

# DEFEROXAMINE INHIBITION OF MALARIA IS INDEPENDENT OF HOST IRON STATUS

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Iron plays a central role in a number of essential biochemical reactions. Since iron deprivation in microbial cultures is regularly associated with inhibition of growth, it has been suggested that hypoferremia may represent an important defense mechanism (1, 2). A number of clinical observations have suggested that iron deficiency may have a beneficial effect in human malaria, and that conversely, iron administration may aggravate malaria (3-5). Likewise, deferoxamine (DF)<sup>1</sup>, a siderophore useful in the management of iron overload, inhibits the *in vitro* growth of *Plasmodium falciparum* (6, 7) and *in vivo* malarial infection in several species of mammals (8, 9). The DF-iron complex, however, is not inhibitory (6, 8). The aim of the present study was to define the mechanism by which DF inhibits malarial infection. In particular, we wished to determine whether this was caused by the induction of iron deficiency in the host, analogous with clinical iron deficiency, or by direct interference with the acquisition of intracellular iron. Our results indicate that the *in vivo* suppression of malaria by DF is independent of host iron status and suggest that interaction with chelatable iron in parasitized erythrocytes may be responsible for the inhibition of infection.

## Materials and Methods

### *Rats*

Female Wistar rats (supplied by Harlen-Olac Ltd., Bicester, Oxon, England) weighing 140-150 g were housed in groups of 4-6 in plastic cages with stainless steel grid lids and fed *ad lib* with RNM3 food (S. D. S., Witham, Essex, England). Blood samplings and injections of <sup>59</sup>Fe were performed through the tail veins. Animals were killed by ether anesthesia or by exsanguination through the abdominal aorta under ether anesthesia.

### *Malaria Infections*

Groups of 12-40 rats were infected with a standard inoculum ( $\sim 3 \times 10^7$ ) of *Plasmodium berghei* (ANKA strain, donated by Dr. Jarra, National Institute of Medical Research, Mill Hill, UK) diluted in 1 ml RPMI and injected via two intraperitoneal injections. Parasitemias were estimated by counting at least 500 cells in methanol-fixed, Giemsa-stained blood films.

### *Radioiron Measurements*

*In vivo* radioiron labeling was performed by diluting <sup>59</sup>FeCl<sub>3</sub> (sp act, 3-20  $\mu$ Ci/ $\mu$ g, Amersham International, Amersham, UK) prepared in 0.005 N HCl and mixed with sufficient

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<sup>1</sup> Abbreviations used in this paper: DF, deferoxamine; MCV, mean corpuscular volume.

sterile 4% sodium citrate to ensure a molar ratio of citrate to iron in excess of 50:1, to a final concentration of 1 or 25  $\mu\text{Ci}/\text{ml}$ . 1 ml of this solution was injected through the tail vein under light ether anesthesia. For the measurement of whole blood radioactivity, 50- $\mu\text{l}$  tail vein samples were collected on alternate days and diluted to 1 ml before counting. The radioactivity of spleen, kidney, weighed portions of the liver, and the diluted blood samples was determined in an automatic gamma counter (model 1282, Pharmacia LKB, Bromma, Sweden). Whole body counts were performed in a Shadow Shield-type total body counter (10) and results were expressed as percent of day 0 radioactivity in the same animal, corrected for  $^{59}\text{Fe}$  decay. In preliminary studies using  $^{51}\text{Cr}$ -labeled erythrocytes (11, 12), blood volume was found to be 6.4 ml per 100 g body weight, and the proportion of circulating blood radioactivity trapped in the liver was 2.4%. These factors were used in subsequent studies to calculate net hepatic and total blood radioactivity. Residual tissue radioactivity was calculated from total body activity minus the combined radioactivity of liver, spleen, blood, and kidney.

Serum iron and total iron binding capacity were determined by the ICSH Iron Panel method (13). Total non-heme iron was determined by the method of Torrance and Bothwell (14). Blood counts and mean corpuscular volume (MCV) measurements were performed in model S + IV counter (Coulter Electronics Inc., Hialeah, FL) with histogram differential. Reticulocytes were estimated by counting 1,000 RBC stained supravivally with brilliant cresyl blue.

#### *Therapeutic Interventions*

*Deferoxamine Treatment.* Deferoxamine (Ciba, Zurich, Switzerland) was freshly dissolved in distilled water and diluted in sterile isotonic saline to provide a final concentration of 50 mg/ml or dilutions thereof as specified in Results. Unless otherwise specified, doses of 1 mg/g body weight per day were administered by subcutaneous injections divided into three daily doses given at 8-h intervals. Injections were started on the day of malaria inoculation (day 0) and continued throughout. In studies in which DF treatment was started on day 7 or 12, the same dose was administered by continuous infusion through a subcutaneous pediatric set secured to the forehead for 3 d and continued thereafter by three daily injections until the end of the study.

*Iron Depletion.* This was produced by raising 3 wk old weanling rats ( $50 \pm 5$  g) on an RM3 Low Iron Diet (S. D. S.) supplied ad lib. This diet is based on skimmed milk powder, sugar, and soybean oil, and has an iron content of 7 ppm. The amounts of other trace metals (ppm) and vitamins (mg/kg) were as follows: Cu, 7; Mn, 3; Zn, 67; Co, 45; Se, 6; vitamin B1, 1.95; B2, 26.0; B6, 2.6; Folate, 0.39; Niacin, 6.5; Pantothenic a., 22.8; Retinol, 59; Calciferol, 6; and Tocopherol, 6. The food was supplemented with 100 mg/kg of paraminobenzoic acid. Control animals of the same weanling cohort were raised on the same diet, supplemented with 144 ppm iron. Studies were begun after 22 d on the special diets, by which time the control and iron-deficient animals weighed  $140 \pm 7$  and  $127 \pm 4$  g, respectively. The effect of the iron-deficient diet on the experimental group was monitored by measurement of serum iron and transferrin saturation. Iron deficiency was confirmed by the development of a microcytic hypochromic anemia (see Table II).

