

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 noninfected 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 that is augmented in acute malaria. Using a laser diffraction technique, we measured red blood cell (RBC) deformability over a range of shear stresses and related this to the severity of anemia in 36 adults with severe falciparum malaria. The RBC deformability 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 RBC deformability 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.

Anemia is an important cause of morbidity and mortality in falciparum malaria.^{1,2} 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 blood cells, and ineffective erythropoiesis.^{3–6} Of these factors, removal of unparasitized red blood cells is the most important, accounting for approximately 90% of the reduction in hematocrit in acute malaria (Price R and others, unpublished data). 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 Fc receptor-mediated clearance.^{7,8} Reduced red blood cell (RBC) deformability is thought to play an important role in the removal of senescent red blood cells from the circulation by the spleen.⁹ Since reduced RBC deformability might also play a role in the clearance of both parasitized and unparasitized red blood cells in malaria, we have measured RBC 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, in western Thailand. Malaria transmission is low in this area with a seasonal peak during the rainy season that starts in late spring.¹⁰ 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.² Exclusion criteria were an age less than 14 years, pregnancy, and previous antimalarial drug

treatment within 24 hr of admission. Previous quinine treatment was checked in a baseline blood sample by the rapid dipstick method in all patients.¹¹ Patients were randomly assigned to treatment with either intravenous quinine dihydrochloride (20 mg salt/kg infused over a 4-hr period followed by 10 mg/kg every 8 hr) followed by oral tetracycline, or intravenous artesunate (2.4 mg/kg initially, then 1.2 mg/kg at 12 and 24 hr 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.³ A control group of 22 healthy age- and sex-matched Thai volunteers provided a blood sample for measurement of RBC deformability.

A second control group comprised 12 adult Dutch travelers who presented with uncomplicated falciparum malaria at the Academic Medical Centre in Amsterdam. This group was treated with either oral sulfadoxine/pyrimethamine or halofantrine. A blood sample was taken on admission and at days 3, 7, 14, 21, and 28 after start of the treatment for assessment of parasitemia, hemoglobin level, and RBC deformability.

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

Laboratory methods. Thick and thin films from peripheral blood were taken on admission and stained with Field's stain for parasite counting.¹² Blood samples were taken every 12 hr for a full blood count, routine biochemistry, lactate, glucose (assessed daily), and assessment of RBC deformability. Immediately after venipuncture, RBC deformability was measured with a laser-assisted optical rotational cell analyzer (LORCA®; Mechatronics, Hoorn, The Netherlands).¹³ With this method a defined shear stress is applied to an RBC suspension in a high viscous medium (5% polyvinylpyrrolidone in phosphate-buffered saline buffer, viscosity = 30 mPa.sec at 37°C) at a constant temperature of 37°C in a small gap between two concentric cylinders. Because

TABLE 1

Mean (SD) clinical and laboratory variables in patients with severe falciparum malaria according to the severity of anemia*

	Severe anemia (Hb \leq 9.0 g/dl) (all patients (n = 17))	Severe anemia (Hb \leq 9.0 g/dl) (microcytosis excluded) (n = 12)	Mild anemia (Hb > 9.0 g/dl) (n = 19)	P†
Age (years)	27 (13)	28 (14)	26 (10)	NS
Fatal cases (n)	3	3	3	NS
Artesunate (n)	9	6	8	NS
Quinine (n)	8	6	11	NS
Parasitemia (%)	6.3 (5.1)	7.4 (5.4)	9.1 (7.5)	NS
Hb (g/dl)	9.1 (2.4)	9.4 (2.6)	13.9 (1.7)	0.03
MCV (fl)	84.4 (8.7)	88.5 (6.1)	88.6 (6.6)	NS
Nadir Hb (g/dl)	7.1 (1.2)	7.3 (1.2)	10.9 (1.4)	0.001
Time after admission to nadir in Hb (hr)	41 (42)	53 (48)	83 (26)	NS
RCD at 30 Pa (EI)	0.574 (0.034)	0.564 (0.036)	0.597 (0.020)	0.002
RCD at 1.7 Pa (EI)	0.233 (0.032)	0.227 (0.032)	0.251 (0.031)	0.05
RCD (30 Pa) at time of lowest Hb (EI)	0.574 (0.032)	0.567 (0.033)	0.598 (0.025)	NS
LDH (mmol/L)	672 (153)	717 (137)	548 (364)	NS
Total bilirubin (μ mol/L)	47.0 (46.0)	57.7 (53.7)	70.1 (114.3)	NS
Direct bilirubin (μ mol/L)	27.4 (35.9)	35.9 (41.7)	46.2 (93.2)	NS
Haptoglobin (g/L)	0.2 (0.6)	0.0 (0.0)	0.1 (0.2)	NS
Serum iron (μ mol/L)	10.4 (9.4)	12.7 (10.4)	6.4 (7.8)	NS
ASAT (U/L)	69 (27)	66 (12)	79 (94)	NS
Creatinine (μ mol/L)	101 (35)	108 (42)	94 (59)	NS
Glucose (mmol/L)	4.5 (1.8)	4.6 (2.0)	3.6 (0.6)	NS
Lactate (mmol/L)	4.8 (3.1)	5.0 (2.7)	3.9 (1.8)	NS

