On the pathophysiology of severe falciparum malaria with special reference to red cell deformability
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Reduced red cell deformability in the pathogenesis of severe falciparum malaria and its restoration by blood transfusion

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Submitted for publication
Abstract

Background. Impairment of the microcirculation is a central feature in the pathogenesis of severe falciparum malaria. In addition to the sequestration of erythrocytes containing the mature stages of the parasite, a reduction in red cell deformability (RCD) is thought to contribute to the derangement of microcirculatory flow. Erythrocytes with reduced deformability are also more likely to be cleared from the circulation by the spleen-a process which is augmented in acute malaria, thus contributing to anaemia. We measured RCD in children with falciparum malaria on the Kenyan coast and related this to the severity of disease and anaemia. We also studied the effect of blood transfusion on RCD.

Methods. Using a laser diffraction technique RCD was measured on admission in 232 consecutive patients with falciparum malaria, of whom 99 had severe disease, 28 had severe anaemia (Hb< 5 g/dl) and 41 had high parasitaemia > 5% (both groups without other signs of severe disease), and 57 had non-complicated malaria. This was compared to 18 children with septicaemia or bacterial meningitis and 57 age matched healthy controls. If children received a blood transfusion because of severe anaemia, the effect on RCD was assessed.

Findings. RCD (as elongation index E.I.) at a shear stress of 1.7 Pa was significantly reduced in falciparum malaria, but not in septicaemic patients, and the reduction was in proportion with the severity of the disease (severe disease E.I. (SD)= 0.217 (0.043), uncomplicated malaria E.I. =0.249 (0.030), healthy controls E.I. = 0.268 (0.022). The reduction in RCD, mainly caused by rigidification of the quantitatively more important unparasitized red cells, was greatest in children with a fatal outcome (E.I.= 0.182 (0.048)). All but two survivors with severe malaria and rigid erythrocytes received a blood transfusion which restored RCD in a predictable way. In addition to the relation with severity of disease, RCD (measured at a shear stress of 1.7 Pa) also strongly correlated with the severity of anaemia (all malaria cases: n=232, r=0.582, p<0.001, severe cases: n=99, r=0.602, p<0.001)

Interpretation. In falciparum malaria the deformability of uninfected erythrocytes is disturbed in proportion with the severity of the disease. Reduced RCD may contribute to impaired microcirculatory flow and a fatal outcome in falciparum malaria. RCD can be improved in a predictable way with blood transfusion. Since a severely reduced RCD has a strong predictive value for mortality, blood transfusion possibly improves disease outcome not only through its beneficial effect on anaemia but also on RCD.
Introduction

Severe falciparum malaria has a mortality of around 10% to 20%, even in circumstances where optimal antimalarial and supportive treatment is available. Its pathogenesis is complex and incompletely understood. Both organ specific pathology and generalised metabolic derangement appear to stem from reduced tissue perfusion. Obstruction of the microcirculation by sequestration of erythrocytes containing the more mature stages of the parasite is usually considered a central factor. However, in adult Thai patients with severe falciparum malaria we showed that reduced RCD, which appeared to be mainly due to rigidification of the quantitatively more important unparasitised red cells, was a strong predictor for fatal outcome. In addition we found that RCD at a high shear stress strongly correlated with the severity of anaemia that developed during the course of the disease, suggesting a causal relationship through splenic entrapment and removal of rigid red cells. Over ninety percent of the world's malaria problem is concentrated in Africa where it is mainly a disease of young children, with coma (cerebral malaria), metabolic acidosis and anaemia as its principle manifestations. We report here the relations between disturbed RCD and severity of disease in children with falciparum malaria.

Methods

Patients and study site

The study was performed at the KEMRI unit attached to Kilifi District Hospital, Kilifi, Kenya. The demographic and socio-economic characteristics of this area have been previously reported. Malaria is stably endemic with seasonal peaks related to the rainy season.

The study was approved by the ethical committee of the Kenyan Medical Research Institute (KEMRI). Informed consent was obtained from the patient's attending relatives.

