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MALARIA PIGMENT AND EXTRACELLULAR IRON

POSSIBLE TARGET FOR IRON CHELATING AGENTS

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Abstract—Extracellular iron is necessary for many biochemical reactions involved in *Plasmodium falciparum* growth and multiplication. The incorporation of radioactive iron taken up by the parasite was found, electrophoretically and via gamma counting, to be mainly associated with the haemozoin only in the presence of the active metabolism of the parasite. The potent antimalarial activity of desferrioxamine, a ferric iron chelating agent, has shown that iron deprivation is inhibitory to the parasite. We propose that the mechanism of action of desferrioxamine in addition to the chelation of iron from the parasitic compartment, chelates iron from the haemozoin crystal resulting in free radical generation and parasite death. The ability of desferrioxamine and not the ferrous iron chelating agent, 2,2′-bipyridyl, to chelate the non-haem iron from the haemozoin structure indicates that the oxidative state of iron associated with the haemozoin structure is ferric in nature.

Iron is an essential micronutrient required by the malaria parasite for many biochemical reactions involved in growth and multiplication. Although the intra-erythrocytic parasite lives in an iron-rich environment, it seems to lack the haem oxygenase enzyme which would allow the parasite to degrade the haem moiety of haemoglobin and utilize the ferrous iron [1]. Instead this ferrous haem becomes oxidized to a toxic ferric form which is then detoxified by the parasite into an inert crystalline material known as malaria pigment or haemozoin. The detoxification occurs in a unique and specialized acidic organelle, the food vacuole, which has evolved to accomplish this important function. It is most likely that the parasite obtains its iron exogenously from the serum transferrin [2, 3].

The importance of the uptake of extracellular iron has lead to the use of iron chelating agents as a new class of antimalarial drugs. Desferrioxamine (DFO§), a ferrous iron chelator, has been shown to have antimalarial activity alone and in combination with other antimalarials both *in vitro* and in clinical trials [4–6]. The cytocidal activity of DFO was reported to only effect the pigmented trophozoites and early schizonts [7]. Thus, the effects of extracellular iron and DFO on the pigmented parasites and haemozoin were investigated in this study.

MATERIALS AND METHODS

RPMI-1640 was obtained from Highveld Biological (Kelvin, South Africa), HEPES from Boehringer Mannheim (Randburg, South Africa), glucose from Merck (Midrand, South Africa), hypoxanthine and gentamicin from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The iron chelating agent, DFO, was obtained from Ciba Geigy (Kempton Park, South Africa) and 2,2′-bipyridyl (BIPY) from Fluka, Labchem (Edenvale, South Africa). Human apotransferrin was obtained from the South African Blood Transfusion Services (Hillbrow, Johannesburg, South Africa). ^{59}Fe as $FeCl_3$ and [^{3}H]-hypoxanthine were purchased from Amersham, Weil (Doornfontein, South Africa).

Culture of Plasmodium falciparum. The chloroquine (CQ)-resistant Gambian FCR-3 strain was cultured in Group O^+ human erythrocytes and suspended in a 5% haematocrit in RPMI-1640, which contained 4 g/L glucose, 44 mg/L hypoxanthine, 50 mg/L gentamicin, 10% group AB^+ human plasma and was buffered with 5.94 g/L HEPES and 2.5% $NaHCO_3$. Cultures were maintained at 37° with a gas mixture of 3% oxygen, 4% carbon dioxide and 93% nitrogen [5]. Synchronous cultures were maintained using 5% D-sorbitol [8]. *In vitro* antimalarial activity of DFO and BIPY was measured by the [^{3}H]hypoxanthine incorporation [5].

Preparation of diferric transferrin. Human apotransferrin was labelled with $^{59}FeCl_3$ as diferric transferrin by standard methods [9]. The diferric nature of the transferrin was confirmed spectrophotometrically, whilst the end concentration of diferric transferrin was determined by radial immunodiffusion [9].