*Iron Loading.* This was produced by the injection of ferric citrate at a dose representing 7.5 mg/kg elemental iron per day, intraperitoneally, starting on day 0 for 10 consecutive days. Previous studies by Awai et al. (15) have shown that this is the highest dose tolerated for the parenteral injection of a soluble iron salt, and that a single injection will produce serum iron levels exceeding the total iron binding capacity of the plasma for  $\sim 4$  h.

#### *Studies on Intracellular Iron*

*Preparation of Cells.* Rats, with parasitemias of at least 25%, were injected intraperitoneally with 25  $\mu\text{Ci}$   $^{59}\text{Fe}$ -citrate and bled 16 h later. When indicated, whole blood was incubated in 9 vol of RPMI 1640 containing 4 g/liter glucose, 2 g/liter  $\text{NaHCO}_3$ , 2mM glutamine, 37.5% Hepes in an atmosphere of 5%  $\text{O}_2$ , 7%  $\text{CO}_2$  in nitrogen at 37°C. No cell lysis was observed during the incubation period.

*Preparation of Cell Lysates.* Cells were washed four times (500 g for 10 min) in PBS and 0.5 ml aliquots resuspended in 2 ml PBS. Cells were lysed by sonication (5 for 1 s), 10% Triton X-100 was then added to give a final concentration of 1%, and they were centrifuged

at 10,000 *g* for 15 min. The pellet contained <10% of the total radioactive counts. Preliminary experiments showed that freezing supernatants resulted in precipitation of hemoglobin, while storage of intact cells at 4°C for up to 48 h did not alter the results. Supernatants were analyzed immediately.

Interaction of reticulocyte iron with DF was tested in two phases. DF was first incubated with washed, intact, radioiron-labeled cells at various concentrations, as specified in Table III, to assess the ability of DF to penetrate the cells. After the incubation and cell lysis, lysates were divided. 3 mM DF was added to one aliquot in order to measure total chelatable iron. The other aliquot was used to measure the fraction of chelatable iron generated in intact reticulocytes.

**Gel Filtration.** Supernatants were analyzed by gel filtration (Ultrogel ACA-34 column [40 cm × 9.0 cm<sup>2</sup>] equilibrated with 0.1 mM Tris, pH 7.6, Pharmacia LKB). The radioactivity of 1.2-ml fractions was determined. The column was precalibrated with blue-dextran, ferritin, hemoglobin, and dinitrophenol.

**Ultrafiltration.** Supernatants (1.5 ml) were also subjected to ultrafiltration using Centricon 30 filters (Amicon Corp., Danvers, MA; mol wt exclusion limit, 30 kD) by centrifugation (fixed rotor) at 5,000 *g* for 20 min according to the manufacturer's instructions. Filtered volumes ranged from 0.25–0.7 ml but were uniform within each batch of results. Comparison of filtered volumes obtained with Centricon 30 and 10 (exclusion limit, 10 kD) showed greater filtrate volumes using Centricon 30, but no increase in total filtrable radioactivity. Radioactivity in the filtrate was counted for 30 min and the total filtrable <sup>59</sup>Fe calculated by using a volume correction.

**Statistics.** As parasite counts varied widely, a non-parametric test (Wilcoxin-rank) was used to confirm differences at the peak day of infection. Errors of radioactivity counts for the estimates of filtrable <sup>59</sup>Fe were calculated from the Poissonian distribution according to the total number of radioactive counts detected.

## Results

### *Effect of DF on Parasite Counts*

Fig. 1 *A* shows the peripheral blood parasite counts from a representative study of six treated rats, receiving 1 mg/g DF per day, subcutaneously, starting on the day of inoculation, and five untreated controls. Fig. 1 *B* shows the composite data from three studies of peak parasitemias in a total of 15 DF-treated rats and 14 controls. Peak parasitemia in untreated controls was reached on days 11–14 after inoculation, and ranged from 52–663 infected cells per 1,000 RBC. In contrast, in DF-treated animals, peak parasitemia was reached on days 4–10 and ranged from 6–40 per 1,000 RBC. Until day 6–7, there was no discernible difference in the parasitemia between the control and DF-treated group. By day 10 and after, the difference in parasite counts in the two groups was significant ( $p < 0.01$ ).

To examine the effects of DF dose on parasite growth in vivo, the results of DF 1 mg/g per day were compared with 0.5, 0.25, and 0.125 mg/g per day in groups of eight animals. Two of the animals receiving DF 1 mg/g per day died of drug toxicity manifested in stunted growth and cachexia, with parasite counts of 1.1 and 1.4%. Peak parasitemia (geometric means) in the untreated group was 18.7% compared with 2.6, 1.9, 3.4, and 2.1% in the 1.0, 0.5, 0.25, and 0.125 mg/g per day groups, respectively. In comparison with untreated controls, all doses were effective ( $p < 0.01$ ) in suppressing infection, and no difference in efficacy between the doses was observed. In contrast, no suppressive effect could be seen when DF treatment (1 mg/g per day) given by continuous infusion was started on day 7 ( $n = 4$ ) or 12 ( $n = 6$ ) of the infection as judged by daily parasite counts and mortality.

