Effects of Malaria Heme Products on Red Blood Cell Deformability

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Abstract. In falciparum malaria, the deformability of the entire erythrocyte population is reduced in proportion to disease severity, and this compromises microcirculatory blood flow through vessels partially obstructed by cytoadherent parasitized erythrocytes. The cause of rigidity of uninfected erythrocytes in known but could be mediated by malaria heme products. In this study, we show that red blood cell deformability (RBC-D), measured by laser-assisted optical rotational cell analyzer, decreased in a dose-dependent manner after incubation with hemin and hydrogen peroxide but not with hemoglobin or β-hematin. Hemin also reduced mean red cell volume. Albumin decreased and N-acetylcysteine (NAC) both prevented and reversed rigidity induced by hemin. Hemin-induced oxidative damage of the membrane seems to be a more important contributor to pathology than cell shrinkage because the antioxidant NAC restored RBC-D but not red blood cell volume. The findings suggest novel approaches to the treatment of potentially lethal malaria.

INTRODUCTION

Obstruction of the microcirculation is central to the pathogenesis of severe falciparum malaria. Autopsy studies show extensive blockage of the microcirculation by sequestered red blood cells containing mature parasites in the latter two-thirds of their intra-erythrocytic development. Flow through partially occluded blood vessels is critically dependent on the rigidity of circulating erythrocytes because these cells must deform considerably to squeeze through the narrowed capillary lumens with cross-sectional areas much less than their own. Although malaria parasites invade red blood cells (RBCs), the majority of erythrocytes, even in lethal malaria, are unparasitized. We have shown previously that the entire red cell population, comprising mainly unparasitized RBCs, becomes rigid in patients with severe malaria, but that this does not occur in bacterial sepsis, and that the degree of reduction in RBC deformability (RBC-D) has a predictive value for a fatal outcome in both adults and children with falciparum malaria in Asia and Africa. In addition, reduced RBC-D is a major determinant of increased splenic clearance of uninfected RBCs during malaria infection, a major contributor to malarial anemia. Anemia is an important cause of death from malaria in young children. Thus reduced RBC-D is a central pathologic process in severe falciparum malaria. Whereas the rigidification of parasitized cells has been characterized in detail, the mechanisms responsible for the loss of deformability in uninfected erythrocytes have not been elucidated.

During maturation, the parasite digests hemoglobin of the host cell, and the toxic heme moiety crystallizes into hemozoin, the malaria pigment, which can be synthesized in vitro as β-hematin. These malaria heme products are released when the mature schizont ruptures at the end of the erythrocytic cycle. In the present study, we investigate the effects of malaria heme products on RBC-D and examine the protective potential of the antioxidant N-acetylcysteine (NAC) in comparison with reduced glutathione as antioxidant. A randomized clinical trial has reported the potential benefit of NAC as adjunctive treatment in severe malaria. The effects of co-incubation of hemin and albumin, as well as hydrogen peroxide alone and in combination with β-hematin, were also studied, as albumin strongly binds heme, and hydrogen peroxide liberates significant amounts of hydroxyl radicals (Fenton reaction) in the presence of Fe²⁺. Hydrogen peroxide is produced during acute malaria infection by activated polymorphonuclear leukocytes and other phagocytic cells.

MATERIALS AND METHODS

β-Hematin, hemin, hemoglobin, NAC, reduced glutathione, hydrogen peroxide, and albumin. β-Hematin was a gift from Dr. D. Monti (ISTM-CNR, Milan, Italy). It was synthesized from a solution of hematin precipitated by the addition of acetic acid. Unreacted hematin was removed by extracting the precipitate twice for 3 hr in 0.1 M sodium carbonate buffer, pH 9.1. The purity of the final product was controlled by infrared spectroscopy. Because β-hematin is insoluble in water, a stack of β-hematin was suspended in phosphate-buffered saline (PBS) by sonication (B. Braun, Degersheim, Switzerland) at 300 Hz for 10 minutes just before each experiment. Hemin (as bovine hemin chloride, Sigma-Aldrich, St. Louis, MO) and hemoglobin (Oxoid Ltd, Basingstoke, England) were dissolved in 0.1 M NaOH/PBS (1:10 v/v). To make the experiments comparable, the concentrations were calculated in heme equivalents, quantified by dissolving an aliquot of the heme products in 1 N NaOH and reading the absorbance at 385 nm.

