, the cells were counterstained with propidium iodide as described above and examined by flow cytometry using two-color analysis. The mean fluorescence intensity measured for infected thalassemic RBCs was compared with that of normal infected RBCs and expressed as the mean relative (thalassemic/normal) fluorescence intensity for each thalassemia type.

Statistical analyses. A nonparametric Mann-Whitney U-test and regression correlation analysis were performed using Statgraphics software, STSc Inc.

RESULTS

Hb genotype. Study in electrophoresis showed that the Hb genotype of 20 RBC donors in \(\beta\)-thalassemia group was \(\beta^{\text{thal}}/\text{HbE}\) and that of the other four donors was \(\beta^{\text{thal}}/\text{HbE}\). Eleven RBC donors in \(\alpha\)-thalassemia group had a genotype of \(\alpha\text{thal}^{+}/\text{HbCS}\) (Constant Spring) and that of the other 9 donors was \(\alpha\text{thal}^-/\alpha\text{thal}^-\), either with (3 cases) or without (6 cases) HbE. Mean values of pack cell volume \(\pm\) SD of the \(\beta\)- and the \(\alpha\)-thalassemia group were 22.5\% \pm 3.6\% and 28.5\% \pm 5.2\%, respectively.

Multiplication and maturation of parasites in thalassemic RBCs. \(\alpha\)-Thalassemic and \(\beta\)-thalassemic RBCs were infected in vitro with the Thai \textit{P. falciparum} strain TM267R. We measured multiplication of TM267R cultured in thalassemic RBCs by determining parasitemia from blood smears at initiation of the culture when the parasites were at mature stage, and 24 hours later, after one schizogonic cycle. The parasitemias obtained from at least two schizogonic cycles were measured and regression correlation analysis were performed using Statgraphics software, STSc Inc.
RBCs were compared with that of normal RBCs, a significantly (P < .001) lower rate of multiplication was observed in all cultures (Table 1). Multiplication of parasites cultured in normal RBCs was increased twofold to fourfold after one schizogenic cycle. RBCs of heterozygous α-thalassemia yielded a multiplication rate that was 80.6% of control. RBCs from heterozygous β-thalassemia showed even lower level of multiplication: 69.8% of control.

Examination of blood smears showed that in both schizogenic cycles, the number of new ring-infected RBCs in heterozygous α- and β-thalassemic RBCs was two to three times less than that observed in cultures with normal RBCs (data not shown). Furthermore, morphology of trophozoites in thalassemic RBCs showed abnormal development (moribund trophozoites), a characteristic that was more prominent in the second schizogenic cycle. The number of nuclei in most of the segmented schizonts in thalassemic RBCs were similar to that found in normal RBCs. However, the cytoplasmic content of parasites was greatly reduced.

We next examined the relationship between variant Hb levels and multiplication of parasites in these RBCs. Using a regression correlation analysis, we found an inverse correlation between the HbF level and multiplication of parasites in the β-thalassemia. RBCs containing higher HbF levels yielded a lower multiplication rate than RBCs that had lower HbF levels (Fig 1A). In the α-thalassemia group, an association between the level of HbH and parasite multiplication was not found. These RBCs yielded various amounts of parasitemia which were independent of the level of HbH. However, multiplication in α-thalassemic RBCs appeared to separate into two groups, one group with higher parasitemia than the other, irrespective of the HbH content (Fig 1B). Results from Hb genotyping showed that the genotype of 9 of the 12 RBC donors providing high parasitemia was αthal1/βCS, and the genotype of the other 3 was αthal1/αthal2 (with or without HbE). Hb genotype of 6 of the RBC donors giving low parasitemia was αthal1/αthal2 (with or without HbE) and the other 2, αthal1/βCS.

Impairment of *P. falciparum* maturation in thalassemic RBCs was also assessed by staining parasite DNA with propidium iodide and examining by flow cytometry. The amount of dye taken up by parasites is proportional to the DNA content. There was a sixfold increase in DNA content as the parasites matured from ring (Fig 2A) to trophozoite/schizont stage (Fig 2B) in normal RBCs in vitro. Most parasites cultured in α- or β-thalassemic RBCs gave a maturation rate similar to that of parasites in normal RBCs as described above (Fig 2, C and D). However, of the total 28 cases, parasites infecting four heterozygous β-thalassemic RBC cultures showed a retarded maturation of the ring stage parasites (Fig 2E) resulting in a marked decrease in the number of trophozoite/schizont-infected RBCs and a lower mean fluorescence intensity of propidium iodide (only a twofold increase in DNA content) than that of the mature stage in normal RBCs (Fig 2F).
Fig 2. Two-color flow cytometric analysis of *P. falciparum* maturation in cultures of normal or thalassemic RBC. *P. falciparum* cultured in normal RBCs: (A) 12% ring stage (R), mean fluorescence intensity (MFI) of propidium iodide (PI) = 50; and 24 hours later, (B) 12% trophozoite/schizont (T/S); MFI of PI = 318; and in a case of heterozygous α-thalassemic RBCs that showed normal growth, (C) 11% R, MFI of PI = 58; and 24 hours later, (D) 13% T/S, MFI of PI = 350; and in a case that showed abnormal growth, (E) 7.4% R, MFI of PI = 43; and 24 hours later, (F) 4% T/S, MFI of PI = 100, U = uninfected RBCs.

