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Hemoglobin serves to protect *Plasmodium* parasites from nitric oxide and reactive oxygen species.

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Abstract

Our understanding of how the host immune response kills *Plasmodium*, the causative agent of malaria, is limited and controversial. One widely held belief is that reactive oxygen species are crucial for controlling parasite replication. One of the hallmarks of the blood-stage malaria is the cyclic rupture of erythrocytes by the parasite, which releases free hemoglobin (Hb) into the circulation. We propose that this free Hb as well as the hemoglobin within the erythrocyte and surrounding the parasite effectively shields *Plasmodium* from reactive oxygen species well in excess of those achievable in vivo.

Introduction

Malaria, a devastating disease that kills over 2 million people each year, is caused by infection with parasites of the genus *Plasmodium*[1,2]. *Plasmodium* is transmitted by the bite of an infected mosquito vector, and this initiates first the liver and then the blood stages of the infection. The liver stage is asymptomatic and is beyond the scope of this paper. The blood stage comprises parasites (merozoites) invading erythrocytes, developing within the erythrocyte, and producing new progeny every 48 or 72 hours, depending on the species. The blood stage of the infection causes the clinical complications, such as fever and chills, as well as the life threatening multi-organ failure (brain, lungs, and kidney). *Plasmodium falciparum* is the most virulent of the species of *Plasmodium* that infect humans, and this species adheres to activated venular endothelium to sequester itself from the lymphoid and filtration organs[3]. Despite considerable research effort, an effective vaccine against *Plasmodium* infection remains elusive perhaps because our understanding of the mechanism(s) underlying parasite killing by the immune system is poorly defined and controversial. The observation by Jensen and colleagues of crisis forms of the parasite (parasites exhibiting deteriorating morphology) immediately prior to declines in parasitemia[4] suggests that a soluble factor is responsible for parasite killing, rather than erythrophagocytosis. One potential effector mechanism that is widely believed to be critical for this parasite killing is the immune production of reactive oxygen species (ROS), including nitric oxide ($NO\cdot$), superoxide, and peroxynitrite. These ROS are produced mainly by activated phagocytic cells, including macrophages and neutrophils, although TH1 cells are also reported to produce $NO\cdot$ during experimental malaria[5].

Nitric oxide, a highly toxic free radical, is produced during the enzymatic conversion of L-arginine to L-citrulline by members of the nitric oxide synthase family (NOS) [6]. To date, three members of the family have been identified, specifically endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1), and inducible NOS (iNOS or NOS2) [7-9]. All three isoforms have similar $NO\cdot$ production rates of about 1μM/min/mg protein [10]. The iNOS isoform is responsible for the high-level production of nitric oxide because it is highly expressed after activation of phagocytes, comprising up to 1% of total protein [11].

Activated phagocytes also produce superoxide by the enzymatic reduction of molecular oxygen by NADPH oxidase (pHox family). Upon activation, the NADPH oxidase is assembled from its membrane and cytosolic pHox subunits (p91, p22, p67, p47, p40) and superoxide is formed inside a phagosome or outside the cell [12]. When both nitric oxide and superoxide are produced in close proximity, e.g. by activated phagocytes, this results in the formation of peroxynitrite by the rapid reaction between nitric oxide and superoxide [10].

Nitric oxide is well established as a major cytotoxic molecule in infectious diseases [13]. Because $NO^{\cdot}$ and ROS are important in the control of other parasitic diseases, it is generally accepted that they play a critical role in killing the malarial parasite. In fact, this hypothesis is supported in all 15 recent reviews addressing the role of nitric oxide or reactive oxygen species in malaria. However, evidence from animal models challenges this notion: mice deficient in the iNOS gene or p91phox gene exhibit similar *P. berghei* parasitemia as controls, indicating that iNOS-derived $NO^{\cdot}$, pHox-derived superoxide, and peroxynitrite do not serve to control parasitemia [14-18]. Further, the role of hemoglobin as a ROS quencher has not been taken into account, leading us to propose that nitric oxide and ROS do not play a significant role in malarial parasite killing.

