Intravenous Ferric Carboxymaltose Accelerates Erythropoietic Recovery From Experimental Malarial Anemia

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Iron restriction has been proposed as a cause of erythropoietic suppression in malarial anemia; however, the role of iron in malaria remains controversial, because it may increase parasitemia. To investigate the role of iron-restricted erythropoiesis, A/J mice were infected with Plasmodium chabaudi AS, treated with intravenous ferric carboxymaltose at different times, and compared with untreated controls. Iron treatment significantly increased weight and hemoglobin nadirs and provided enhanced reticulocytosis and faster recovery, compared with controls. Our findings challenge the restrictive use of iron therapy in malaria and show the need for trials of intravenous ferric carboxymaltose as an adjunctive treatment for severe malarial anemia.

Malaria remains a leading cause of childhood mortality associated with complications, such as severe malarial anemia (SMA), which is particularly common among infants in areas with high transmission intensity. SMA arises when an accelerated destruction of erythrocytes stays uncompensated because of malaria-induced erythropoietic suppression. Iron-restricted erythropoiesis has been proposed as a cause of the latter on the basis of the observation of elevated hepcidin, restricted erythropoiesis has been proposed as a cause of the former [9]. Here, we show that ferric carboxymaltose treatment accelerates erythropoietic and clinical recovery from experimental malarial anemia without unwanted effects on parasite multiplication.

METHODS

Experimental Infection and Treatment

Eight-week-old, male, pathogen-free A/J mice (Harlan) kept under standard conditions were infected intraperitoneally with 10^4 Plasmodium chabaudi AS from a frozen stock through a single passage in C57Bl/6j mice [10]. The P. chabaudi AS strain was obtained from University of Reading, United Kingdom, originally derived from the parasite strain collection at University of Edinburgh, United Kingdom.

All experiments complied with Danish and European guidelines for animal research and were approved by the national board for animal studies. The mice were treated intravenously with a 3-day course of 600 µg iron daily as ferric carboxymaltose (Ferinject; Vifor Pharma). Treatment was started either at inoculation (days 0–2; n = 10), at patent parasitemia (days 5–7; n = 15), or immediately prior to the hemoglobin nadir (days 9–11; n = 10). Untreated mice (n = 15) were included as controls. Five mice were removed from the control and inoculation (days 0–2) groups at day 9 for pilot studies (R. E. Sharp, L. Maretty, unpublished data, May 2010).

Outcome Measures

Hemoglobin concentration was determined in whole blood with use of a plate-based version of AHD-575 spectrophotometry validated in our laboratory [11].
Parasites and reticulocytes were counted using flow cytometry essentially as described previously [12]. In brief, 2 μL of blood was suspended in 98 μL of heparinized phosphate-buffered saline (PBS; 300 U/mL); 10 μL of this mixture was transferred to a well containing 200 μL of PBS with acridine orange (0.5 μg/mL), incubated for 15 minutes, and acquired on a MXP-500 flow cytometer (Beckman Coulter). Data were analyzed using FlowJo software (TreeStar) by gating red blood cells (RBCs) on light scatter, followed by enumeration of normocytes, parasitized RBCs, and reticulocytes based on DNA/RNA content. Of note, in our laboratory we have observed improved performance of this assay on resolution of parasite and reticulocyte populations, using an increased incubation time [12]; this may be of future value for similar studies in the field (L. Maretty, unpublished data, April 2010). Because we do not know of any previous validation studies of this assay on reticulocyte counting performance in the current context, a comprehensive evaluation was conducted using microscopy (Supplementary Methods). Reticulocyte fractions were converted to cell concentrations by multiplication with RBC concentrations obtained from hemoglobin concentrations (Supplementary Methods).

Statistical Analyses
The data were analyzed using a mixed-effects model (SAS, version 9.2; SAS Institute) with individual mice and treatment groups included as random and fixed effects, respectively. All mice were treated as controls until their first treatment. Weights were normalized to baseline. \( P \) values < .05 were considered statistically significant. The reported experiments were a repeat of an initial pilot study showing essentially identical results for the day 5–7 treatment regimen (n = 10).

RESULTS
We used a standard experimental model of malarial anemia to study the effect of intravenous ferric carboxymaltose treatment on erythropoiesis, parasitemia, and weight as a marker of disease severity [10, 13–15]. As expected, all mice exhibited a steep increase in parasitemia on days 7–9 and a marked decrease in weight and hemoglobin concentration until the nadir, occurring at approximately day 11 after inoculation (Figures 1 and 2A–D).

Treatment during the early clinical course (day 5–7) significantly prevented the decrease in hemoglobin concentration \( (P = .03) \) (Figure 1B) and induced higher reticulocyte counts \( (P < .0001) \) (Figure 1C) on day 11, compared with controls. Similar effects were observed for treatment provided during severe signs of malaria (days 9–11), although the difference in hemoglobin levels did not reach statistical significance until day 13 \( (P < .0001) \) (Figure 1B). Finally, hemoglobin levels recovered more rapidly in treated than untreated mice \( (eg, \) treatment days 5–7, difference in mean hemoglobin concentration \( \pm \) standard error of the mean on day 13 was 1.6 ± 0.3 mM; \( P < .0001) \).

Ferric carboxymaltose had no adverse effects on parasitemia and disease progression. On the contrary, treatment on days 5–7 or days 9–11 resulted in significantly higher weights, compared with controls on days 11–15 and days 13–19, respectively \( (P < .05) \) (Figure 1A). No significant differences in parasite count were observed between individual treatment groups and controls during peak parasitemia and when parasitemia recovered after day 15 \( (P > .13) \) (Figures 1D and 2D).

