Diminished organelle motion in murine Kupffer cells during the erythrocytic stage of malaria

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Parasitized erythrocytes are ingested by murine hepatic macrophages during malaria infection. We non-invasively monitored how this altered the motion of intracellular phagosomes in Kupffer cells using magnetometry. Submicrometric $\gamma$Fe$_2$O$_3$ particles were injected prior to malaria infection. They were cleared from the blood, primarily by Kupffer cells, and retained within their phagosomes. The mice were periodically magnetized. After removing this external magnet, the aligned iron particles created a remnant magnetic field (RMF) which then decayed (relaxation), reflecting the motion of particle-containing phagosomes. After baseline measurements of relaxation, the mice were injected intravenously with Plasmodium chabaudi-parasitized or normal murine red blood cells (RBCs). During the next 15 days, relaxation measurements, parasitaemia and haematocrit values were monitored. At 6 days post injection with $3 \times 10^7$ parasitized RBCs, relaxation rates had decreased. At this time, all mice had parasitaemias greater than 58 per cent and haematocrits less than 20 per cent. At day 7, while the parasitaemias were declining, the rate of relaxation continued to decrease. Throughout the experiment, relaxation remained constant in animals injected with normal RBCs. Electron microscopy revealed Kupffer cells filled with damaged and parasitized erythrocytes, and haemoglobin degradation pigment. We conclude that ingestion and metabolism of parasitized erythrocytes by liver macrophages during malaria infection decreases their organelle motion with likely consequences of compromised host defences.

Keywords: erythrophagocytosis; phagolysosomes; cell motility; magnetometry

1. INTRODUCTION

Malaria remains one of the leading causes of morbidity and mortality throughout the world. It is caused by infection with a protozoan belonging to the genus Plasmodium. The life cycle of malaria parasites is complex and includes an erythrocytic cycle that is responsible for much of the pathology associated with Plasmodium infection. This cycle involves the invasion of circulating erythrocytes by the parasite. This invasion leads to alterations of the red blood cell (RBC) surface, which signals macrophages to remove parasitized erythrocytes from the circulation and to destroy them. Adhesion to endothelial cells is also increased. Both phagocytosis of RBCs and enhanced adhesion is partially mediated by changes in the deformability of RBCs containing Plasmodium chabaudi [1]. Moreover, there are alterations in the membranes of non-parasitized erythrocytes during malaria which encourages macrophages to identify additional RBCs to be eliminated [2,3]. If the parasites in the infected red cells evade or suppress host defences, asexual division of the parasite occurs within these blood cells leading to the formation of additional parasites. After several rounds of division, the new parasites and the contents of the infected RBC are released upon its lysis. These parasites then further invade uninfected RBCs and initiate another erythrocytic cycle. This repetitive intraerythrocytic cycle of invasion–multiplication–release–invasion leads to large quantities of foreign intravascular materials such as parasites, parasitized RBCs (pRBCs) and RBC debris from burst erythrocytes.

Kupffer cells, positioned along the hepatic sinuses, make up 80–90% of the body’s tissue macrophages with direct access to the circulation [4]. They are largely responsible for clearing the bloodstream of damaged and senescent RBCs, foreign debris, particulate materials, blood-borne pathogens, endotoxin and other potentially damaging materials. This phagocytic activity is central to the role of the liver in host defences generally and to the body’s specific response to malaria [5–7]. Thus, it is important to characterize how Kupffer cells are affected by a malaria infection.

Magnetometric measures of organelle motility have been developed and applied in our laboratory to assess the functional status of Kupffer cells in vivo [8–11]. This allows us to non-invasively monitor and
quantitate changes in the intracellular motion of organelles containing magnetic particles. We have previously shown that toxins or overloading a cell’s cytoplasm with even non-toxic material can significantly compromise its ability to function normally [12–14]. For example, the ability to ingest and kill bacteria may be compromised when organelle motion is reduced [12]. Others have described changes in in vitro magnetometric measurements of organelle motility and cytoskeletal mechanical properties, which correlate with macrophage cytotoxicity. Magnetic particles have been used to study the effects of cigarette smoke on human alveolar macrophages recovered from healthy and from patients with chronic lung diseases [15], and to assess the cytotoxic potentials of arsenic compounds [16]. These methods have also been used to demonstrate the effects of ultrafine particles on macrophage function that require cytoskeletal integrity such as migration, phagocytosis and intracellular transport of phagosomes [17].

