Plasmodium Falciparum Infection flares up during Iron Therapy

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ABSTRACT

The interactions between falciparum malaria and iron deficiency anemia (IDA) are complex and bi-directional. Malaria causes acute anemia by destroying both infected and uninfected red blood cells (RBCs) whereas persistent sub-clinical infection causes a milder anemia of infection by blocking iron recycling to the bone marrow2. Conversely, once established, IDA protects both pregnant women and children from malaria. In addition, supplemental iron, given alone or in combination with other micronutrients, predisposes children to malaria and other serious adverse outcomes. Iron homeostasis has been implicated in regulating liver stage P. falciparum infection; in murine studies, erythrocytic stage malaria infection initiates hepcidin-mediated hepatic hypoferremia, which blocks superinfections by sporozoites from competing plasmodial strains. Mathematical modeling suggests that this can explain the low levels of super infections in young children, but this mechanism cannot account for observed reductions in the risk of primary malaria infection in children with IDA. It has also been speculated that transient peaks in non-transferrin-bound iron caused by administration of highly absorbable iron supplements could promote intra-erythrocyte parasite growth or bacterial septicemia (a common cause of death in malaria patients but definitive evidence is absent. A large body of clinical and epidemiological evidence has accumulated which clearly demonstrates that host iron deficiency is protective against falciparum malaria and that host iron supplementation may increase the risk of malaria. Although many effective antimalarial treatments and preventive measures are available, malaria remains a significant public health problem, in part because the mechanisms of malaria pathogenesis remain obscured by the complexity of the relationships that exist between parasite virulence factors, host susceptibility traits, and the immune responses that modulate disease. Here we review (i) the clinical and epidemiological data that describes the relationship between host iron status and malaria infection and (ii) the current understanding of the biological basis for these clinical and epidemiological. To determine the

effect of IDA on the growth of erythrocytic stage P. falciparum, we enrolled donors with and without IDA from a non-malaria endemic Donors were classified as IR (hemoglobin (Hgb) >11 g dl−1, mean corpuscular volume (MCV) >80 fL, Ferritin >12 ng ml−1) or as IDA (Hgb <11 g dl−1, MCV <80 fL, Ferritin <12 ng ml−1) . Non-anemic donors with low-iron stores (Hgb >11 g dl−1, Ferritin <12 ng ml−1) were excluded. P. falciparum (strains 3D7, Dd2 and FCR3-FMG) were grown in either RBCs from the IR (n=10) or IDA (n=7) donors in up to three consecutive 96 h growth assays . We observed that parasite growth rates were reduced in RBCs from IDA donors as compared with growth in RBCs from IR donors by 48.8% (standard deviation (SD) ±23.9), 34.3% (s.d.±22.2) and 50.0% (s.d.±20.4) for strains 3D7, Dd2 and FCR3-FMG, respectively. These findings clearly show that P. falciparum propagation is reduced within RBCs from IDA individuals, but that variability may exist in the degree to which different P. falciparum isolates are affected by IDA.

Keywords: Malaria, iron, iron deficiency Anemia, Plasmodium falciparum, iron supplementation

