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Characterising the effect of antimalarial drugs on the maturation and clearance of murine blood-stage *Plasmodium* parasites in vivo

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ABSTRACT

The artemisinins are the first-line therapy for severe and uncomplicated malaria, since they cause rapid declines in parasitemia after treatment. Despite this, in vivo mechanisms underlying this rapid decline remain poorly characterised. The overall decline in parasitemia is the net effect of drug inhibition of parasites and host clearance, which competes against any ongoing parasite proliferation. Separating these mechanisms in vivo was not possible through measurements of total parasitemia alone. Therefore, we employed an adoptive transfer approach in which C57BL/6J mice were transfused with *Plasmodium berghei* ANKA strain-infected, fluorescent red blood cells, and subsequently drug-treated. This approach allowed us to distinguish between the initial drug-treated generation of parasites (Gen_0), and their progeny (Gen_1). Artesunate efficiently impaired maturation of Gen_0 parasites, such that a sufficiently high dose completely arrested maturation after 6 h of in vivo exposure. In addition, artesunate-affected parasites were cleared from circulation with a half-life of 6.7 h. In vivo cell depletion studies using clodronate liposomes revealed an important role for host phagocytes in the removal of artesunate-affected parasites, particularly ring and trophozoite stages. Finally, we found that a second antimalarial drug, mefloquine, was less effective than artesunate at suppressing parasite maturation and driving host-mediated parasite clearance. Thus, we propose that in vivo artesunate treatment causes rapid decline in parasitemia by arresting parasite maturation and encouraging phagocyte-mediated clearance of parasitised RBCs.

1. Introduction

There were an estimated 214 million cases of malaria in 2015, resulting in 438,000 deaths (World Health Organization (WHO), 2015b). Artesunate (an artemisinin derivative) is the recommended treatment for patients with complicated malaria, and is associated with improved survival among those admitted to hospital with severe malaria (Dondorp et al., 2010; Newton et al., 2013; World Health Organization, 2015a). However, growing parasite resistance to artesunate and its partner drugs has prompted the search for alternative therapies (Dondorp et al., 2009; Phyo et al., 2012; Amaratunga et al., 2016). When assessing antimalarial efficacy in vivo, key metrics include the time to clearance of parasitemia (Jiang et al., 1982), the parasite reduction ratio (White, 1997; Marquart et al., 2015), and more recently the rate of clearance of parasitemia (Flegg et al., 2011; Abdulla et al., 2015). The usefulness of these metrics became most apparent when they affirmed the use of highly effective artemisinins, which were originally found to elicit very rapid elimination of parasitemia in patients compared with other antimalarial drugs (Jiang et al., 1982; White, 1994; Hien et al., 1996). More recently, the detection of slower declines in parasitemia after treatment with artemisinins played a critical role in the early detection of artemisinin resistance (Dondorp et al., 2009; Phyo et al., 2012).

Despite the well-established utility of these measures for assessing drug efficacy, the underlying mechanisms driving a decline in parasitemia after treatment are not well understood. Here, we hypothesise that a reduction in parasite numbers after treatment involves at least two major concurrent processes

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(Dogovski et al., 2015; Hastings et al., 2015): firstly, an impairment of parasite development that hinders further proliferation (Wilson et al., 2013), and secondly, host-mediated clearance of drug-affected parasites from circulation, most likely by phagocytic cells in the spleen (Chotivanich et al., 2002). Thus far, it has not been possible to separate and measure these two processes, either clinically or otherwise, in vivo.

In this paper, we used a mouse model of infection and an adoptive transfer approach to make quantitative assessments of parasite maturation and host clearance during the first 24 h after treatment with artesunate. This approach allowed us to study a single generation of infected parasites and separate the process of host clearance from parasite maturation and proliferation. We found that the normal progression of parasite maturation was rapidly arrested after treatment, suppressing further parasite proliferation over the first 24 h. We also measured the clearance rate of drug-affected early-stage and late-stage parasites from circulation, and showed that clearance of drug-affected early-stage parasites is largely mediated by host phagocytic cells. Finally, we also explored the same processes after treatment with an alternative antimalarial drug, mefloquine (MQ). Thus, our combined experimental and analytical methodology in mouse models of malaria has permitted greater insight into the in vivo effects of antimalarial drugs on parasite replication and subsequent host clearance.

2. Materials and methods

2.1. Mice and ethics

Female C57BL/6J mice aged 6–12 weeks were purchased from the Animal Resource Centre (Canning Vale, Perth, WA, Australia) and maintained under conventional conditions. This study was carried out in strict accordance with guidelines from The National Health and Medical Research Council of Australia (NH&MRC), as detailed in the document Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition, 2004. All animal procedures and protocols were approved (A02-633M) and monitored by the QIMR Berghofer Medical Research Institute Animal Ethics Committee, Australia.