The following groups of children were studied:

Severe malaria. All children with a positive blood film for P. falciparum and one or more of the following clinical indicators of severe malaria were admitted to the Kenya Medical Research Institute (KEMRI) high dependency ward: (i) coma, defined as failure to localise a painful stimulus; (ii) impaired consciousness, defined as a Blantyre coma score less than 5 but able to localise a painful stimulus; (iii) prostration (the inability to sit unaided in older children or breast feed in those less than 1 year); (iv) respiratory distress, defined as the presence of deep breathing, indrawing or nasal flaring in the absence of crackles, bronchial breathing or chest X-ray changes indicating acute respiratory infection.

Inpatient malaria cases with good prognosis. Patients with falciparum parasitaemia accompanied by severe anaemia (Hb ≤ 5 g/dl) or patients with a parasitaemia above 5%,
without further signs of distress were not admitted to the research unit, because of the previously reported low risk of death in this subgroup despite fulfilling the WHO criteria for severe disease.\(^6\)

**Non severe malaria.** Children with uncomplicated falciparum malaria that were seen at the outpatient clinic of Kilifi District Hospital, not fulfilling any of the above criteria.

**Severe non malarial disease.** Children admitted to the KEMRI high dependency ward with culture positive septicaemia or bacterial meningitis.

**Community controls.** Healthy children from rural communities around Kilifi who were involved in a separate cross sectional epidemiological study.

**Procedures**

A detailed medical history and a physical examination was performed on all patients admitted to the hospital. For children admitted to the high dependency ward baseline investigations included: full blood count (Coulter MS2) including mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC) of the red cells, thick and thin blood films Giemsa-stained and counted for asexual forms of *P. falciparum*, venous blood gas analysis (CIBA Corning Diagnostics), venous glucose and lactate (Analox Instruments), creatinine (Beekman Instruments) and haemoglobin electrophoresis. Patients were treated with intravenous quinine followed by pyrimethamine/sulfadoxine (Fansidar\textsuperscript{TM}). Transfusion (20 ml/kg whole blood) was given for severe anaemia (Hb< 5 g/dl) accompanied by either respiratory distress or hyperparasitaemia (≥20% peripheral parasitaemia). All children unable to take sufficient fluids orally were given a minimum of 75 ml/kg/day of intravenous 0.18% saline in dextrose with additional 0.9% saline given to children who were acidic or who had signs of dehydration. Further supportive treatment has been described in full elsewhere.\(^8\) For children seen in outpatients and for the low risk group admitted to the main paediatric ward baseline investigations comprised full blood count and microscopic examination of a thick blood film. Treatment was with oral pyrimethamine sulfadoxine and chloroquine.

Red cell deformability was measured on fresh 25 microlitre samples of whole blood stored in EDTA by ektacytometry using a Laser Assisted Rotational Cell Analyser (LORCA)\(^9,10\). RCD was corrected to a MCV of 80 fl using the regression lines describing the correlation between MCV and RCD in the healthy control group at the different shear stresses as described previously.\(^11,12\) Red cell deformability was assessed at a range of shear stresses from 0.3 Pa to 30 Pa. Shear stresses of 1.5 Pa and above are encountered at the arterial side of the circulation and in the capillaries.\(^13\)

In anaemic children who met the above criteria for transfusion RCD was also measured before and after blood transfusion for severe anaemia. The measured improvement in RCD
after transfusion was compared with the calculated improvement, based on the RCD of the transfused blood and the estimated proportion of the transfused erythrocyte mass compared to the patient's erythrocyte mass.

Statistics

Statistical analysis was performed using SPSS 8.0 statistical package (SPSS Corporation, Chicago, IL, USA). Correlation between normal distributed variables were calculated with the method of Pearson. Comparisons between more than 2 groups were assessed by ANOVA followed by Student's t-test with correction for multiple comparisons (Bonferroni) for normal distributed variables. The method of Mann-Whitney was used for comparison of non normal distributed data. In the analysis of continuous outcome variables that depended on more than one explanatory variable, a multiple regression model was constructed.

Results

Clinical details

A total of 232 children with falciparum malaria were studied. Sixty-four had uncomplicated malaria. Of 69 children in the low risk inpatient malaria group 28 had severe anaemia (Hb≤ 5 mmol/l) and 41 had a parasitaemia over 5% and the data from these children is presented separately in the analysis. Of the 99 malaria patients admitted to the high dependency unit with severe malaria, 40 children had cerebral malaria, 33 had an impaired consciousness but were not comatose, and 26 children were only prostrated. Nineteen (19%) had a parasitaemia above 20% and 28 children (28%) had respiratory distress. In the patients fulfilling the criteria for severe malaria, severe anaemia (Hb≤ 5g/dl) was present in 33 patients (33%) of whom 31 received a blood transfusion. There were 3 very young children who showed HbS/HbF on haemoglobin electrophoresis. All three survived. In the group with severe malaria, no child had a sickle cell trait, whereas 5 children in the group with uncomplicated malaria and 5 in the control group showed this electrophoresis pattern. In the group with severe malaria 11 patients died (11%), all children with cerebral malaria. Clinical and laboratory details of this group are shown in table 1.

