On the pathophysiology of severe falciparum malaria with special reference to red cell deformability
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Low resistance to shear stress of *Plasmodium falciparum* rosettes questions their pathophysiological relevance

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Abstract

Rosette formation in falciparum malaria has been proposed as an important pathophysiological mechanism in the sequelae leading to obstruction of the microcirculation in severe and in particular cerebral malaria. However, rosetting of uninfected red cells to parasitised erythrocytes is an in vitro phenomenon. The pathophysiological significance will depend on their ability to withstand shear forces that are encountered in the human circulation. In this study rosettes formed by 3 different strains of P. falciparum were subjected to increasing, well defined, shear stresses. Very low shear stresses of 0.1 and 0.5 Pa, representative of those encountered in post-capillary venules, disrupted 48% and 62% of the rosettes formed, respectively. At a shear stress of 2 Pa, as encountered at the arterial side of the circulation, 91% of all rosettes were disrupted. At higher shear stresses, as expected in the capillaries, practically no rosettes remained stable. The results are in contrast with earlier reports claiming that rosettes are much more resistant to shear stress. The probable causes of this discrepancy are discussed. This finding questions the rheological significance of rosette formation in the pathogenesis of microvascular obstruction in severe falciparum malaria. However, it can not be excluded that rosettes under physiological conditions, suspended in human plasma, are more resistant to physiological shear forces.
Introduction

The pathological hallmark of severe falciparum malaria is the sequestration of *P. falciparum*-infected red cells in the microvasculature of the brain and other vital organs, causing obstruction and reduction of microvascular blood flow \(^1,2,3\). Several other factors can contribute to this microvascular obstruction. We have suggested the importance of reduced red cell deformability of mainly the uninfected erythrocytes as an important factor, since this phenomenon was strongly correlated with mortality \(^4\). The formation of rosettes has also been proposed as an important factor in the pathophysiology of severe, in particular cerebral, falciparum malaria \(^5\)–\(^9\), although the clinical relationship between rosette formation and disease severity has been questioned \(^10\)–\(^11\). Rosetting is defined as the adherence of two or more uninfected red cells to a *P. falciparum*-infected red cell containing a mature parasite (trophozoite to schizont stage). Their possible role will depend on their ability to withstand shear stresses that are encountered in the human circulation. The Laser-assisted Optical Rotational Cell Analyser (LORCA) \(^12\) provides the opportunity to apply a well defined shear stress on an erythrocyte suspension. The aim of this study was to determine whether the adherent properties which manifest *in vitro* as rosette formation would be able to resist shear stresses encountered in the circulation.

Material and methods

Parasite culture

Three Thai *P. falciparum* strains, TM267R (laboratory strain), MHRP11 (wild isolate), and P98-5 (wild isolate) were maintained in O positive human red cells, using malaria culture medium (RPMI-1640 medium supplemented with 25 mM of HEPES; pH 7.4, 10% non-immune human AB serum and 1 mg/ml of gentamicin) \(^13\). The parasites were cultured at 37°C in 5% CO\(_2\) plus air. The culture was continued until the parasites matured to the trophozoite stage. Rosettes were determined under light microscopy by dropping 20 ml of parasite culture on a glass slide with a cover slip and the number of rosettes was expressed as the percentage of rosettes per 100 infected red cells.

Rosette disruption under shear stress conditions

A well defined shear stress was applied to the erythrocyte suspension in culture with the use of the LORCA (Mechatronics, Hoorn, The Netherlands). This machine consist of two concentric cylinders with a well defined gap width. The cell suspension can be transferred to this gap. The outer cylinder rotates at very constant speeds. Knowing the gap width, the rotation speed and the viscosity of the red cell suspension in the culture medium, the
applied shear stress exerted on the erythrocytes can be calculated accurately. The viscosity of the cell suspension was measured with a Contraves LS30 viscometer (Contraves Ltd, USA) at 37°C. A stepwise increased shear stress was applied to the erythrocyte suspension containing the rosettes. After 1 minute the machine was stopped and a sample was taken from the gap and the percentage of rosette formation was determined immediately microscopically on a glass slide and expressed as the percentage disruption in comparison with the static situation. The applied shear stress was increased stepwise and the procedure repeated.

In a second experiment the culture medium was 1:1 (v/v) mixed with Dextran with a MW of 40.000 (Sigma Chemical Co Ltd, Poole, U.K.) and a concentration of 50% (w/v) to increase the viscosity of the suspension medium as described previously \[^{14}\], and the same procedure was repeated as described above. As a control experiment uninfected red cells were resuspended in either RPMI or RPMI 1:1 mixed with 50% Dextran MW 40.000. The red cells were subsequently exposed to the same shear stresses as described above, and the number of aggregates were determined microscopically. Besides, red cell aggregation of the same suspensions were measured with a LORCA aggregometer as described previously \[^{12}\].