We have shown that the remnant magnetic field (RMF) generated after a brief external magnetization of ferrimagnetic particles contained in phagosomes and phagolysosomes of macrophages decreases over time. This phenomenon, called relaxation, is attributed to rotation (misalignment) of particle-containing phagosomes and phagolysosomes [8,18,19] as well as phagosomal transport along microfilaments and microtubules [20,21]. The latter report also confirms that relaxation requires an intact cytoskeleton and active ATP production.

The purpose of this study was to explore whether malaria-induced accelerated red cell ingestion and breakdown in the liver would alter the motion of particle-containing phagosomes and phagolysosomes in Kupffer cells. We used the murine malaria pathogen P. chabaudi, and monitored three parameters during the erythrocytic stage of malaria infection: (i) the extent of red cell destruction as reflected by decreases in haematoctrit, (ii) in vitro Kupffer cell cytoplasmic motility using magnetometry, and (iii) the ultrastructural appearance of Kupffer cells.

2. MATERIAL AND METHODS

2.1. Animals and particles

Commercial mouse chow may contain magnetic contaminants that interfere with magnetometric measurements. Thus, all mice were fed a non-magnetic purified diet [11] for 7 days prior to the start of the experiments. All experimental animals were injected intravenously (iv) via the tail vein with a suspension of magnetic iron oxide (γFe₂O₃) at 5 mg kg⁻¹. This dose of inert ferrimagnetic particles has been shown to cause no pathological changes in Kupffer cells in previous magnetometric studies [10,22]. The particles were prepared by combustion of iron pentacarbonyl vapours and then collected on paper filters [23]. These iron oxide particles are 0.5 μm agglomerates of 0.05–0.1 μm subunits and are easily recognized using electron microscopy by their crystalline electron-dense appearance [22,23].

2.2. Plasmodium chabaudi

Plasmodium chabaudi (stabilate LUMP 1309; London School of Hygiene and Tropical Medicine, London, UK) were maintained by weekly intraperitoneal passage in female BALB/c mice. The mice were kept on a reverse light cycle to optimize parasite synchronization and allow schizogony to occur during the daytime. The extent of parasitaemia was assessed by light microscopy of methanol-fixed, Giemsa-stained (Sigma, St Louis, MO, USA) thin blood smears taken through tail vein puncture. Parasitaemia values and the number of RBCs per microlitre of blood were calculated. Parasitaemia was defined as the number of parasites per 100 intact RBCs. Parasites in 500 RBCs were counted per smear and the value expressed as a percentage of RBC numbers. Mice were used for parasite collection and subsequent injection into naive mice when parasitaemia values reached 45–50%. Blood was collected by right ventricular cardiac puncture, mixed with Hank’s balanced salt solution, then passed over a CF-11 column to eliminate white blood cells and platelets. The pRBC suspension was intravenously injected at 3 × 10⁷ pRBCs per mouse.

2.3. Haemozoin and β-haematin

Since a significant feature of malaria infection is the production of haemozoin, an insoluble crystalline product of RBC breakdown [24,25], we explored whether the presence of haemozoin could contribute to the measured magnetic fields over the liver. Haemozoin from murine malaria-infected RBCs and β-haematin prepared in vitro were kindly provided by Dr Margaret Rush, Harvard School of Public Health (Boston, MA, USA). Haemozoin and β-haematin were then dried, weighed and embedded in resin for magnetometric measurements. This was performed to confirm that haemozoin or β-haematin are not ferrimagnetic, and thus, unable to contribute to the RMF measured in the liver in vivo.