* Hb = hemoglobin; MCV = mean cell volume; RCD = red blood cell deformability; Pa = Pascal; EI = elongation index; LDH = lactate dehydrogenase; ASAT = aspartate aminotransferase.

† P values for significance of difference between patients with severe and mild anemia, with the exclusion of patients with microcytic anemia.

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 $EI = (\text{length of the long axis} - \text{length of the short axis}) / (\text{length of the long axis} + \text{length of the short axis})$. 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.¹⁴ Higher shear stresses are also likely to occur in the sinusoids of the spleen where red blood cells have to squeeze through the small intercellular gaps. Poor reproducibility has been a major drawback in earlier methods of measuring RBC deformability by filtration, but it appears to be very good with ektacytometry.^{15,16}

In the patients that were included in 1996, the mean cell volume (MCV) was measured with a Coulter Counter (Coulter, Inc., Hialeah, FL). Because of the lack of availability of the Coulter machine in 1995, the MCV was calculated from the mean cell diameter (MCD) during this year. The MCD was assessed by light microscopy by averaging 200 red blood cells in a thin smear. The MCV was calculated from these values by using a nomogram.¹⁷ The thin smear was also checked for the appearance of target cells and other RBC abnormalities. Hemoglobin electrophoresis was performed on the admission blood samples. Admission plasma samples were also stored at -20°C for subsequent measurement of bilirubin, transaminases, lactate dehydrogenase, and serum iron.

Statistical methods. Statistical analyses were carried out

using SPSS 6.1 statistical programs (SPSS Corporation, Chicago, IL). 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

A total of 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 (hemoglobin level = 3.3 g/dl), another (hemoglobin level = 7.8 g/dl) received a blood transfusion 24 hr 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 (\pm SD) time to fever clearance in severe malaria was 67 (\pm 30) hr and the corresponding time to parasite clearance was 68 (\pm 19) hours. The mean RBC deformability at admission at a shear stress of 30 Pa of all patients, expressed as EI (SD), was 0.586 (0.030) (range = 0.508–0.624), and was significantly lower than the RBC deformability in healthy controls (EI [SD] = 0.608 [0.005], $P < 0.05$).

Red blood cell deformability and anemia. Figure 1 shows the correlation between the mean RBC deformability

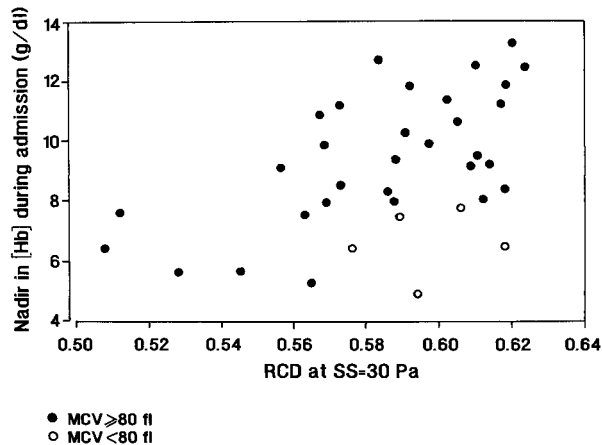


FIGURE 1. Correlation between admission values of mean red blood cell deformability (RCD) at a shear stress (SS) of 30 Pa and the lowest hemoglobin (Hb) level reached during the time of hospitalization in patients with severe falciparum malaria ($n = 36$, correlation coefficient = 0.49, $P = 0.002$). When patients with a microcytic anemia (mean red blood cell volume < 80 fl, open circles) are excluded, the correlation between the two variables becomes much stronger ($n = 31$, correlation coefficient = 0.64, $P < 0.001$). MCV = mean cell volume.

on admission and the nadir value in the hemoglobin level during hospitalization. The admission RBC deformability 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 the RBC deformability was measured at a high level of shear. At a lower shear stress of 1.7 Pa, the correlation coefficient between RBC deformability and the degree of anemia was 0.38 ($P < 0.02$) for all patients (Figure 2).