INTRODUCTION

Iron deficiency is a condition in which there is insufficient iron in the body to maintain normal physiologic functions. Iron deficiency can be categorized into three stages: iron deficiency without anemia, iron deficiency with mild anemia, and iron deficiency with severe anemia. Iron deficiency anemia occurs when iron stores are exhausted and the supply of iron to tissue is compromised; this condition is defined as anemia with biochemical evidence of iron deficiency. Iron deficiency is most prevalent and severe in young children and women of reproductive age, but can also occur in older children, adolescents, adult men, and the elderly. It is estimated that 50% of pregnant women and 40% of preschool children in the developing world are iron deficient (WHO | Assessing the iron status of populations, Kassebaum et al., 2004)1. Often, iron deficiency develops slowly and is not clinically diagnosed until severe anemia is apparent (Stoltzfus, 2003). Studies suggest that iron deficiency impairs the growth, cognition, and neurological development of children from infancy through adolescence, impairs immune function, and is associated with increased morbidity rates (De-Regil et al., 2011 t 2013; Wang et al., 2013. Iron deficiency during pregnancy is associated with multiple adverse outcomes for both mother and infant, including increased risk of hemorrhage, sepsis, maternal mortality, perinatal mortality, and low birth weight (Peña-Rosas et al., 2011,2012). Iron deficiency anemia can be a direct cause of death or contribute indirectly. For example, during child birth an anemic mother cannot afford to lose more than 150 mL of blood, compared with a healthy mother who can lose up to 1 liter of blood and still survive2. Thus, the WHO recommends iron supplementation for all men, women, and children in areas where malnutrition is prevalent (WHO | Guidelines on food fortification with micronutrients, 2006). Host iron metabolism is intimately linked to the host response to infection and inflammation. In the face of infection and inflammation, the human host protein hepcidin becomes elevated and initiates signalling which results in reduced iron absorption into the body along with the redistribution of body iron stores. As a consequence many of the biomarkers utilized to assess host iron status are sensitive to both iron as well as infection3. For example, low serum ferritin (serum ferritin reflects total body iron reservoirs) is indicative of iron deficiency. However, ferritin is also an acute phase protein which is elevated in the context of infection, and as a result is not a reliable marker of human iron status in the presence of infection or inflammation. Like serum ferritin, transferrin saturation and transferrin receptor levels are biochemical markers of human iron status that are also sensitive to infection and inflammation. As a result evaluating an individual's iron status during an infection has proven difficult (Aguilar et al., 2012), and the scientific community has struggled to establish formal guidelines. In 2012 malaria caused an estimated 207 million infections and over 600,000 deaths; 90% of these deaths occurred in sub-Saharan Africa, and 77% occurred in children under five (WHO | World Malaria Report, 2013). At least five species of the eukaryotic Apicomplexan parasite from the genus Plasmodium cause malaria in humans with Plasmodium falciparum being the most common and deadly. Following the bite of a malaria parasite infected mosquito, the sporozoites stage of the parasite enters the bloodstream and travels to the liver, where it subsequently infects liver hepatocytes. Malaria replication in the liver is asymptomatic. Next, the merozoite form of the parasite leaves the liver and enters into circulation to infect host red blood cells (RBCs) 4. During the erythrocytic stage of infection, the parasite repeatedly invades, replicates within, and egresses from host RBCs. This erythrocytic stage of infection is responsible for all symptoms of disease (Miller et al., 2013), and the severity of disease is directly associated with parasite burden (Chotivanich et al., 2000; Dondorp et al., 2005)5. A wide range of symptoms can be observed in malaria patients. Clinically however, malaria is categorized as either uncomplicated or complicated. Complicated malaria is further divided into three overlapping syndromes: cerebral malaria, severe anemia, and metabolic acidosis. The clinical syndrome observed in each individual patient is influenced by multiple variables: parasite species, host immune status, and genetic background, as well as the use and timing of antimalarial drugs (Taylor et al., 2010).