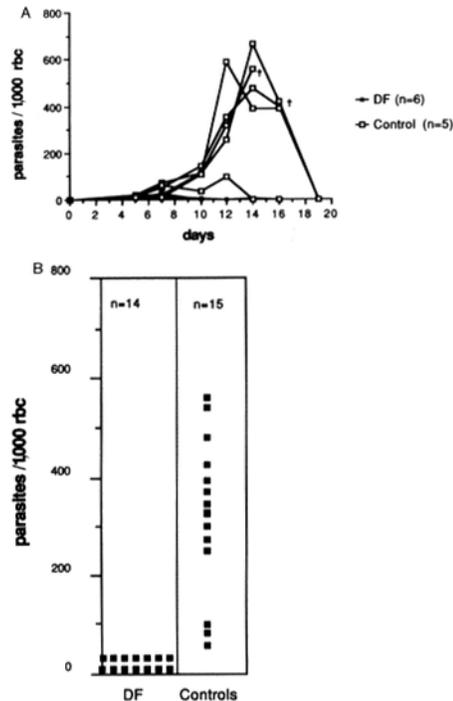


FIGURE 1. (A) Individual parasite counts in control and DF-treated rats. DF discontinued on day 19. Counts between days 19 and 28 did not exceed 0.01% in either group. (B) Combined results of three studies representing peak parasitemia in individual rats.

#### *Effect of DF and Malaria on Host Iron Status*

To examine the effect of DF treatment on host iron status, four groups of 5–6 rats were divided into those receiving (a) no treatment (normal); (b) DF only (normal + DF); (c) malaria; or (d) malaria + DF. 24 h before inoculation (day –1) all animals were given 1  $\mu\text{g}^{59}\text{Fe}$ -ferric citrate by intravenous injection. Groups (c) and (d) were infected on day 0. All animals were killed on day 12. Results of these experiments are presented in Table I and Fig. 2.

The effect of DF treatment on normal animals is shown by the comparison of the first two experimental groups (a and b). There was a marked reduction in body weight but there was no effect on hemoglobin concentration, MCV, numbers of reticulocytes, serum iron level, or transferrin saturation. Hepatic non-heme iron was reduced by 56%. There was a marked effect of DF on total body radioactivity, with excretion of 25% of the injected  $^{59}\text{Fe}$  in excess of spontaneous excretion in untreated controls. As shown by the organ radioactivity on day 12, this excretion was recruited from hepatic and residual iron stores, whereas blood radioactivity was unaffected by DF treatment.

The effect of *P. berghei* infection on iron status is shown by comparison of groups a and c. Infection resulted in stunted growth and a striking increase in splenic size (4.55 vs. 0.41 g). By day 12, all infected animals had severe anemia with an increase in MCV and reticulocytosis. The increase in MCV was the composite effect of surviving old RBC with an MCV of 56 fl and the emergence of young RBC with an MCV of  $\sim 100$  fl. Transferrin saturation was slightly increased, but there was no significant increase in hepatic non-heme iron. The percent reduction in blood  $^{59}\text{Fe}$

TABLE I  
Effect of DF and Malaria on Host Iron Status

	(a) Normal	(b) Normal + DF	(c) Malaria	(d) Malaria + DF
<i>n</i>	5	6	6	6
Day 0				
Weight (g): Body	140.0 ± 7.4	137.0 ± 4.1	137.0 ± 3.0	141.0 ± 3.7
Day 12				
Weight (g): Body	159.0 ± 9.1	132.0 ± 14.2	140.6 ± 7.1	135.0 ± 2.2
Liver	6.6 ± 0.5	5.7 ± 1.1	7.6 ± 1.0	7.5 ± 1.0
Spleen	0.4 ± 0.03	0.5 ± 0.2	4.6 ± 0.4	2.0 ± 0.3
Parasitemia	—	—	32.1 ± 14.9	0.13 ± 0.1
Iron status				
Hb	13.5 ± 0.9	14.3 ± 1.2	5.2 ± 1.4	8.2 ± 0.5
HCT	37.1 ± 1.8	39.2 ± 3.1	16.8 ± 2.8	22.7 ± 1.5
MCV	56.3 ± 1.0	55.2 ± 0.7	85.5 ± 14.2	59.8 ± 2.8
Retics (%)	2.7 ± 0.5	2.0 ± 0.9	46.3 ± 13.4	11.4 ± 5.1
Se Fe (μM)	55.7 ± 8.3	76.0 ± 14.1	59.1 ± 14.4	84.8 ± 26.2
TIBC (μM)	84.6 ± 8.9	92.7 ± 11.6	76.5 ± 23.1	117.8 ± 9.9
Saturation (%)	65.6 ± 3.6	82.3 ± 12.9	81.0 ± 22.2	71.0 ± 0.2
Liver Fe (μg)	1,237 ± 191.1	544.4 ± 44.5	1,327.5 ± 82.8	671.4 ± 47.1
(%) Organ distribution <sup>59</sup> Fe				
Blood	54.5 ± 7.7	56.2 ± 11.5	17.7 ± 5.7	31.0 ± 2.0
Liver	18.1 ± 2.0	3.7 ± 1.0	28.6 ± 1.6	7.1 ± 0.8
Spleen	0.8 ± 0.1	0.8 ± 0.3	17.8 ± 3.7	7.8 ± 1.1
Kidney	1.3 ± 0.2	1.0 ± 0.4	1.7 ± 0.5	0.5 ± 0.1
Residual	12.9 ± 7.1	0 ± 4.0	18.3 ± 2.7	12.4 ± 1.8
Excretion	13.8 ± 2.1	38.3 ± 2.8	15.9 ± 2.5	41.2 ± 1.1

was identical to the reduction in hematocrits, indicating equal destruction of young (<sup>59</sup>Fe-labeled) and old, unlabeled erythrocytes. The loss of blood radioactivity is accounted for by a simultaneous increase in hepatic and splenic radioactivity. The lack of increase in spontaneous <sup>59</sup>Fe excretion and low kidney radioactivity exclude significant hemoglobinuria associated with the severe hemolysis.