In vitro *effect of DFO on the parasite haemozoin.* Before use in an experiment the parasitized erythrocytes were washed with RPMI-1640 to remove traces of plasma transferrin, iron and gentamicin. Appropriate erythrocyte controls were prepared in parallel with the parasitized erythrocytes. RPMI-1640, 2.5% $NaHCO_3$ and [^{59}Fe]diferric transferrin

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§ Abbreviations: BIPY, 2,2′-bipyridyl; CQ, chloroquine; DFO, desferrioxamine; HEPES, *N*-2-hydroxy-ethyl-piperazine-*N*′-2-ethane-sulphonic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate.

were added to the parasites (10% haematocrit, 10% parasitaemia). Once the parasites had progressed to trophozoites, the parasitized erythrocytes were washed in RPMI-1640. The supernatants were collected for counting in a Packard Autogamma counter (Model 5650, Packard Instruments, Downers Grove, IL, U.S.A.). At this stage either the haemozoin was isolated from the pellet or the parasites were further treated with DFO. If the pellet was to be further treated, it was resuspended in RPMI-1640 and 2.5% $NaHCO_3$ and evenly aliquoted out. Various concentrations of DFO were added to each aliquot. The erythrocyte and parasitized erythrocyte controls were treated appropriately. After a 2 hr incubation period at 37°, the cells were centrifuged. The pellets were washed with RPMI-1640 and the supernatants collected for gamma counting. In preparation for haemozoin isolation, the pellets were resuspended in RPMI-1640 and frozen at −70°.

Haemozoin isolation. Thawed tubes were centrifuged at 2000 rpm for 10 min at 10° to collect the isolated parasites. Two microlitres of the supernatant and isolated parasites were collected for gamma counting. The pellets were washed with RPMI-1640 and 0.25 M sucrose/5 mM Tris solution (pH 7.4) until no haemoglobin was visible. The isolated parasites were lysed by five cycles of freezing and thawing. To the lysed parasites 1 mL of sodium dodecyl sulphate–phosphate-buffered saline buffer (0.1% SDS to 10% PBS) was added before the tubes were vortexed and centrifuged at 4000 rpm for 10 min at 10°. The haemozoin pellets were washed with the SDS–PBS buffer as before until only pure haemozoin crystals remained as judged by Giemsa staining.* The haemozoin pellets were then gamma counted for 1 min.

Electrophoresis. A 6–20% gradient polyacrylamide gel was run on the haemozoin and erythrocyte haemoglobin at 95 V for 4 hr at 4°. A phosphate buffer of pH 11.2 with SDS in the cathode buffer was used in the vertical electrophoretic apparatus.* Two lanes containing haemoglobin and haemozoin were sliced into 2 mm pieces and gamma counted for 1 min. The remaining lanes of haemoglobin and haemozoin were first fixed in destaining solution (5% acetic acid, 30% methanol and 65% distilled water) for approximately 3 hr, then placed in Coomassie Brilliant Blue overnight and destained until the background became clear.

Effect of DFO and BIPY on isolated radiolabelled haemozoin. Isolated radiolabelled haemozoin was aliquoted out and gamma counted for 1 min (i.e. initial pellet). The various iron chelators (prepared in a phosphate buffer of pH 5.0) were added to the aliquoted haemozoin and vortexed. After a 30 min incubation period at room temperature, the tubes were centrifuged at 4000 rpm for 15 min at 10°. The collected supernatants and pellets (i.e. final pellet) were then gamma counted and finally expressed as percentages of the initial pellet.

* Hempelmann E, de Almeida M and Havlik I, A simplified method for the separation of malaria pigment from *Plasmodium falciparum*. Submitted, 1992.

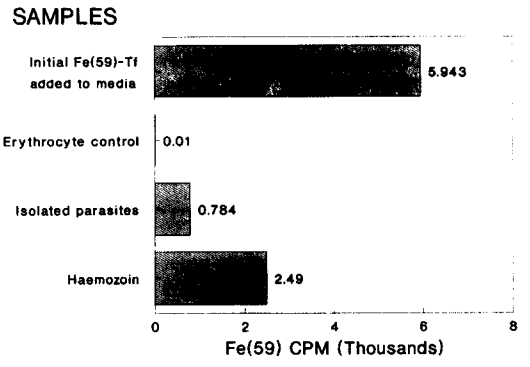

Fig. 1. The distribution of radioactive ^{59}Fe during the isolation of haemozoin from trophozoites which had been incubated at 37° in the presence of diferric radiolabelled transferrin. Counts were determined by gamma counting 2 μL of sample for 1 min.