The Role of Hemoglobin

Hemoglobin (Hb) is released when *Plasmodium* ruptures the erythrocyte and our preliminary experimental data suggests that the plasma concentration of free Hb on day-6 of *P. berghei* infection (when the animal becomes moribund) can reach 100μM. The in vivo effects due to free natural molecular hemoglobin in blood are well defined in the research community interested in the development of molecular hemoglobin based oxygen carrying blood substitutes [19,20]. Free hemoglobin is a powerful in vivo scavenger of $NO^{\cdot}$, leading to vasoconstriction and impaired microvascular blood perfusion that, in turn, are major determinants of tissue and organism survival [21]. Free hemoglobin is almost 1,000-fold more efficient at scavenging $NO^{\cdot}$ than Hb packaged in RBCs [22], so this free Hb may be protective by significantly quenching ROS before they can diffuse into the erythrocyte. Besides free Hb, *Plasmodium* resides in a parasitophorus vacuole within the erythrocyte and is surrounded by about 0.5 fmol Hb. The combination of free molecular Hb in blood plasma and Hb in the erythrocyte likely protects the parasite because of complex ROS scavenging reactions.

The chemical reactions of $NO^{\cdot}$ with Hb depend on the form and state of the Hb molecule. When Hb passes through the lung, it changes from the taut or T state without oxygen (deoxyHb) to the relaxed or R state with oxygen bound to heme moiety (oxyHb)[23,24]. In the venous microcirculation where sequestered parasites such as *P. falciparum*, and murine-infecting *P. berghei*, and *P. yoelii* reside, about 60% of Hb within erythrocytes is oxyHb and 40% is deoxyHb. In the arterial microcirculation, most of the Hb is oxyHb (>99%). $NO^{\cdot}$ reacts with the oxygen in oxyHb generating metHb ($Hb(Fe^{3+})$) and nitrate (Box 1, reaction-1). $NO^{\cdot}$ also reacts with deoxyHb forming $Hb(Fe^{2+})NO$ (Box 1, reaction-2), which in turn reacts with oxygen to

form metHb and nitrate (Box 1, reaction-3). Further, oxyhemoglobin is capable of quenching superoxide, to form molecular oxygen, hydrogen peroxide, and methemoglobin (Box 1, reaction-4) [25]. Oxyhemoglobin can also rapidly react with peroxynitrite, to ultimately form nitrate and methemoglobin (Box 1, reaction-5) [26]. Thus, hemoglobin effectively renders exogenous free radicals incapable of directly damaging the parasite.

Box 1. Chemical reactions of hemoglobin and reactive oxygen species.

$$Hb(Fe^{2+})O_2 + NO^{\cdot} \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (1)$$

$$Hb(Fe^{2+}) + NO^{\cdot} \rightarrow Hb(Fe^{2+})NO \quad (2)$$

$$Hb(Fe^{2+})NO + O_2 \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (3)$$

$$Hb(Fe^{2+})O_2 + 2H^+ + O_2^{\cdot -} \rightarrow Hb(Fe^{3+}) + O_2 + H_2O_2 \quad (4)$$

$$Hb(Fe^{2+})O_2 + OONO^- \rightarrow \text{intermediates} \rightarrow Hb(Fe^{3+}) + O_2 + NO_3^- \quad (5)$$

Hemoglobin acts not only as a scavenger of radical species, but it can undergo redox transitions to higher oxidation states, making it a potent oxidant[27]. First, oxyhemoglobin can slowly autooxidize, forming methemoglobin and superoxide and the latter can form hydrogen peroxide, via dismutation. However, this process within the erythrocyte is balanced within the erythrocyte by the presence of superoxide dismutase, catalase, and methemoglobin reductase [28,29]. Second, both oxyhemoglobin and methemoglobin can react with peroxides to form the highly oxidant ferryl species, which damages many biomolecules [30]. Again, the presence of catalase within the erythrocyte is likely to mitigate this. Third, hemoglobin may be able to act as a Fenton reagent, catalyzing the formation of the hydroxyl radical from hydrogen peroxide [31,32], but it is likely that the heme must be free (i.e., released from the hemoglobin molecule) for this to occur [33,34]. Finally, recently Gladwin and Rifkind have proposed that deoxyHb within the erythrocyte can reduce nitrite, resulting in $NO^{\cdot}$ production [35,36]. However, this reaction is limited by the influx of nitrite into the erythrocyte and has a low yield of $NO^{\cdot}$, which may bind to the excess deoxyHb according to Box 1, reaction-2.