Results

Parasitaemias of the parasite cultures prepared for the assays were between 3% and 7%. The median percentage (range) of rosettes of strain TM267R, MHRP11 and P98-5 was 50% (48 to 52%), 45% (42 to 48%) and 35% (33 to 37%), respectively. Aggregation (rouleaux formation) of the red cells could be distinguished easily from rosettes by microscopic examination.

The viscosity of the red cell suspension in RPMI with 10% human serum was 0.89 mPa.s. Herewith the applied shear stresses could be calculated. Rosettes suspended in culture medium were easily disrupted at low shear stresses in all three rosette forming strains studied (figure 1). At a shear stress of 0.1 Pa, 48% (18%) of the rosettes formed were disrupted (mean, SEM). At shear stresses of 0.5, 1 and 2 Pa the percentage disruption of the rosettes (mean, SEM) was 62% (19%), 81% (11%) and 91% (6%), respectively. At shear stresses of 3 Pa and above almost no rosettes could be observed.

A 50% dextran 40 (MW 40.000) solution added 1:1 (v/v) to the culture medium (supplemented with 10% human serum) increased the viscosity of the medium to 7 mPa.s. Dextran 40 had no effect on rosette formation under static conditions: median (range) rosette formation was 50% (47% to 53%) before, and 49% (47% to 51%) after resuspension in the dextran solution. Also, dextran 40 did not promote the aggregation of red cells,
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both under static conditions on a glass slide or as measured with a LORCA aggregometer (data not shown). However, dextran 40 did make rosettes considerably more resistant to shear stresses (figure 1). When the erythrocyte suspension containing dextran 40 were subjected to a high shear stress of 7 Pa, 57% (SEM 16%) of the rosettes were being disrupted.

**Figure 1.**
Percentage disruption (compared to the static situation) of rosettes subjected to increasing levels of shear stress. Values are mean (SEM) of 3 experiments in 3 different rosette-forming strains of *P. falciparum*.
- • = rosettes suspended in malaria culture medium containing 10% serum.
- ■ = rosettes suspended in 50% dextran 40 (MW 40,000) solution added 1:1 (v/v) to the culture medium

**Discussion**

Rosette formation is an *in vitro* phenomenon. Falciparum malaria parasites have to be cultured *ex vivo* in order to reach the mature trophozoite stages at which rosette formation occurs. The *in vivo* significance of rosetting is therefore difficult to assess. The pathophysiological significance of rosetting will depend on their resistance to shear forces that are encountered in the human circulation. The shear stresses encountered at the arterial side of the circulation and the capillaries are high (around 1.5 up to 15 Pa.), whereas those in the post-capillary venules are much lower, 0.1-0.5 Pa. The pathophysiological significance of rosetting will depend on their resistance to shear forces that are encountered in the human circulation. The shear stresses encountered at the arterial side of the circulation and the capillaries are high (around 1.5 up to 15 Pa.), whereas those in the post-capillary venules are much lower, 0.1-0.5 Pa. Lower shear forces result from lower flow velocities and the greater vessel diameters at the venular side.
of the circulation. Nash et al. have reported that rosettes were able to withstand shear forces up to 1.6 Pa. However, in their study rosettes were suspended in dextran MW 40,000 to increase the viscosity of the suspension medium and as we show in the present study, the addition of dextrans increases the resistance of *P. falciparum* rosettes to shear stress considerably. Being large molecules, dextrans with a MW above 50,000 are known to induce red cell aggregation by forming bridges between the adjacent red cells depending on their molecular weight and concentration. Although dextran MW 40,000 does strengthen rosettes, it is not capable of inducing aggregation, as shown in this and previous studies.

In the absence of dextrans, we found that shear stresses similar to those encountered in the post capillary venules were sufficient to disrupt more than 50% of rosettes suspended in culture medium containing 10% serum. This could be related to the composition of culture medium that is quite different from human plasma. However, the fragility of rosettes suspended in human plasma is hard to assess because of disturbing red cell aggregation (rouleaux formation) in the presence of fibrinogen. In all clinical studies relating rosette formation to severity of disease, rosettes suspended in malaria culture medium (containing 10% serum) are studied. The pathophysiological significance of rosettes assessed in this way can be questioned.

This would be in accordance with several clinical studies that did not find a correlation between the occurrence of a rosette forming strain and disease severity. Moreover, not only *P. falciparum*-infected red cells adhere to the uninfected red cells but also *P. ovale*, *P. malariae* and *P. vivax*-infected red cells form rosettes; infections without significant compromise of the microcirculation. This makes an important pathophysiological role unlikely. In conclusion, this study shows that rosettes formed in an *in vitro* culture of *P. falciparum* are easily disrupted at low shear stresses. This questions the *in vivo* rheological significance of rosette formation in the pathogenesis of microvascular obstruction in severe falciparum malaria. However, strengthening of rosettes by plasmatic factors in the *in vivo* situation can not be excluded.

References

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