RESULTS

Incubation with radiolabelled transferrin

When the parasites were incubated in the presence of diferric transferrin, there was a progressive uptake of ^{125}I-transferrin and cellular iron accumulated in the cultured parasites (data not shown). There was a significant uptake of ^{59}Fe into the isolated parasites (13.2%) compared to the erythrocyte control (0.17%). Approximately 41.9% of the total radio-iron added was associated with the haemozoin after 10 washes with the SDS–PBS buffer (Fig. 1). The incorporation of radio-iron into haemozoin was confirmed by electrophoresis. As shown in Fig. 2, the major radioactive peak corresponds exactly to the position of the haemozoin. No protein was detected with the haemozoin band. When non-labelled haemozoin was isolated and incubated at a pH of 5.0 and at room temperature in the presence of $^{59}FeCl_3$, all the iron remained in the supernatant (data not shown).

In vitro *effect of DFO on the haemozoin*

The IC_{50} of DFO was determined to be 11.4 μM. Two hours incubation of radiolabelled parasites with increasing concentrations of DFO was associated with decreased radioactivity in the isolated haemozoin (Fig. 3). The final parasitaemia for each DFO concentration did not vary significantly from the control after the 2 hr incubation period.

Effect of DFO and BIPY on isolated labelled haemozoin

Labelled haemozoin incubated at the pH of the food vacuole (pH 5.0) and in the presence of various DFO concentrations resulted in the removal of the ^{59}Fe into the DFO supernatant (Fig. 4a). Whilst the presence of BIPY, even at concentrations up to approximately two and a half times the BIPY IC_{50} of 30.6 μM, did not remove ^{59}Fe from the haemozoin pellet (Fig. 4b).