While the intraerythrocytic hemoglobin may generate oxidants and radicals, the extent of exposure of the *Plasmodium* parasite to oxidant stress is mitigated by the presence of superoxide dismutase, catalase, and methemoglobin reductase within the erythrocyte. However, Clark et al argue that the intraerythrocytic environment is not favorable to the parasite and that the requirement of 3% oxygen tension for parasite culture in vitro is also indicative of the parasite being susceptible to oxidant stress [37]. Indeed, in vivo the *Plasmodium* parasite sequesters itself on the venous endothelium, where oxygen tension is low. However, hypoxic conditions actually result in greater radical production by hemoglobin autooxidation [38]. This observation,

combined with the nitrite reductase role of deoxyHb proposed by Gladwin and Rifkind, suggests that oxidative stress may be increased when the parasite sequesters. A more likely explanation for parasite sequestration in venules is that cell adhesion molecules are increased under proinflammatory conditions in venules but not arterioles. To combat ROS exposure, *Plasmodium* has adapted by acquiring host superoxide dismutase [39] and possibly synthesizing its own [40]. We conclude that within the erythrocyte hemoglobin is more likely to act as a ROS scavenger than a source of oxidant stress, thus serving to protect the parasite.

Maximal in vivo levels of $NO^{\cdot}$ and ROS during malaria

We envision two maximal scenarios for exposure of *P. falciparum* parasitized erythrocytes to $NO^{\cdot}$: (1) a parasitized erythrocyte passing through the splenic shunt between arterioles and venules in the red pulp and between activated red pulp macrophages[41] and (2) a sequestered parasite adherent in the venous microcirculation to activated endothelial cell with a macrophage adherent close by. For the case of the splenic red pulp, the erythrocyte passes within 20-30μm of two layers of macrophages[41]. Assuming a monolayer of cells surrounding the shunt, the nitric oxide production of the macrophages should be about 2μM[42]. However, the splenic red pulp is filled with erythrocytes in close proximity to the macrophages, resulting in tremendous $NO^{\cdot}$ scavenging capacity that likely would prevent the majority of the $NO^{\cdot}$ from reaching the parasitized RBC. Thus, the effective dose of nitric oxide experienced by the parasite is likely lower than the 1μM.

The likely worst-case scenario for the parasite occurs when the erythrocyte is sequestered and there is an activated macrophage in very close proximity (Figure 1). The first source of $NO^{\cdot}$ is the activated venous endothelial cell, which can produce approximately 0.17 fmol/hr of $NO^{\cdot}$ in venules exposed to shear stress of 1.8 dyne/cm^2 [43]. In arterioles, the blood flow results in a 1-10% cell-free region near the luminal surface of the endothelium where nitric oxide is only scavenged by the reaction with oxygen, resulting in a nitric oxide half-life in the range of several minutes to hours, depending on the nitric oxide and oxygen concentrations [44]. In venules, on the other hand, no axial migration of erythrocytes is seen[45], so $NO^{\cdot}$ is quenched by Hb throughout the vessel. While models estimate that the arterial endothelium can maintain approximately 100nM $NO^{\cdot}$ at its luminal surface primarily due to the cell-free region [46], a fraction of this concentration is likely at the luminal surface of venules because of the presence of erythrocytes and free hemoglobin. In C57BL/6 mice, we have detected a marked increase in plasma free hemoglobin on day-6 of *P. berghei* infection, as compared to healthy animals. Therefore, the bulk of the $NO^{\cdot}$ will come from the area of direct contact of the erythrocyte with the single endothelial cell. Based on the endothelial cell to erythrocyte surface area ratio of ~50, as well as the fact that half of the 0.17 fmol/hr $NO^{\cdot}$ produced will diffuse away from the lumen, we estimate that 1.7 amol/hr $NO^{\cdot}$ will be bioavailable within the RBC (0.17/50/2 fmol/hr). If the *P. falciparum* infected erythrocyte is sequestered for 24 hours, this yields 40.8 amol of nitric oxide, which is well below of the 2fmol of heme within an RBC. Thus, it appears that the

endothelium alone cannot produce sufficient levels of nitric oxide to overcome the quenching by the hemoglobin inside the erythrocyte.

