On the pathophysiology of severe falciparum malaria with special reference to red cell deformability
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Red blood cell deformability as a predictor of anemia in severe falciparum malaria


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Abstract

Decreased erythropoiesis and increased clearance of both parasitized and non-infected erythrocytes both contribute to the pathogenesis of anemia in falciparum malaria. Erythrocytes with reduced deformability are more likely to be cleared from the circulation by the spleen- a process which is augmented in acute malaria. Using a laser diffraction technique red blood cell deformability (RCD) was measured over a range of shear stresses and related to the severity of anemia in 36 adults with severe falciparum malaria. RCD at a high shear stress of 30 Pa, similar to that encountered in the splenic sinusoids, showed a significant positive correlation with the nadir in hemoglobin concentration during hospitalization ($r=0.49$, $p<0.002$). Exclusion of five patients with microcytic anemia strengthened this relationship ($r=0.64$, $p<0.001$). Reduction in RCD resulted mainly from changes in unparasitized erythrocytes. Reduced deformability of uninfected erythrocytes at high shear stresses and subsequent splenic removal of these cells may be an important contributor to the anemia of severe malaria.
Introduction

Anemia is an important cause of morbidity and mortality in falciparum malaria. The pathogenesis of anemia in malaria is multifactorial and incompletely understood. It is thought to result from a combination of parasitized erythrocyte destruction at schizont rupture, accelerated removal of both parasitized and unparasitized red cells, and ineffective erythropoiesis. Of these factors, removal of unparasitized red cells is the most important, accounting for approximately 90% of the reduction in hematocrit in acute malaria. We have shown previously that the threshold for splenic removal of heat damaged or antibody coated erythrocytes in acute malaria is lowered, suggesting enhancement of both mechanical filtrative function and also Fc receptor mediated clearance. Reduced red blood cell deformability (RCD) is thought to play an important role in the removal of senescent red cells from the circulation by the spleen. Since reduced RCD might also play a role in the clearance of both parasitized and unparasitized red cells in malaria, we have measured red blood cell deformability in relation to the development of anemia in severe falciparum malaria.

Patients and methods

Study Site

The study was carried out during the rainy season months from May until July in both 1995 and 1996, in the provincial hospital of Mae Sot, Tak province, western Thailand. Malaria transmission is low in this area with a seasonal peak during the rainy season which starts in late spring. Severe disease occurs at all ages. Multiple drug resistance is an increasing problem in this area.

Patients and clinical procedures

Consecutive adult patients admitted to Mae Sot Hospital with severe falciparum malaria were included, providing that written informed consent for blood sampling was obtained from the patients or their attendant relatives. Disease severity was classified according to standard criteria. Exclusion criteria were: age below 14 years, pregnancy and previous antimalarial drug treatment within 24 hours of admission. Previous quinine treatment was checked in a baseline blood sample by the rapid dipstick method in all patients. Patients were randomly assigned to treatment with either intravenous quinine dihydrochloride (20 mg salt/kg infused over 4 hours followed by 10 mg/kg 8-hourly).
followed by oral tetracycline or intravenous artesunate (2.4 mg/kg stat, then 1.2 mg/kg at 12 and 24 hours and then daily) followed by mefloquine in a comparative study, the results of which will be published elsewhere. Full supportive care was given as described previously. A control group of 22 healthy age and sex matched Thai volunteers provided a blood sample for RCD measurement.

A second control group comprised twelve adult Dutch travelers who presented with uncomplicated falciparum malaria at the Academic Medical Centre in Amsterdam. This group was treated with either oral sulphadoxine/pyrimethamine or halofantrine. A blood sample was taken on admission and at day 3, 7, 14, 21 and 28 after start of the treatment for assessment of parasitemia, hemoglobin level, and RCD.

This investigation was part of studies approved by the Ethical and Scientific Review Subcommittee of the Ministry of Public Health, Thailand. The study in Dutch travelers was approved by the medical ethical committee of the Academic Medical Centre, Amsterdam.

