**Plasmodium falciparum** Clearance Is Pitting-Dependent With Artemisinin-Based Drugs but Pitting-Independent With Atovaquone-Proguanil or Mefloquine

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Pitting, the removal of dead parasites from their host erythrocyte, has been studied in patients with severe malaria treated parenterally with quinine or artesunate, and was recently shown to contribute to delayed hemolysis, a frequent adverse event of artesunate. We quantified pitting in 81 travelers treated with oral antimalarial therapy. Pitting rate was high (55.8%) with artemisinin-based combinations, but <10% with the nonartemisinin drugs quinine, mefloquine, and atovaquone-proguanil. This may, in part, explain the slower parasite clearance in patients treated with antimalarial drugs lacking an artemisinin component, as well as the absence of posttreatment hemolysis with these drugs.

**Keywords.** malaria parasite clearance; pitting; atovaquone-proguanil; artemisinin derivatives combination therapy; postartemisinin delayed hemolysis.

The World Health Organization recommends intravenous artesunate and oral artemisinin-based combination therapies (ACTs) for the first-line treatment of severe and uncomplicated malaria, respectively [1]. Mefloquine, quinine, and atovaquone-proguanil (AP) are used in specific situations, such as treatment of travelers with uncomplicated malaria where ACTs are unavailable, or patients with multidrug-resistant malaria in Southeast Asia. The mechanisms of *Plasmodium falciparum* clearance with artemisinin derivatives were explored in patients with severe malaria, but much less is known for antimalarial drugs other than artemisinins. Pitting in severe malaria is a spleen-specific process whereby dead parasites are expelled from their host erythrocytes [2–4]. After pitting has occurred, once-infected red blood cells (pitted red cells) return to the circulation but have a reduced lifespan [4, 5] sometimes causing postartesunate-induced hemolysis [4, 6]. Pitting has been successfully quantified in patients with severe malaria by immunofluorescence [7], flow cytometry [3], and, more recently, by semiquantitative titration using dipsticks [6].

While several studies analyzed pitting in patients with severe malaria treated with artesunate or quinine, explorations in patients treated with oral antimalarials are still limited [3, 5, 8, 9]. The proportion of parasitized red blood cells transformed into pitted red cells during the 3–7 days following artesunate treatment (pitting rate) was generally >60% in patients with severe malaria [3]. However, pitting rates were lower (generally <30%) with quinine, the only nonartemisinin antimalarial drug evaluated so far for its ability to induce pitting. We assessed pitting rates in French travelers treated for malaria with ACTs (artemether-lumefantrine [AL], dihydroartemisinin-piperaquine [DP]) and with AP, mefloquine, and quinine.

**MATERIALS AND METHODS**

**Patient Surveillance Program and Treatment**

Forms, data, and samples from all patients were collected in the setting of an observational program implemented by the National Reference Center for Malaria (CNR). The Ile de France II Institutional Review Board approved this approach as a nonresearch process (Article L1121-1 of the French Code for Public Health) embedded in the surveillance missions of the CNR, officially empowered to collect information and biological samples. Patients provided consent according to a procedure common to all French National Reference Centers (available at: http://www.invs.sante.fr/Espace-professionnels/Centres-nationaux-de-reference/Textesreglementaires). The study was conducted in accordance with the Declaration of Helsinki.

**Parasite Clearance and Pitting Rates**

Pitting rates were determined using flow cytometry as previously described [2, 3] on peripheral blood samples from French travelers with uncomplicated malaria and patients with hyperparasitemia (>4%) who received treatment with DP, AL, AP, mefloquine, or quinine in the context of their medical care. The dosing was according to manufacturer recommendations: AL 120 mg/20 mg tablets given orally twice a day for 3 days (total 6 doses), the first 2 doses given 8 hours apart, based on body
weight (5 to <15 kg: 1 tablet; 15 to <25 kg: 2 tablets; 25 to <35 kg: 3 tablets; ≥35 kg: 4 tablets) or DP 320 mg/40 mg given orally once a day for 3 days (7 to <13 kg: 0.5 tablet; 13 to <24 kg: 1 tablet; 24 to <36 kg: 2 tablets; 36–75 kg: 3 tablets; >75 kg: 4 tablets). The 3 other drug regimens were either AP 250 mg/100 mg based on body weight (11–20 kg: 1 tablet; 21–30 kg: 2 tablets; 31–40 kg: 3 tablets; >40 kg: 4 tablets) given orally once a day for 3 days; mefloquine 25 mg/kg given orally (15 mg/kg initially followed by 10 mg/kg 12 hours later); or the oral formulation of quinine 8 mg/kg of body weight 3 times a day for 7 days.