Any scenario involving a macrophage is highly dependent on the relative positioning of the macrophage and erythrocyte with respect to the flow (Figure 1). The macrophage must be extremely close and up-stream to have an effect, but if the macrophage touches the parasitized erythrocyte, it will likely phagocytize the erythrocyte and the parasite will not be able to replicate because its progeny merozoites cannot reach new erythrocytes. An activated macrophage sustains up to 10 fmol/hr of $NO\cdot$ production and 1 fmol/hr of superoxide production for several hours [42]. This yields an $NO\cdot$ concentration of about 1μM of $NO\cdot$ near the surface of the macrophage [42], but the resultant flow-driven $NO\cdot$ "plume" is rapidly scavenged by the surrounding erythrocytes and free hemoglobin. Thus, it is unlikely that the sequestered erythrocyte will experience any marked elevation of $NO\cdot$ levels and it will remain sheltered from nitric oxide by erythrocytic hemoglobin.

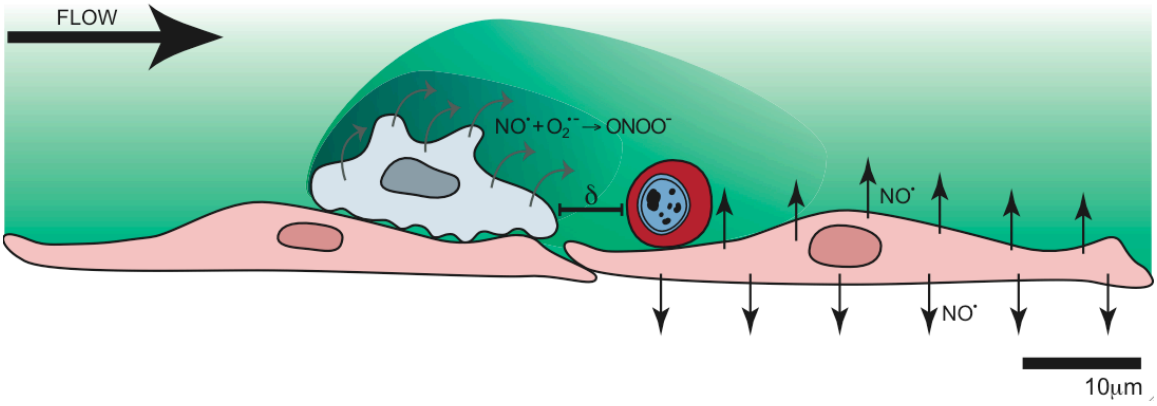

Figure 1. Exposure of parasitized erythrocytes to reactive oxygen species derived from either endothelial cells or from an activated adherent monocyte/macrophage. $NO\cdot$: nitric oxide; $O_2^{\cdot-}$: superoxide; $ONOO^-$: peroxynitrite. δ: distance between the upstream monocyte/macrophage and the parasitized erythrocyte. Flow: blood flow in venules. [Color figure: Green shading represents, nitric oxide; Blue: peroxynitrite.]

Activated macrophages also produce superoxide, but at a 10-fold lower level than nitric oxide[47]. Due to the much higher $NO\cdot$ levels and the fact that both radicals come from the same source, all of the superoxide rapidly reacts with the nitric oxide, yielding ~1.5 nM peroxynitrite at the macrophage surface. In addition, Hb in other erythrocytes and plasma Hb quench superoxide, making the likelihood of superoxide reaching the parasite almost nil.