DF treatment in infected animals (group *d*), had a dual effect. On one hand it suppressed the manifestations of the infectious process. Thus, splenomegaly, anemia, and all secondary effects of hemolysis such as reticulocytosis, increased MCV, reduced blood radioactivity, and reciprocal <sup>59</sup>Fe accumulation in spleen and liver were less pronounced in the DF-treated group. On the other hand, it promoted iron excretion. Thus, all the effects of DF observed in normal animals, such as increased radioiron excretion and reduced storage iron documented both by <sup>59</sup>Fe and non-heme iron measurements, could be discerned in infected animals as well. Comparison of groups *b* and *d* reveals that malaria did not alter the magnitude of these changes. The suppressive effect of DF on malaria was achieved without any reduction in transferrin saturation or interference with hemoglobin production, and with only a moderate effect on hepatic non-heme iron stores.

#### Effect of Host Iron Status on Malaria.

The putative effect of host iron status on the rate of malaria progression was studied by the induction of iron deficiency or iron overload.

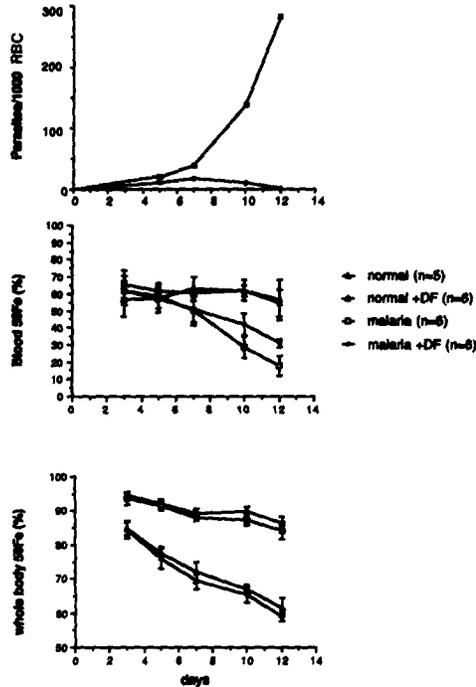


FIGURE 2. Mean parasitemia (geometric), whole body  $^{59}\text{Fe}$  and blood  $^{59}\text{Fe}$  counts (mean  $\pm$  1 SD in DF-treated and control animals. For identification of groups see Table I.

*Iron Deficiency.* Table II documents the severity of iron deficiency in weanlings raised on an iron-deficient diet (iron, 7 ppm) for 22 d, compared with controls raised on the same diet supplemented with iron at 140 ppm. The hematocrit, MCV, and body weight on day 0 and 10 of the study were all significantly lower in the iron-

TABLE II  
*Effects of Iron Deficiency*

	Control	Iron Deficient
<i>n</i>	5	7
Day 0		
Weight (g): Body	144.0 $\pm$ 7	127.0 $\pm$ 4.4
Reticulocytes (%)	4.4 $\pm$ 0.3	3.2 $\pm$ 1.1
Hematocrit	46.4 $\pm$ 1.7	24.7 $\pm$ 5.7
MCV	59.6 $\pm$ 1.3	54.0 $\pm$ 2.9
Day 7		
Reticulocytes (%)	6.9 $\pm$ 2.4	26.7 $\pm$ 20.3
Day 10		
Hematocrit	17.8 $\pm$ 3.6	9.7 $\pm$ 1.4
Hb (g/dl)	5.3 $\pm$ 0.9	3.2 $\pm$ 0.3
MCV (fl)	94.7 $\pm$ 7.8	67.8 $\pm$ 2.7
Serum Fe ( $\mu\text{M}$ )	90.6 $\pm$ 22.2	14.9 $\pm$ 3.7
TIBC ( $\mu\text{M}$ )	95.4 $\pm$ 24	88.5 $\pm$ 11.5
Saturation (%)	96.1 $\pm$ 2.3	17.3 $\pm$ 5.9
Parasitaemia (%)	35.7	30.0
Deaths	0	2

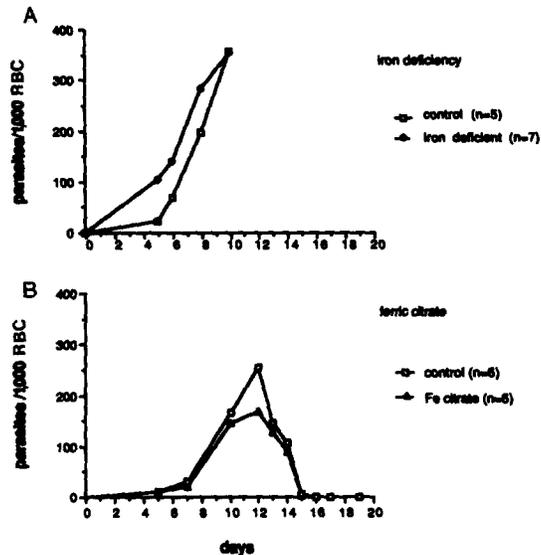


FIGURE 3. Mean parasitemia in two studies comparing (A) chronic iron deficiency and (B) acute iron loading with ferric citrate with the corresponding control groups.

deficient group. In spite of the hemolysis associated with malaria and manifested in the further aggravation of anemia, transferrin saturation in iron-deficient rats at the conclusion of the study was only 17% compared with 96% in the control group. Throughout the entire study, iron-deficient rats had higher parasite counts when expressed as percent infected RBC (Fig. 3 A). However, this difference is not corrected for the lower red cell counts in the anemic iron-deficient group. Peak parasitemia on the last day of study was nearly identical in both groups.