NAC was obtained from Celltech Group PLC (Slough, England) as Parvolex for injection, containing 20% NAC w/v. The drug was dissolved in sterile distilled water (1:4) before use. Reduced glutathione (Sigma-Aldrich) was dissolved in PBS shortly before use. Hydrogen peroxide (Merck KGaA, Darmstadt, Germany) 35% (v/v) was diluted 10-fold in distilled water and further diluted in PBS just before use. Albumin (Sigma-Aldrich) was dissolved in PBS to a final concentration of 20 g/L (303 μM).
Red blood cell deformability. RBC-D was measured by ektacryometry using a laser-assisted optical rotational cell analyzer (LORCA, Mechatronics, Hoorn, The Netherlands). With this method, a defined shear stress is applied to a RBC suspension in a viscous medium (5% polyvinylpyrrolidone in PBS buffer) at a constant temperature of 37°C, in a small gap between two concentric rotating cylinders. A laser beam directed through the erythrocyte suspension forms an elliptic diffraction pattern, which is directly proportional to the mean ellipticity of the RBC population. The unit of deformability is “elongation index” (EI) defined as the length of the long axis minus the short axis divided by the length of the long axis plus the short axis of the deformability pattern. 

RBC-D was assessed over a range of shear stresses. A shear stress of 1.7 Pa corresponds to those encountered in capillaries; at this level of shear, the RBC-D depends mainly on membrane rigidity, internal viscosity, and surface-to-volume ratio. A shear stress of 30 Pa is supraphysiologic, but it gives information on the contribution of cell geometry, especially changes in surface-to-volume ratios, in RBC-D.

Blood sample preparation. Fresh blood from healthy donors was centrifuged at 2,500 rpm at 4°C for 5 minutes, and plasma anduffy coat were removed. The same donors were used for each series of experiments. All experiments were performed 5 times, except where otherwise indicated. Packed RBCs were resuspended to a hematocrit of 5% in PBS alone (control) or containing β-hematin, hemin, or hemoglobin in different concentrations. The effects of co-incubation of hemin with either 2% albumin or different concentrations of hydrogen peroxide were studied using the same experimental setup. In a different group of experiments, NAC was added to the suspension medium over a range of concentrations, either simultaneously or 2–4 hr after start of incubation with β-hematin or hemin.

RBC-D in each sample was measured repeatedly up to 8 hr after the start of incubation. In every experiment, a blood smear was prepared on a glass slide to assess erythrocyte morphology. Mean red cell volume (MCV) and mean red blood cell hemoglobin content (MCH) were assessed by an ADVIA 120 hematology system (Bayer HealthCare LLC, Diagnostics Division, Tarrytown, NY).

Statistical analysis. RBC-D of the treated erythrocytes was expressed as percentage of control. Multiple groups and observations over time were compared by analysis of variance, followed by post-hoc comparisons between groups with Bonferroni correction for multiple comparisons, using SPSS statistical software package 11.0 (SPSS Inc., Chicago, IL). Paired data were analyzed by the paired t test.

RESULTS

The deformability of RBCs of healthy donors (N = 10), expressed as mean (SD) elongation index (EI), was 0.250 (0.06) at 1.7 Pa and 0.580 (0.03) at 30 Pa.

Effects of hemoglobin, β-hematin, and hemin on RBC-D. The effects of hemoglobin, hemin, and β-hematin in concentrations of 75, 150, and 300 μM on RBC-D was assessed after 4 hr of incubation (Figure 1). At concentrations up to 300 μM, neither hemoglobin nor β-hematin affected RBC-D significantly. Hemin (Fe²⁺-protoporphyrin IX) decreased RBC-D in a concentration- and time-dependent manner (Figures 1 and 2), irrespective of the blood donor (P < 0.001 by ANOVA). The rigidifying effect of hemin observed at high shear stress (30 Pa) was less pronounced: after 4 hr of incubation with 75 μM hemin, the mean (SD) reduction in RBC-D was 12% (6%) of control; this compared with a mean (SD) 23% (6%) reduction in RBC-D at a shear stress of 1.7 Pa. For further experiments, a hemin concentration of 75 μM was chosen, because higher concentrations induce significant hemolysis.

Inhibition of the effects of hemin on RBC-D by NAC and glutathione. Co-incubation of hemin (75 μM) with NAC in 3 different concentrations ranging from 0.06 to 0.6 mM, at a shear stress of 1.7 Pa, largely prevented in dose- and time-dependent ways the rigidification of the RBCs (P < 0.001 for dose and time by ANOVA). This effect became apparent
after 2 hr of incubation and was most prominent after 4 hr (Figure 2). The effect was also observed at high levels of shear stress (data not shown).