Laboratory methods

Thick and thin films from peripheral blood were taken on admission and stained with Field’s stain for parasite counting. Blood samples were taken 12 hourly for full blood count, routine biochemistry, lactate, glucose (assessed daily) and assessment of red blood cell deformability (RCD). Immediately after venepuncture RCD was measured with a Laser-assisted Optical Rotational Cell Analyzer (LORCA®, Mechatronics, Hoorn, The Netherlands). With this method, a defined shear stress is applied to a red cell suspension in a high viscous medium (5% polyvinylpyrrolidone in PBS-buffer, viscosity 30 mPa.s at 37°C) at a constant temperature of 37°C, in a small gap between two concentric cylinders. Because of the applied shear stress caused by rotation of the outer cylinder, the cells elongate and align themselves in the fluid layer. A laser beam is directed through the fluid layer and forms a diffraction pattern on a screen behind it. The ellipticity of this diffraction pattern is directly proportional to the mean ellipticity of the red blood cells. This ellipticity is described by the elongation index (EI) defined by the formula: $EI = \frac{(\text{length of the long axis} - \text{length of the short axis})}{(\text{length of the long axis} + \text{length of the short axis})}$. This is determined by computer analysis of the diffraction pattern. Red blood cell deformability was assessed at a range of shear stresses from 1.7 Pa. to 30 Pa. Shear stresses of 1.7 Pa. and above are encountered in the capillaries. High shear stresses are also likely to occur in the sinusoids of the spleen where red cells have to squeeze through the small intercellular gaps. Poor reproducibility has been a major drawback in earlier methods of measuring RCD by filtration, but appears to be very good with ektacytometry.

In the patients that were included in 1996 mean cell volume (MCV) was measured with a Coulter Counter. Because of the lack of availability of the Coulter machine in 1995, MCV
were calculated from the mean cell diameter (MCD) during this year. MCD was assessed light-microscopically, by averaging 200 red cells in a thin smear. MCV was calculated from these values by using a nomogram. The thin smear was also checked for the appearance of target cells and other red cell abnormalities. Hemoglobin electrophoresis was performed on the admission blood samples. Admission plasma samples were also stored at -20°C for later measurement of bilirubin, transaminases, LDH and serum iron.

Statistical methods

Statistical analyses were carried out using SPSS 6.1 statistical programmes (SPSS Corporation, Benelux). Comparisons of means were made by Student’s t-test, with a Bonferroni correction for multiple comparisons if applicable. Non normally distributed parameters were compared by the Mann-Whitney-U test. Correlations were assessed by the method of Pearson for normally distributed variables, and the method of Spearman for the remainder. A multiple regression analysis was performed to determine the most discriminating prognostic parameters in predicting the severity of the anemia, defined as the lowest hemoglobin level that was reached during admission.

Results

In total 42 patients with severe malaria were included in the study. Six patients were excluded from further analysis because they were given blood transfusions shortly after admission: one was thalassemic with a severe microcytic anemia (Hb 3.3 g/dl), another (Hb 7.8 g/dl) received a blood transfusion 24 hours after admission because of unstable hemodynamics partly related to atrial fibrillation. Four patients received exchange transfusion because of severe illness. Six patients (17%) subsequently died. Clinical and laboratory details are shown in table 1. The mean (SD) time to fever clearance in severe malaria was 67 (±30) hours and the corresponding time to parasite clearance was 68 (±19) hours. The mean RCD at admission at a shear stress of 30 Pa. of all patients, expressed as El (SD), was 0.586 (0.030), range 0.508–0.624, and was significantly lower than the RCD in healthy controls (El (SD)= 0.608 (0.005), p<0.05).