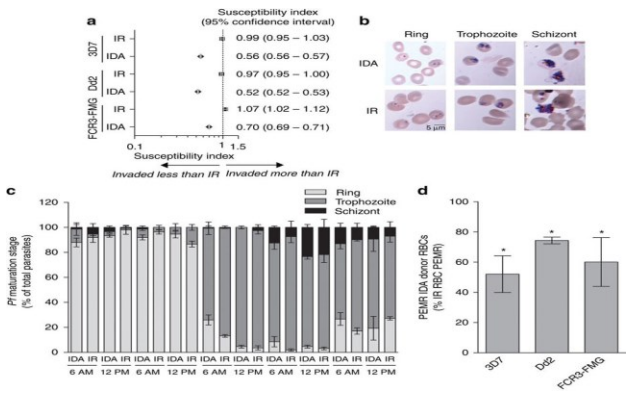
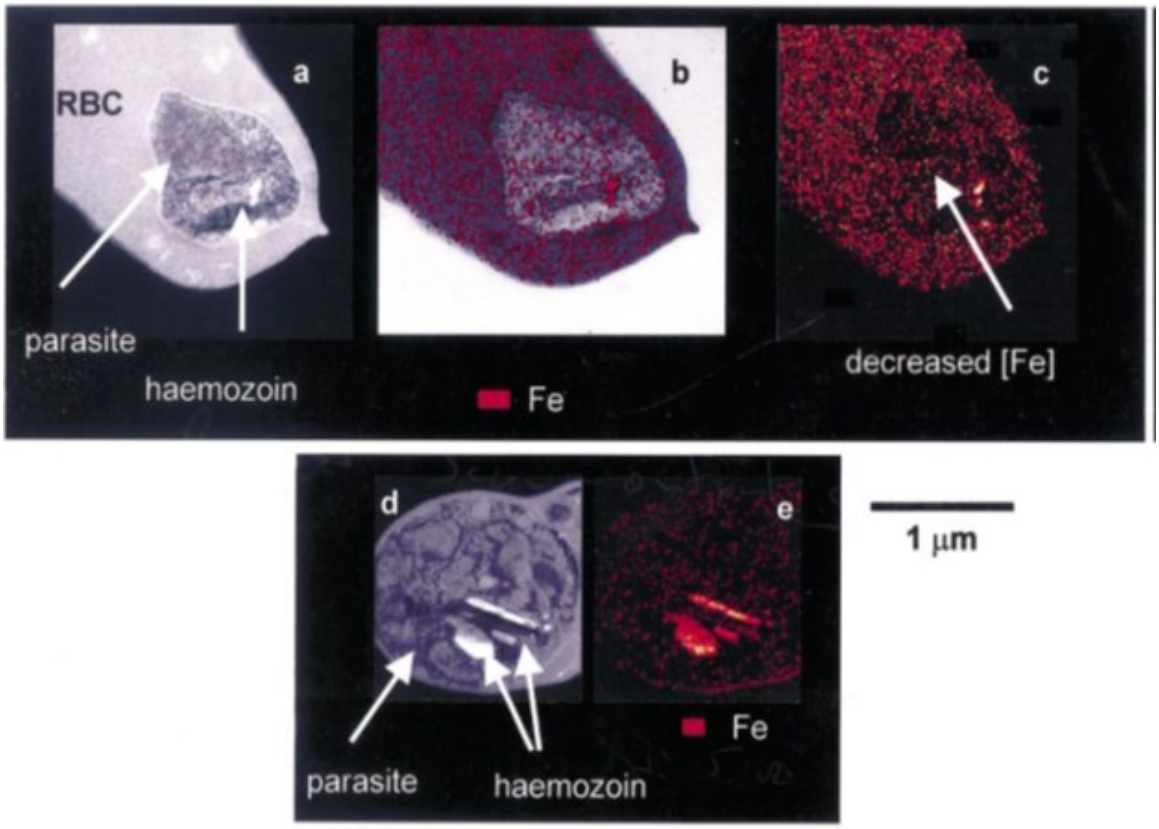


Figure – 1

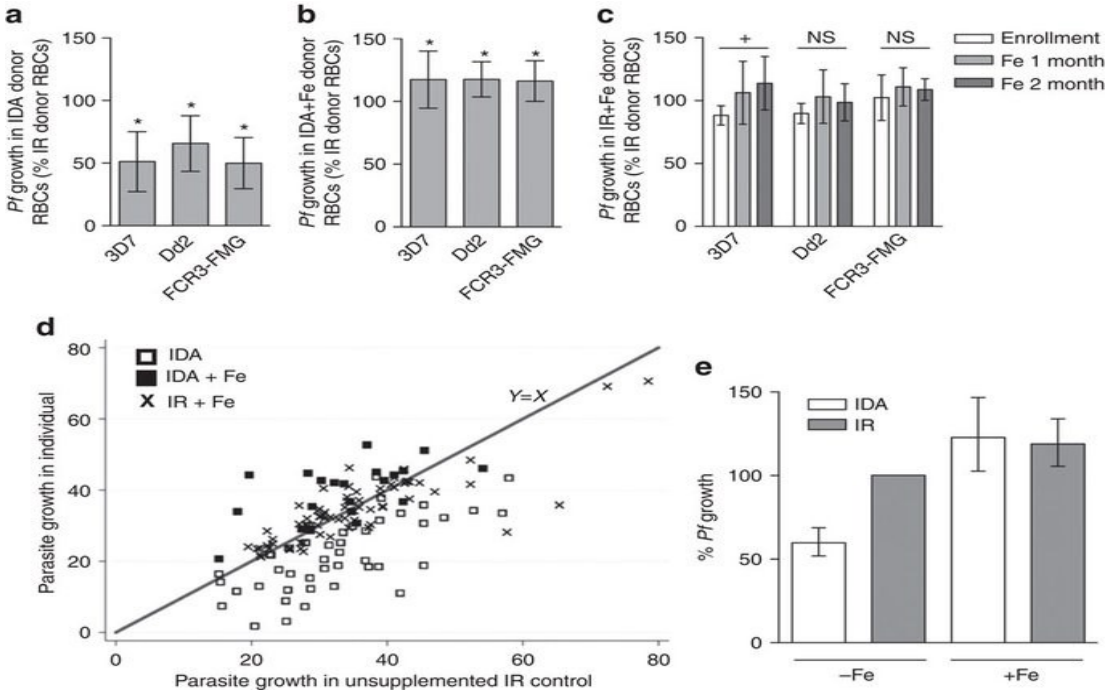
P. falciparum growth is reduced in iron-deficient RBCs and iron supplementation eliminates growth attenuation