2.4. Experimental design

All mice were injected intravenously with a suspension of γFe₂O₃ at 5 mg kg⁻¹. At 3 and 7 days, magnetometric measurements were used to establish baseline parameters. The following day (day 8), mice were randomly assigned to two groups. One group received an iv injection of 3 × 10⁷ pRBCs per mouse (n= 6) or 2 × 10⁷ non-parasitized red cells (npRBCs) per mouse (n = 10). The dose of npRBCs was chosen for our control group as it was approximately equal to the volume of pRBCs used in the experimental group. The amounts of iv-injected pRBCs and npRBCs were estimated at 0.27 and 0.18 per cent of circulating RBCs in normal mice, respectively. The estimates were based on published haematological parameters in rodents [26,27]. After injection of npRBCs or pRBCs, magnetometric measurements were repeated in each mouse at 1, 3, 6, 7, 8, 10 and 15 days post injection. At each time point, the animals were anaesthetized with vaporized halothane to permit the magnetization and subsequent magnetometric measurement. At the
same time points, blood was collected to assess the level of parasitaemia. Haematocrits were determined before iv injection of pRBC, and at 6, 8 and 15 days after. Parasitaemia was evaluated in thin Giemsa-stained blood smears taken from the tail vein.

2.5. Magnetometry

When intravenously injected, ferrimagnetic γFe₂O₃ particles are quickly removed from the circulation by the macrophages lining the blood-filled spaces, primarily in the liver [11,22]. The half-life is less than 1 min [22]. Once ingested, the particles can soon be found within phagosomes and phagolysosomes. During magnetic relaxation measurements, each mouse was anaesthetized with vaporized halothane (Webster Veterinary Supply, Inc., Sterling, MA, USA). An external magnet (0.2 T, 2 × 2 × 0.5 cm) was placed on the ventral body surface over the liver for 2 min, which magnetized and aligned the magnetic particles in the Kupffer cells in the liver. The magnet was then removed and the RMF coming from the iron particles in the liver was immediately measured with a fluxgate magnetometer (Magnetscop F 1.067; Foerster Instruments, Corapolis, PA, USA). This was done by quickly placing the mouse in a cylindrical MolyPermalloy shield (to minimize external magnetic noise) and by presenting the mouse liver area to the flux gate magnetometer. The RMF was followed for 20 min, the baseline was checked at specified intervals (every 5 s for the first min, every 30 s during the next 4 min, and every minute till the end) to check for any drift. Over this 20 min period, we observed a progressive decrease in RMF. This decrease is caused by the random progressive rotation of the particle containing organelles away from their initial aligned state.

Organelle motion is produced by activity of the cytoskeleton (especially microfilaments) and results in a process called ‘relaxation’ [10,18,28]. Magnetic field strength was recorded and analyzed to characterize each relaxation curve. Since the peak initial field strength (B₀) immediately after magnetization cannot be measured directly because of an approximate 5 s delay needed to move the mouse from the magnetizing field into the shielded cylinders where the magnetometer probe is located, it has to be estimated. The initial relaxation rate (λ₀) was derived by fitting the first 1 min of data to an exponential equation \( B = B₀ \times e^{−λ₀t} \) and extrapolating back to zero time to obtain \( B₀ \) [8,29]. This equation represents the best fit for the data during the first minute. The purpose of \( λ₀ \) is to estimate \( B₀ \), the magnetic field strength at the very beginning of relaxation that in turn is proportional to the amount of magnetic material present. It is also a measure of initial decay rate for the first minute post magnetization. The 20 min curve is not a single exponential [18]. We defined the relaxation time, \( t_{3/4} \) as the time (in seconds) for the calculated \( B₀ \) to decay to 75 per cent of the initial value (figure 1). After two baseline measurements of relaxation were obtained at 3 and 7 days post injection of γFe₂O₃, the mice were randomly divided into two groups. One received uninfected erythrocytes; the other group received parasitized erythrocytes. Magnetometric data were analysed using MAGPLOT software developed by us.