The peroxynitrite resultant from the reaction of superoxide and nitric oxide produced by the macrophage is a highly reactive radical and at physiological pH has an extremely short half-life[47]. This results in a very short diffusion distance and near-zero concentration (<0.2nM) at

10μm from the macrophage. Thus, a sequestered erythrocyte is unlikely to be exposed to significant concentrations of peroxynitrite (<1nM) and peroxynitrite is quenched by oxyhemoglobin, to form metHb, nitrite, and oxygen. Based on the low production and its scavenging by hemoglobin, we propose that peroxynitrite does not play a significant role in parasite killing in vivo.

Current evidence in support of $NO^{\cdot}$ and ROS mediated *Plasmodium* killing

Nitric oxide is reportedly produced at high levels during the course of *P. falciparum* malaria in humans [48]. Further, the blood from patients with uncomplicated malaria contains high levels of iNOS mRNA, with the increased expression primarily in monocytes, as compared to those with severe malaria [49]. One concern with this study is that the two patient groups also received different drug treatments, and anti-malarial agents have been shown to affect monocyte function [50]. Several studies report that polymorphisms in the promotor region of the NOS2 gene (CCTTT repeat, G-945C or Lambarene mutation, and C-1173T) result in higher baseline NOS activity in the PBMCs and are associated with protection from malaria [51-54]. On the one hand, these investigators propose that $NO^{\cdot}$ killing of the parasite explains this genetic association with protection from malaria. On the other hand, the results have been inconsistent [55-58] and we speculate that these findings may be explained by the possible anti-inflammatory, homeostatic, and vasodilatory roles of $NO^{\cdot}$, rather than direct parasite killing. Nitric oxide is a potent anti-inflammatory molecule that plays a role in *P. falciparum* sequestration, so increased $NO^{\cdot}$ may down-regulate endothelial activation and CAM expression[59]. Further, $NO^{\cdot}$ quenching by Hb during sickle cell crisis results in vasoconstriction, which can be attenuated by $NO^{\cdot}$ gas or sodium nitroprusside treatment[60]. The reduced NO bioavilability during sickle cell anemia may also be responsible for the observed increase sVCAM-1 expression[60]. Similarly, NO quenching by hemoglobin is likely to occur during malaria and increased $NO^{\cdot}$ production may ameliorate microcirculatory complications. Finally, it is possible that the chronic exposure to increased levels of free hemoglobin and NO quenching during sickle cell anemia serve as an adaptation mechanism, contributing to the resistance to malaria conferred by sickle cell anemia.

Several in vitro co-culture studies, are also cited as evidence for $NO^{\cdot}$ and ROS killing the malarial parasite. One in vitro study [61] reports that IFN-γ-treated macrophages kill *P. falciparum* during co-culture, and this parasite killing is markedly reduced by L-NMMA, a NOS inhibitor. However, another in vitro study with cultured *P. falciparum* reports that a saturated solution of nitric oxide does not kill the parasite [62].

Similarly, superoxide reportedly kills *P. falciparum* when produced in vitro by monocyte-derived-macrophages [63,64]. Peroxynitrite also reportedly kills *P. falciparum* in vitro[65].

However, these studies do not take into account the role of free Hb in the in vivo situation. Ockenhouse et al. use indirect methods (^{3}H-hypoxanthine incorporation which is incorporated when the parasite replicates its DNA at trophozoite stage) to assess parasitemia[64]. Because co-culture of *P. falciparum* with activated monocyte-derived macrophages shortens the replication

time from 48 hours to 24 hours, it is likely that lower ^{3}H-hypoxanthine incorporation reflects poor labeling despite the presence of viable parasites because the parasites are past the stage of DNA incorporation when the radiolabel is added[66]. Fritsche et al. only detect parasite killing when they add to the parasite-macrophage co-cultures, which are already producing ROS, additional supraphysiological concentrations (1mM) of SIN-1 (3-morpholino-sydnonimine, a NO and superoxide donor which react to produce peroxynitrite) for 24 hours, resulting in >1mM cumulative peroxynitrite production[65].