REVIEW



Iron is an essential nutrient for nearly every living organism including humans and the malaria parasite. Iron impacts a broad range of biological processes; including host and parasite cellular function, erythropoiesis and immune function. The capacity of iron to fluctuate between two oxidation states, ferrous (Fe2+) and ferric (Fe3+), makes it indispensable for many critical biological processes, including DNA replication, cellular respiration, and oxygen transport. However, the same useful biphasic properties of iron which make it indispensable also contribute to its high cytotoxicity. As a result the human host tightly regulates iron availability and usage. Access to iron is particularly important in the context of host-pathogen interactions6. When confronted with infection and inflammation the human host reallocates its iron reservoirs in an effort to deprive invading pathogens of iron. The human protein hepcidin—a rheostat of systemic iron homeostasis—signals the body to decrease absorption of iron in the proximal duodenum and orchestrates the movement of iron from serum into storage within the liver and macrophages (Roy, 2013)7. As a result of reduced serum iron, erythropoiesis—a process exquisitely sensitive to iron levels—slows in the face of infection as well as inflammation. The human host's active reduction in bioavailable iron protects against a wide range of pathogens (Armitage et al.,2011). Not surprisingly, as many pathogens require access to host iron sources to survive and grow, pathogens have evolved sophisticated iron acquisition systems, and the iron acquisition systems of many bacterial and fungal species have been well described (Skaar, 2010). By comparison how the malaria parasite acquires, regulates, and utilizes iron remains a mystery. Iron is essential for the survival of the malaria parasite. The parasite multiplies 8–32 times in the course of a single intra-erythrocytic lifecycle. Iron is an essential cofactor for the DNA replication enzyme ribonucleotide reductase, and as a result iron is required to fuel this rapid intra-erythrocytic proliferation (Rubin et al., 1993)21. Iron is also utilized by the parasite for pyrimidine (Krungkrai et al., 1990; Van Dooren et al., 2006) and heme biosynthesis (Sato and Wilson, 2002; Dhanasekaran et al., 2004; Sato et al., 2004; Nagaraj et al., 2008, 2009, and 2010). As with the human host, the malaria parasite must also balance its need for iron against the cytotoxicity of iron. The malaria parasite metabolizes host hemoglobin in its acidic digestive vacuole in order to acquire necessary amino acids; however, as discussed below, the parasite does not utilize the iron in host heme. Plasmodium aspartic and cysteine proteases degrade host hemoglobin and release large quantities of toxic iron-laden heme (Goldberg et al., 1990; Subramanian et al., 2009). Apicoblast parasites neutralize the cytotoxic heme produced during hemoglobin metabolism by sequestering the heme in an inert crystal, hemozoin (Rudzinska et al., 1965; Chugh et al., 2013)20. Despite neutralizing a substantial portion of host heme into hemozoin, some residual heme remains free and becomes oxidized, generating free oxygen radicals (Francis et al., 1997). The parasite possesses powerful thioredoxin and glutathione systems to maintain intracellular redox equilibrium (Jortzik and Becker, 2012). However, even when these redox systems are functioning at full capacity, oxidative stress significantly increases as the parasite matures and replicates within host erythrocytes (Fu et al., 2010. In fact, many antimalarials, including artemisinin, appear to target the parasite's

ability to detoxify reactive oxygen species (ROS) (Rosenthal and Meshnick, 1996; Klonis et al., 2013; Ariey et al., 2014)22. For example, it was recently found that mutations in PF3D7_1343700 (Kelch) can confer resistance to artemisinin. The authors speculate that these mutations cause a disruption of the parasite's ability to detoxify ROS because the efficacy of artemisinin depends on its ability to generate oxygen radicals and some kelch-containing proteins in other Organisms have been shown to be involved in the regulation of cytoprotection (Ariey et al., 2014).