Parasitemia and percentage of pitted red cells were assessed by flow cytometry (Accuri C5, BD Biosciences, Le Pont de Claix, France) as described elsewhere [3, 4]. In brief, samples were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS), then incubated with a polyclonal hyperimmune serum [2] at 1:10 in a suspension 1% AlbuMAX II in PBS (Life Technologies) after permeation with Triton X-100 for 30 minutes (Sigma-Aldrich). Samples were then washed and incubated with secondary antibody (goat antihuman immunoglobulin G) coupled with Alexa-Fluor 568 (Life Technologies) and SYBR green (Life Technologies) for DNA labeling before analysis. For each patient included in the analysis, 1–3 samples were available during the first week of treatment and analyzed for percentage of parasitemia and once-infected erythrocytes. In case of discrepant parasitemia between flow cytometry and optical microscopy provided by the hospital (which occurred for 5 samples), manual counting of parasitemia on immunofluorescent slides, prepared as described, was used to resolve the contradiction [2, 3].

Pitting data from a patient were included in the analysis if pitting rate had been quantified both at baseline (ie, prior to the administration of any antimalarial treatment) and at least once between day 3 and day 7. Exclusion criteria included initial parasitemia ≤0.4% (as the quantification of pitting at these very low parasitemia levels is expected to be less specific or accurate), history of blood transfusion in the last 2 months, or splenectomy. Subjects who had blood transfusion after measuring post-treatment pitting rates were included in the analysis, but any pitting rate determined after blood transfusion was censored.

Statistical analyses were performed using GraphPad Prism 6 software. Day 0 and day 3–7 variables were compared by the Mann–Whitney test or by analysis of variance for multiple comparisons. Differences with *P* < .05 were considered significant.

**RESULTS**

Of 181 patients for whom blood samples were collected in the context of the national surveillance program, 81 had at least one determination of pitting rate before and after treatment with either an artemisinin combination (n = 20): AL (n = 18) or DP (n = 2), or antimalarial regimens devoid of an artemisinin component (n = 61): AP (n = 30), mefloquine (n = 5), or quinine (n = 26) (Figure 1 and Table 1). Baseline pitting rate was <8% in all treatment groups: 0% for AL, DP, and AP; 2% for quinine; and 7% for mefloquine (Table 1). The peak pitting increase on days 3–7 was significantly higher with ACTs than with non-ACT regimens (54.4% vs 8.36%, respectively; *P* < .0001; Figure 2).

Even though a rise in pitting rate from day 0 to days 3–7 (normalized against initial parasitemia) was also observed for
non-ACT regimens, reaching 7.2% at days 3–7 for AP, 9.7% for quinine, and 9.7% for mefloquine (Figure 2), it was much lower than observed with ACTs, with a 3-fold increase observed for AP (P < .0001), 2-fold increase for quinine (P = .10), and 1.7-fold increase for mefloquine (P = .11), compared to a 40-fold increase for ACTs (P < .0001). Posttreatment pitting rates at days 3–7 were 56.5% and 49.6% for AL and DP, respectively (Table 1). Overall, patients treated with ACTs had a significantly greater increase in median posttreatment pitting compared to AP, quinine, or mefloquine (Figure 2A). Parasite clearance was 90% complete (18/20) in patients treated with ACTs and 70% complete (38/54) in non-ACT treatments at day 3. All patients included in the analysis had complete parasite clearance by day 7 and all recovered without sequelae.