Haemozoin and haematin particles were analysed for ferrimagnetic properties using the magnetometry apparatus designed for in vitro studies described earlier [13]. Our purpose was to confirm that these iron-containing pigments were not ferrimagnetic—they have no RMF. The apparatus measures the magnetic field from samples over time. Ferrimagnetic particles produce RMF when magnetic directions of the particles were aligned using a magnetic field strength of approximately 125 mT from a transient current pulse in Helmholtz coils that surround the sample holder. The RMF is then measured with fluxgate detectors in a magnetically shielded apparatus. Ferrimagnetic particles such as γFe₂O₃ or Fe₃O₄ produce and maintain RMF upon external magnetization. Other forms of iron such as haemoglobin, ferritin, haemosiderin or malarial pigments do not.

2.6. Electron microscopy

Liver tissue from mice infected with \( 3 \times 10^7 \) pRBCs per mouse was collected at 7 days post inoculation, when the rate of relaxation had decreased significantly. Tissue samples were fixed by intraparenchymally perfusing a small piece of liver first with heparinized saline, then with 2.5 per cent glutaraldehyde in 0.03 M potassium phosphate buffer. Small (1 mm) cubes of fixed tissue were subsequently post-fixed in 1 per cent osmium tetroxide and stained en bloc with 0.5 per cent uranyl acetate. Blocks were then dehydrated through a series of alcohol solutions and embedded in epoxy resin. Eighty nanometer thick tissue sections were cut with a diamond knife, collected on 200 mesh grids, post-stained with uranyl acetate and lead citrate, and examined in a Phillips 300 electron microscope.
2.7. Statistical analysis

All values are means ± standard error (s.e.). Two group comparisons were analysed with Student’s t-test. Changes in parameters over time were analysed with a one-way analysis of variance (ANOVA). Differences between baseline and each time point were evaluated using Fisher’s post hoc test. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Effects of P. chabaudi infection on parasitaemia and haematocrits

The time course of the development of parasitaemia in mice infected with P. chabaudi (3 × 10⁷) is shown in figure 2. Plasmodium chabaudi were seen in erythrocytes as early as day 1 post injection. The percentage of RBCs infected with the parasite rose sharply after day 3, reaching 59 per cent by day 6. At this time, the extent of RBC destruction was pronounced, with the haematocrit dropping to less than 20 per cent (figure 2). By day 10, nearly all parasites were eliminated from the blood stream, but the anaemia persisted. Increased haematocrits were seen by day 15. No mortality occurred during the entire observation period.

3.2. Effects of P. chabaudi infection on organelle motion of Kupffer cells

Figure 1 shows a typical baseline magnetic relaxation in Kupffer cells prior to infection with P. chabaudi. Relaxation is clearly not characterized by a single time constant. It is most rapid during the first minute, and then slows, and finally there is a plateau. We believe this relaxation curve reflects two kinds of heterogeneity that produce this multi-exponential character. The γFe₂O₃ particles themselves are heterogeneous in size. Smaller ones have greater mobility than larger ones, especially as they interact with cell structures. Secondly, there is probably heterogeneity in terms of magnetic particle location. Some may be in larger aggregates in phagosomes which have coalesced. Others are in smaller membrane-bound phagosomes which may have greater mobility.

After the animals were infected with 3 × 10⁷ pRBCs, the rate of relaxation in Kupffer cells was significantly reduced. Averaged normalized relaxation curves before and after malaria infection are shown in figure 3. At day 1, relaxation parameters were similar to those of the baseline. From day 3 on, relaxation slowed until day 7 when relaxation was slowest. At later time points, relaxation started to return towards baseline. Recovery of relaxation to pre-infection level was not complete at day 15, the last time we examined the animals. The initial rate of decay (λ₀) also showed a time-dependent slowing of relaxation in infected mice (figure 4). The lowest λ₀ was observed around day 7 post infection, consistent with the data in figure 3. Likewise, another relaxation parameter, the t₁/4 increased from baseline of 182.2 ± 6.2 s to 1005.1 ± 149.3 s at day 7, more than a fivefold increase (figure 5). The following day, the t₁/4 began to return towards baseline.
of haematocrit and the mononuclear phagocyte system. Regression analysis thus reflects the loss of erythrocytes from the circulation due to enhanced erythrophagocytosis of infected cells. Decreases in haematocrit (circulating RBC numbers) and the consequences of haemoglobin degradation. Regression analysis of $t_{1/4}$ and haematocrit over the course of malaria infection showed a significant inverse correlation ($r^2 = 0.92$, $p < 0.05$). As the haematocrit decreases, relaxation slowed as indicated by increasing $t_{1/4}$. The lowest haematocrits and highest $t_{1/4}$ were at 6 and 8 days after injection of pRBCs.

