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EFFECT OF DIETARY IRON SUPPLEMENTATION ON THE COURSE OF *PLASMODIUM CHABAUDI* MALARIA IN WEANLING MICE

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ABSTRACT

The hypothesis that iron repletion may enhance parasite multiplication and aggravate malaria infection in iron-deficient hosts was tested in a murine model of dietary iron deficiency and iron supplementation. Weanling C57Bl/6J mice were fed diets containing either 15mg iron/kg diet (Group D, n=20) or 50 mg iron/kg diet (Group N, n=12). After 30 days, when hemoglobin levels (Hb in g/100ml; Mean ± SD) were significantly lower in Group D (13.7 ± 1.2) than in from Group N (15.4 ± 2.0), 16 mice from Group D and 8 mice from Group N were inoculated intraperitoneally with 10^4 intraerythrocytic *Plasmodium chabaudi* (AS strain) malaria parasites. Four animals from each group remained as non-infected controls. At the time of inoculation, 8 mice from Group D (thereafter Group DS) and all animals from Group N (thereafter Group NS) were transferred to a diet containing 140 mg iron/kg, whereas 8 mice from Group D were maintained on the original iron-deficient diet. At the peak of parasitemias (day 10-11 post-infection), anemia was less severe in mice from Group DS if compared to those from Group D (Hb: 4.5 ± 1.1 *vs.* 3.4 ± 1.0), but more severe than in those from Group NS (5.6 ± 1.8). However, the time course of parasitemias was similar in all groups. Mortality was higher among mice from Groups D and DS (87.5%) than in those from Group NS (37.5%; Fisher's exact test, $p = 0.0104$). These data indicate that neither iron deficiency suppressed nor iron repletion enhanced *P. chabaudi* multiplication in weanling mice. Moreover, dietary iron supplementation induced hemoglobin response in iron-deficient mice but did not increase their ability to resist severe malarial anemia.

Key words: Anemia, Iron supplementation, *Plasmodium chabaudi*, Malaria

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Further details of data are available from the corresponding author.

INTRODUCTION

Although high rates of malaria and iron deficiency anemia are commonly seen together in developing countries (1), scientific evidence of a biological relation between them remains controversial (2). It is well known that malaria may cause severe anemia due to the massive rupture of parasitized red blood cells (PRBC) and concurrent phenomena such as autoimmune hemolysis, reticuloendothelial hyperfunction and diserythropoiesis (3). However, the widespread administration of iron supplements in malaria-endemic areas has been suggested to exacerbate some previously asymptomatic malaria infections in semi-immune children (2,4,5). These findings were originally explained by: (a) the increased availability of young erythrocytes, the preferential host cell for most malaria parasites, induced by iron supplementation; (b) the dependence of parasites on adequate host iron reserves, and (c) an inhibitory effect of microcytosis secondary to iron deficiency on the growth of intraerythrocytic parasites (2). In contrast, subsequent well-controlled field studies have shown no association between host iron status and malaria susceptibility (6,7). More recently, oral iron supplementation has been used to improve hematological recovery in malaria patients (8) and to prevent severe anemia in malaria-exposed children (9).

Experimental malaria models have also provided contradictory results. An early study showed strong protection against mortality from infection with unspecified strain of *Plasmodium chabaudi* infection in iron-deficient inbred NFR/N mice (10). When surviving iron-deficient animals were given oral iron supplementation, they presented parasite recrudescences (9). More recent data, using a non-lethal rodent malaria model, indicate that the multiplication of *Plasmodium berghei* in young rats is neither suppressed by dietary iron deficiency nor enhanced by either oral or parenteral iron supplementation (11,12). Here we have examined the effect of iron deficiency and dietary iron supplementation on parasite multiplication and host survival in a well-standardized model of severe rodent malaria, *P. chabaudi* AS infection in C57Bl/6J weanling mice.