DISCUSSION

The mechanism by which *P. falciparum* acquires

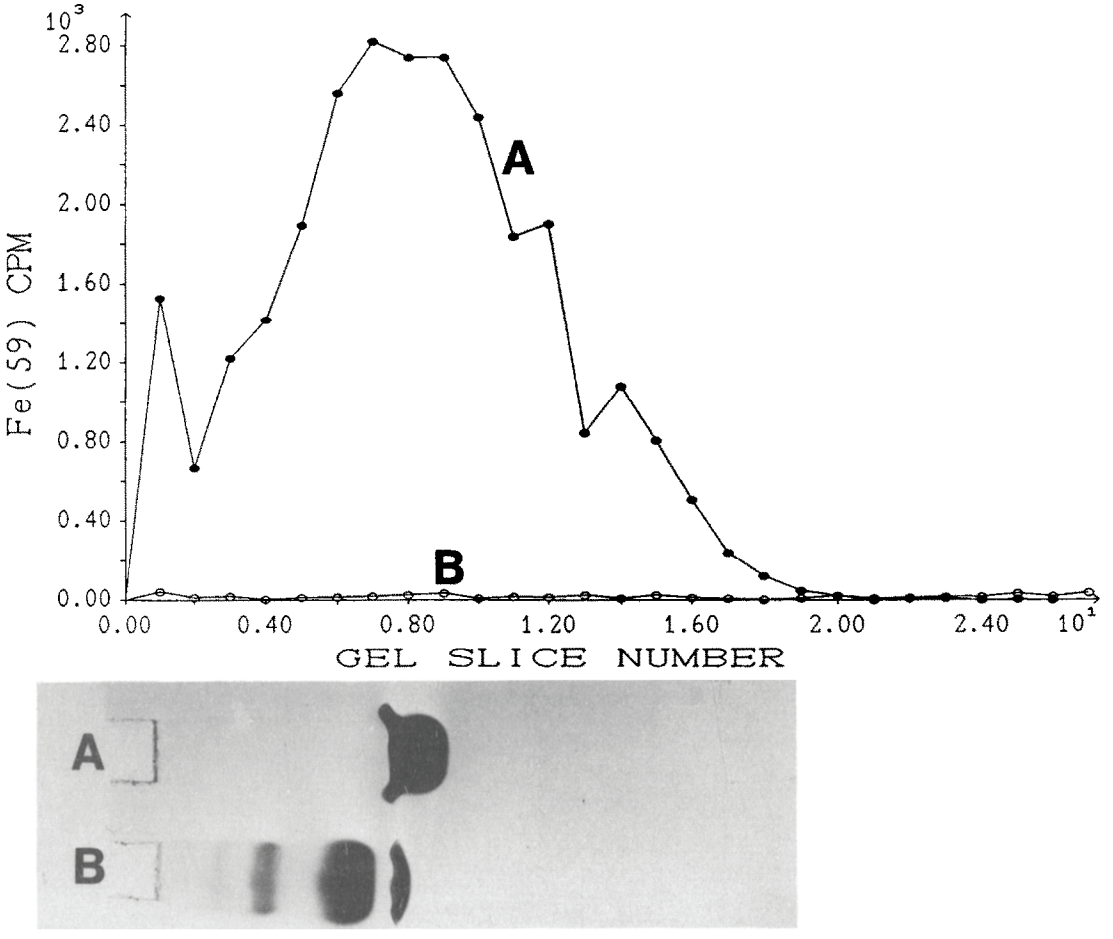

Fig. 2. Results of 6–20% gradient electrophoresis on haemozoin (A) and erythrocyte haemoglobin (B) obtained from trophozoites and erythrocytes, which had been incubated in the presence of ^{59}Fe diferric transferrin. The 2 mm thick gel slices gamma counted for 1 min are shown (top) to the corresponding 6–20% gradient electrophoretic gel (bottom) with the parasite haemozoin band (A) and erythrocyte haemoglobin (B).

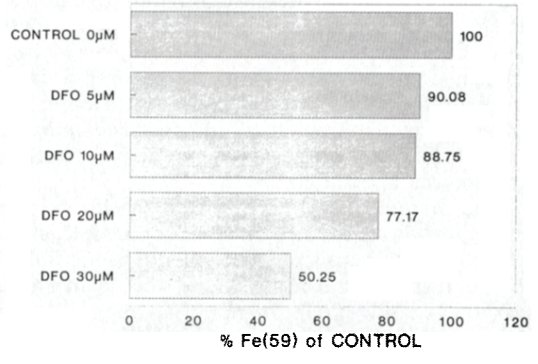

Fig. 3. Mature trophozoites which had been incubated in the presence of ^{59}Fe diferric transferrin were further incubated with various DFO concentrations for 2 hr at 37°. The *in vitro* effects of DFO on the isolated haemozoin ^{59}Fe content are expressed as a percentage of the control haemozoin.

iron has been well documented and is of both biological and clinical interest. Proliferating cells normally take up iron bound to transferrin via receptor-mediated endocytosis. However during red cell maturation, all transferrin receptors are lost, thereby preventing further uptake of iron into the erythrocyte. It has been proposed that the parasite regenerates or synthesizes a neo-antigen which is localized in the erythrocyte membrane to function as a "pseudo-transferrin receptor", thus facilitating the uptake of the transferrin-bound iron [1, 3]. The presence of the parasite transferrin receptors has been contested in studies using diferric radiolabelled transferrin, which have found that extracellular iron is taken up into the parasitized erythrocytes by pinocytosis of non-specifically bound transferrin [2]. The latter protein is then catabolized instead of being recycled, whilst the iron is selectively retained and incorporated into several proteins. The studies done in our laboratory again confirm that the parasite utilizes extracellular serum transferrin iron for its proliferation.