**Iron Loading.** This was achieved by daily intraperitoneal injections of 7.5 mg iron/kg supplied as ferric citrate, on 10 consecutive days starting on day 0. As shown in Fig. 3 B, iron loading to a cumulative dose representing eight times the hepatic iron stores of normal rats, had no effect on the timing or magnitude of parasite counts compared with normal controls.

#### *Effect of DF on Parasitized Reticulocytes*

Since host iron status was largely unaffected by therapeutic doses of DF, whereas gross variations in iron status induced by other means had no effect on the progression of malaria, we examined the possibility that DF may interact directly with chelatable iron in parasitized erythrocytes. The putative existence of chelatable iron was investigated by gel filtration of hemolysates obtained from parasitized erythrocytes labeled *in vivo* by the intravenous injection of  $^{59}\text{Fe}$ -citrate 16 h previously. The hemolysate was preincubated with 70  $\mu\text{M}$  DF before separation on Ultrogel ACA-34 columns in order to prevent the retention of loosely bound chelatable iron on the column. As shown in Fig. 4 A, in addition to a major peak of labeled hemoglobin (84.5%), the existence of a void volume fraction (5.8%), ferritin (7.7%), and a low molecular weight radioiron complex (2.0%) could be demonstrated in parasitized erythrocytes. Control experiments showed that DF-treated lysates prepared from uninfected cells, radiolabeled 6 d previously, only showed a single hemoglobin peak.

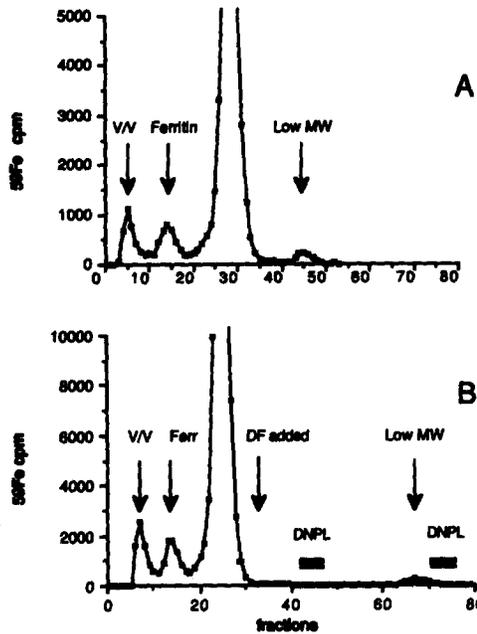


FIGURE 4. Gel filtration (Ultragel ACA-34) of lysates obtained from parasitized RBC labeled *in vivo* with  $^{59}\text{Fe}$  16 h previously. The major peak represents hemoglobin. Arrows indicate void volume (vol/vol), and positions of ferritin (*Ferr*) and low molecular weight (*Low MW*) peaks. Deferoxamine (*DF*, 70  $\mu\text{M}$ ) was added to the lysate before gel filtration (*A*) or to the column after the elution of the hemoglobin peak (*B*). (*B*) Dinitrophenol (*DNPL*) was added as a low molecular weight marker first to the lysate, and second with *DF*. The total radioactivity of the lysate was 53,000 (*A*) and in 118,000 cpm (*B*). Total recovery in the eluates was 95.5 and 91.0%, respectively.

To distinguish between labile iron present *a priori* in parasitized reticulocytes or produced *in vitro* by the interaction of *DF* with either ferritin or hemoglobin iron, the lysate was subjected to chromatography without preincubation with *DF*. As shown in Fig. 4 *B*, only the labeled void volume fraction, ferritin, and hemoglobin were eluted initially from the Ultragel column. However, subsequent washing with 70  $\mu\text{M}$  of *DF* resulted in the elution of a chelated radioiron fraction similar to that described in Fig. 4 *A*, representing chelatable iron retained temporarily on the column. This finding proves that the labile iron is not derived from the *in vitro* interaction of *DF* with ferritin or hemoglobin iron, since neither has been exposed to *DF* at any time, and supports the *a priori* existence of a chelatable, labile iron pool in parasitized reticulocytes.

To study further the interaction between labeled parasitized reticulocytes and *DF*, a simplified method was used using Amicon filters that retain molecules >30,000 daltons. Table III describes the effect of *DF* incubation on the magnitude of the chelated and chelatable radioiron fraction as measured by Amicon filtration. Both increasing the concentration or increasing the time of *DF* incubation resulted in an increased efficiency of radioiron chelation in intact cells. Conversely, there was less radioiron remaining in the cell lysate for additional chelation. Since no radioiron was present in the incubation medium, the gradual increase with time in total chelatable iron implies the generation of chelatable iron from an existing intracellular  $^{59}\text{Fe}$  pool. We found no evidence for the release of chelated intracellular iron into the incubation medium.

To show that *DF* was able to interact *in vivo* with labile intracellular iron, 0.3 mg/g of *DF* was injected subcutaneously 2 h before killing an infected animal (45% parasitemia) labeled 16 h previously with  $^{59}\text{Fe}$ . The fraction of filtratable iron found in the radiolabeled cells was 0.55% compared with a total chelatable fraction of 1.1%.

TABLE III  
*Effects of DF Concentration and Time on the Amount of Iron Chelated in Parasitized RBC*

	Percent of total $^{59}\text{Fe}$ at DF concentration ( $\mu\text{M}$ ) of:*			
	0	50	200	500
Intact RBC	0.06 $\pm$ 0.01	0.33 $\pm$ 0.01	0.76 $\pm$ 0.02	1.07 $\pm$ 0.02
Lysate	0.74 $\pm$ 0.02	0.73 $\pm$ 0.02	0.60 $\pm$ 0.03	0.39 $\pm$ 0.03
Total	0.80 $\pm$ 0.02	1.06 $\pm$ 0.02	1.36 $\pm$ 0.02	1.46 $\pm$ 0.03
	Percent of total $^{59}\text{Fe}$ at DF treatment duration (min) of:†			
	10	50	140	330
Intact RBC	0.59 $\pm$ 0.03	1.14 $\pm$ 0.03	1.28 $\pm$ 0.03	1.86 $\pm$ 0.03
Lysate	0.71 $\pm$ 0.03	0.1 $\pm$ 0.05	0.47 $\pm$ 0.07	0
Total	1.30 $\pm$ 0.04	1.24 $\pm$ 0.03	1.75 $\pm$ 0.04	1.86 $\pm$ 0.03

\* All incubations for 180 min.