METHODS AND MATERIALS

Animals and diets

All animal procedures were conducted in accordance with the Brazilian regulations of the College of Animal Experimentation. Thirty-two male weanling C57Bl/6J mice (mean body weight, 7.0 g) bred in the animal facilities of the Faculty of Medicine (University of São Paulo) were maintained on a 12h light cycle at 22–25°C in sterilized plastic cages with epoxy-coated grid covers. They were allowed free access to demineralized water and isocaloric and isoproteic diets prepared in our laboratory according to the Committee on Laboratory Animal Diets (13), except for the use of iron-free mineral mix. Ferrous sulfate ($FeSO_4.7H_2O$, Merck®) was added to the basal diet to provide 50 mg iron/kg diet for the normal iron diet and 140mg iron/kg diet for the iron-supplemented diet. The iron content of the diets and of the saline perfused livers was determined, after wet digestion (HNO_3:$HclO_4$, 5:1) by atomic absorption spectrophotometry (14).

Mice were randomly allocated to two groups, according to the iron concentration of their diets: Group D were fed the iron-deficient diet (n=20) and Group N were fed the normal-iron diet (n=12). After 30 days, 16 mice from Group D and 8 mice from Group N were infected with *P. chabaudi,* whereas 4 animals from each group remained as non-infected controls. At the time of parasite inoculation, 8 animals from Group D and all animals from Group N were transferred to the iron-supplemented diet (thereafter Groups DS and NS, respectively), whereas 8 animals from Group D were maintained on their original iron-deficient diet. To minimize blood loss, only tail vein blood samples were collected to measure hemoglobin concentrations (Hb, by the cyanmethemoglobin method) and parasitemias.

Parasite and parasitemia assessment

The AS strain of *Plasmodium chabaudi chabaudi* used in this experiment was originally obtained from Prof. David Walliker (University of Edinburgh, Edinburgh, Scotland) and cryopreserved as stabilates in liquid nitrogen. A frozen sample was thawed and injected into a C57Bl/6J mouse in order to prepare fresh inoculum. The animals were infected by intra-peritoneal injection with 10^4 PRBC suspended in 0.1 ml of pyrogen-free saline. Parasitemias were monitored by counting at least 200 erythrocytes in methanol-fixed, Giemsa-stained thin smears prepared with tail blood and reported as percentage of parasitized red blood cells.

Statistical analysis

The nonparametric tests of Kruskal-Wallis and Mann-Whitney were sequentially used to compare continuous data and extended Fisher's exact test(Freeman-Halton test)was used to compare proportions in three groups. Significance was defined at the 95% level.

RESULTS AND DISCUSSION

After 30 days of dietary iron deficiency, body weights in mice from Group D (mean ± SD, 19.3 ± 1.5 g) were statistically lower than those in mice from Group N (21.7 ± 1.8 g). At that time, Group D animals had significantly lower Hb concentrations (13.7 ± 1.2 *vs.* 15.4 ± 2.0; mean ± SD, g/100 ml) and lower liver iron stores(27.0±14.1 vs 51.9±4.7 mean ± SD, μg/g). These data indicate that mild dietary iron deficiency was produced, with an acceptable range of growth suppression (~10%) in rodents exposed to the iron-deficient diet. Dietary iron deficiency is usually obtained in mice by iron restriction imposed to weanling animals (12, 15). Mice are relatively resistant to mild dietary iron deficiency (16), but the growth rate of weanling animals exposed to severely iron-deficient diets may be affected due to decreased diet consumption (17). For these reasons, we have used here a model of mild dietary iron deficiency (15 mg iron/kg diet) to avoid higher growth deficits in iron-deficient mice. At the end of the experiment (day 59), iron-deficient and normal iron non-infected controls, when fed with iron-supplemented diets for 30 days, showed similar Hb levels (14.1±1.4 g/ 100ml and 13.8±1.3 g/100ml, respectively) and iron liver stores of 73.0 ± 25.5 and 56.5 ± 5.4 μg/g, respectively, without statistical difference. The hepatic iron stores in previously iron-deficient mice might be related to higher iron absorption, as described by Sorbie & Valberg (16).

Parasitemias and mortality rates are shown in Figure 1. Parasitemia peaked between day 10 and 11 post-infection, and reached mean peak values above 50%. The time course of parasitemias and the magnitude of their peaks were not significantly different among the experimental groups. Mortality reached 87.5% in previously iron-deficient mice (Groups D and DS), regardless of the access to iron-supplemented diet during infection (Group DS) (Figure 1A and 1B). In contrast, mice from Group NS showed a lower mortality rate (37.5%) (Fisher's exact test, $p = 0.0104$).

