Excess Release of Ferriheme in G6PD-Deficient Erythrocytes: Possible Cause of Hemolysis and Resistance to Malaria

By Stephen K. Janney, J. Heinrich Joist, and Coy D. Fitch

Hemoglobin in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes is abnormally vulnerable to oxidative denaturation, which may release ferriheme, a known cytolytic agent. We found 13.3 nmol of ferriheme in G6PD-deficient erythrocyte membranes (per gram of total erythrocyte hemoglobin) using a spectrophotometric assay, as compared to 9.8 in normal membranes (P < .05). After incubation of erythrocytes with 260 μmol/L menadione, an oxidant drug, the values increased by 37.4 nmol in G6PD-deficient membranes and by 26 in normal membranes (P < .005), indicating increased hemoglobin denaturation. To verify that hemoglobin denaturation in G6PD-deficient erythrocytes releases ferriheme in a form available to interact with other ligands, [14C]-chloroquine binding to intact erythrocytes was measured. With an initial concentration of 5 μmol/L chloroquine in a medium containing no menadione, an excess of 14.8 nmol of chloroquine was bound in G6PD-deficient erythrocytes (per gram of hemoglobin) as compared to normal erythrocytes (P < .005). In the presence of 250 μmol/L menadione, chloroquine binding increased by 17.9 nmol in G6PD-deficient and by 7.2 in normal erythrocytes (P < .005). These results indicate that ferriheme becomes available to interact with endogenous ligands and, thus, to mediate menadione-induced hemolysis in patients with G6PD deficiency. Furthermore, the increase in ferriheme may mediate the selective toxicity of menadione for Plasmodium falciparum parasites growing in G6PD-deficient erythrocytes. Ferriheme release in response to the intraerythrocytic oxidant stress introduced by malaria parasites also may account for the resistance to malaria afforded by G6PD deficiency.

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When Plasmodium falciparum infects glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes, parasite maturation is delayed and impaired. Presumably, the oxidant stress introduced by the parasite is sufficient to overwhelm the antioxidant capacity of G6PD-deficient erythrocytes, leading to the accumulation of free radicals and other toxic substances. Ferriheme may be one of these toxic substances. Hemoglobin in G6PD-deficient erythrocytes undergoes denaturation during oxidant stress. Oxidative denaturation may release ferriheme, and ferriheme is a potent lytic agent for erythrocytes and malaria parasites. The term ferriheme is used to identify iron(III) protoporphyrin IX. Both hemin, which is iron(III) protoporphyrin IX chloride, and hemat, which is iron(III) protoporphyrin IX hydroxide, are potent hemolytic agents.

In this study, we examined ferriheme release in erythrocytes in the presence or absence of the oxidant drug, menadione. This drug precipitates hemolytic episodes in patients with G6PD deficiency, and it accentuates the inhibition of P. falciparum parasite development in G6PD-deficient erythrocytes. Moreover, high concentrations of menadione release sufficient amounts of ferriheme in normal erythrocytes to cause hemolysis. When ferriheme is released intracellularly or added exogenously, it is available to interact with cellular constituents, to lyse erythrocytes, and to bind chloroquine with high affinity (Kd of 10^-6 to 10^-7 mol/L). In mature mammalian erythrocytes, chloroquine does not bind with high affinity unless ferriheme has been released from hemoglobin. Consequently, it is possible to estimate ferriheme release in intact erythrocytes by measuring chloroquine binding and correcting for low-affinity binding to normal erythrocytic constituents.

