The Mechanisms of Parasite Clearance after Antimalarial Treatment of \textit{Plasmodium falciparum} Malaria

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Studies were conducted to determine how malaria parasites are cleared from the blood after antimalarial treatment. Neither artesunate nor quinine decreased parasitized red cell deformability or increased antibody binding. In acute falciparum malaria, ring-infected erythrocyte surface antigen (RESA) was observed in erythrocytes without malaria parasites (RESA–red blood cell [RBC]), indicating prior parasitization. In uncomplicated malaria, RESA-RBC numbers increased significantly ($P = .002$) within 24 h of starting artesunate but rose much more slowly (7 days) after quinine treatment. In severe malaria, RESA-RBC increased significantly ($P = .001$) within hours of starting artesunate but not with quinine treatment ($P = .43$). RESA-RBCs were not produced after drug treatment of malaria parasite cultures in vitro. Rapid malaria parasite clearance after treatment with artemisinin derivatives results mainly from the extraction of drug-affected parasites from host erythrocytes—presumably by the spleen. This explains why the fall in hematocrit after treatment of hyperparasitemia is often less than that predicted from loss of parasitized cells.

Antimalarial treatment with artemisinin or one of its derivatives, is associated with a more rapid decline in parasitemia than with other antimalarials [1]. The rate of fall exceeds that associated with inhibition of schizogony alone. This suggests that these drugs induce rapid changes in the circulating ring stage–infected erythrocytes that allow recognition by the host-defense system. Using the 2 most widely used treatments of severe malaria, quinine and artesunate, we have investigated 3 potential mechanisms that might explain this phenomenon: drug-induced changes in infected erythrocyte deformability, antibody binding, or “pitting”—that is, removal of intraerythrocytic parasites without host red cell destruction.

Methods

The Thai \textit{Plasmodium falciparum} strain, TM 267R, was maintained in continuous culture, as described elsewhere [2]. The $IC_{50}$ values for TM 267R, determined by inhibition of $^3$H hypoxanthine uptake, were $756 \times 10^{-9}$ M (244 ng/mL) for quinine and $13 \times 10^{-9}$ M (5.3 ng/mL) for artesunate. To investigate the effects of antimalarial drugs on red blood cell (RBC) deformability (RCD), a synchronized culture of ring or trophozoite-infected RBCs, concentrated to 50% parasitemia by using sorbitol lysis, was resuspended at 2% hematocrit in malaria culture medium containing 0.001, 0.01, 0.1, 1, and 5 $\mu$g/mL of either quinine dihydrochloride or artesunate adjusted to a pH of 7.3–7.4. Infected RBCs without drugs and uninfected RBCs with and without the drugs acted as controls. RBC deformability was assessed after incubation for 5, 15, 30, and 60 min and 4 h at 37°C in 5% CO$_2$ plus air. Each experiment with controls was performed in duplicate on 3 different occasions. RCD was measured under different conditions that mimic the vascular shear stresses encountered in vivo, by use of using the laser-assisted optical rotational cell analyzer (Mechatronix Instruments, Amsterdam), as described elsewhere [3]. The average deformability of RBCs, assessed by laser diffraction, is expressed as an elongation index (EI), the average ratio of the horizontal to the longitudinal erythrocyte axes. A low value of EI indicates reduced RCD. The 2 main EIs of interest are at 1.7 and 3.3.

Antibodies bound on the surface of \textit{P. falciparum}–infected RBCs were quantitated by incubating synchronized-washed \textit{P. falciparum} (Brazilian strains UF and ITO and Thai strain TM 267R) cultures with antibody (IgG fraction of malaria hyperimmune serum from adult Kenyans living in an area of stable high transmission; KS) for 30 min before and after exposure to artesunate (5–1000 ng/mL).
for 4 h under in vitro culture conditions. RBCs from the same donor were incubated with naive serum (European serum; ES) or with buffer (1% bovine serum albumin [BSA] in RPMI-1640) as controls. The infected RBCs were labeled sequentially with 1:25 antihuman rabbit IgG in buffer for 30 min and 1:25 swine antirabbit IgG-fluorescein isothiocyanate (FITC). All RBC suspensions were washed 3 times and resuspended in 1% BSA in PBS. Then, by use of flow cytometry, infected RBCs were sorted by ethidium bromide labeling of parasite DNA, and surface immunofluorescence labeling was quantitated for 10,000 infected RBCs. Controls were RBCs without added drugs.