Red blood cell deformability and anemia

Fig. 1 shows the correlation between the mean red blood cell deformability on admission and the nadir value in hemoglobin level during hospitalization. The admission RCD at a shear stress of 30 Pa. correlated significantly with the degree of anemia (defined as the absolute hemoglobin concentration) that developed during admission (n=36, Pearson r=0.49, p<0.002). This correlation was strongest when RCD was measured at a high level
<table>
<thead>
<tr>
<th>severe anemia</th>
<th>severe anemia (Hb&lt;9.0 g/dl) (all patients)</th>
<th>severe anemia (Hb&lt;9.0 g/dl) (microcytosis excluded)</th>
<th>mild anemia (Hb&gt;9.0 g/dl)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (years)</td>
<td>27 (13)</td>
<td>28 (14)</td>
<td>26 (10)</td>
<td>n.s.</td>
</tr>
<tr>
<td>fatal cases (n)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>n.s.</td>
</tr>
<tr>
<td>artesunate (n)</td>
<td>9</td>
<td>6</td>
<td>8</td>
<td>n.s.</td>
</tr>
<tr>
<td>quinine (n)</td>
<td>8</td>
<td>6</td>
<td>11</td>
<td>n.s.</td>
</tr>
<tr>
<td>parasitemia (%)</td>
<td>6.3 (5.1)</td>
<td>7.4 (5.4)</td>
<td>9.1 (7.5)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>9.1 (2.4)</td>
<td>9.4 (2.6)</td>
<td>13.9 (1.7)</td>
<td>0.03</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>84.4 (8.7)</td>
<td>88.5 (6.1)</td>
<td>88.6 (6.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>nadir Hb (g/dl)</td>
<td>7.1 (1.2)</td>
<td>7.3 (1.2)</td>
<td>10.9 (1.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>time after admission to nadir in Hb (h)</td>
<td>41 (42)</td>
<td>53 (48)</td>
<td>83 (26)</td>
<td>n.s.</td>
</tr>
<tr>
<td>RCD at 30 Pa. (El)</td>
<td>0.574 (0.034)</td>
<td>0.564 (0.036)</td>
<td>0.597 (0.020)</td>
<td>0.002</td>
</tr>
<tr>
<td>RCD at 1.7 Pa. (El)</td>
<td>0.233 (0.032)</td>
<td>0.227 (0.032)</td>
<td>0.251 (0.031)</td>
<td>0.05</td>
</tr>
<tr>
<td>RCD (30 Pa.) at time of lowest Hb (El)</td>
<td>0.574 (0.032)</td>
<td>0.567 (0.033)</td>
<td>0.598 (0.025)</td>
<td>n.s.</td>
</tr>
<tr>
<td>LDH (mmol/l)</td>
<td>672 (153)</td>
<td>717 (137)</td>
<td>548 (364)</td>
<td>n.s.</td>
</tr>
<tr>
<td>total bilirubin (µmol/l)</td>
<td>47.0 (46.0)</td>
<td>57.7 (53.7)</td>
<td>70.1 (114.3)</td>
<td>n.s.</td>
</tr>
<tr>
<td>direct bilirubin (µmol/l)</td>
<td>27.4 (35.9)</td>
<td>35.9 (41.7)</td>
<td>46.2 (93.2)</td>
<td>n.s.</td>
</tr>
<tr>
<td>haptoglobin (g/l)</td>
<td>0.2 (0.6)</td>
<td>0.0 (0.0)</td>
<td>0.1 (0.2)</td>
<td>n.s.</td>
</tr>
<tr>
<td>serum iron (µmol/l)</td>
<td>10.4 (9.4)</td>
<td>12.7 (10.4)</td>
<td>6.4 (7.8)</td>
<td>n.s.</td>
</tr>
<tr>
<td>ASAT (U/l)</td>
<td>69 (27)</td>
<td>66 (12)</td>
<td>79 (94)</td>
<td>n.s.</td>
</tr>
<tr>
<td>creatinine (µmol/l)</td>
<td>101 (35)</td>
<td>108 (42)</td>
<td>94 (59)</td>
<td>n.s.</td>
</tr>
<tr>
<td>glucose (mmol/l)</td>
<td>4.5 (1.8)</td>
<td>4.6 (2.0)</td>
<td>3.6 (0.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>lactate (mmol/l)</td>
<td>4.8 (3.1)</td>
<td>5.0 (2.7)</td>
<td>3.9 (1.8)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table 1. Mean (SD) clinical and laboratory variables in patients with severe falciparum malaria according to the severity of anemia. P-values for significance of difference between patients with severe and mild anemia, with the exclusion of patients with microcytic anemia.
Figure 1. Correlation between admission values of mean red blood cell deformability at a shear stress of 30 Pa and the lowest hemoglobin level reached during the time of hospitalisation, in patients with severe falciparum malaria (n=36, correlation coefficient =0.49, p=0.002). When patients with a microcytic anemia (mean red cell volume < 80 fl, open circles) are excluded, the correlation between the two variables becomes much stronger (n=31, correlation coefficient =0.64, p<0.001). RCD= red blood cell deformability, EI= elongation index, MCV= mean cell volume, SS= shear stress, Pa= Pascal.