† All incubations with 600  $\mu\text{M}$  DF.

## Discussion

It has been suggested that iron deprivation is an important defense mechanism limiting the growth of invading organisms (1, 2). A number of clinical observations suggest that iron administration may aggravate the risk associated with malarial infection (3-5). The relative shortage of intracellular hemoglobin in iron-deficient RBC may be responsible for limiting parasite growth (16). Clinical evidence, however, for the protective effect of iron deficiency or the deleterious effect of iron supplementation in human malaria is not conclusive (17, 18).

In view of the possible beneficial effects of iron depletion, DF, a siderophore that binds iron with an affinity of  $10^{31} \text{ M}^{-1}$ , has been studied as a possible antimalarial agent. DF inhibits the growth of *P. falciparum* cultured in vitro at a concentration of 15  $\mu\text{M}$  (6), possibly by interfering with the acquisition of iron from transferrin (19, 20). Other studies have confirmed the in vitro inhibition of *P. falciparum* by DF, but have failed to demonstrate the uptake of transferrin iron by the parasitized RBC, or the inhibition of parasite growth by the removal of iron from the culture medium (7). The present study demonstrates that DF, given every 8 h suppresses *P. berghei* infections in rats, and recent similar studies on mice with *P. vinckei* (8) and on monkeys with *P. falciparum* (9) have shown that it is possible to suppress malarial infection in vivo by the continuous supply of DF, using implanted subcutaneous osmotic pumps or frequent daily injections. The failure of single daily injections to suppress infection, shown in our preliminary studies and in those of Fritch et al. (8), demonstrates that continuous exposure to the drug is essential.

In the present study, we have attempted to define the site of action of DF. As an iron chelator, DF might inhibit malaria in vivo by two mechanisms; firstly, by the induction of iron deficiency, which will limit the amount of iron available to the parasite by reducing plasma transferrin iron or intracellular hemoglobin; or secondly, by direct interference with the acquisition of intracellular iron by the parasite. We elected to study these questions in the rat because iron requirements and the internal recycling of iron in this species have been characterized in great detail in previous studies (11, 12).

The present data clearly show that the therapeutic in vivo effect of DF cannot

be attributed to the induction of an iron-deficient state in the host. Serum iron, transferrin saturation, hemoglobin production, and red cell indices remained unchanged after 12 d of continuous DF treatment at doses exceeding eight times the minimal daily dose required to suppress malaria infection *in vivo*. These findings exclude the possibility that DF inhibition of malaria is achieved by reducing the amount of available plasma iron or intracellular hemoglobin. Further evidence against the relevance of host iron status for *in vivo* malaria proliferation has been provided by our studies in iron-deficient and iron-loaded animals, showing no effect on death rates or parasite counts. These findings appear to conflict with those of Harvey et al. (21) who found a mild, protective effect of iron deficiency in mice infected by *P. chabaudi*. However in that study, the iron deficient diet was started only 3 d before infection, and no data were provided to document iron deficiency.

By exclusion, therefore, the most likely explanation for the *in vivo* therapeutic effect of DF is a direct interaction with the parasitized erythrocyte. This possibility is supported by the ability of DF to inhibit the *in vitro* growth of malaria irrespective of extracellular iron concentrations (7); the rapidity with which very low doses of DF are able to inhibit *in vivo* malaria in monkeys (9) and the rapid rebound rise in parasitemia in mice after the withdrawal of DF (8). If DF acts by depriving the parasite of intracellular iron, it follows that a chelatable pool of iron exists in parasitized erythrocytes, and that DF must be able to penetrate RBC.

In mature erythrocytes, practically all iron is hemoglobin bound and therefore not available for chelation. In contrast, in erythroblasts and reticulocytes, in which hemoglobin synthesis is still active, there is a continuous uptake of iron by internalization of the transferrin-transferrin-receptor complex (22). Evidence supporting the existence of a low molecular weight intracellular iron pool has been reviewed by Jacobs (23). Such a pool functions as an intermediate between extracellular iron and the intracellular iron-containing proteins. Although the exact chemical nature of the pool is unknown, it should all be available for DF chelation. In reticulocytes, several distinct patterns of interaction between iron-chelating drugs and the intracellular chelatable pool have been identified. Some chelators, such as EDTA, are unable to enter the cells. Others, such as pyridoxal isonicotinoyl hydrazone (PIH), readily enter reticulocytes, and the chelated iron complex is extruded by an energy-dependent process (24). Finally, some very potent chelators such as N,N'-ethylenebis(*o*-hydroxyphenylglycine) readily chelate intracellular iron, but the chelated complex is unable to cross the cellular membrane and accumulates within the cell (25). DF shows only a limited uptake by normal reticulocytes, and such iron as is chelated by DF intracellularly is unable subsequently to leave the cell (25). However, parasitized RBC are, in general, more permeable to malaricidal metal-chelating drugs than healthy reticulocytes (26, 27), and the uptake of DF by parasitized erythrocytes has been directly demonstrated in a recent study using  $^{14}\text{C}$ -deferoxamine (28).