**Pitting.** The removal of intraerythrocytic malaria parasites (“pitting”) without RBC destruction was assessed in vivo by immunostaining peripheral blood smears for the ring-infected erythrocyte surface antigen (RESA), as described elsewhere [4]. Seventy-five patients with severe malaria [5] (65 treated with artesunate and 10 with quinine) and 17 patients with uncomplicated falciparum malaria (10 treated with artesunate and 7 with quinine) were studied. Fully informed consent from the patients or their relatives was obtained for serial blood sampling. In uncomplicated malaria, samples were taken before treatment and then on days 1, 3, 5, 7, 14, 21, and 28 after treatment. In severe malaria, samples were taken at 0, 8, 16, and 24 h and then on days 4, 7, 14, 21, and 28. The presence of *P. falciparum* antigens in or on erythrocytes was assessed by immunostaining with FITC-labeled AB* RBC preabsorbed polyclonal immune serum and an FITC-labeled human monoclonal antibody (MAb) 3G2 directed specifically against RESA [6]. Sera from healthy nonimmune donors were used as controls. In order to investigate whether RESA-positive unparasitized RBCs can arise ex vivo, parasites in patients’ blood (n = 6) were cultured continuously at 0.1% parasitemia in RPMI-1640 supplemented with 10% human AB serum. To investigate drug effects, *P. falciparum* (TM 267R) was incubated with artesunate (1 μg/mL or >100× the IC<sub>50</sub>). RBCs were sampled daily and stained for RESA for 2 weeks from these cultures.

**Statistical analysis.** Normally distributed paired data were compared using Student’s *t* test. The change in fluorescence density after drug exposure was compared using the Wilcoxon signed rank test. Correlations were assessed by the linear regression. All analyses were performed separately for severe and uncomplicated malaria. SPSS for Windows (SPSS, Chicago) was used for all analyses.

**Results**

**Effects of quinine and artesunate on RCD.** The mean (SD) EI of RBCs from 17 healthy Thai donors was 0.23 (0.02) at 1.7 Pa (low shear stress) and 0.61 (0.01) at 30 Pa (high shear stress). RBCs infected with *P. falciparum* showed a progressive decrease of the EI at both low and high shear stresses. The mean (SD) EIs of ring-, trophozoite-, and schizont-infected RBCs were 0.22 (0.03), 0.19 (0.03), and 0.16 (0.03) at 1.7 Pa and 0.58 (0.03), 0.52 (0.03), and 0.48 (0.08) at 30 Pa. Quinine (0.001–5 μg/mL) or artesunate (0.001–5 μg/mL) exposure for ≦4 h did not affect uninfected RBC deformability. Incubation of ring-infected RBCs with artesunate for ≧2 h resulted in a 20% inhibition of the malaria induced reduction of baseline EI at 1.7 Pa (P = .005), whereas quinine had no effect even after 8 h. Artesunate incubation for ≧2 h resulted in a mean EI for trophozoite-infected RBCs 38% higher than in the drug-free controls (P = .01), compared with a 19% increase with quinine (P = .01). At high shear stresses, the effects of the drugs were usually small and not significant.

Mean fluorescein intensity of infected RBCs (both ring and trophozoite stages) incubated with hyperimmune serum was considerably higher than those incubated with normal ES or buffer reflecting antibody binding to RBC-surface malarial antigens. The mean fluorescence intensity of synchronized tiny rings (0–6 h), small rings (6–12 h), large rings (12–18 h), or more mature trophozoites was not changed significantly after incubation with artesunate (5–1000 ng/mL) for 4 h (P = .07).

**Evidence for pitting—in vivo study.** Mean parasitemia and hematocrit values in the severe malaria group (n = 75) were 5.3% and 32% and in the uncomplicated group (n = 17) 1.4% and 34%, respectively. Median (range) parasite clearance times were 52 (30–108) h and 58 (13–96) h, respectively. Thin blood films showed staining with the pattern characteristic of RESA on all ring-infected RBCs and some uninfected RBCs (RESA-RBC), in marked contrast to adjacent unstained cells (figure 1). Pretreatment RESA-RBC numbers were significantly higher in severe malaria than in uncomplicated malaria; geometric means (95% confidence interval [CI]) were 26,140 (21,193–32,248) versus 9141 (5099–16,387), P = .001. In uncomplicated malaria, RESA-RBC numbers increased significantly 24 h after treatment with artesunate (P = .002), coincident with >90% parasite clearance. There was a slower rise (7 days) after treatment with quinine (P = .002; figure 2A). In 5 of the 10 patients, N<sub>max</sub> occurred within 24 h after treatment with an artemisinin derivative. In contrast, after quinine, most patients (6/7) had not reached N<sub>max</sub> until 7 days after treatment (figure 2A). Therefore, RESA-RBC numbers declined exponentially in both treatment groups, with a half-life of ≈2 days, until none could be detected after 28 days.