The formation of haemozoin enables the parasite

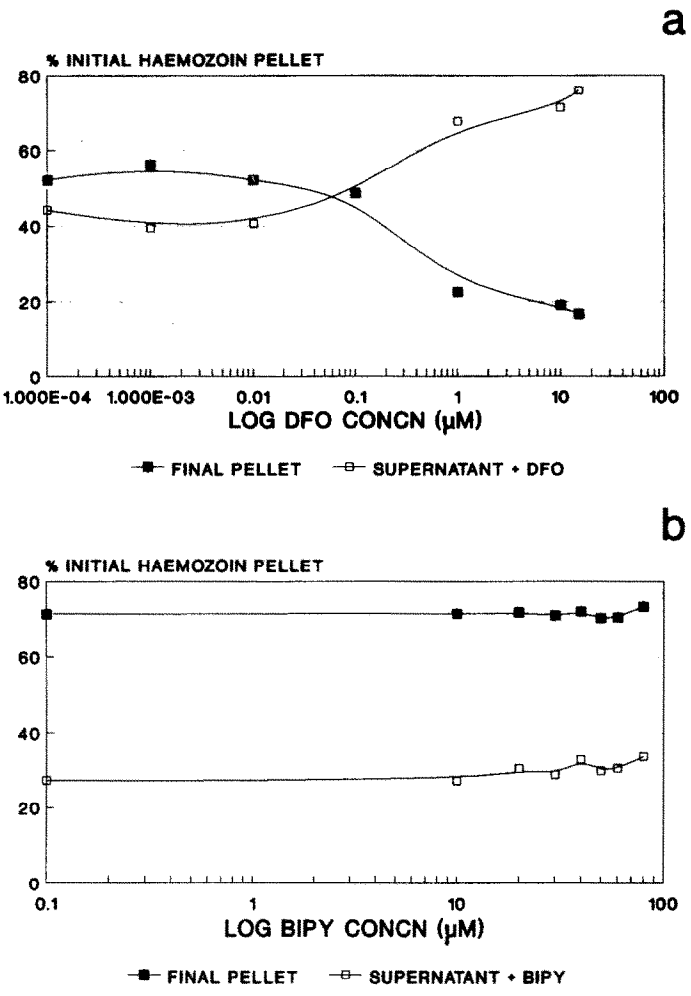

Fig. 4. The effects of various concentrations of DFO (a) and BIPY (b) on isolated ^{59}Fe-labelled haemozoin after being incubated at a pH of 5.0 for 30 min at room temperature. The results are expressed as a percentage of the initial pellet for each concentration.

to continue digesting haemoglobin without having to contend with the toxic ferric form of haemin and the resultant production of superoxide. Haemoglobin ingestion occurs early in the trophozoite stage to meet the amino acid requirements. It is at this stage that haemozoin crystals become visible under light microscopy. In order to determine whether the uptake of extracellular iron and the formation of haemozoin were interrelated; parasites were incubated in the presence of diferric radiolabelled transferrin from merozoite, early rings until mid-late trophozoites. As shown in Figs 1 and 2, there was significant incorporation of the extracellular ferric iron into the haemozoin crystals, which was detected electrophoretically and by gamma counting the pure haemozoin crystals. The radioactivity associated with the haemozoin was present even after 10 washes with SDS buffer, indicating that its presence was not due to non-specific binding of the labelled transferrin molecule to the haemozoin crystal. In addition, gel electrophoresis showed that no protein was associated with the haemozoin.

The incorporation of the iron into the haemozoin occurs only *in vitro*, as when isolated haemozoin was incubated in the presence of radioactive iron at the food vacuole pH of 5.0, there was no radioactivity associated with the haemozoin. The radioactive iron remained in the supernatant, even after a 24 hr incubation period. This indicates that in order for the extracellular iron to be incorporated into the haemozoin, the presence of an active parasitic metabolism is required.

The presence of a ferric iron in the haemozoin structure is in accordance with the proposed structure that the crystal consists of a polymer of haems linked between the central ferric ion of one haem and a carboxylate side-group oxygen of another [10]. We propose that the methyl groups situated around the ferriprotophorphyrin IX structure are oxidized to carboxyl groups. These in turn bind extracellular iron to form the complex structure of the haemozoin crystal.