Evidence against the role of $NO^{\cdot}$ and ROS killing of the malarial parasite

Direct evidence using animal models of malaria argues against the role of $NO^{\cdot}$ in parasite killing. Although $NO^{\cdot}$ is produced at high levels during experimental malaria, mice deficient in the iNOS gene exhibit similar *P. berghei* and *P. chabaudi* parasitemia as controls, indicating that iNOS-derived $NO^{\cdot}$ and peroxynitrite are not required in parasite killing [14-16]. Treatment with the iNOS inhibitor aminoguanidine does not alter the time course of *P. chabaudi* or *P. berghei* parasitemia [14,16,67]. However, a compensatory mechanism by one or both of the remaining NOS genes in these mice may account for the lack of an effect. Injection of killed *Propionibacterium acnes* prior to *P. chabaudi* infection results in a marked (>100 fold) increase in $NO^{\cdot}$ production that is sustained for more than a week, but this high level of $NO^{\cdot}$ has no detectable effect on the time-course of parasitemia in mice when compared to similarly treated iNOS knockout mice lacking the elevated $NO^{\cdot}$ production [16].

Similar to the lack of detectable in vivo killing of malarial parasites by $NO^{\cdot}$, the results from animal models also question the in vivo role of superoxide and peroxynitrite in parasite killing. P-mice (a strain of mice that have a spontaneous mutation in the pHox gene, resulting in reduced superoxide production) and p91pHox knockout mice have similar *P. chabaudi* and *P. berghei* parasitemias as controls [17,18], indicating that superoxide and peroxynitrite are not required for parasite killing.

In fact, the results obtained from both the NOS and pHox deficient animals can be explained by the ROS quenching capability of hemoglobin. Because hemoglobin is present at such a high concentration within the blood, it can easily quench even the elevated ROS production that occurs during malaria [68-72], resulting in the effective amount of ROS reaching the parasite being be near zero. Thus, modulating ROS production via gene knockout or inhibitor treatment cannot further lower the already low effective dose of ROS the parasite is exposed to.

Finally, we assessed the ability of ROS to kill the *P. berghei* parasites in a defined ex vivo situation with careful analysis of hemoglobin oxidation state[73] (manuscript in review). Parasitized RBCs (pRBC) were treated ex vivo with 1.5μM NO, 15μM NO, 150μM NO, 1.2mM NO, 1.5mM NO, or 1mM SIN-1 for 10 minutes and the ROS-treated inoculum (1 million pRBC) were injected into groups of mice. Supraphysiological NO treatments up to and including 150μM do not reduce parasite viability in the inocula. The 150μM $NO^{\cdot}$ treatment was bioactive inside

the erythrocyte, because it converts all of the oxyhemoglobin to methemoglobin. The fact that the parasite remains viable, even if treated with NO· (up to 150μM) or peroxynitrite (up to 220μM cumulative production) far in excess of the hemoglobin concentration indicates that (1) the theoretical in vivo levels of ROS are insufficient to kill and (2) *Plasmodium* likely has an intrinsic protective mechanisms to deal with exogenous (immune) ROS.

Summary

Despite its complex redox chemistry, hemoglobin, which is a potent NO· and ROS scavenger and is present in abundance around the parasite, is likely to protect the malarial parasite. The high levels of free hemoglobin in the plasma during the course of disease are likely to minimize the ability of NO· and ROS to enter the erythrocyte. Intraerythrocytic hemoglobin is also likely to be protective, because oxidant stress relating to hemoglobin redox chemistry is enzymatically controlled within the red cell. Finally, the malarial parasite has developed mechanisms to cope with the oxidant nature of the free heme it produces, such as packaging it in the hemozoin granule and confiscating erythrocyte superoxide dismutase.

While NO· does not kill the parasite, it may protect against the development of disease. NO· plays important roles in maintaining the homeostasis of (1) the immune system, (2) the endothelium, and (3) coagulation system, while pathologic activation of these systems is required for malarial pathogenesis [3,74,75]. Thus, NO·'s function may be as an anti-oxidant and protective molecule [76], as well as a potent anti-inflammatory molecule that minimizes *P. falciparum* sequestration by down-regulating endothelial activation and CAM expression[59].

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