On the pathophysiology of severe falciparum malaria with special reference to red cell deformability

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Chapter 3

Prognostic significance of reduced red cell deformability in severe falciparum malaria

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Abstract

Severe falciparum malaria is associated with microvascular obstruction resulting from sequestration of erythrocytes containing mature stages of the parasite. As reduced red cell deformability (RCD) can contribute to impaired microcirculatory flow, RCD was measured in 23 patients with severe falciparum malaria (7 of whom died subsequently), 30 patients with uncomplicated malaria and 17 healthy controls. RCD, measured by ektacytometry, was significantly reduced in severe malaria and was particularly low in all fatal cases. At a low shear stress of 1.7 Pa, a red cell elongation index below 0.21 on admission to the hospital predicted fatal outcome with a sensitivity of 100% (C.I. 59%-100%) and a specificity of 88% (C.I. 61%-98%). The reduction in red cell deformability appeared to result mainly from changes in unparasitized erythrocytes. Reduced deformability of unparasitized red cells in severe falciparum malaria may contribute to impaired microcirculatory flow and a fatal outcome.

Introduction

Infection with *Plasmodium falciparum* remains a major cause of death in the tropics, with an annual global mortality of 1-2 million people and a mortality rate in severe malaria of 15%-30% [1, 2]. The sequestration of red cells containing the mature forms of the parasite in the microcirculation of the vital organs is considered to be the essential pathological feature of the infection, but precisely how this causes death is not known. Sequestration interferes with microcirculatory flow and tissue metabolism and may focus the release of host or parasite derived toxins to the vital organs [3]. The cytoadherent parasitized red cells impede the passage of the uninfected red cells, which are forced to deform more than usual in their transit through the microcirculation. As reduced RCD has been associated with impaired tissue perfusion in other conditions, we investigated the relationship between RCD and disease severity in falciparum malaria [4].

Patients and methods

The study was carried out in May and June 1995 in the provincial hospital of Mae Sot, Tak province, Thailand. Malaria transmission is low in this area with a seasonal peak during the rainy season which starts in late spring [5]. 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 acute falciparum malaria were included, providing that written informed consent was obtained from the patients or their attendant relatives. Disease severity was classified according to standard criteria [2]. Exclusion criteria were: age below 14 years, pregnancy and a history of previous antimalarial drug treatment within 24 hours of admission. Previous quinine treatment was checked in a baseline blood sample by a rapid quinine dipstick method in all patients [6]. A full clinical examination was performed on admission and all details were recorded on a standard form. In cases of cerebral malaria a lumbar puncture was performed to exclude other causes of altered consciousness. 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 with 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 which will be published elsewhere. Full supportive care was given as described previously [2]. If necessary patients were transferred to an intensive care unit for mechanical ventilation, peritoneal dialysis, or hemodynamic support and monitoring. This

investigation was part of studies approved by the Ethical and Scientific Review Sub-committee of the Ministry of Public Health, Thailand.

Laboratory methods

Thick and thin films from peripheral blood were taken on admission and stained with Field's stain for parasite counting [7]. Baseline blood samples were taken for full blood count, glucose, lactate, and routine biochemistry. Hb-electrophoresis was assessed from stored frozen samples (-20°C). Mean red cell diameter (MCD) and red cell morphology were evaluated in a thin smear using a callibrated light microscope. Red cell deformability was measured immediately by ektacytometry using a Laser-assisted Optical Rotational Cell Analyser (LORCA®, Mechatronics, The Netherlands)[8]. Blood samples from healthy Thai adults of the same age range were used as controls. With this method a defined shear stress is applied to a red cell suspension in a high viscous medium (5% polyvinylpyrrolidone in PBS-buffer) at a constant temperature of 37°C, in a small gap between two concentric rotating cylinders. Because of the applied shear stress the cells elongate and align themselves in the fluid layer, thus forming a grid. A laser beam is directed through the fluid layer and forms a diffraction pattern behind it. The ellipticity of this diffraction pattern is directly proportional to the mean ellipticity of the red blood cells (i.e. the amount that the normally discoid erythrocyte is deformed). The unit of deformability is the elongation index (EI) defined by the length of the long axis minus the short axis devided by the length of the long axis plus the short axis of the deformability pattern. This is determined by computer analysis of the diffraction pattern, using iso-intensity lines for curve fitting. Red cell deformability was assessed at 3 shear stresses (1.7 Pa, 9.5 Pa and 30 Pa.) corresponding approximately to shear stresses encountered in vivo in respectively the venules, the arterioles and capillaries, and in arteries with significant stenosis [9]. Reproducibility was a major drawback in former filtration methods measuring RCD, but is very good with ektacytometry [4, 8, 10].