Similarly to co-incubation with NAC, reduced glutathione also prevented rigidification of RBCs by hemin (75 μM). After 4 hr of co-incubation, glutathione at 0.2 mM improved RBC-D [as % from control, mean (SD)] from 86.5% (7.8%) up to 95.2% (3.7%, \( P = 0.34 \)) and 96.6% (7.2%, \( P = 0.43 \)), respectively.

Because RBC-D as measured by ektacytometry depends on the viscoelastic properties of the membrane as well as on RBC geometry, changes in mean blood cell volume (MCV) induced by hemin were assessed, as were the modifying effects of NAC on this. After 4 hr of incubation with hemin at concentrations of 37.5 and 75 μM, MCV decreased [mean (SD)] 4.5% (0.6%) and 7.8% (1.3%), respectively (\( P < 0.001 \)). A similar decrease in MCV was observed after 1 hr of incubation. The heme-induced change in MCV was not prevented by co-incubation with NAC. After 4 hr, the mean (SD) MCV was 89.8% (0.9%) of control values for co-incubated RBCs versus 91.5% (0.6%) of RBCs incubated with hemin alone (\( P = 0.06 \)). Co-incubation of hemin (75 μM) with reduced glutathione at 0.2 or 2 mM also did not restore MCV: at 0.2 mM, the MCV [mean (SD)] was 91.0% (0.8%) of control (\( P = 0.47 \)), and at 2 mM, the MCV was 94.2% (3.1%) of the control value (\( P = 0.38 \)).

Reversal of the effects of hemin on RBC-D by NAC. To explore if NAC could not only prevent but also reverse the rigidifying effects of hemin on RBC-D, NAC (0.6 mM) was added together with hemin at 2 and 4 hr after the start of incubation with hemin (Figure 3). NAC was able to reverse the rigidifying effects of hemin, even when added 2 or 4 hr after the start of incubation with hemin (\( P < 0.0001 \) by ANOVA). The timing of the addition of NAC (0, 2, or 4 hr after hemin) did not significantly affect the extent of restoration in RBC-D.

Modifying effect of albumin on deformability of erythrocytes exposed to hemin. Because human plasma contains a significant concentration of albumin and albumin is a strong hemin-binding protein, we assessed the modifying role of albumin on the rigidifying effects of hemin. Incubation with albumin for 4 hr improved RBC-D by (mean, SD) 13.7%, 0.1% (\( P < 0.001 \)). The simultaneous incubation of hemin (75 μM) with 2% albumin (303 μM) partly prevented the rigidifying effects of hemin. The mean (SD) RBC-D (as % of control) after co-incubation with albumin was 89.1% (8.6%), compared with 75% (8%) after 4 hr of incubation with hemin alone (\( P < 0.01 \)).

Co-incubation with albumin prevented hemin-induced reduction in MCV. The MCV after 4 hr of co-incubation of hemin (75 μM) with albumin was not different from control: mean (SD), 100.4% (1.1%) (\( P = 0.43 \)), compared with a reduction in MCV to 91.5% (0.6%) in RBCs incubated with hemin alone (\( P < 0.001 \)).

Effect of hydrogen peroxide and antioxidants on RBC-D. Hydrogen peroxide is produced by host leukocytes during acute malaria infection and potentially increases the oxidative effects of ferric ions in β-hematin on the RBC membrane through the production of lipid peroxyl species and derived hydroperoxides.\(^{15} \) Hydrogen peroxide alone, at concentrations ranging from 100 to 1,000 μM, reduced RBC-D in a dose-dependent manner (\( P < 0.001 \) for \( \text{H}_2\text{O}_2 \) concentration by ANOVA). After 4 hr of incubation, the mean (SD) of RBC-D was reduced to 95.8% (10.8%) with 100 μM \( \text{H}_2\text{O}_2 \) and to 88.1% (7.2%) of control with 1,000 μM \( \text{H}_2\text{O}_2 \) (\( P = 0.01 \)) (Figure 4). In contrast with hemin, \( \text{H}_2\text{O}_2 \) at concentrations from 100 to 1,000 μM did not significantly reduce MCV: at 1,000 μM \( \text{H}_2\text{O}_2 \), the mean MCV was 96.8% (SD 3.0%) (\( P = 0.27 \)).