In severe malaria, the maximum numbers of RESA-RBC/μL (N<sub>max</sub>) of each patient ranged from 10,550 to 1,094,227/μL. From figure 2B, RESA-RBC/μL increased significantly 8 h after starting artesunate (P = .001) but not with quinine (P = .43). This corresponded to a median (range) of 4.83 (0–36)–fold rise, compared with 1.64 (0–7.04)–fold after quinine. After artesunate treatment, 50% of subjects had reached maximum values of RESA-RBC within 8 h. Circulating RESA-RBCs were cleared more slowly than were malaria parasites, but by days 21–28 (n = 21) no RESA-RBCs were detected. RESA-RBC numbers did not change after treatment with quinine; for 6 of 10 patients, the N<sub>max</sub> occurred at admission (figure 2B). The parasitemia on admission was correlated positively with the N<sub>max</sub> (r = .56, P = .001) in uncomplicated but not in severe malaria (r = .025, P = .9), but the fall in parasitemia and rise...
Figure 1. Ring-infected erythrocyte surface antigen–positive staining of unparasitized red blood cells (*) and an adjacent parasitized cell. The intracellular malaria parasite is stained by ethidium bromide (arrow).

in RESA-RBC after treatment were not correlated significantly in either group ($P > .5$).

Evidence for pitting—in vitro study. Pooled hyperimmune sera and MAb 33G2 showed the same characteristic pattern of membrane staining of $P$. $falciparum$ ring–infected RBCs. Neither reacted with $P$. vivax–infected RBCs, normal RBCs, or uninfected RBCs in cases of in vitro parasite cultures. No RESA-positive uninfected RBCs appeared in parasite cultures after artemunate was added (.001–1 mg/mL).

Discussion

Parasite multiplication can be remarkably efficient in a non-immune patient with acute malaria. Multiplication rates of $\leq 20$-fold per asexual cycle have been observed in volunteers and patients receiving malaria therapy [$7, 8$]. This approaches the number of merozoites per developed schizont and indicates little or no host removal of parasites during the expanding phase of the infection. After antimalarial treatment, particularly with drugs affecting ring-form parasites, there is an abrupt and dramatic change. Parasites that hours earlier were circulating unrecognized by the host are now identified and cleared. This is most marked after treatment with the artemisinin derivatives. Clearance is related presumably to antimalarial-induced damage to the parasite, but it is not known how the injured or dead intraerythrocytic parasite signals its presence to the host, nor is the final route of removal. In this series of experiments, 3 possible mechanisms for drug-induced change to circulating parasitized erythrocytes were examined using the 2 drugs most widely used for the treatment of severe malaria: artemunate, which induces rapid ring-form clearance, and quinine, which does not [$8, 9$].

The first mechanism examined was the possibility that artemunate induces a decrease in parasitized RBC deformability. In the presence of heme Fe$^{3+}$, these drugs generate carbon-centered free radicals that could damage the RBC membrane or cytoskeleton and thereby increase the rigidity of the infected RBC. The spleen has a lowered recognition threshold for the removal of rigid RBCs in malaria [$10$]. The reverse was found; artemunate, by acting on young ring-form parasites, prevented their development to more rigid mature trophozoites and thereby attenuated the reduction in deformability associated with continued parasite growth. Quinine, which acts predominantly on the mature trophozoites, had much less effect. The second possibility examined was that artemunate induces changes either in the parasite or in the RBC directly, which lead to increased antigenicity (either through increased expression of parasite antigens or the uncovering of cryptic host an-
tigens) and thus increased opsonization. The splenic threshold for removal of antibody-coated RBCs is also lowered in acute malaria [11, 12]. Again, this proved incorrect. No evidence for increased ring-form parasitized erythrocyte antigenicity after antimalarial drug exposure was found.

The spleen plays a central role in circulating parasitized RBC clearance. After antimalarial treatment with artesunate in spleenectomized patients, parasite clearance is delayed considerably—although the parasites appear pyknotic and are usually not viable on culture. We reported elsewhere [4] that parasites are removed from circulating RBCs in falciparum malaria without destruction of the erythrocytes, a process described elsewhere in experimental Simian malaria and known as “pitting” [13]. This process is presumably similar to the normal function of the spleen to remove residual nuclear or particulate material from RBCs without hemolysis. The present study suggests that splenic pitting is the main route of removal of circulating parasites after antimalarial treatment. Parasite clearance after artesunate treatment is associated with a significant rise in the number of “once-parasitized” RBCs, readily identified by their immunofluorescent staining with antibody against the \( P. falciparum \)–ring erythrocyte surface antigen. The spleen presumably recognizes the dying or dead intraerythrocytic parasites and removes them as it does host nuclear remnants. The central role of the spleen in parasite removal is further supported by the failure of artesunate to induce once-parasitized cells in vitro. Perhaps the spleen recognizes the drug-affected parasite’s simple loss of normal intraerythrocytic motility as it becomes more spherical and particulate, whereas the healthy motile intraerythrocytic parasite may be regarded as similar to a leukocyte.

After quinine treatment, there was often no significant rise in once-parasitized RBCs above the pretreatment level. This is consistent with the known stage specificity of this drug, which does not prevent significantly the development of ring-form parasites or their subsequent sequestration in the deep vasculature away from splenic removal [14]. These data also explain how, in some patients with hyperparasitemia, the parasite count falls considerably more than the RBC count after antimalarial treatment.

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References