Statistical methods

Statistical analyses were carried out using SPSS 6.1 statistical programmes (SPSS Corporation, Benelux). Normally distributed data were analysed using Student's t-test and analysis of variance, with application of the 'least significant difference'-method for multiple comparisons. The Mann Whitney test was used to compare non-normally distributed variables. Correlations were assessed by the method of Pearson for normally distributed variables, and the method of Spearman for the remainder. A multiple logistic regression model was used (Foward Logistic Regression, SPSS 6.1 statistical programmes) to determine the most discriminating prognostic indicators and their relative contributions in predicting outcome (death or survival).

Results

Clinical details

A total of 23 patients with severe malaria were included in the study. The comparison groups comprised 30 adult patients with uncomplicated falciparum malaria and 17 healthy volunteers. In the severe malaria group 12 had cerebral malaria, 4 developed pulmonary oedema requiring assisted ventilation, 1 patient became anuric and was dialysed, and 1 patient developed nosocomial pneumonia. Seven patients (30%) subsequently died (of whom 4 received quinine and 3 artesunate). There were no deaths in the group with uncomplicated malaria. Clinical and laboratory details are shown in table 1. The mean (SD) time to fever clearance in severe malaria was 68.5 (54.8) hours and the corresponding time to parasite clearance was 63.4 (24.2) hours. Within the group of severe patients MCD of the red cells did not differ significantly between survivors and fatal cases. There were 5 patients with severe malaria and severe microcytosis (MCD<6.0 μm) who all survived. Of

Table 1. Admission clinical and laboratory variables in 23 patients with severe malaria in the provincial hospital of Mae Sot, Tak province, Thailand.

Variable	Survivors* (n=16)	Fatal cases* (n=7)	Significance of difference (p)
Age	28±8	28±14	n.s.
Days with fever prior to admission	3.5±1.8	4.3±1.8	n.s.
Pulse rate (per min)	114±11	109±15	n.s.
Blood pressure (mmHg)			
Systolic	108±12	104±14	n.s.
Diastolic	61±13	63±20	n.s.
Temperature (°C)	38.9±9	38.2±1.4	n.s.
Median coma score	15	8	p=0.004 †
Packed cell volume (%)	31±12	31±9	n.s.
Parasitaemia (%)	6±6	10±11	n.s.
Creatinine (mg/dl)	1.15±0.22	2.35±2.36	p=0.029 †
Lactate (mmol/L)	4.4±2.4	13.1±9.2	p=0.019 †
Glucose (mmol/L)	9.5±6.3	8.9±3.5	n.s.
MCD** (μm)	6.2±0.5	6.2±0.2	n.s.
RCD‡ at SS§=1.7 Pa (EI°)	0.252±0.033	0.189±0.014	p=0.0001
RCD‡ at SS§=9.5 Pa (EI°)	0.507±0.035	0.450±0.034	p=0.0014
RCD‡ at SS§=30 Pa (EI°)	0.588±0.029	0.547±0.033	p=0.0077

* Values expressed as mean ± standard deviation
† Comparisons by Mann-Whitney U test; all other comparisons by Student's t-test.
‡ RCD = red cell deformability
** MCD = mean cell diameter of red blood cells
§ SS = shear stress
° EI = elongation index

these 5 patients 4 were likely to suffer from thalassemia (high HbA2 levels and target cells in the thin smear). RCD at 1.7 Pa varied between 0.24 and 0.28 in this group. One patient who expired was later shown to have had a HbE hemoglobinopathy. MCD of the red cells was 6.0 mmm and RCD at 1.7 Pa was 0.20 in this patient. Six patients with intravascular hemolysis were found to be negative for G6PD deficiency.