2.2. Parasites and infections

Plasmodium berghei ANKA (*Pb*A) and *Plasmodium yoelii* XNL (*Py*XNL) strains were used in all experiments after a single in vivo passage in wild type C57BL/6J mice (Animal Resource Centre). Transgenic *Pb*A-GFP strains were maintained as previously reported (Haque et al., 2011b). All donor mice were infected with either 10^5 (*Pb*A) or 10^4 (*Py*XNL) infected red blood cells (RBCs) i.v. via the lateral tail vein.

2.3. Adoptive transfer of donor RBCs

The adoptive transfer of donor parasites was carried out as previously described (Khoury et al., 2015). Donor parasitised RBCs (pRBCs) were collected from mice infected with *Pb*A or *Py*XNL by cardiac punctures. Heparinised blood was washed twice in Ca^{2+}/Mg^{2+}-free PBS (PBS-A), and stained in CellTrace™ Far Red DDAO-SE (Life Technologies, USA) according to manufacturer's instructions. Briefly, 50 μg of CellTrace™ were dissolved for 10 min in 25 μl of DMSO. This was added to 5 ml of resuspended blood in PBS-A. Blood was stained in the dark, at room temperature with constant rolling for 15 min, and then washed twice in 10× volumes of PBS-A. Successful labelling of RBCs was confirmed by flow cytometry using an LSRII Fortessa analyzer (BD Biosciences, Australia) and FlowJo software (Treestar, CA, USA). CellTrace™-labelled blood was resuspended in 2 ml volumes per donor mouse, and injected in 200 μl volumes via i.v. injection using a 26 G needle.

2.4. Drug preparation

Sodium artesunate (Guilin Pharmaceutical Co., Ltd., Guilin, Guangxi, China) was prepared according to the manufacturer's instructions, diluted in 0.9% saline (Baxter, Australia), and administered to mice immediately after donor parasites were transfused, and for some mice, a second time 12 h later. Doses ranged from 10 μg to 1000 μg (corresponding to 0.5 mg/kg to 50 mg/kg) and were administered in 200 μl volumes via i.p. injection.

Mefloquine hydrochloride (Lariam®) (Roche, Basel, Switzerland) was dissolved to a stock concentration of 25 mg/ml in ultrapure water (Milli-Q®, Millipore) containing 10% v/v DMSO (Sigma, USA) for 30 min at room temperature with constant agitation. This was further diluted just before administration to mice at doses indicated above, and given via i.p. injection in 200 μl volumes.

2.5. Phagocyte depletion in vivo

Host phagocytes were depleted in vivo with a single i.v. injection (via a lateral tail vein using 26 G needles) of 200 μl of clodronate-containing liposomes (www.clodronateliposomes.com) 3 days prior to transfusion of labelled blood and antimalarial drug treatment.

2.6. In vitro culturing of peripheral blood

Peripheral blood was collected and diluted 1:40 in culture medium (Roswell Park Memorial Institute medium (RPMI), 20% FBS, 1 U/ml of heparin sodium). A 200 μl volume of diluted blood was plated per well in 96-well flat bottom plates (Corning, USA). Plates were covered, then flushed for 30 s with 5% CO_2, 5% O_2, 90% N_2 in a closed secondary container which was then sealed. Cultures were incubated at 37 °C for the specified times. Cells were then resuspended by gentle pipetting immediately before staining for flow cytometric analysis. Separate cultures were maintained for each ex vivo time point.

2.7. Flow cytometric analysis of blood

Forward scatter (FSC) and side scatter (SSC) were used to distinguish RBCs from other cell types. Plotting the FSC-Area (FSC-A) and the FSC–Height (FSC–H) allowed the exclusion of doublets (events recorded by the flow cytometer that are the result of two cells being detected simultaneously). For imaging flow cytometry, the aspect ratio and area of channel 1 (bright field; BF) were used to distinguish RBCs from other cell types and exclude doublets. Focused events were then selected by plotting of the gradient RMS feature of channel 1 (first camera; BF) and the gradient RMS feature of channel 9 (second camera; BF). A flow cytometric method, adapted from various research groups (Apte et al., 2011; Klonis et al., 2011; Malleret et al., 2011), was employed to simultaneously detect adoptively transferred (CellTrace™-labelled) RBCs, to distinguish DDAO-SE^{pos} (donor) from DDAO-SE^{neg} (recipient) RBCs, and to determine the developmental stage of pRBCs (Supplementary Figs. S1, S2). Briefly, a single drop of blood from a tail bleed was diluted and mixed in 200 μl of RPMI medium containing 5 U/ml of heparin sulphate. Diluted blood was simultaneously stained for 30 min in the dark at room temperature with an anti-mouse erythroid cell antigen TER119-APC antibody (2 μg/ml; BioLegend, USA), cell-permeant RNA/DNA stain, Syto84 (5 μM; Life Technologies) and with DNA stain, Hoechst 33342 (10 μg/ml; Sigma). Staining was quenched with 10 volumes of RPMI medium,

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