To test whether administration of ferric carboxymaltose prior to disease development is a risk factor for severe disease, we treated a third experimental group around the time of inoculation (days 0–2). This caused a significant decrease in parasitemia on day 7 \( (P < .0001) \) (Figures 1D and 2D), followed by a small, significant increase in parasitemia on day 9, compared with control mice \( (P = .03) \); after that, no differences were observed. In line with the other treatment regimes, treatment on days 0–2 improved erythropoietic and clinical recovery, however, with significantly improved hemoglobin levels from day 9 \( (P = .03) \) (Figure 2B) and a delayed reticulocytosis, compared with the other treatment groups (Figures 1C and 2C).

DISCUSSION
Our findings unanimously showed that ferric carboxymaltose treatment during the clinical course of experimental malaria accelerated erythropoietic recovery. More specifically, iron treatment reversed the progression of anemia by stimulation of a robust reticulocyte response and was associated with faster clinical recovery as estimated by weight.

Epidemiological studies on the interaction between host iron status, oral iron supplementation, and malaria-associated mortality have produced conflicting results, ranging from protection from severe anemia to increased mortality associated with an increase in malaria susceptibility, the latter especially in iron-replete individuals [5, 7, 8].

We observed no effects on parasite proliferation of ferric carboxymaltose treatment during the clinical course of infection. To further evaluate the safety aspect of ferric carboxymaltose treatment, 1 group of mice was subjected to iron treatment already at the time of inoculation. This caused a highly significant decrease in parasitemia, followed by a temporary, modest increase in parasitemia in the treated group. Our current experiment does not allow for any inference on the cause of this initial depression in parasitemia. However, we speculate that the increase in parasitemia observed on day 9 is likely to have been secondary to the temporary inhibition of parasite growth on day 7, leaving more
RBCs for reinvasion as the inhibitory effect of ferric carboxymaltose (FC) decays [14]. Despite these oscillations in parasitemia, this treatment regimen was associated with favorable hematological and clinical outcomes. However, the reduced initial parasite densities, along with the early increase in hemoglobin levels and delayed reticulocyte response, suggest that treatment effects in this group may be partly secondary to a reduced parasite burden.

Previous studies have reported elevated hepcidin levels, reduced plasma iron levels, and impaired iron incorporation into hemoglobin in malaria-infected humans [1–3]. These data, together with the observation of erythropoietin nonresponsiveness in both human and murine malaria, suggests a role for iron-restricted erythropoiesis [1, 9, 13]. Whether erythroid iron supply constitutes a determinant for the suppressed erythropoietic response to malarial anemia has not been studied directly. Given the marked increase in hemoglobin and reticulocyte levels with no effects on parasite densities in the late treatment groups, our data strongly suggest that iron does constitute a limiting factor in erythropoiesis at the recovery stage of malaria. However, no effect of iron treatment was observed during peak parasitemia, and because little is known on the exact route of iron from intravascular FC to the erythron, additional studies are needed to fully understand these effects and to identify treatment modifiers, such as host- or parasite-derived factors that determine the erythropoietic response to iron during acute malaria [15]. Finally, given the large geographical overlap

**Figure 1.** Response to ferric carboxymaltose (FC) treatment at clinical stages of *Plasmodium chabaudi* AS infection in A/J mice. Two groups of male A/J mice were infected with $10^4$ *P. chabaudi* AS parasites and subjected to FC treatment at parasite patency (FC days 5–7) or prior to the hemoglobin nadir (FC days 9–11). Similarly infected, untreated mice were included as controls. Iron treatment was associated with a significant increase in weight (A), hemoglobin level (B), and reticulocyte concentration (C). No differences in parasite densities were observed (D). *Statistically significant difference ($P < .05$) between treatment group designated by color and controls inferred using a mixed-effects model.
between malaria endemicity and nutritional iron deficiency, additional studies will also investigate the effects of FC treatment in experimental malarial anemia complicated by iron deficiency.

In conclusion, our findings demonstrate a role for iron homeostasis in malaria-induced erythropoietic suppression. The favorable clinical outcome challenges the view of iron treatment as detrimental in malarial anemia. Patients with SMA commonly present to healthcare facilities with limited access to safe blood transfusion, which renders the option of attaining rapid hematological recovery with a safe iron preparation highly clinically relevant. We acknowledge the limitation of intravenous drug administration in peripheral healthcare facilities but still find its use more accessible than blood transfusion. Additional studies on the efficacy and safety of ferric carboxymaltose treatment for malarial anemia are warranted in preparation of clinical trials.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all

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Figure 2. Response to ferric carboxymaltose (FC) treatment at inoculation in Plasmodium chabaudi AS–infected A/J mice. One group of male A/J mice was infected with 10^4 P. chabaudi AS parasites and subjected to FC treatment around inoculation (FC days 0–2). Similarly infected, untreated mice were included as controls. Iron treatment was associated with significantly lower parasitemia on day 7; this trend reversed on day 9 (D). Hemoglobin concentration (B) was significantly elevated from day 9, whereas weight (A) and reticulocyte concentration (C) reached statistical significance on day 13.

*Statistically significant difference (P < .05) between treatment group and controls.
supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Casper Hempel, Trine Staalsøe, Brian Della-Valle, Grethe Gomme, and Marjan Yosefi for invaluable discussions. The *P. chabaudi* AS strain was a kind gift of Tracey Lamb (University of Reading, United Kingdom).

Financial support. This work was supported by The Danish Council for Independent Research (Medical Sciences; grant number 271-08-1003) and Vífor Pharma. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of this manuscript.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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