**MATERIALS AND METHODS**

Blood was withdrawn by venipuncture from black male patients with G6PD deficiency who were hospitalized at the John Cochran Veterans Administration Medical Center after informed consent was obtained from them. Healthy adult male volunteers provided normal blood. An aliquot of blood was anticoagulated with EDTA and used for cellulose acetate hemoglobin electrophoresis to exclude common hemoglobinopathies and for G6PD measurements. The remainder of the blood was immediately mixed with an equal volume of tris(hydroxymethyl)aminomethane (Tris) medium (141 mmol/L NaCl, 20 mmol/L glucose, and 10 mmol/L Tris, pH 7.4) containing 175 U/mL of heparin. Erythrocytes were sedimented by centrifugation and washed three times with Tris medium to remove plasma and Buffy coat before preparing suspensions for incubation with or without menadione and chloroquine, alone or in combination. Prior to incubation, the hemoglobin concentrations of these erythrocyte suspensions were measured by the cyanmethemoglobin method to serve as a common reference for expression of results. Incubations were carried out for 60 minutes at 37°C using 12.5% (by volume) suspensions of erythrocytes in Tris medium in the absence (control) or presence of menadione. A stock suspension of 5 mmol/L menadione in Tris medium containing 0.08% Tween 80 (Sigma, St. Louis) was prepared and sonicated immediately prior to dilution in the incubation mixture.

To measure membrane ferriheme content, erythrocytes were hemolyzed using 30 vol of a hypotonic medium (5 mmol/L NaCl, 10 mmol/L Tris, pH 8.0) at 4°C. The membranes were sedimented by centrifugation at 20,000 g for 20 minutes at 4°C and washed five times with the hypotonic medium. Ferrhieme bound to membranes prepared in this way has the spectral characteristics of hemichrome.
and has been used as an indicator of hemoglobin denaturation.\textsuperscript{31,32} The ferriheme content of the membranes was measured spectrophotometrically after dissolving them in 2.5% sodium dodecyl sulfate (SDS).\textsuperscript{13} This method measures total ferriheme, including any heme present in denaturated hemoglobin (eg, Heinz bodies) or hemochrome bound to or trapped in these washed membrane preparations.

As in previous studies, chloroquine binding to washed, intact erythrocytes was measured after incubation with ring-labeled 3-\textsuperscript{[14C]}-chloroquine (2.36 \textsuperscript{mCi}/\textsuperscript{mmol}; New England Nuclear, Boston).\textsuperscript{13,18} The erythrocytes were separated from the medium by centrifugation, and the chloroquine content of each was determined radiochemically after quantitative extraction into heptane under alkaline conditions.\textsuperscript{18} The initial concentration of chloroquine in the incubation medium (5 \textsuperscript{mmol}/L) was chosen because ferriheme released intracellularly from hemoglobin binds chloroquine with a dissociation constant of approximately 10\textsuperscript{-6}mol/L.\textsuperscript{13} Normal erythrocytes bind chloroquine with low affinity (dissociation constant of approximately 10\textsuperscript{-7} mol/L),\textsuperscript{13} attributable to other erythrocyte constituents such as hemoglobin and methemoglobin.

RESULTS

Under control conditions, the ferriheme content measured spectrophotometrically was greater in isolated membranes from G6PD-deficient erythrocytes than from normal erythrocytes (Fig 1). The values were 13.3 $\pm$ 2.8 (mean $\pm$ 1 SD) mmol of ferriheme per gram of erythrocyte hemoglobin for G6PD-deficient and 9.8 $\pm$ 2.4 for normal membranes ($P < .05$, Student’s $t$ test). After incubation with 250 \textsuperscript{mmol}/L menadione, these values increased by 37.4 to 50.7 $\pm$ 6.1 for G6PD-deficient and by 26 to 35.8 $\pm$ 3.9 for normal membranes ($P < .001$). Since the spectrophotometric method measures total ferriheme, these results only confirm that menadione denatures intracellular hemoglobin\textsuperscript{25}; they do not provide quantitative information about the concomitant release of ferriheme in a form available to interact with erythrocytic constituents.