3.3. Magnetometry of haemozoin and $\beta$-haematin

Our data show that iron-containing pigments abundant in the livers of malaria-infected mice are not ferrimagnetic. A 1.79 mg sample of $\gamma$Fe$_2$O$_3$ produced a $B_0$ of 78.52 nT. However, when we measured the RMF of 13.12 mg of haemozoin or 121.56 mg of $\beta$-haematin, the measured $B_0$ values were indistinguishable from background levels. There was neither detectable RMF from haemozoin nor $\beta$-haematin analysed in vitro. Thus, we believe that changes in magnetic field observed over the liver in infected mice reflect only magnetic fields from previously injected iron oxide particles.

3.4. Electron microscopy

At day 7 post injection of pRBC, when observed parasitaemia was highest, we found that the Kupffer cells were filled with erythrocytes in various stages of digestion. Hepatic macrophages also contained dark pigment and cellular debris (figure 7a). Furthermore, the mitochondria cristae were slightly disorganized (figure 7b). pRBCs, which had escaped phagocytosis, were found within the hepatic sinusoids (figure 7c). The tracer $\gamma$Fe$_2$O$_3$ particles were also seen within phagosomes and phagolysosomes in Kupffer cells (figure 7a).

4. DISCUSSION

The parasitic invasion and subsequent lysis of erythrocytes during malaria infection results in the...
accumulation of pRBCs, malaria pigment (haemozoin) and cellular debris, such as lipid membrane of erythrocytes in the blood. Kupffer cells are resident macrophages in the sinusoids of the liver with access to the circulating blood. These cells have a high capacity to ingest circulating cellular debris, altered or ageing cells, and most pathogens. Recognition of infected or altered red cells is mediated both by changes in surface antigens as well as by adjacent decreases in the deformability of RBCs [1].

The phagocytic function of these cells constitutes a major line of defence during a malaria infection as they clear the blood of circulating materials generated during malaria infection. Damage and subsequent reduction of Kupffer cell function can lead to increased persistence of pathogenic particles within the circulating blood. Moreover, when Kupffer cell activity is compromised, the lungs are placed at increased risk of injury from other blood-borne pathogens. In the present study, we observed that P. chabaudi infection results in significant parasitaemia and red cell destruction that eventually resolves. Infected mice survived and developed many of the familiar secondary features of the disease, such as splenomegaly and hepatomegaly.

Examination of liver tissue by electron microscopy showed that the Kupffer cells contained massive numbers of red cells, and red cell debris. This extensive erythrophagocytosis by liver macrophages is consistent with findings in other studies [30,31]. The macrophages ingest pRBCs by either non-opsonic (via CD36) or opsonic (via Fc receptor) mechanisms. They also ingest npRBCs and remnants of ruptured circulating RBCs [32–36]. In fact, for each pRBC, an average of 8.5–12.6 uninfected RBCs are destroyed [37,38] during malaria. Likewise, a murine model showed that uninfected RBCs are lost far more frequently than pRBCs [31]. This substantial red cell loss is one mechanism contributing to the anaemia observed in infected patients. It may also contribute to the development of macrophage dysfunction [39,40].