The correlation between admission RBC deformability at high shear and the severity of subsequent anemia was even stronger ($r = 0.64$, $P < 0.001$) when five cases with microcytic anemia (MCV < 80 fl) were excluded from the regression analysis (Figure 1). All patients with microcytic anemia had a hemoglobin concentration < 9.0 g/dl and showed relatively more deformable red blood cells at these low hemoglobin levels than did the remaining patients. The cause of the microcytic anemia was iron deficiency (serum iron level = 2.3 mmol/L, iron binding capacity = 75 mmol/L) in one case, and thalassemia in the other four, who had normal serum iron levels, target cells in the blood smear, and (in two cases) high hemoglobin A₂ levels on electrophoresis.

There was no significant correlation between the change in hemoglobin concentration and the change in RBC deformability over the same period of time. Red blood cell deformability at the time of the nadir in the hemoglobin level was not significantly different from the RBC deformability on admission (Table 1). Six patients with macroscopic hemoglobinuria had similar RBC deformability values at a shear stress of 30 Pa as the remaining patients (mean [SD] RBC deformability = 0.588 [0.019] compared with 0.586 [0.032]). None of these hemoglobinuric patients were deficient for glucose-6-phosphate dehydrogenase.

The group of 12 Dutch travelers that returned from the

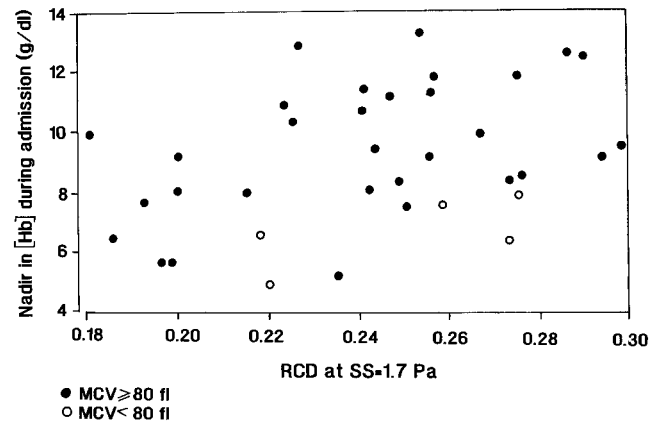


FIGURE 2. Correlation between admission values of mean red blood cell deformability (RCD) at a shear stress (SS) of 1.7 Pa and the lowest hemoglobin (Hb) level reached during the time of hospitalization in patients with severe falciparum malaria ($n = 36$, correlation coefficient = 0.38, $P = 0.02$). MCV = mean cell volume.

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. The RBC deformability was only slightly decreased with a mean (SD) EI of 0.595 (0.009) at a shear stress of 30 Pa. Nevertheless, in this group the percentage improvement in hemoglobin concentration during the four-weeks follow-up correlated significantly with the percentage improvement in the RBC deformability over the same time period ($r = 0.67$, $P = 0.018$). Moreover, the linear regression line describing the correlation between these two parameters nearly crossed the zero point (% improvement in hemoglobin level = 1.5% + 3.5 × % improvement in RBC deformability). The RBC deformability started to normalize two weeks after the start of treatment.

Factors related to anemia. Besides RBC deformability, 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, not significant). In a multiple regression analysis (forward stepwise regression) with the variables listed in Table 1 as explanatory variables, the RBC deformability at admission (at a shear stress = 30 Pa) was the only parameter that contributed significantly to the model (adjusted $R^2 = 0.39$, $B = 45.1$, $SE(B) = 10.1$, $P = 0.0001$, $F = 20.0$).

