Bleomycin-detectable iron in plasma from *Plasmodium vinckei vinckei*-infected mice

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(1) Plasma from mice heavily parasitized by *Plasmodium vinckei vinckei* was found to contain micromolar levels of iron as detected by the 'bleomycin assay' (slightly modified) of Gutteridge et al. [(1981) Biochem. J. 199, 263–265]. Uninfected mouse plasma contained little or no bleomycin-detectable iron. (2) Plasma ultrafiltrate from infected mice contained no bleomycin-detectable iron, indicating that such iron was associated with the protein/macromolecule fraction. (3) We speculate that this iron could catalyse reduction of peroxides in vivo and thus play a role in malaria pathology.

Malaria *Bleomycin* *Iron detection* *Free radical* *Tissue damage*

1. INTRODUCTION

Malaria is caused by intra-erythrocytic parasites that induce hemolysis and extensive damage to parenchymatous organs and endothelial cells [1]. It has been proposed that this tissue damage, and also the appearance of degenerating parasites ('crisis forms') within erythrocytes during the natural course of the infection, are mediated by soluble factors secreted by phagocytes [2]. These crisis forms can be artificially induced by injection of oxidative drugs such as alloxan, *t*-butyl hydroperoxide, divicine and H_2O_2 [3–7]. Both this type of drug [4,8], and activated macrophages [9,10], have been shown to place lethal oxidative stress on intra-erythrocytic parasites in vitro. These observations support the suggestion [3] that reactive oxygen species contribute to the host's immune response to malaria.

The observation that the anti-parasitic and hemolytic activity of these drugs is blocked by the iron chelator desferrioxamine [3–5] raises the possibility that iron, by catalysing the reduction of H_2O_2 and organic peroxides, plays a significant role in the tissue damage seen in malaria, which is similar to that found in iron overload conditions [11]. Iron-dependent damage to DNA by bleomycin (Blm) has been used to determine levels of catalytic iron in biological samples [12,13]. Blm binds to DNA and chelates available Fe^{3+} (K_a = 10^{15} [14]). Chemical reduction of the chelated iron to Fe^{2+} in the presence of oxygen causes oxidative damage to DNA and the formation of thiobarbituric acid-reactive material [15,16].

Here we report the detection, using a slight modification of this method, of potentially catalytic iron in plasma from *Plasmodium vinckei vinckei*-infected mice.

2. MATERIALS AND METHODS

CBA/CaH mice of either sex and 7–9 weeks of age were used throughout. *P. vinckei vinckei* (strain V52, from F.E.G. Cox) was maintained by i.p. injection of 10^6 parasitized erythrocytes. Blood was drawn into heparin (20 units/ml).

Pure Blm B_2 was the generous gift of Dr N. Hart, Division of Applied Organic Chemistry, CSIRO, Melbourne. Blenoxane was from Bristol Labs, Bristol, England, calf thymus DNA (type I)

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and mouse hemoglobin (type X) from Sigma, L-ascorbic acid (Analar) from BDH Chemicals and Chelex-100 (200–400 mesh) from BioRad.

The assay for catalytic iron, based on Blm-induced damage to DNA [12], was modified slightly. Pure Blm B_2 was used instead of the mixture of Blm A_2 and B_2 types found in blenoxane or Blm sulfate. Solutions containing chelex 100 were centrifuged (10000 × *g*, 30 min, 4°C) before use. $FeCl_3$ standards were made up freshly in chelex 100-treated H_2O and added within 3 min to incubation tubes containing Blm. Samples were preincubated for 5 min before addition of ascorbic acid, followed by a 2 h incubation at 37°C. It was found necessary to centrifuge samples (1500 × *g*, 10 min) after HCl/thiobarbituric acid boiling treatment before spectrophotometric determinations. Absorbance changes were determined by the difference spectrum $A_{532-600}$ against a blank containing all components except Blm and sample. Samples were assayed in duplicate in the presence and absence of Blm. Plasma from 4–5 mice (parasitemia 75–90%) was ultrafiltered using an Amicon Centrifree micropartition system (1500 × *g*, 2 h, 4°C, Sorvall SS-34 rotor, angle 34°). Hemoglobin was assayed as described [17].