To evaluate ferriheme release, chloroquine binding to intact erythrocytes was measured (Fig 1). Under control conditions, 69.5 $\pm$ 8.6 mmol of chloroquine were bound per gram of hemoglobin in G6PD-deficient erythrocytes and 54.7 $\pm$ 4.0 mmol were bound in normal erythrocytes. Under control conditions, the ferriheme content measured spectrophotometrically was greater in isolated membranes from G6PD-deficient erythrocytes than from normal erythrocytes (Fig 1). The values were 13.3 $\pm$ 2.8 (mean $\pm$ 1 SD) mmol of ferriheme per gram of erythrocyte hemoglobin for G6PD-deficient and 9.8 $\pm$ 2.4 for normal membranes ($P < .05$, Student’s $t$ test). After incubation with 250 \textsuperscript{mmol}/L menadione, these values increased by 37.4 to 50.7 $\pm$ 6.1 for G6PD-deficient and by 26 to 35.8 $\pm$ 3.9 for normal membranes ($P < .001$). Since the spectrophotometric method measures total ferriheme, these results only confirm that menadione denatures intracellular hemoglobin\textsuperscript{25}; they do not provide quantitative information about the concomitant release of ferriheme in a form available to interact with erythrocytic constituents.

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DISCUSSION

These results indicate that there is excess release of ferriheme in G6PD-deficient erythrocytes and that oxidant stress further increases this excess. To use chloroquine binding to estimate the amount of ferriheme available to interact with endogenous ligands, it is necessary first to correct for low-affinity binding to normal erythrocytic constituents.\textsuperscript{13} The magnitude of this correction is approximated by the binding of chloroquine to normal erythrocytes under control conditions since normal erythrocytes bind little if any chloroquine with high affinity.\textsuperscript{13,18} Using the value for normal erythrocytes as an approximation of low-affinity binding, G6PD-deficient erythrocytes bound an excess of 14.8 mmol of chloroquine per gram of hemoglobin under control conditions, and this excess increased by 17.9 to 32.8 mmol after the stress of 250 \textsuperscript{mmol}/L menadione. The corresponding excess of chloroquine binding for normal erythrocytes stressed with menadione was 7.2 mmol. Assuming that 1 molecule of chloroquine binds two molecules of ferriheme, as has been determined in equilibrium dialysis experiments,\textsuperscript{17} it is possible to calculate that G6PD-deficient erythrocytes stressed with menadione contain 65.6 mmol of ferriheme per gram of erythrocyte hemoglobin in a form available to interact with endogenous ligands. The corresponding value for normal erythrocytes stressed with menadione is 14.4.

Since oxidatively stressed G6PD-deficient erythrocytes release an excess of ferriheme, we conclude that ferriheme is available to mediate the hemolysis induced by menadione in G6PD-deficient patients\textsuperscript{18} and the selective toxicity of mena-
EXCESS FERRIHEME IN G6PD-DEFICIENT RBC

dione for malaria parasites growing in G6PD-deficient erythrocytes.\(^2\) Release of ferriheme may also be involved in the pathogenesis of hemolytic episodes caused by other oxidant drugs in G6PD-deficient patients. In addition, G6PD-deficient erythrocytes may be particularly vulnerable to the intraerythrocytic oxidant stress produced by malaria parasites, which metabolize NADPH\(^3\) and generate \(\text{H}_2\text{O}_2\).\(^4\) The resultant release of ferriheme could inhibit intraerythrocytic development of malaria parasites in G6PD-deficient erythrocytes, providing an explanation for the mechanism of reduced parasitemia that has been observed, and thus could explain genetic selection for G6PD deficiency.

Even in the absence of abnormal oxidant stress from drugs or malaria parasites, enough ferriheme may eventually accumulate within some erythrocytes to cause their destruction. Thus, an excess of ferriheme could have contributed to the decreased erythrocyte survival observed by Brewer and associates in black individuals with G6PD deficiency who were not in hemolytic crisis.\(^7\) Furthermore, since there is evidence of a decrease in G6PD activity with increasing age of normal erythrocytes, it is possible that ferriheme accumulation may contribute to the destruction of senescent erythrocytes.

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