[Figure-2] P. falciparum growth is reduced in iron-deficient RBCs and iron supplementation eliminates growth

OBSERVATIONS

As iron deficiency and iron supplementation of iron deficient individuals profoundly alters erythropoiesis, RBC physiology, and RBC population structure, we hypothesized that iron deficiency and iron supplementation directly impact the disease causing erythrocytic stage of P. falciparum infection. This approach eliminated the influence of acquired and innate immunity to malaria, haemoglobinopathies and concurrent inflammation. Our study reveals that RBCs from donors with IDA confer malaria protection by impairing P. falciparum invasion and intra-erythrocyte propagation. This protective effect was reversed when donors with IDA received iron supplementation8. We go on to show that when iron-deficient RBCs are replaced with iron-replete (IR) RBCs in vitro (as occurs in individuals with IDA following iron supplementation) the susceptibility to P. falciparum infection is increased. These findings support well-described clinical patterns of differential susceptibility to malaria. Taken together, they indicate that therapeutic iron supplementation conspires with host iron status to mediate host RBC susceptibility to malaria infection by altering the dynamic structure of the host's RBC population. Iron deficiency and malaria have similar global distributions, and frequently co-exist in pregnant women and young children19. Where both conditions are prevalent, iron supplementation is complicated by observations that iron deficiency anemia protects against falciparum malaria, and that iron supplements increase susceptibility to clinically significant malaria, but the mechanisms remain obscure. Here, using an in vitro parasite culture system with erythrocytes from iron-deficient and replete human donors, we demonstrate that Plasmodium falciparum infects iron-deficient erythrocytes less efficiently. In addition, owing to merozoite preference for young erythrocytes, iron supplementation of iron-deficient individuals reverses the protective effects of iron deficiency. Our results provide experimental validation of field observations reporting protective effects of iron deficiency and harmful effects of iron administration on human malaria susceptibility9. Because recovery from anemia requires transient reticulocytosis, our findings imply that iron supplementation should be accompanied by effective measures to prevent falciparum malaria. Of this, 88³9% is in the form of haemozoin. To determine the effect of IDA on the growth of erythrocytic stage P. falciparum, we enrolled donors with and without IDA from a non-malaria endemic area through our US-based hospital clinic18. Donors were classified as IR (haemoglobin (Hgb) >11 g dl−1, mean corpuscular volume (MCV) >80 fL, ferritin >12 ng ml−1) or as IDA (Hgb <11 g dl−1, MCV <80 fL, ferritin <12 ng ml−1). Non-anaemic donors with low-iron stores (Hgb >11 g dl−1, ferritin <12 ng ml−1) were excluded. P. falciparum21 (strains 3D7, Dd2 and FCR3-FMG) were grown in either RBCs from the IR (n=10) or IDA (n=7) donors in up to three consecutive 96 h growth assays . We observed that parasite growth rates were reduced in RBCs from IDA donors as compared with growth in RBCs from IR donors by 48.8% (standard deviation (SD) ±23.9), 34.3% (SD ±22.2) and 50.0% (s.d.±20.4) for strains 3D7, Dd2 and

FCR3-FMG, respectively .These findings clearly show that P. falciparum propagation is reduced within RBCs from IDA individuals, but that variability may exist in the degree to which different P. falciparum isolates are affected by IDA. The interactions between falciparum malaria and iron deficiency anemia (IDA) are complex and bi-directional[10].

DISCUSSION

Iron supplementation has clear nutritional benefits for children and pregnant women2, but iron is also an essential nutrient for most pathogens and as a result is a critical mediator of host–pathogen interactions. Activation of the host innate immune system by the malaria parasite or other infectious organisms triggers reduction in iron absorption, redistribution of existing iron stores and decreases erythropoiesis, which effectively limits the availability of iron to invading pathogens22. It is unknown neither what host iron (mosquito or human) P. falciparum is able to access and utilize nor how the parasite circumvents the host's attempt to restrict iron. It has been previously postulated that as occurs with other pathogens, iron deficiency inhibits P. falciparum infection via iron deprivation. Although the malaria parasite may find iron less readily available in an iron-deficient host, our work reveals an alternate cellular mechanism by which iron deficiency may protect against malaria11. Our study of the relationship between iron deficiency, iron supplementation and erythrocytic stage P. falciparum infection highlights how by altering the dynamics of the human hosts RBC population iron deficiency and iron supplementation shape erythrocytic stage P. falciparum infection.