Red cell deformability

RCD and severity of disease

All measurements were corrected for MCV 11,12. RCD at a shear stress of 1.7 Pa or 3.0 Pa was significantly reduced in children with falciparum malaria in proportion with the severity of the disease (table 2, fig. 1). The most rigid erythrocytes were found in children with severe malaria. In addition to children with impaired consciousness, prostration or respiratory
<table>
<thead>
<tr>
<th>Variable</th>
<th>Survivors (n=88)</th>
<th>Fatal cases (n=11)</th>
<th>Significance of difference (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>2.4 (1.6)</td>
<td>2.0 (1.1)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>9.8 (3.4)</td>
<td>8.7 (1.8)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Days with fever prior to admission</td>
<td>2.9 (1.5)</td>
<td>2.9 (1.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>38.7 (1.4)</td>
<td>38.2 (1.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Blantyre Coma Scale (median)</td>
<td>3</td>
<td>2</td>
<td>0.001</td>
</tr>
<tr>
<td>Abnormal breathing pattern (%)</td>
<td>26%</td>
<td>36%</td>
<td>n.s.</td>
</tr>
<tr>
<td>Parasitaemia (/μl)</td>
<td>69348 (17414)</td>
<td>78582 (2717)</td>
<td>n.s.</td>
</tr>
<tr>
<td>(geometric mean and coefficient of variance)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinin (μmol/l)</td>
<td>71 (38)</td>
<td>82 (38)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>4.6 (3.0)</td>
<td>7.2 (3.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Base excess (mmol/l) (n=83)</td>
<td>-7.9 (6.1)</td>
<td>-15.3 (7.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.0 (2.8)</td>
<td>4.3 (3.1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>6.5 (2.4)</td>
<td>5.5 (3.1)</td>
<td>n.s.</td>
</tr>
<tr>
<td>WBC (10⁹/μl)</td>
<td>13.6 (8.6)</td>
<td>27.8 (24.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>RCD at SS=1.7 Pa (El)</td>
<td>0.221 (0.041)</td>
<td>0.182 (0.048)</td>
<td>0.004</td>
</tr>
<tr>
<td>RCD at SS=9.5 Pa (El)</td>
<td>0.480 (0.048)</td>
<td>0.463 (0.051)</td>
<td>n.s.</td>
</tr>
<tr>
<td>RCD at SS=30 Pa (El)</td>
<td>0.577 (0.039)</td>
<td>0.566 (0.048)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table 1: Admission clinical and laboratory variables in children with cerebral malaria in Kilifi, Kenya. All data as mean (SD), except where otherwise indicated. RCD= red cell deformability, SS= shear stress, El= elongation index.

Figure 1. Mean (SD) red cell deformability (RCD) on admission in children with malaria compared to children with sepsis/meningitis and healthy age matched controls. El= elongation index.
Reduced red cell deformability in the pathogenesis of severe malaria
distress, RCD was also markedly impaired in children with severe anaemia (Hb≤ 5 g/dl). A
smaller, but still significant reduction in RCD was found in patients with uncomplicated falciparum malaria. In children with severe septicaemia or meningitis RCD was comparable
with the values found in patients with non-severe malaria, although not significantly reduced
compared to healthy controls because of the smaller numbers.

Within the group with severe falciparum malaria, children with a fatal course of their
disease had significantly less deformable erythrocytes than children who survived (table 1, fig. 3). In children with severe malaria there was a significant negative correlation between
the RCD at SS= 1.7 Pa and plasma lactate concentrations at admission (r= -0.36, p<0.01)
and a significant positive correlation with the base excess as calculated by blood gas analysis
(r=0.34, p<0.01).