A major advantage of our magnetometry method is the ability to make repeated non-invasive measurements in the same animal, and thus follow the time course of the changes in Kupffer cell organelle motility. Using this methodology, we found that the ultrastructural changes observed in Kupffer cells during a malaria infection were associated with significant in vivo slowing in the relaxation rate of Kupffer cells. The most dramatic decrease in relaxation rate correlated with increased RBC destruction consistent with elevated parasitaemia levels and low haematocrit levels. These magnetometric changes reflect a reduction in the motion of particle-containing phagosomes caused by diminished forces generated by the cytoskeleton as well as increased cytoplasmic viscosity [13,28]. Results from previous studies in our laboratory suggest that decreases in cytoplasmic movement as observed magnetometrically are correlated with decreased phagocytic and antimicrobial function of the same cell in vivo [12]. This may explain the human data that demonstrate the frequent association of bacteremia in patients presenting with clinical malaria [41–45]. Magnetometric studies of Kupffer cells in different models of Kupffer cell loading have been reported [12]. Other interventions such as ischaemia during liver

**Figure 7.** Electron micrographs of liver from mice infected with P. chabaudi at a dose of $3 \times 10^7$ pRBCs. (a) A Kupffer cell at 7 days post infection containing large amounts of red cell debris (cd), erythrocytes and iron oxide particles (arrow). (b) A Kupffer cell at 7 days post infection showing RBCs and malaria pigment (mp) within the cytoplasm. (c) Plasmodium-infected RBCs (pe) within the hepatic sinusoid. At this time, the parasitaemia value was 35%. 

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transplantation or cirrhosis of the liver also alter Kupffer cell function.

The precise mechanism for the depressed Kupffer cell phagocytic and antimicrobial function during the blood-stage of malaria remains to be clarified. In our study, we were unable to separate out which of the phagocytized materials was most responsible for the cell’s dysfunction. Is it the RBC, the parasite, or haem degradation products that impair the macrophage? In vitro studies have demonstrated that the phagocytosis of malaria-infected RBCs interferes with the ability of macrophages to kill the bacteria [46,47]. However, depressed host defences for bacterial infections have also been observed in non-malaria conditions in which there are altered erythrocytes and extensive phagocytosis of erythrocytes in humans and animals [48–50]. Free iron is also known to be cytotoxic. Extensive metabolism of haemoglobin may result in toxic levels of iron ions. Commins et al. [51] demonstrate a clear dose-dependent depression of phagocytic function and hydrogen peroxide production following the ingestion of IgG-coated RBCs. The authors contend that it was the phagocytosis of the RBC contents that caused the macrophage dysfunction, since ingestion of the same amount of red cell ghosts did not have the same effect on macrophage function. This suggests a toxic role of haemoglobin metabolism. Others [52,53] have reported similar results. However, more recently, Wilhelm et al. [54] showed that even the washed erythrocyte membranes can inhibit macrophage function. In contrast, other investigators have demonstrated that it is the accumulation of the parasite by-product (i.e. haemozoin, iron) in macrophages, which leads to the paralysis of these critical phagocytic cells [55,56]. Understanding the pathogenesis of macrophage dysfunction associated with malaria may help improve treatment and reduce the morbidity and mortality related to this disease. The precise mechanisms responsible for altered phagocytic motility in this model are unknown. Previous studies showed that relaxation requires an intact cytoskeleton, and energy production [13,15,20]. How erythropagocytosis of malaria-infected RBCs decreases relaxation in Kupffer cells may be related to inflammatory responses to haem degradation products, decreased ATP production and resulting decreases in forces available to move organelles. It is also possible that forces generated by the cytoskeleton are maintained, but are opposed by significant increases in ingested materials.

Results from this study have shown that magnetometry is a useful way to non-invasively follow the progression of malaria-induced hepatic macrophage function in vivo. We believe that the factors contributing to observed reduction in the Kupffer cell organelle motion occurred for multiple reasons: (i) the physical load of RBCs and other particulates within the cytoplasm of the Kupffer cell, (ii) macrophage toxicity caused by the parasite itself or its products, and (iii) the consequences of breaking down a large amount of haemoglobin leading to excessive iron and associated free radicals. This model incorporating repeated non-invasive assessment of Kupffer cell organelle motility can be used to test the efficacy of diverse therapies.

Animal protocols for this study were approved by Harvard Medical Area Animal Care Committee of Harvard University, Female Balb/C mice were obtained from Charles River Laboratory, Inc., Wilmington, MA, USA.

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