Host Iron available to erythrocytic stage P. falciparum. Host iron immediately available to the erythrocytic stage of P. falciparum include serum and intra-erythrocytic iron. Serum iron ranges from 10 to 27 µM. Transferrin bound iron is the predominant

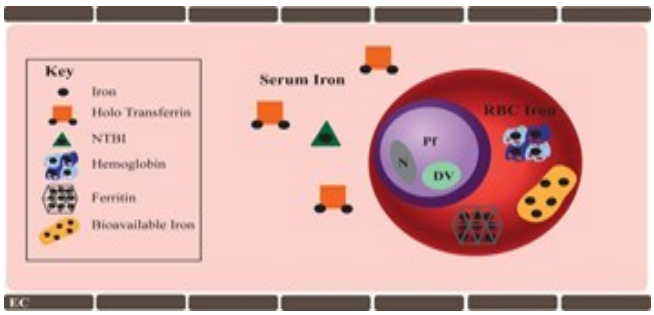


Figure-3

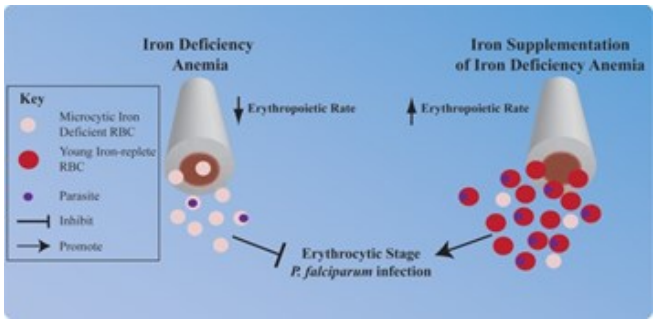


Figure-4

RESULTS

Malaria growth is reduced in RBCs from individuals with IDA. To determine the effect of IDA on the growth of erythrocytic stage P. falciparum, we enrolled donors with and without IDA from a non-malaria endemic area through our US-based hospital clinic. Donors were classified as IR (haemoglobin (Hgb) 411 g dl_1, mean corpuscular volume (MCV) 480 fL, Ferritin 412 ng ml_1) or as IDA (Hgb o11 g dl_1, MCV o80 fL, Ferritin o12 ng ml_1) (Table 1). Non-anemic donors with low iron stores (Hgb 411 g dl_1, ferritin o12 ng ml_1) were excluded. P. falciparum (strains 3D7, Dd2 and FCR3-FMG) were grown in either RBCs from the IR (n¼10) or IDA (n¼7) donors in up to three consecutive 96 h growth assays (Supplementary Fig. 1)12. We observed that parasite growth rates were reduced in RBCs from IDA donors as compared with growth in RBCs from IR donors by 48.8% (standard deviation (SD) ±23.9), 34.3% (SD ±22.2) and 50.0% (SD±20.4) for strains 3D7, Dd2 and FCR3-FMG, respectively (Fig. 1a). These findings clearly show that P. falciparum propagation is reduced within RBCs from IDA individuals, but that variability may exist in the degree to which different P. falciparum isolates are affected by IDA. Malaria growth is increased in RBCs from iron-supplemented donors. Given field evidence that supplementation of children with 12.5 mg of iron (1–1.5 mg kg_1) and 50 ng of folic acid may potentiate the risk of malaria9, we next investigated the effects of iron supplementation of IDA and IR individuals on in vitro growth of erythrocytic stage P. falciparum17. We first collected RBCs from IDA patients who were receiving iron supplementation (IDAþFe); these individuals met the above criteria for IDA and were receiving either high-dose oral ferrous sulfate (60 mg elemental irons orally three times per day (9–