Results of the present study extend the observations on the interaction of DF with intact reticulocytes to parasitized RBC. Our studies of *in vivo* labeled heavily parasitized reticulocytes indicate that ~1-2% of the total  $^{59}\text{Fe}$  is located in a chelatable iron fraction, which, similar to the labile iron pool found in cultured Chang cells (23), is retained on gel filtration columns but can readily be eluted by DF. With increasing concentrations of extracellular DF or with increasing duration of incubation, a proportionately greater part of the available iron in intact reticulocytes is

chelated by DF until little or no additional chelation is achieved by subsequent incubation of the lysate with a huge excess of added DF. These findings confirm that DF is able to penetrate parasitized reticulocytes and indicate that it can interact with a chelatable iron pool. As shown in our study, the chelatable iron of parasitized reticulocytes is available for interaction with DF both in vivo and in vitro.

The design of these studies does not permit any conclusion as to the origin of this iron pool in parasitized erythrocytes. Since *P. berghei* is a reticulocyte-seeking parasite, it is possible that the chelatable iron is derived from the continued uptake of transferrin iron by these reticulocytes, or by putative transferrin-receptor synthesised in mature parasitized RBC (29–31). The present study, however, showed a gradual increase with time in the fraction of labeled chelatable iron in the absence of measurable extracellular  $^{59}\text{Fe}$ , indicating the generation of chelatable iron from an existing intracellular pool. The pool is likely to be derived from hemoglobin; malarial parasites exert oxidant stress on the host erythrocyte (32–34) and it has recently been shown by Gutteridge (35) that  $\text{H}_2\text{O}_2$  and other peroxides are able to release iron from hemoglobin by oxidative degradation.

Although our study provides evidence for the direct interaction of DF with a chelatable iron pool in the parasitized reticulocyte, it yields little information on the manner in which this may result in the inhibition of parasite proliferation. We have shown that the suppressive effect of DF only occurred after 5 d or more of continuous treatment and could not be shown at all if treatment was started after 7 d of infection. This slow rate of action may reflect, as already discussed, the limited ability of DF to penetrate erythrocytes. Low concentrations of DF may be inadequate to compete with the increasing amounts of intracellular iron associated with the increasing immaturity of reticulocytes found in the terminal phase of infection. Alternatively, intracellular iron chelation itself may only exert a slow parasitocidal effect. Some antimalarial iron chelators, such as 8-hydroxyquinoline, are parasitocidal by forming an extracellular complex with iron that is subsequently internalized to produce a rapidly lethal intracellular reaction (26, 36). Other compounds, such as rhodotorulic acid, mycobactin, and probably DF, exert their antimalarial effect through the deprivation of intracellular iron from the parasite. The iron requirements of the parasite is at present unknown.

Studies of the mechanisms of the parasitocidal effects of DF may have important implications for the pathophysiology of malaria and the development of new strategies for antimalarial therapy. In spite of the effectiveness of DF in suppressing malaria in experimental animals, it is unlikely that it will be suitable for the management of clinical malaria. Its poor oral absorption, high price, and relatively slow rate of red cell penetration make it an unlikely candidate for clinical use. However, a new generation of orally effective iron chelators are already available (25), and exploration of their antimalarial efficacy is one of the obvious objectives of future research.

### Summary

The mechanism whereby deferoxamine (DF) inhibits the growth of malaria parasites was studied in rats infected with *Plasmodium berghei*. Peak parasitemia was 32.6% (day 14) in untreated controls and 0.15% (day 7) in rats receiving 0.33 mg/g in 8 hourly DF injections, subcutaneously. DF inhibition of parasite growth was achieved without any reduction in transferrin saturation or hemoglobin synthesis and with

only a partial (56%) depletion of hepatic iron stores. Dietary iron depletion resulted in anemia (hematocrit 25 vs. 46%), microcytosis (MCV 54 vs. 60 fl), and reduced transferrin saturation (17 vs. 96%) without any effect on infection (peak parasitemia 30 vs. 36%). Similarly, parenteral iron loading with ferric citrate over 10 d (75 mg iron/kg) failed to aggravate infection. In a search for evidence of direct interaction between DF and parasitized erythrocytes, gel filtration and ultrafiltration was performed on hemolysates obtained from in vivo <sup>59</sup>Fe-labeled parasitized erythrocytes. This showed that 1.1–1.9% of the intracellular radioiron was located in a chelatable, labile iron pool. Incubation of intact cells with 0–500 μM DF resulted in a proportional increase in intracellular iron chelation, and the chelation of all available labile intracellular iron was completed within 6 h. These observations indicate that the severity of *P. berghei* infection in rats and its in vivo suppression by DF are independent of host iron status and suggest that DF inhibition of malaria involves intracellular chelation of a labile iron pool in parasitized erythrocytes.