Red cell deformability

Red cell deformability was reduced in proportion to disease severity (table 2) . The most striking difference was between fatal cases with severe malaria and survivors (fig 1). Severely reduced RCD (EI<0.21) at a shear stress of 1.7 Pa predicted fatal outcome with a sensitivity of 100% (C.I. 59%-100%) and a specificity of 88% (C.I. 61%-98%). Parasitaemia on admission did not correlate either with RCD (r= -0.08, p=0.6) or with survival. In a multiple

Variable	Study group		
	uncomplicated malaria	severe malaria	healthy controls
n	30	23	17
age	27±10	28±10	32±7
RCD* at SS†=1.7 Pa (EI‡)	0.270±0.027	0.232±0.041	0.284±0.017
RCD* at SS†=9.5 Pa (EI‡)	0.520±0.020	0.489±0.043	0.544±0.011
RCD* at SS†=30 Pa (EI‡)	0.602±0.016	0.576±0.035	0.617±0.010

* RCD = red cell deformability
† SS = shear stress
‡ EI = Elongation index

Table 2. Mean (±SD) red cell deformability on admission in 30 patients with uncomplicated falciparum malaria, 23 patients with severe falciparum malaria and 17 healthy controls, provincial hospital of Mae Sot, Tak province, Thailand.
Differences in RCD at all levels of shear are significant ($p<0.05$) between all study groups (ANOVA), except RCD at SS=1.7 Pa in uncomplicated malaria compared to healthy controls.

logistic regression analysis, with the parameters listed in table 1 as variables, RCD at a shear stress of 1.7 Pa was the strongest predictor of mortality (Wald statistic=4.5). The only other variable which contributed significantly to the model was the Glasgow Coma Score on admission (Wald statistic 2.1). There was a significant correlation between admission values for plasma lactate levels and RCD (r= -0.44, p=0.04). There was no correlation between the MCD of the red cells and RCD at 1.7 Pa. In fatal cases the RCD on admission was not significantly different from the RCD 2 to 12 hours before death (RCD, as mean ±SD, at 1.7 Pa respectively 0.186±0.018 and 0.197±0.039). There was no significant change in RCD during the time of admission (up to 168 hours).

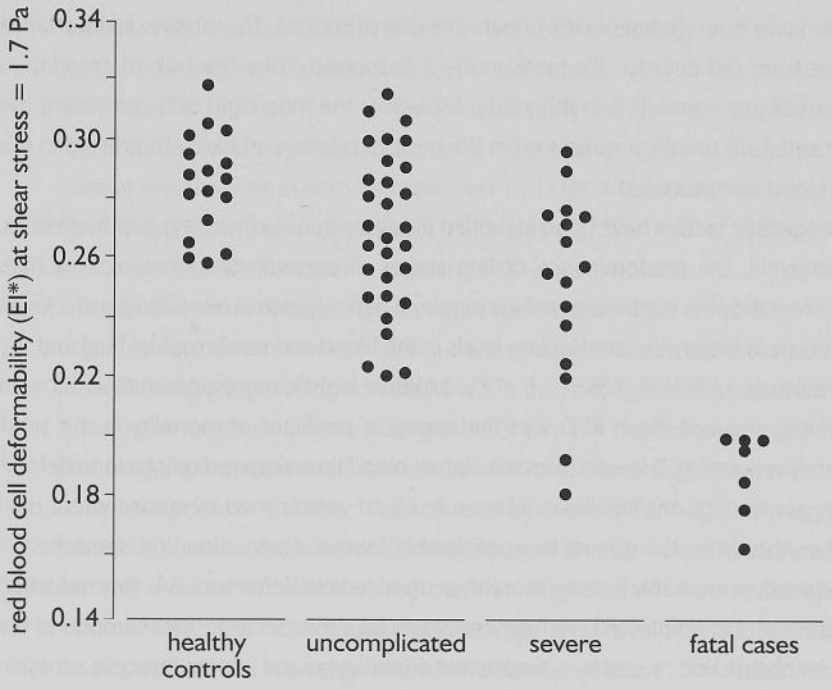

Figure 1. Red cell deformability (admission values) of patients with falciparum malaria compared to healthy controls. *EI = elongation index.