To further investigate whether the rigidifying effects of hydrogen peroxide are indeed mediated by its oxidative properties, we added the antioxidants NAC and reduced glutathione. NAC at 0.06 and 0.6 mM, as well as glutathione at 0.2 and 2 mM, significantly reduced the rigidifying effects of hydrogen peroxide at 100, 500, and 1,000 μM (Table 1).

Effects of β-hematin or hemin in combination with hydrogen peroxide on RBC-D. Addition of β-hematin (150 μM as heme equivalents) did not further decrease RBC-D in the hydrogen peroxide-treated RBCs (Figure 4). RBC-D 4 hr after co-incubation of β-hematin and 1,000 μM hydrogen peroxide was 83.8% (11.0%), not different from incubation with
hydrogen peroxide alone ($P = 0.31$). Similarly, RBC-D (as mean % of control, SD) after co-incubation for 4 hr with 75 μM hemin and H$_2$O$_2$ at 100 μM (88.7%, 6.9%), 500 μM (90.5%, 4.6%), and 1,000 μM (88.5%, 4.9%) was not significantly different from incubation with hemin alone (87.9%, 5.9%).

### DISCUSSION

During intra-erythrocytic growth, *Plasmodium falciparum* ingests and digests 60–80% of the host cell’s hemoglobin, its major source of nutrition.15,16 In this digestive process, heme is produced as a toxic oxidative byproduct. Heme (Fe$^{3+}$ protoporphyrin IX) is to a large extent detoxified in the acid food vacuole by demerization and crystallization to the brown black malaria pigment or hemozoin, where it can be readily seen under light microscopy.7,17 Inhibition of heme detoxification is central to the action of antimalarial drugs such as chloroquine.18 Hemozoin is released into the circulation at the moment of schizont rupture. As much as 0.2–20 g of hemozoin can be produced per 48-hr asexual cycle of the infecting *P. falciparum* population. Phagocytosis of hemozoin has been shown to mediate immunosuppression by inhibiting dendritic cell activity19 and to modulate the production of cytokines.20 In addition, through adhesion to the membrane, β-hematin can exert a pro-oxidant activity, although its oxidative properties are much less than those of free hemin, through steric hindrance of the heme moiety, making it less available to participate in redox reactions.14,21,22

In this study, we did not find a significant effect on RBC-D of the synthetic malaria pigment, β-hematin, even at high concentrations. This is consistent with findings from previous studies showing that β-hematin in intact erythrocytes was not oxidative toward erythrocyte membrane protein sulphydryl groups and did not cause lipid peroxidation.14 An earlier report showing a slight decrease in RBC-D at high concentrations of β-hematin can be explained by the release of free hemin from β-hematin in the presence of hydroxide.23 We also investigated whether β-hematin in the presence of hydrogen peroxide reduces RBC-D. During acute infections, including malaria, activated neutrophils and monocytes produce O$_2^-$ (superoxide) by the one-electron reduction of oxygen at the expense of NADPH, and most of this O$_2^-$ reacts to form hydrogen peroxide.24 Hydrogen peroxide generates hydroxyl free radicals in the presence of Fe$^{3+}$ through the Fenton reaction, which can damage the erythrocyte membrane.25

### TABLE 1

<table>
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<th>[H$_2$O$_2$] (μM)</th>
<th>No antioxidant</th>
<th>N-Acetylcyesteine</th>
<th>Glutathione</th>
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<td>0.6 mM</td>
<td>0.2 mM</td>
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<td>94.8%</td>
<td>101.5% †</td>
<td>97.1% †</td>
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<td>(2.0%)</td>
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<td>99.3% †</td>
<td>97.9% †</td>
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<td>(1.5%)</td>
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<td>98.9% †</td>
<td>96.9% †</td>
</tr>
<tr>
<td></td>
<td>(3.2%)</td>
<td>(0.7%)</td>
<td>(2.1%)</td>
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† $P < 0.05$.