Cytoadherence and rosette formation of infected thalassemic RBCs. To observe whether adherence properties of *P. falciparum* grown in thalassemic RBCs will be different from that grown in normal RBCs, cytoadherence and rosetting of these infected RBCs were compared. Cytoadherence was between 400 and 700 normal infected RBCs/100 live, unfixed endothelial cells and rosette formation was between 25% and 40% of mature infected RBCs. Cytoadherence of both infected heterozygous α- and β-thalassemic RBCs was significantly (*P* < .001) reduced when compared with that of infected normal RBCs. Cytoadherence of infected heterozygous α-thalassemic RBCs (39.5% of control binding) was slightly lower than that of infected RBCs of heterozygous β type (45.3% of control binding) (Table 1). Similarly, rosetting of the infected thalassemic RBCs was also reduced significantly in both types of heterozygous thalassemic RBCs (*P* < .001) when compared with control cultures (Table 1). Heterozygous α- and heterozygous β-thalassemic RBCs yielded similar numbers for rosette formation (42.4% and 39.2% of control, respectively).

Reactivity of MoAb 33G2 with TM267R. To reveal the reactivity of MoAb 33G2 with infected RBCs, the MoAb was tested with unfixed TM267R in immunofluorescence assay and showed a specific staining of the pigment containing infected RBCs (Fig 3). The fluorescence staining was not observed after treating the infected RBCs with ≥50 μg/mL trypsin, confirming that the antigen was exposed on the surface of the RBCs. Reactivity of the MoAb with infected RBCs was elaborated further in an in vitro cytoadherence inhibition assay. The result showed that MoAb 33G2 also inhibited cytoadherence of TM267R giving 41% and 30% inhibition at 1:2 and 1:4 dilution, respectively.

Antigen expression in the membrane of infected thalassemic RBCs. To investigate whether antigen expression of *P. falciparum* can be altered by thalassemic RBCs we determined the level of antigens associated with the membrane of
ring- and mature-infected RBCs by using pooled immune serum and a *P. falciparum* specific MoAb 33G2. Table 2 shows the mean relative fluorescence intensity of antibody binding to infected RBCs determined by immunofluorescence staining and flow cytometry. Staining with MoAb 33G2, the mean relative fluorescence intensity in mature infected β-thalassemic RBCs was significantly (P < .01) reduced compared with that obtained from mature infected normal RBCs. The mean relative fluorescence intensity in α-thalassemic RBCs was also reduced compared with mature infected normal RBCs; however, this was not statistically significant (P > .05). With MoAb 33G2, ring-infected α- and β-thalassemic RBCs had similar levels of mean relative fluorescence intensity compared with that obtained from ring-infected normal RBCs. When stained with pooled immune serum, ring- and mature-infected α- or β-thalassemic RBCs also showed similar or slightly higher levels of mean relative fluorescence intensity compared with those obtained from infected normal RBCs.

**DISCUSSION**

We infected α- and β-thalassemic RBCs with a *P. falciparum* strain, TM267R, that characteristically rosetted with uninfected RBCs and cytoadhered with live, unfixed endothelial cells. The TM267R strain was chosen so that we could assess the effect of thalassemic RBCs on maturation and multiplication of the parasite, and on the expression of cytoadherence and rosetting properties of the parasite that may confer protection against malaria in thalassemia. As previously suggested, we observed that thalassemic RBCs were susceptible to merozoite invasion as indicated by an increased parasitemia at the end of the schizogonic cycle; however, rate of multiplication of parasites cultured in all thalassemic RBCs was lower than that obtained in normal RBC. Recent work has related the reduced deformability of thalassemic RBCs as a cause of resistance to parasite invasion. Further, the level of sialic acids of the thalassemic RBC membrane is 25% less than in normal RBCs and is distributed in an uneven manner. Deficiency of sialic acids in the RBC membrane appears to be associated with resistance to parasite invasion.