<table>
<thead>
<tr>
<th>Falciparum malaria</th>
<th>Sepsis or bacterial meningitis</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Severe malaria</td>
<td>Non-severe malaria</td>
</tr>
<tr>
<td></td>
<td>Good prognosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inpatient malaria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe anaemia</td>
<td>High parasitaemia</td>
</tr>
<tr>
<td>n</td>
<td>99</td>
<td>28</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>2.5 (1.9)</td>
<td>1.6 (1.1)</td>
</tr>
<tr>
<td>Hb (SD) (g/dl)</td>
<td>6.4 (2.5)</td>
<td>3.6 (0.9)</td>
</tr>
<tr>
<td>RCD (El) at SS=1.7 Pa</td>
<td>0.217 (0.043)</td>
<td>0.198 (0.030)</td>
</tr>
<tr>
<td>RCD (El) at SS=3.0 Pa</td>
<td>0.317 (0.048)</td>
<td>0.306 (0.035)</td>
</tr>
<tr>
<td>RCD (El) at SS=9.5 Pa</td>
<td>0.490 (0.044)</td>
<td>0.477 (0.046)</td>
</tr>
<tr>
<td>RCD (El) at SS=30 Pa</td>
<td>0.584 (0.036)</td>
<td>0.576 (0.040)</td>
</tr>
<tr>
<td>Hb&lt; 5 g/dl excluded</td>
<td>n=66 excluded</td>
<td>41</td>
</tr>
<tr>
<td>RCD at S S=1.7 Pa</td>
<td>0.233 (0.031)</td>
<td>excluded</td>
</tr>
<tr>
<td></td>
<td>0.256 (0.022)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Mean (SD) red cell deformability (RCD) at different shear stresses (SS) on admission in children with falciparum malaria compared to children with sepsis/meningitis and healthy age matched controls
RCD and anaemia

Children with severe anaemia accompanying their malaria, but no impaired consciousness, also had a severely reduced RCD, comparable to children with cerebral malaria (table 2, fig. 1). This was related to the fact that in all children studied with falciparum malaria a strong and highly significant correlation was present between RCD at admission and haemoglobin levels (fig. 2). This correlation was strongest when RCD was regarded at a shear stress of 1.7 Pa (for all malaria cases: n=232, r=0.582, p<0.001, for severe cases: n=99, r=0.602, p<0.001) but also at higher shear stresses up to 30 Pa. This correlation was not present in the healthy control group. In a stepwise multiple regression analysis with the admission haemoglobin level as dependent variable and RCD (at a shear stress of 1.7 Pa), MCV, the presence of abnormal haemoglobins on electrophoresis, and parasitaemia as parameters, RCD contributed most to the equation (Standardized regression coefficient (Beta)= 0.624, t=10.4 with p<0.001). MCV (Beta= 0.224, t=4.1 with p<0.001) and parasitaemia (Beta= 0.149, t=2.6, p= 0.01) also contributed, giving an overall adjusted R-square of 0.374.

Because of this strong correlation between RCD and anaemia, a subanalysis was made for all patients with Hb values above 5 g/dl in order to evaluate if RCD was related to severity of disease independent of anaemia (table 2). In this subgroup RCD was significantly reduced in children with severe malaria and to a lesser extend, but still significantly in uncomplicated malaria cases.
Reduced red cell deformability in the pathogenesis of severe malaria

Figure 3. Red cell deformability in patients with severe falciparum malaria, according to disease outcome, values before and after transfusion. RCD= red cell deformability, SS= shear stress.

Improvement of RCD by blood transfusion

Blood transfusion had a clear beneficial effect on RCD as is shown in figure 3. The mean improvement in RCD at a SS of 1.7 Pa after blood transfusion was 0.046 (SD 0.030). The RCD after transfusion could be predicted by the RCD of the donor blood and the recipient (RCD after transfusion = 0.60 * RCD (patient blood) + 0.40 * RCD (transfused blood)). This calculated value predicted fairly well the observed RCD after transfusion, with a mean (SD) difference between the observed and calculated value of 0.010 (0.019) giving a 95% limit of agreement of −0.028 to 0.048.

A measured admission RCD at a shear stress of 1.7 Pa below E.I. = 0.20 gave a relative risk for a fatal outcome of 7.1 (95% C.I. 2.1 till 24.8). However, of the 19 surviving patients with very rigid erythrocytes, 17 received a blood transfusion that improved the mean RCD considerably. When these post-transfusion RCD values are considered, a RCD below E.I.=0.20 at a shear stress of 1.7 Pa gave a relative risk for a fatal outcome of 12.8 (95% C.I. 3.8 till 43.6).