DISCUSSION

Anemia in acute falciparum malaria is caused by increased destruction of both infected and noninfected erythrocytes and decreased erythropoiesis. In severe malaria, anemia develops rapidly. The decrease in hemoglobin concentration is often considerably greater than could be accounted for by destruction of parasitized cells only. Anemia results largely from accelerated RBC destruction.^{3-6,18} Labeling studies have shown rapid clearance of uninfected red blood cells by the spleen.³ The importance of the enhanced clearance of uninfected cells is illustrated by the lack of corre-

lation between parasitemia and the severity of the anemia evident in this and previous studies.^{1,19} The mechanism for this enhanced clearance of uninfected red blood cells remains to be elucidated. Evidence of an immune-mediated mechanism is unconvincing, although a role for antibody-mediated clearance cannot be ruled out since the spleen in acute malaria shows a lowered threshold for clearance of erythrocytes coated with immunoglobulins, and it therefore may be difficult to demonstrate increased antibody binding in circulating erythrocytes.^{7,8,20,21}

In this study, the RBC deformability on admission in patients with severe falciparum malaria was significantly lower than in healthy controls. Red blood cells infected with *Plasmodium falciparum* parasites become progressively less deformable as the intra-erythrocytic parasites mature.^{22,23} However, the mean RBC deformability obtained with LORCA is a summation of the RBC deformability of all the RBC fractions in the peripheral blood, with contributions to the overall value that are proportional to their size (Streekstra GJ, 1994. *A Bi Plane Rheoscope for the Measurement of Red Cell Deformation and Orientation in a Couette Flow*. Thesis, University of Utrecht, Utrecht, The Netherlands). Since the majority of red blood cells even in severe malaria is uninfected, the reduction in RBC deformability in the patients in this study results mainly from changes in the unparasitized erythrocytes.

Reduced RBC deformability does not result from a non-specific response to severe infections. In a group of 14 septicemic patients in the intensive care unit of the Academic Medical Centre in Amsterdam, there was no correlation between RBC deformability and severity of anemia and the mean (SD) RBC deformability at 30 Pa, expressed as the EI, was 0.594 (0.020) (range = 0.574–0.614).

This study shows a clear correlation between the RBC deformability on admission and subsequent anemia in severe falciparum malaria. Reduced RBC deformability 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.⁹ The relationship between reduced RBC deformability and anemia was most prominent at the shear stresses encountered normally in the spleen where red blood cells have to squeeze through the small intercellular gaps in the sinusoids of the spleen (width = 0.5–2 μ m).²⁴ In a recent study, we showed that in severe falciparum malaria, RBC deformability at a lower shear stress of 1.7 Pa correlated strongly with mortality, suggesting impairment of microcirculatory flow by rigid red blood cells.²⁵ This shear stress corresponds with that encountered in the capillaries (average diameter = 3–5 μ m).¹⁴

The mean RBC deformability did not change significantly during hospitalization, which was generally up to seven days after the start of treatment. Longer follow-up, to study if the improvement in RBC deformability is related 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 four-week period correlated closely with the improvement in RBC deforma-

bility over the same time period. These findings further support a causal relationship between the two parameters.

The correlation between RBC-D 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 nonsignificant decrease in RBC deformability at 30 Pa compared with healthy controls (mean [SD] = 0.600 [0.020] and 0.608 [0.005], respectively, Figure 1). The RBC deformability can be reduced in both iron deficiency and thalassemia.^{26,27} 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 hemoglobin E and hemoglobin Constant Spring (hemoglobin CS) disease studied in Bangkok, only 10 had a normal MCV > 80 fl. Seven had hemoglobin CS (4) or hemoglobin E thalassemia (3) with a normal RBC deformability at 30 Pa and slight or no anemia (mean hemoglobin levels = Hb 11.5 g/dl and 13.4 g/dl, respectively) (Dondorp AM, unpublished data).

The mechanisms underlying the reduction in RBC deformability of uninfected cells in severe malaria are not known. Mohan and others showed damage of the uninfected erythrocyte membrane through lipid peroxidation in *P. falciparum* cocultured with blood monocytes.²⁸ Nauman and others have reported on a heat-labile exoantigen produced by *in vitro* cultures of *P. falciparum* that binds reversibly to normal red blood cells and reduces their deformability.²⁹ We also think that a soluble factor produced by the parasite is the most likely cause of a reduction in RBC deformability 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 blood cells, an increase in temperature up to 41°C did not reduce the RBC deformability of normal erythrocytes *in vitro* as measured by LORCA, suggesting that fever was not a major contributor to this effect. The relative roles of systemic host factors or endothelial cell dysfunction in reducing RBC deformability is not known.

In conclusion, this study shows a strong predictive value of admission RBC deformability at a high shear level for the severity of the anemia that develops in the course of severe falciparum malaria. Since the reduction in RBC deformability is caused mainly by rigidification of nonparasitized erythrocytes, this correlation could be an explanation for the increased splenic clearance of noninfected erythrocytes.

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