Table 1. Demographic, Clinical, and Parasitological Characteristics of Malaria Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AP (n = 30)</th>
<th>AL (n = 18)</th>
<th>DP (n = 2)</th>
<th>Mefloquine (n = 5)</th>
<th>Quinine (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>41 (29–50)</td>
<td>42 (31–50)</td>
<td>28 (10–45)</td>
<td>9 (6.5–37)</td>
<td>44 (37–55)</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>15 (50%)</td>
<td>15 (63%)</td>
<td>0 (0)</td>
<td>2 (40%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Time to presentation, days symptomatica</td>
<td>3 (2–4)</td>
<td>3 (2–6.5)</td>
<td>4.5 (2–7)</td>
<td>6 (2–13)</td>
<td>4 (3–6)</td>
</tr>
<tr>
<td>Baseline parasitemia, %</td>
<td>2.7 (1.8–3.7)</td>
<td>3.8 (2.7–5.0)</td>
<td>9.3 (6.6–23)</td>
<td>3.7 (1.9–5.4)</td>
<td>6.3 (3.8–8.8)</td>
</tr>
<tr>
<td>Baseline parasitemia, estimated density/µL</td>
<td>108 000 (72 000–148 000)</td>
<td>152 000 (108 000–200 000)</td>
<td>372 000 (20 000–724 000)</td>
<td>148 000 (76 000–216 000)</td>
<td>252 000 (152 000–352 000)</td>
</tr>
<tr>
<td>Baseline pitting, uncorrecteda</td>
<td>0 (0–0.08)</td>
<td>0 (0–0.05)</td>
<td>0 (0–0)</td>
<td>0.1 (0.05–1.3)</td>
<td>0 (0–0.2)</td>
</tr>
<tr>
<td>Baseline pitting normalized to initial parasitemiaa</td>
<td>0 (0–2.4)</td>
<td>0 (0–1)</td>
<td>0 (0–0)</td>
<td>7 (1.1–26)</td>
<td>2 (0–9.4)</td>
</tr>
<tr>
<td>Mean Δ change in pitting, D0 to D3–7c</td>
<td>7.2 ± 2.7</td>
<td>56.5 ± 12.05</td>
<td>49.6 ± 9.6</td>
<td>9.7 ± 4.9</td>
<td>9.7 ± 2.5</td>
</tr>
<tr>
<td>Median Δ change in pitting, D0 to D3–7 (Min, Max)</td>
<td>6.2 (–10, 66.7)</td>
<td>46.15 (0, 140)</td>
<td>49.55 (40, 59.1)</td>
<td>8.7 (–4, 23)</td>
<td>5.6 (0, 33.4)</td>
</tr>
</tbody>
</table>

Dataset includes 30 patients treated with AP, 18 with AL, 2 with DP, 5 with mefloquine, and 26 patients treated with quinine. Parasite density (per µL) was estimated based on parasitemia percentage obtained by flow cytometry analysis.

Abbreviations: AL, artemether-lumefantrine; AP, atovaquone-proguanil; D0, day 0; D3–7, days 3 through 7; DP, dihydroartemisinin-piperaquine.

aMedian (interquartile range).
bMean (95% confidence interval).
cMean ± standard error. For any given patient with >1 pitting data between days 3 and 7, the average of all collected data points for that period was used.

Figure 2. A, Percentage rise in pitting for artemisinin-based combination therapies (ACTs) vs non-ACT drug regimens, with median, interquartile range, and 5%–95% range shown. B, Comparison between ACTs and other antimalarials. All patients eventually showed complete parasite clearance. C, Pitting kinetics in patients treated with AL (artemether-lumefantrine) vs atovaquone-proguanil (AP). The kinetics of parasitemia was expressed in all patients as a proportion of the initial parasitemia ([parasitemia at a given time point / parasitemia at hour 0 of treatment] × 100). The kinetics of pitted red cells (pitting rate) was expressed similarly, after normalization against initial parasitemia ([fraction of pitted red cells at time X / fraction of infected erythrocytes at day 0] × 100).
DISCUSSION