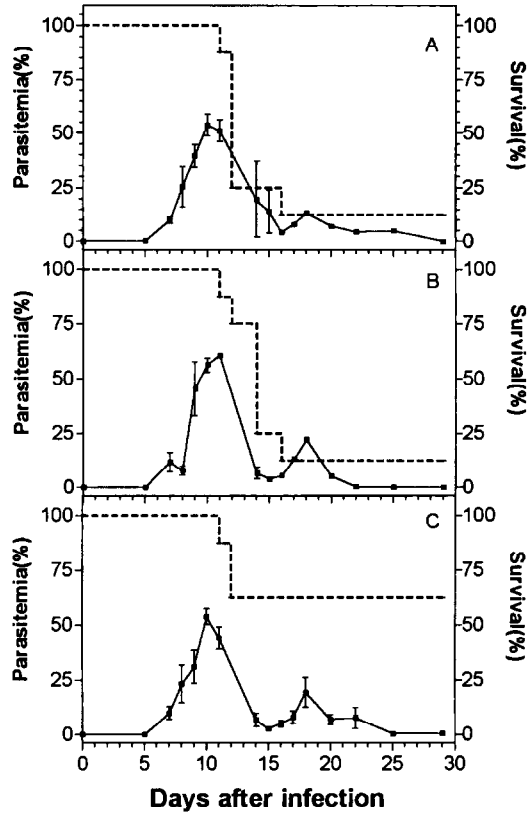

FIG. 1 – *Plasmodium chabaudi* parasitemia and mortality rate in mice fed with different iron regimens. Animals in (A) were fed iron-deficient diet for 30 days and maintained on the same diet during infection; animals in (B) were fed iron-deficient diet for 30 days and then transferred to iron-supplemented diet during infection; animals in (C) were fed normal iron diet for 30 days and then transferred to iron-supplemented diet during infection. Parasite counts are reported as mean and standard error of the mean. The time course of parasitemia was similar in all groups. Mortality was higher among mice in (A) and (B) (87.5%) than in those from (C) (37.5%); Fisher's exact test, $p = 0.0104$.

The high mortality of iron-deficient mice precludes formal analyses of parasite recrudescence among them, but it is worth mentioning that recrudescence were observed in

surviving animals from all groups. At day 10 post-infection, mean Hb concentrations (g/100 ml) among mice from Group D were significantly lower than those of all other groups (Group D: 3.4 ± 1.0; Group DS: 4.5 ± 1.1; Group NS: 5.6 ± 1.8; mean ± SD, $p < 0.05$). These data show that the iron deficiency before infection was the major determinant of host mortality in this model of severe murine malaria. Hb levels, but not mortality, were affected by dietary iron supplementation given to previously iron-deficient animals.

The mortality rate in iron-deficient mice (Groups D and DS) was unusually high, regardless of dietary iron supplementation during infection in Group DS. These results can be ascribed to the severe anemia induced by malaria infection, the major cause of mortality in this model of rodent malaria. Resistance to *P. chabaudi* AS infection is closely associated, in different strains of mice, with their ability of enhancing erythropoietic responses during infection (18). Iron depletion impairs erythropoiesis, and also depresses host immune responses, increasing the susceptibility to several infections (19). Despite this, protection against lethal. *P. chabaudi* infection was previously reported in iron-deficient NFR/N mice (10). This previous study used commercial, severely iron-deficient diets, which can be deficient in other essential nutrients that affect the growth of malaria parasites (20), in a nonstandard model of murine *P. chabaudi* (of unspecified strain) infection. Further, previous studies have failed to show major effects of iron deficiency and iron supplementation on the course of non-lethal *P. berghei* infection in young rats (11,12). The role of iron status in malaria should be analyzed carefully both in experimental models and in human patients, due to large variation in the severity and prevalence of iron deficiency, severe in some countries or less intense in others, with malaria agents inducing different forms of disease, depending on adequate experimental design for each expected situation. In conclusion, we showed that dietary iron deficiency leads to anemia and high mortality in *P. chabaudi*-infected weanling mice, and that dietary iron supplementation during infection may improve hemoglobin levels but does not improve survival of previously iron-deficient hosts in our experimental model.

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