Using flow cytometry to measure DNA content, we found that in most infected thalassemic RBCs (except for five cases of β-thalassemia), maturation rates based on DNA synthesis of the ring-form parasite into the trophozoite/schizont stage were similar in thalassemic and normal RBCs. This observation was confirmed by microscopy showing that the segmented schizont in infected thalassemic RBCs contained similar numbers of nuclei compared with control RBCs. However, the protein-rich cytoplasm of the parasite in thalassemic RBCs was scanty, suggesting retardation of maturation. This may be caused by the fact that intraerythrocytic parasites use Hb as the primary amino acid resource. However, in thalassemic RBCs, the unmatched globin chains precipitate, and this abnormal linkage form of Hb may be resistant to parasite protease as has been reported previously.

Hb genotype of thalassemic RBCs in the majority (20

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**Table 2. Determination of *P. falciparum* Antigen Expression on the Surface of Infected Thalassemic RBCs**

<table>
<thead>
<tr>
<th>Thalassemia Type</th>
<th>Immune Serum</th>
<th>MoAb 33G2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>R</td>
</tr>
<tr>
<td>α-Heterozygous</td>
<td>20</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>(NS)*</td>
<td></td>
<td>(NS)</td>
</tr>
<tr>
<td>β-Heterozygous</td>
<td>24</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>(NS)</td>
<td></td>
<td>(NS)</td>
</tr>
</tbody>
</table>

*The relative (thalassemic:normal) value of fluorescence intensity for each thalassemia sample was calculated and expressed as the mean relative intensity for each thalassemia type (mean ± SD). R and T/S were ring- and trophozoite/schizont-infected RBC, respectively. Abbreviation: NS, not significant (P > .05).*

* Statistical analysis was performed between the control and each thalassemia group as described in Table 1, P value is shown in parenthesis.
cases) of the β-thalassemia population studied was β°thal/ 
HbE. This suggests that the difference in HbF levels in the 
β-thalassemic RBCs might be the factor affecting maturation 
and multiplication of the parasite. Regression correla-
tion analysis showed that in RBCs with higher HbF content, 
a lower parasite multiplication was observed. This is consis-
tent with a previous report that as little as 7 pg/cell of HbF 
can retard maturation of parasites and that the degree of 
retardation is correlated with the level of HbF in the RBC. 
In α-thalassemia we found no consistent evidence for im-
pairment of parasite multiplication in association with the 
level of HbH. With Hb genotyping, a similar number of 
α-thalassemic RBCs might be the factor affecting matura-
tion rate. However, a defect in multiplication could be 
caused by other factors such as the presence of oxidant stress 
that has been shown to correlate with reduced multiplica-
tion of parasites in α- and β-thalassemic RBCs. 

Cytoadherence and rosette formation of *P. falciparum* have been shown to be involved in the pathogenesis in severe falciparum malaria. These biologic properties are related to parasite proteins, some of which are covalently associated with cytoskeletal components of the RBC membrane. In thalassemic RBCs, abnormal interactions between cytoskeletal components have been postu-
lated as a cause of reduced deformability of the RBC. Ab-
normal clustering of transmembrane proteins and defects in the spectrin binding site of the cytoskeleton in thalassemic RBC membranes have been recently reported. Such aberrant associations in thalassemic RBC membranes may underlie defective deposition of parasite proteins that could result in impairment of cytoadherence/rosette formation and hence reduce clinical manifestation and severity of malar-
ia in thalassemia. In the present study, both cytoadhe-
erence and rosetting of infected thalassemic RBCs were, in fact, significantly reduced in heterozygous thalassemia of both α and β type. The mechanisms underlying these reduc-
tions are not clear. However, previous studies have shown that cytoadherence mechanisms involve multireceptor-li-
gand interactions, yet the mechanism responsible for ro-
setting is unclear. Our recent work has shown that rosetting of an infected RBC involves ABO blood group antigens of the RBC. In the malarious host, sequestration involving cytoadherence to small vessel endothelium is thought to allow *P. falciparum* to evade clearance by the reticuloen-
dotheial system and in particular the spleen. Thus, the observed reduction of in vitro cytoadherence and rosetting of infected thalassemic RBCs may predict in vivo a reduced ability of infected RBCs to sequester in the deep vascula-
ture, thus providing a mechanism in thalassemia by which the host is protected against the severe manifestations of falciparum malaria. 

The membranes of infected RBCs show a number of para-
derived proteins. Some of these proteins are de-
tectable on unfixed infected RBCs by anti-*P. falciparum* specific antibodies and their presence has been shown to correlate with the cytoadherence of infected RBC to endothelial cells. Using cytoadherence noninhibitory-pooled immune sera, we found that both ring- and trophozoite/ 
schizont-infected thalassemic RBCs had a similar or slightly higher level of membrane-associated antigens than did normal infected RBCs. This is consistent with a recent report of increased levels of antibody binding to trophozoite/schiz-
ont-infected thalassemic RBCs by using Gambian immune sera. However, in our assay, the difference in anti-

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