In our *in vitro* system, hydrogen peroxide reduced RBC-D in a dose-dependent manner, but the effect was only significant at high concentrations that are unlikely to be reached *in vivo*. The addition of hydrogen peroxide to β-hematin did not result in an additional decrease in RBC-D, which makes it unlikely that β-hematin is affecting RBC membrane deformability, even in the presence of H$_2$O$_2$. In spite of this, previous studies showed that, after exposure to β-hematin, erythrocytes destabilize and become more sensitive to hemolytic agents such as H$_2$O$_2$ or hypotonic medium.14 Hemoglobin is released at schizont rupture. Massive hemolysis of uninfected erythrocytes occurs in “black water fever,” which occurs in ~8% of adults with severe malaria treated with quinine or artesunate.26 The extent of intravascular hemolysis in malaria patients without overt black water fever has not been well documented, but free hemoglobin concentrations may reach the millimolar range.27 Although hemoglobin is potentially oxidative through its heme moiety, in our study hemoglobin did not significantly reduce RBC-D, even at high concentrations. Free heme is also released into the circulation at schizont rupture, although this has not been quantified accurately. In normal conditions, heme-binding proteins such as hemopexin and albumin will remove most of the intravascular-released heme,28 but in pathologic situations of increased hemolysis, as in malaria, high plasma concentrations up to 20 μM have been proposed, although it should be noted that this does not represent free hemozine.29 Physiologically relevant is the free heme concentration, which is not known in patients with severe malaria. The free heme concentration used in this study is likely to be higher than achieved *in vivo*. In our study, hemin reduced RBC-D in a concentration-dependent manner. Previous studies have shown a dose- and time-dependent incorporation of hemin into intact erythrocytes, causing mechanical disruption of the erythrocyte membrane, oxidation of sulphydryl groups, and to a lesser extent lipid peroxidation.14 Membrane lipid peroxidation can cause cross-linking of membrane components, leading to decreased membrane deformability.30 Malonyldialdehyde (MDA), a secondary product of lipid peroxidation, is able to rigidify RBCs in micromolar concentrations through cross-linking of membrane components containing amino groups.31 Peroxidant injury initiated in the membrane can be extended to the membrane skeleton proteins, notably spectrin. Oxidation of its sulphydryl groups reversibly cross-links spectrin via disulfide bonds, thereby reducing RBC-D.32 An additional finding of our study was that hemin also reduced erythrocyte volume (MCV) without changing the intracellular hemoglobin content (MCH). This volume change affects the surface area-to-volume ratio of the erythrocyte, causing a decrease in deformability.33 Oxidative agents can cause cell shrinkage through stimulation of the Ca$^{2+}$-dependent Gardos channel.34 This increases the passive K$^+$ permeability, which will cause the loss of K$^+$ together with H$_2$O from the erythrocyte. Hemin also impairs the erythrocyte’s ability to maintain cation gradients.35 However, in our study, incubation of erythrocytes with the oxidant H$_2$O$_2$ did not significantly reduce MCV, and the addition of the antioxidants (NAC or glutathione) did not reverse the shrinking effects of hemin on the erythrocytes. The effect of hemin on MCV thus cannot be explained only by an oxidative mechanism; mechanical damage to the membrane could also play a
The detrimental effects of hemin on RBC-D could be prevented and reversed by the addition of the antioxidants NAC or reduced glutathione to the suspension medium. This is consistent with an *ex vivo* study in patients with noninsulin-dependent diabetes showing that NAC can counteract the oxidative changes in the spectrin cytoskeleton of erythrocytes. The antioxidant effects of NAC depend on its reducing thiol group, and rate constants depend on the oxidants that are scavenged. In our experiments, the maximum effect was reached within 4 hr of incubation. The concentrations used in the experiments are high but are in the expected range achieved during treatment with high-dose NAC, as recommended in paracetamol poisoning, where plasma concentrations between 0.2 and 0.5 mM are reached. The findings of this study suggest a promising new option for therapeutic intervention, aimed at improving microcirculatory flow in severe malaria and preventing RBC destruction. A pilot study in 30 patients with severe malaria on the Thai–Burmese border showed that NAC reduced plasma lactate clearance times by half. Plasma lactate is a crude measure of vital organ oxygenation and thus reflects the microcirculatory status; it is a strong predictor for mortality in severe malaria. Currently, a large and more detailed study evaluating the effects of NAC on RBC-D, microcirculatory flow, and outcome in patients with severe malaria is underway.

In conclusion, this study shows that hemin can compromise the physiologically important deformability of erythrocytes. The effect is mediated partly through a reduction in cell volume but more importantly through oxidative damage to the erythrocyte membrane. The antioxidants NAC and glutathione can restore the rigidifying effects of hemin. This might provide new modalities of intervention in patients with severe malaria, where RBC-D is decreased in relation to disease severity.

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