3. RESULTS AND DISCUSSION

Fig.1 shows a representative assay standard curve, using $FeCl_3$ for Blm-dependent damage to DNA. The linearity found at low concentrations of $FeCl_3$ is in agreement with others [12], although the magnitude of the response is somewhat lower. This deviation may be a function of the particular type of Blm used. The reactivity of different Blm types is illustrated by the standard curve using blenoxane (fig.1), which contains approx. 70% Blm A_2 and 30% Blm B_2.

Normal mouse plasma, like normal human serum [13], contained little or no detectable Fe^{3+} (table 1a), whereas plasma from mice carrying a high parasite load (75–70% of red cells infected) contained significant amounts (88–150 μM). Chelation by heparin could have artificially lowered these readings, but they are still appreciable. It is unlikely that these high levels represent free ionic iron alone. To see if we were detecting protein-bound iron, plasma from control and *P. vinckei vinckei*-infected mice was ultrafiltered

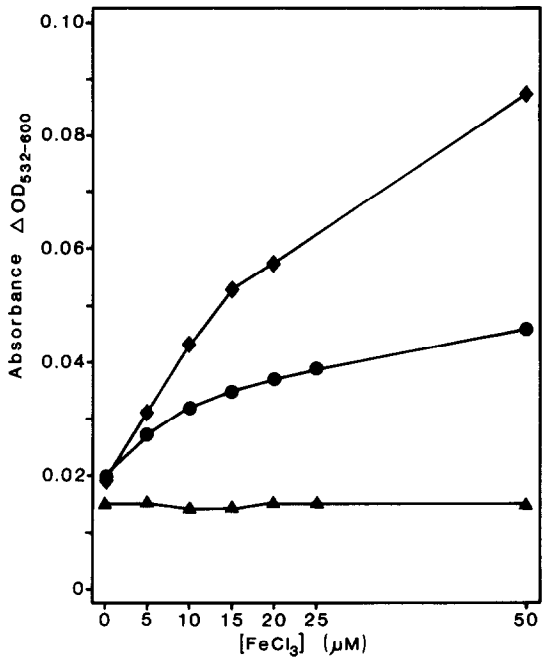

Fig.1. Bleomycin-Fe^{3+} dependent degradation of DNA. Dose-response curve for the $FeCl_3$-dependent formation of thiobarbituric acid-reactive material from DNA in the presence of Blm. Samples were assayed as described in section 2. (▲) Control, no bleomycin; (●) Blm B_2; (◆) blenoxane.

Table 1

Bleomycin B_2-detectable Fe^{3+} in malaria plasma

(a) Plasma		
Control mice		3.3 ± 4.4 (6)
Infected mice		118.2 ± 28.8 (5)
(b) Ultrafiltration		
Sample	Control	Infected
Plasma	0	88
Ultrafiltrate	3	4
Non-ultrafiltrable material	5	73

Bleomycin-detectable Fe^{3+} was determined as described in section 2. (a) Plasma from control and *P. vinckei vinckei*-infected mice. (b) Ultrafiltration of plasma from control and *P. vinckei vinckei*-infected mice. Ultrafiltrate was >99% hemoglobin and protein-free. Values are expressed as micromolar Fe^{3+}, mean ± SD (*n*)

before assaying it for Blm-detectable iron. Negligible amounts were found in the filtrate or plasma residue from uninfected mice or the plasma filtrate from infected animals. In contrast, the plasma residue from infected mice contained about 80% of the total Blm-detectable iron found in unfractionated plasma (table 1b), suggesting that the iron found in plasma from infected animals is associated with a protein or macromolecule complex(es), where the binding constant is similar to, or less than, that of the iron-Blm complex. Pure mouse hemoglobin did not release iron to bleomycin (not shown). Some of this bound iron evidently has at least one co-ordination site occupied by an easily displaceable ligand, such as H_2O [18], since it can act catalytically to reduce peroxides [3,4,8].

Within these constraints, it is suggested that the iron detected by the Blm assay in plasma from malaria-infected mice could catalyze reduction of peroxides in vivo to products that might be partially responsible for the tissue damage associated with the infection.

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