Figure 2. Correlation between admission values of mean red blood cell deformability at a shear stress of 1.7 Pa and the lowest hemoglobin level reached during the time of hospitalization, in patients with severe falciparum malaria (n=36, correlation coefficient =0.38, p=0.02). Open circles: MCV < 80 fl, closed circles: MCV ≥ 80 fl. RCD= red blood cell deformability EI= elongation index, MCV=mean cell volume, SS= shear stress, Pa= Pascal.
Chapter 5

of shear. For comparison: at a lower shear stress of 1.7 Pa, the correlation coefficient between RCD and the degree of anemia was 0.38 (p<0.02) for all patients (fig. 2).

The correlation between admission RCD at high shear and the severity of subsequent anemia was even stronger (r=0.64, p<0.001) when five cases with microcytic anemia (mean cell volume < 80 fl) were excluded from the regression analysis (fig. 1). The patients with microcytic anemia all had a hemoglobin concentration below Hb<9.0 g/dl and showed relatively more deformable red cells at these low hemoglobin levels than did the remaining patients. The cause of the microcytic anemia was considered to be iron deficiency (serum iron 2.3 µmol/l, iron binding capacity 75 µmol/l) in one case, and thalassemia in the other four, who had a normal serum iron, target cells in the blood smear and (in two cases) high HbA₂ levels on electrophoresis.

There was no significant correlation between the change in hemoglobin concentration and the change in RCD over the same period of time. Red blood cell deformability at the time of the nadir in hemoglobin level was not significantly different from the RCD on admission (table 1). Six patients with macroscopic hemoglobinuria had similar RCD values at a shear stress of 30 Pa as the remaining patients; (mean RCD 0.588 (±0.019) compared to 0.586 (±0.032)). None of these hemoglobinuric patients were G6PD deficient.

The group of twelve Dutch travelers that returned from the tropics with uncomplicated malaria did not have high parasitemias (mean(SD) 1.5% (1.3%)) and developed only mild anemia, with a mean (SD) nadir in hemoglobin concentration of 12.5 (1.3) g/dl RCD was only slightly decreased with a mean (SD) elongation index of 0.595 (0.009) at a shear stress of 30 Pa. Nevertheless, in this group the percentage improvement in hemoglobin concentration during the 4 weeks follow-up correlated significantly with the percentage improvement in the RCD over the same time period (r=0.67, p=0.018). Moreover the linear regression line describing the correlation between these two parameters almost crossed the zero point (%improvement in Hb = 1.5% + 3.5 * %improvement in RCD). RCD started to normalize 2 weeks after the start of treatment.

Factors related to anemia

Besides RCD, none of the other clinical or laboratory variables listed in table 1 showed a significant correlation with the degree of anemia during the course of the disease. In particular the admission parasitemia was not a predictor of anemia (correlation coefficient=0.13, n.s.). In a multiple regression analysis (forward stepwise regression) with the variables listed in table 1 as explanatory variables, RCD at admission (at shear stress = 30 Pa.) was the only parameter that contributed significantly to the model (adjusted r²=0.39, B=45.1, SE(B)=10.1, p=0.0001, F=20.0).
Discussion

Anemia in acute falciparum malaria is caused by increased destruction of both infected and non-infected erythrocytes and decreased erythropoiesis. In severe malaria anemia develops rapidly. The fall in hemoglobin concentration is often considerably greater than could be accounted for by destruction of parasitised cells only. Anemia results largely from accelerated red cell destruction. Labeling studies have shown rapid clearance of uninfected red cells by the spleen. The importance of the enhanced clearance of uninfected cells is illustrated by the lack of correlation between parasitemia and the severity of anemia evident in this and previous studies. The mechanism for this enhanced clearance of uninfected red cells remains to be elucidated. Evidence of an immune mediated mechanism is unconvincing, although a role for antibody mediated clearance cannot be ruled out as the spleen in acute malaria shows a lowered threshold for clearance of erythrocytes coated with immunoglobulins, and it may be difficult therefore to demonstrate increased antibody binding in circulating erythrocytes.