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#### References

1. Weinberg, E. D. 1974. Iron and susceptibility to infectious disease. *Science (Wash. DC)* 184:952.
2. Kochan, I. 1973. The role of iron in bacterial infections with special consideration of host-tubercle bacillus interaction. *Curr. Top. Microbiol. Immunol.* 60:1.
3. Byles, A. B., and A. D'sa. 1970. Reduction of reaction due to iron dextran infusion using chloroquine. *Br. Med. J.* 3:625.
4. Murray, M. J., A. B. Murray, M. B. Murray, and C. J. Murray. 1978. The adverse effect of iron repletion on the course of certain infections. *Br. Med. J.* 2:1113.
5. Oppenheimer, S. J., F. D. Gibson, S. B. MacFarlane, J. B. Moody, C. Harrison, A. Spencer, and O. Bunari. 1986. Iron supplementation increases prevalence and effects of malaria: report on clinical studies in Papua New Guinea. *Trans. Roy. Soc. Trop. Med. Hyg.* 80:603.
6. Raventos-Suarez, C., S. Pollack, and R. L. Nagel. 1982. Plasmodium falciparum: inhibition of in vitro growth by desferrioxamine. *Am. J. Trop. Med. Hyg.* 31:919.
7. Peto, T. E. A., and J. L. Thompson. 1986. A reappraisal of the effects of iron and desferrioxamine on the growth of plasmodium falciparum in vitro: the unimportance of serum iron. *Br. J. Haematol.* 63:273.
8. Fritch, G., J. Treumer, D. T. Spira, and A. Jung. 1985. Plasmodium vinckei: suppression of mouse infections with desferrioxamine B. *Exp. Pathol. (Jena)* 60:171.
9. Pollack, S., R. N. Rossan, D. E. Davidson, and A. Escajadillo. 1987. Desferrioxamine suppresses Plasmodium falciparum in aotus monkeys. *Proc. Soc. Exp. Biol. Med.* 184:162.
10. Warner, G. T., and R. Oliver. 1966. A whole-body counter for clinical measurements utilizing the 'shadow shield' technique. *Phys. Med. Biol.* 11:83.
11. Hershko, C., J. Cook, and C. A. Finch. 1974. Storage iron kinetics VI. The effect of inflammation on iron exchange in the rat. *Br. J. Haematol.* 28:67.
12. Cook, J. D., C. Hershko, and C. A. Finch. 1973. Storage iron kinetics. V. Iron exchange in the rat. *Br. J. Haematol.* 25:695.
13. International Committee for Standardization in Hematology. Expert Panel on Iron. 1971. Proposed recommendations for measurement of serum iron in human blood. *Br. J. Haematol.* 20:451.

14. Torrance, J. D., T. H. Bothwell. 1968. A simple technique for measuring storage iron concentrations in formalized liver samples. *South Afr. J. Med. Sci.* 33:9.
15. Awai, M., Y. Yamanai, J. Kuwashima, and S. Satimaru. 1982. Induction mechanism of diabetes by ferric nitriloacetate injection. In *The Biochemistry and Physiology of Iron*. P. Saltman and J. Hegenauer, editors. Elsevier/North Holland, Amsterdam. 543-554.
16. Nurse, G. T. 1979. Iron, the thalassaemias, and malaria. *Lancet*. 2:938.
17. Harvey, P., P. Heywood, M. C. Nesheim, J. P. Habicht, and M. Alpers. 1987. Iron repletion and malaria. *Fed. Proc.* 46:1161. (Abstr.).
18. McGregor, I. A. 1982. Malaria: nutritional implications. *Rev. Infect. Dis.* 4:798.
19. Pollack, S., and J. Fleming. 1984. Plasmodium falciparum takes up iron from transferrin. *Br. J. Haemat.* 58:289.
20. Pollack, S. 1983. Malaria and iron. *Br. J. Haemat.* 53:181.
21. Harvey, P. W. J., R. G. Bell, and M. C. Nesheim. 1985. Iron deficiency protects inbred mice against infection with Plasmodium chaubaudi. *Infect. Immun.* 50:932.
22. Huebers, H. A., and C. A. Finch. 1987. Transferrin and transferrin receptors. *Physiol. Rev.* 67:520.
23. Jacobs, A. 1977. Low molecular weight intracellular iron transport compartments. *Blood*. 50:433.
24. Huang, A. R., and P. Ponka. 1983. A study of the mechanism of action of pyridoxal isonicotinoyl hydrazone at the cellular level using reticulocytes with non-heme <sup>59</sup>Fe. *Biochem. Biophys. Acta.* 757:306.
25. Ponka, P., R. W. Grady, A. Wilczynska, and H. M. Schulman. 1984. The effect of various chelating agents on the mobilization of iron from reticulocytes in the presence and absence of pyridoxal isonicotinoyl hydrazone. *Biochim. Biophys. Acta.* 802:477.
26. Scheibel, L. W., G. G. Stanton. 1986. Antimalarial activity of selected aromatic chelators. IV. Cation uptake by plasmodium falciparum in the presence of oxines and siderosomes. *Mol. Pharmacol.* 30:364.
27. Ginsburg, H., R. Gorodetsky, and M. Krugliak. 1986. The status of zinc in malaria (Plasmodium falciparum) infected human red blood cells: stage dependent accumulation, compartmentation and effect of dipicolinate. *Biochim. Biophys. Acta.* 886:337.
28. Fritch, G., and A. Jung. 1986. <sup>14</sup>C-desferrioxamine B uptake into erythrocytes infected with Plasmodium falciparum. *Z. Parasitenk. D.* 72:709.
29. Rodriguez, M. H., and M. Jungery. 1986. A protein on Plasmodium falciparum-infected erythrocytes functions as a transferrin receptor. *Nature (Wash. DC).* 324:388.
30. Haldar, K. H., C. L. Henderson, and G. A. M. Cross. 1986. Identification of the parasite transferrin receptor of Plasmodium falciparum-infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. *Proc. Natl. Acad. Sci. USA.* 83:8565.
31. Pollack, S., and V. Schnelle. 1988. Inability to detect transferrin receptors on P. Falciparum parasitized red cells. *Br. J. Haemat.* 68:125.
32. Eaton, J. W., J. R. Eckman, E. Berger, and H. S. Jacobs. 1976. Suppression of malaria infection by oxidant-sensitive host erythrocytes. *Nature (Wash. DC).* 264:758.
33. Pasvol, G., and D. J. Weatherall. 1980. The red cell and the malarial parasite. *Br. J. Haemat.* 46:165.
34. Clark, I. A., and N. H. Hunt. 1983. Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infect. Immun.* 39:1.
35. Gutteridge, J. M. C. 1986. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 201:291.
36. Scheibel, L. W., and A. Adler. 1980. Antimalarial activity of selected aromatic chelators. *Mol. Pharmacol.* 18:320.