When intra-erythrocytic parasites are deprived of non-haem ferric iron *in vitro* by the addition of the

ferric iron chelator, DFO, abnormal growth and cell death are observed [11]. The antimalarial mechanism of action of DFO was initially thought to be chelator-mediated depletion of serum iron, but DFO does not have the capacity of removing iron from serum transferrin [12]. Reports now indicate that it is a prerequisite for the antimalarial activity of DFO to be taken up into the parasite [13, 14]. The antimalarial activity of DFO is related to its ability to enter the parasitic compartment and not due to the chelation of extra- and intracellular iron necessary for parasite growth. From the kinetic parameters of DFO it has been shown that DFO is distributed evenly between lysosomes (42.1%) and the cytosol (52.7%), possibly due to the weak base property of DFO [15]. In addition, stage-dependent studies of DFO on the malaria parasite have shown that DFO is cytostatic to the ring and non-pigmented trophozoite stages; whilst cytocidal to the pigmented trophozoites and early schizonts [7].

The *in vitro* effect of DFO on the haemozoin observed in this study seems to indicate that the presence of haemozoin is required for the antimalarial activity of DFO. This is shown from the fact that as the DFO concentrations increased, the amount of radioactivity associated with the haemozoin decreased. In addition, DFO has a potent chelating action directly against the haemozoin. This is seen after the incubation of isolated radiolabelled haemozoin and DFO at the food vacuole pH. At concentrations greater than 0.06 μM DFO the amount of radioactivity associated with the chelator increased substantially.

Thus, the possible disruption of the haemozoin crystal by DFO would release toxic haemin which is lytic to the parasite [16]. The presence of excess ferric iron released from the crystal would result in the production of hydrogen peroxide, superoxide and hydroxyl radicals, which would in turn promote membrane damage via lipid peroxidation, enzyme and DNA damage [17, 18]. It has previously been reported that DFO and Fe^{2+} act as an oxygen radical generating system by accelerating the auto-oxidation of iron with the production of hydrogen peroxide and hydroxyl radicals [19]. The production of these toxic free radicals and the release of a small proportion of haemozoin haemin would be enough to be cytocidal to the parasite. In addition, the antimalarial activities of DFO alone seem to include the chelation of iron from the parasitic compartment that is required for essential biochemical reactions. Included in these is the necessity of iron for the iron-dependent ribonucleotide reductase enzyme involved in DNA synthesis [20].

The inability of the ferrous iron chelator BIPY to chelate or affect the haemozoin crystal when incubated in the presence of isolated labelled haemozoin illustrates that the iron associated with the crystal is ferric in nature. From the antagonistic effect between BIPY and DFO, it was previously concluded that the antimalarial mechanism of action of iron chelators is not solely dependent on iron deprivation to kill the parasite [21]. Here it is possible that the antagonism may be due to the fact that BIPY prevents the availability of Fe^{2+} for redox cycling and free radical formation by DFO.

Recently, it has been reported that a possible site of action for CQ may be a novel enzyme, haem polymerase, which is thought to be responsible for catalysing haemozoin production [22]. It has also been observed that there is an additive effect when DFO and CQ are combined *in vitro* and in clinical trials [5, 6]. This additive effect may be explained by the fact that DFO and CQ react differently on haemozoin, where CQ may prevent the formation of haemozoin, whilst DFO may have a direct effect on the structure. In addition, it has recently been reported that the selective toxicity of artemisinin (qinghaosu) to the malaria parasites, may contribute to the reaction between artemisinin and haemin within the parasite to generate toxic activated oxygen byproducts [23].

In conclusion, it seems that extracellular ferric iron may not only be essential for the parasite biochemical reactions, but also as a stabilizing factor for the formation of haemozoin. In addition, the chelating ability of DFO on the haemozoin may be a vital mechanism of action against the late, pigmented parasites. It is becoming more evident that haemozoin may be the structure of the malaria parasite to which new antimalarial drugs could be targeted.

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