In this study the RCD on admission in patients with severe falciparum malaria was significantly lower than in healthy controls. Red blood cells infected with P. falciparum parasites become progressively less deformable as the intra-erythrocytic parasites mature. However, the mean red blood cell deformability obtained by the LORCA is a summation of the RCD of all the red cell fractions in the peripheral blood, with contributions to the overall value that are proportional to their size. Since the majority of red cells even in severe malaria is uninfected, the reduction in RCD in the patients in this study results mainly from changes in the unparasitized erythrocytes.

Reduced RCD does not result from a non-specific response to severe infections. In a group of fourteen septicaemic patients in the intensive care unit of the Academic Medical Centre, Amsterdam, there was no correlation between RCD and severity of anemia and the mean (SD) RCD at 30 Pa, expressed as EI was 0.594 (0.020), range 0.574–0.614.

This study shows a clear correlation between the RCD on admission and subsequent anemia in severe falciparum malaria. Reduced RCD and anemia could both be independent markers of overall disease severity, but a causal relationship seems more likely i.e. clearance of less deformable red blood cells from the circulation by the spleen, a mechanism that is also thought to account for the clearance of senescent erythrocytes. The relation between reduced RCD and anemia was most prominent at the shear stresses encountered normally in the spleen where red cells have to squeeze through the small intercellular gaps in the sinusoids of the spleen (width of 0.5–2 μm). In a recent study we showed that in severe falciparum malaria, RCD at a lower shear stress of 1.7 Pa correlated strongly with mortality.
suggesting impairment of microcirculatory flow by rigid red cells. This shear stress corresponds with that approximately encountered in the capillaries.

The mean RCD did not change significantly during hospitalization, which was generally up to 7 days after start of treatment. Longer follow-up, to study if the improvement in RCD relates to recovery from anemia, was unfortunately not possible in the Thai patients. However, follow up in a group of 12 Dutch travelers with uncomplicated falciparum malaria showed that the improvement in hemoglobin levels over a 4 weeks period correlated closely with the improvement in RCD over the same time period. These findings further support a causal relationship between the two parameters.

The correlation between RCD and the severity of anemia that developed in patients with severe malaria was even stronger when the patients with microcytic anemia, resulting from either a hemoglobinopathy or iron deficiency, were excluded. The few patients with microcytic anemia showed a slight but non-significant decrease in RCD at 30 Pa, compared to healthy controls (respectively 0.600 (0.020) and 0.608 (0.005), mean (SD), (fig.1). RCD can be reduced in both iron deficiency and thalassemia. We were not able to genotype all patients but it is unlikely that thalassemia was a significant confounder in the normocytic malaria patients. Of 95 well defined patients with various forms of α- and β-thalassemia and HbE and HbConstant Spring (HbCS) disease studied in Bangkok, only 10 had a normal MCV above 80 fL. Seven of these had HbCS (4) or HbE-thalassemia (3) with a normal RCD at 30 Pa and slight or no anemia (mean Hb 11.5 g/dl and 13.4 g/dl, respectively) (unpublished observations).

The mechanisms underlying the reduction in red blood cell deformability of uninfected cells in severe malaria are not known. Mohan et al. showed damage of the uninfected erythrocyte membrane through lipid peroxidation in P. falciparum co-cultured with blood monocytes. Nauman et al. have reported on a heat labile exoantigen produced by in-vitro cultures of P. falciparum which binds reversibly to normal red cells and reduces their deformability. We also think that a soluble factor produced by the parasite is the most likely cause of a reduction in RCD in patients with falciparum malaria. Preliminary data show that plasma from patients with acute falciparum malaria mixed with healthy donor red blood cells, can rigidify these cells. Also, supernatant from a P. falciparum culture seems to be able to rigidify healthy donor red blood cells, but the exact mechanism remains to be elucidated. Although heat damages red cells, an increase in temperature up to 41°C did not reduce RCD of normal erythrocytes in-vitro as measured by LORCA (data not shown), suggesting that fever was not a major contributor to this effect. The relative roles of systemic host factors or endothelial cell dysfunction in reducing RCD is not known.

In conclusion this study shows a strong predictive value of admission red blood cell deformability at a high shear level, for the severity of the anemia that develops in the
Red blood cell deformability and anemia in severe malaria

course of severe falciparum malaria. Since the reduction in RCD is mainly caused by
rigidification of non-parasitized erythrocytes, this correlation could be an explanation for
the increased splenic clearance of non-infected erythrocytes.

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