Discussion

During their passage through the microcirculation, red cells must undergo considerable deformation as their diameter (7.5 μm) exceeds the average midpoint diameter of the capillaries (3μm-7μm). Red cell deformability is therefore an important determinant of microvascular blood flow [11]. Red blood cells infected with *P. falciparum* parasites become progressively less deformable as the intra-erythrocytic parasites mature [12, 13]. Early studies showed that the "filterability" of red cells in uncomplicated malaria was reduced, suggesting that uninfected red cells might also be less deformable, although the relationship of red cell filterability to the rheological conditions encountered in-vivo is uncertain [14, 15]. The present study shows that red blood cells in patients with acute falciparum malaria are less deformable than in healthy subjects and that this rigidity increases with increasing severity of the infection. The red cell deformability estimate obtained by the LORCA is a summation of the RCD of all the red cell fractions, with contributions to the overall value that are proportional to their size (Streekstra GJ, A bi plane rheoscoop for the measurement of red cell deformation and orientation in a Couette flow. Thesis, University of Utrecht, August 1994). Since the majority of red cells even in severe malaria is uninfected, this reduction in

RCD results mainly from changes in the unparasitized erythrocytes. The relative unimportance of the parasitized red cells to this measurement is supported by the lack of correlation between parasitaemia and RCD in this study. Moreover the most rigid cells containing the mature parasites are usually sequestered in the microcirculation and are not present in the peripheral blood samples used.

Several prognostic factors have been identified in severe malaria including depth of coma, hyperparasitaemia, the predominance of late stages of parasite development or a high proportion of neutrophils containing malaria pigment, hypoglycaemia, elevated plasma levels of tumour necrosis factor, elevated lactate levels in the blood and cerebrospinal fluid and the severity of acidosis [2, 3, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25]. Multiple logistic regression analysis showed that a markedly reduced mean RCD was the strongest predictor of mortality in this small series. Severely reduced RCD lowers microcirculatory blood flow since red cells have to deform in order to pass through the smaller capillaries. In blood vessels lined by cytoadherent rigid parasitized erythrocytes, there must be considerable luminal obstruction. Yet some flow is often maintained, presumably by even more than usual red cell deformation [3]. Any reduction in the deformability of uninfected erythrocytes would be expected to further compound the microvascular obstruction caused by cytoadherent erythrocytes and intererythrocytic adhesion (rosetting).This effect would be greatest in the tissues of vital organs such as the brain where sequestration is greatest. This mechanism could also contribute to the lactic acidosis by inducing anaerobic glycolysis. Host tissues are quantitatively the most important source of lactate in severe malaria [3, 21]. The predictive value of lactic acidosis for mortality in malaria and the correlation between venous lactate concentrations and reduced mean RCD, could be explained by this causal relation. Reduced RCD is not an epiphenomenon related to direct effects of the lactate ion or acidaemia, as acidification of the suspension medium (to pH=6.9) with lactate does not reduce RCD significantly at any shear stress as measured with the LORCA (M.R. Hardeman, unpublished observation).

The mechanisms underlying the reduction in red cell deformability of uninfected cells in severe malaria are not known. There was no significant increase in RCD during the time of admission, also not shortly after recovery, suggesting irreversible dammage to the uninfected red cell. In this study we could not follow the patients after recovery. We have observed that normalisation of mean RCD in non-immune Dutch travelers with falciparum malaria took 2 to 4 weeks (personal observations). Reduction in RCD was most significant at the lower shear stresses. Changes in the flexibility of the red cell membrane are likely to be an important factor, since RCD at low shear stresses is very susceptible to membrane changes [26]. Nauman et al. have identified a heat labile exoantigen produced by in-vitro cultures of *P. falciparum* which binds reversibly to normal red cells and reduces their deformability [27]. We have also found that soluble products of *P. falciparum* in culture reduce the RCD of

normal erythrocytes (data not shown). Red cell morphology and mean red cell diameter was not an important parameter of red cell deformability in this study. Although RCD is diminished in thalassemia [28], in the present study the level of disturbance is smaller than found in the fatal cases. 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 role of systemic host factors or endothelial cell malfunction in reducing RCD is not known.

If reduced red cell deformability is a cause rather than an effect of potentially lethal organ dysfunction in severe malaria, then measures to correct this abnormality may save lives. Exchange transfusion is widely used in the management of severe malaria, although it has never been clear how it might be of benefit [29]. The parasitized red cells causing pathology, are sequestered and not available for exchange. However, removal of rigid unparasitized cells and their replacement by more deformable new erythrocytes would provide a plausible explanation for the apparent benefit from this treatment. Patients with severely reduced RCD form a subgroup that might benefit from exchange transfusion.

In conclusion this study shows that the mean RCD is an important predictor of, and may be a contributor to mortality in severe falciparum malaria. This reduction in mean RCD is mainly due to a reduction in the RCD of unparasitized erythrocytes.

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