Possible causes of reduced RCD

To study the influence of possible unfavourable cell geometry (surface to volume ratio in relation to internal viscosity), RCD was also measured at a low (280 mosm) and a high (320 mosm) osmolality of the PVP suspension medium. However, in the children with severe
malaria the mean RCD deteriorated both at low and high osmolality of the suspension medium (data not shown). Also, MCHC did not correlate with RCD. There was no significant correlation between the parasitaemia at admission and the RCD at any shear stress.

Discussion

We report here a clear correlation between the reduction in mean RCD as measured at admission and severity of disease and disease outcome in children with falciparum malaria. This correlation was strongest at shear stresses that are encountered at the arterial site of the circulation and in the capillaries. Although red blood cells infected with *P. falciparum* parasites become progressively less deformable as the intra-erythrocytic parasites mature, the reduction in mean RCD is mainly caused by the quantitatively more important unparasitized red cells, since the red cell deformability estimate obtained by the LORCA is a summation of the RCD of all the red cell fractions 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). Erythrocytes have to deform to be able to pass through capillaries with a diameter that is smaller than its own. We hypothesise that a severe reduction in mean RCD contributes significantly to the impairment of microcirculatory flow in blood vessels that are already partially blocked by cytoadherent rigid parasitized erythrocytes. Such a reduction in microcirculatory flow is probably the main cause of the metabolic (predominantly lactic) acidosis which is a major predictor of poor outcome in severe malaria. This is supported by the strong correlation between venous lactate concentrations, base excess and reduced mean RCD that we observed.

A reduction in RCD below an E.I. of 0.20 at a shear stress of 1.7 Pa gave a relative risk of 7.1 for a fatal outcome. However, all but two survivors with a RCD below this threshold received a blood transfusion because of concomitant severe anaemia. This procedure improved RCD considerably and in a predictable way. The fact that a severely reduced RCD has a strong predictive value for mortality and RCD can be restored with blood transfusion raises the important possibility that blood transfusion in this study improved disease outcome not only through its beneficial effect on anaemia but also on RCD.

In addition to the relation between RCD and severity of disease, this study shows a clear and strong correlation between the mean RCD of the erythrocytes and the severity of anaemia in children with severe falciparum malaria. In a multiple regression analysis RCD was the best predictor of the haemoglobin levels of all laboratory parameters included. In severe malaria anaemia develops rapidly, resulting largely from accelerated destruction of both parasitized and unparasitized red cells. Labeling studies have shown rapid clearance
of uninfected red cells by the spleen. We hypothesise that rigid unparasitized erythrocytes are filtered by the spleen and in this way removed from the circulation. In our previous studies in Thai adults with severe falciparum malaria the strongest correlation between RCD and anaemia was found at higher shear stresses. By contrast the maximum effect in this study was at more "physiological" levels of shear. Lower levels of shear might be present in spleens of children, that have a different histological architecture. This could also be an explanation for the more severe anaemia observed in children since changes in the red cell membrane will first affect RCD at the lower shear stresses.

The mechanism by which RCD is reduced in malaria is not known. Shrinking or swelling of the erythrocytes by changing the osmolality of the suspension medium did not improve RCD in patients with severe falciparum malaria, suggesting that an unfavourable cell geometry is not the cause of rigidification of the unparasitized red cells. It is therefore more likely that this is due to changes in the red cell membrane. 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. Preliminary data show that plasma from patients with acute falciparum malaria mixed with healthy donor red blood cells, can rigidify these cells (data not shown), but the exact mechanism remains to be elucidated. In conclusion: mean RCD in falciparum malaria is reduced in proportion to the severity of the disease, and is a strong predictor for mortality. In addition reduced RCD correlates with the severity of anaemia. We suggest that the quantitatively important rigidified uninfected red cells contribute to impaired microcirculatory flow in addition to the sequestered parasitized erythrocytes. Blood transfusion improves RCD and possibly improves disease outcome not only through its beneficial effect on anaemia but also on RCD. Further research to elucidate the mechanisms leading to rigidification of the unparasitized red cells is needed to come to rational therapy aiming at improving RCD.

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