We characterized the rates of pitting in malaria patients treated with either ACTs or other oral antimalarials. The observations confirm that pitting is strongly related to the mode of action of artemisinin derivatives. Despite large interindividual variations, pitting rates were higher following treatment with artemisinin-based combinations compared to other drugs, namely mefloquine, AP, and quinine. Patients treated with mefloquine were predominantly nonimmune children in whom parasite clearance is pitting-dependent when exposed to artesunate [3], so the low pitting rates observed in this group are unlikely to be related to their young age. Each non-ACT drug regimen tested showed a markedly lower (1.7- to 3-fold) rise in pitting rates, compared to the 40-fold rise observed with ACTs. We thus confirm that pitting contributes only mildly to parasite clearance following treatments with AP, mefloquine, and quinine. Drug-induced parasite clearance by pitting is potentially beneficial in hyperparasitemic patients with severe malaria and patients with anemia, because it rapidly clears parasites from the circulation, and spares a substantial biomass of infected red blood cells (RBCs) from destruction during the initial posttreatment phase [4]. Whether artemisinin-resistant parasites are less susceptible to clearance by pitting will require specific investigations. Along the same line, whether pitting influences posttreatment gametocyte carriage has not been explored yet.

Our data confirm that pitting is almost completely artemisinin-specific. This may be related to the fact that artemisinin derivatives act not only on sequestered mature forms of P. falciparum but also on circulating ring forms. Pitting occurs when infected RBCs cross interendothelial slits in the spleen and therefore can affect only infected RBCs exposed to the drug while they are still in circulation. However, when pitting has been replicated in vitro, it was only observed on ring forms of P. falciparum [3], which suggests that determinants other than presence in circulation—such as the small size of the remnant to expel—may also limit pitting to ring forms. Drugs like quinine, mefloquine, and AP, which act mainly on sequestered mature parasites forms, induce longer parasite clearance time than do ACTs. It is also possible that other factors might affect parasite clearance for drugs such as atovaquone, whose absorption is dependent on coadministration with a high-fat meal.

Treatment with AP results in longer parasite clearance, which raised concerns for higher risk of treatment failure. However, the high cure rate in travelers [10] suggests that the delayed parasite clearance post-AP may be due to lack of pitting in the spleen rather than defective parasite killing. Pitting is absent in splenectomized patients treated for malaria by artemisinins, but the prolonged circulation of red cells containing dead parasite remnants (“hearse RBCs”) in these patients does not correlate with an obvious increase in treatment failure. Dead parasites persist in circulation for several weeks after therapy before their clearance takes place [7]. In a few patients, high rates of pitting were observed on admission. In these cases, we suspect a prior treatment likely with ACTs, which are widely available in Africa, although this could not be confirmed from the information available in the database. Taken together, these observations underline that, in the specific context of infection with P. falciparum, parasite clearance defined by the disappearance of parasitized RBC from the peripheral blood is generally but not always an accurate marker of drug efficacy and positive outcome. Red blood cells containing mature parasites disappear from the circulation, but this “clearance” reflects the pathogenic sequestration of live parasites in small vessels. Conversely, RBCs containing dead parasites may stay in circulation without harmful consequences. In both cases, the observation of parasite numbers in circulation poorly reflects the kinetics of the whole biomass of live parasites in the body.

We suggest that drug-induced pitting should be evaluated early in the development of new generations of antimalarial molecules such as spiroindolones [11], which have an effect on ring circulating forms. This would help better grasp their mode of action in vivo, which helps predict the risk of posttreatment hemolysis observed in severe [4, 12–14] and more recently in uncomplicated malaria [9, 15]. In the context of artemisinin resistance in Southeast Asia characterized by slow parasite clearance, a specific study on the role of pitting may explain how parasites initially escape clearance mechanisms. Measuring the degree of pitting during and after the treatment of malaria can potentially explain some of the variability in parasite clearance times and contribute to evaluations of drug efficacy and safety, spleen function, and the risk of postartesunate-induced hemolysis.

Notes

Acknowledgments. We thank all patients and their families for participating in this study; Liliane Cicéron for her help in this study; and Pattaraporn Vanachayangkul for her assistance with GraphPad Prism.

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The manuscript has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The investigators have adhered to the policies for protection of human subjects as prescribed in Army Regulation 70-25.

Financial support. Travel and subsistence support were provided by the United States Army Medical Research and Materiel Command (to M. W.). This work was supported by the French Institut National de la Santé Et de la Recherche Médicale.
(INSERM), the GR-Ex laboratory of excellence, and the Bill & Melinda Gates Foundation (to P. A. N.) and by the Assistance Publique–Hôpitaux de Paris (to O. M.). C. C. was supported by the French Ministry of Research and C. R was supported by a grant from the GR-Ex laboratory of excellence.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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