12.6 mg kg_1)) or intravenous iron (at a dosage determined by their personal physician using the following equation: Dose¼0.0442 [desired Hgb—observed Hgb] _LBWþ [0.26_ LBW]). IDAþFe group Hgb values ranged from 6.6 to 9.8 g dL_1 and MCV values ranged from 75 to 98 fL. Additionally reticulocyte counts and red cell distribution width (RDW) were elevated; and average mean corpuscular hemoglobin concentration (MCHC), total iron, and Ferritin values were greater than that of the IDA group but still lower than that of the IR group (Table 1). Together, these values are indicative of an erythropoietic response to iron supplements, but not full recovery from IDA. Comparison of the growth rate of P.falciparum (strains 3D7, Dd2 and FCR3-FMG) within RBCs from the IDAþFe donors to the growth rate of parasites within RBCs from IR donors revealed increases in P. falciparum growth of 17.3% (s.d.±22.7), 17.6% (s.d.±14.0) and 26.3% (s.d.±16.1) for 3D7, Dd2 and FCR3-FMG in RBCs from IDAþFe donors (Fig. 1b)23. We additionally assessed the effect of iron supplementation of IR individuals on P. falciparum growth. For this study, IR individuals donated blood at enrollment (baseline) and were then prescribed daily oral iron supplementation (325 mg ferrous sulfate). Iron-supplemented IR study

participants (IRþFe) subsequently returned at one and two months following initiation of daily iron supplementation to donate blood. At each donation (enrollment, 1 month and 2 months), the growth rate of P. falciparum (strains 3D7, Dd2 and FCR3-FMG) within RBCs from IRþFe donors was determined and then compared with the corresponding parasite growth rates within RBCs from a nonsupplemented IR donor. Compared with RBCs from IR donors, we observed in RBCs from IRþFe donors that 1 month of iron supplementation increases of 17.5% (s.d.±16.1), 11.3% (s.d.±15.7) and 6.6% (s.d.±8.1) in growth for 3D7, Dd2 and FCR3-FMG, respectively. There was no change in parasite growth rate in RBCs collected 1 and 2 months after administering iron supplements (Fig. 1c). Clinical studies in different field sites have reported that iron deficiency correlates with protection from malaria. In Malawian children, baseline iron deficiency was associated with significant reductions in the subsequent risks of both parasitemia (45%) and malaria (51%) 13. Our results—that iron-deficient RBCs impair parasite propagation in vitro are consistent with these clinical findings, and provide valuable insight into a cellular mechanism for the observations made in the clinical setting. In our study of P. falciparum growth in RBCs from IDA donors, we reveal that RBCs from IDA donors are refractory to P. falciparum invasion and support a lower PEMR but that parasite maturation is normal. There are multiple physiological differences between iron-deficient and IR RBCs that may contribute to the impaired invasion into and replication within iron-deficient RBCs24. The low temperatures or solvent-free conditions present during and after freeze-drying would also not favor conversion of haematin into # 2002 Biochemical Society haemozoin14. The observed fraction of haemozoin iron in the sample thus almost certainly is representative of that in the live trophozoite.

CONCLUSION

Malaria causes acute anemia by destroying both infected and uninfected red blood cells (RBCs)1, whereas persistent sub-clinical infection causes a milder anemia of infection by blocking iron recycling to the bone marrow2. Conversely, once established, IDA protects both pregnant women and children from malaria. In addition, supplemental iron, given alone or in combination with other micronutrients, predisposes children to malaria and other serious adverse outcomes. Given the relationship between iron, heme, and ROS, it is possible that perturbations in host iron regulation might also affect the malaria parasite's redox equilibrium15. Iron responsive proteins (IRPs) and their accompanying iron responsive elements are critical for maintaining cellular iron homeostasis in the human host. IRPs and iron responsive elements are responsible for mobilizing iron when demands are high and moving iron into storage when excess iron may promote ROS formation (Hentze et al., 2010). Loyevsky et al. identified and characterized a P. falciparum IRP, the expression of which was affected by iron starvation as well as iron supplementation (Loyevsky et al., 2001, 2003; Hodges et al., 2005).The inescapable conclusion arising from these data is that haemozoin (b-haematin) is the only detectable iron species.From the magnitude of the statistical scatter in the data we can confidently state that at least 95% of the iron observed within the trophozoite is incorporated into haemozoin. Although it is likely that freeze-drying will break down cell membranes and has the potential to disturb species within the parasite, it is not possible for haemozoin to be re-assembled from chemically degraded haematin. This means that any such degradation processes that may occur must be, at most, a minor component of the overall haematin processing in the parasite16. Overall, the available evidence supports a link between (i) iron deficiency and protection from malaria infection and (ii) iron supplementation and increased risk of malaria. However, there is still much to be learned. Furthermore, study of the competition between the malaria parasite and the human host for iron can serve as a translational model to identify critical molecular mechanisms of P. falciparum pathogenesis.

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