Factors Contributing to the Development of Anaemia in Plasmodium falciparum Malaria: What about Drug-Resistant Parasites?

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Summary
A major manifestation of complicated malaria especially among children is severe anaemia, the pathogenesis of which is not well understood. Among other factors, suppression of the bone marrow’s response to erythropoietin, which is rapidly reversed after successful treatment of the malaria, has been implicated in its pathogenesis. Since resolution of malaria restores erythropoiesis, we hypothesized that drug-resistant strains of Plasmodium falciparum would increase the risk of severe anaemia developing from initially uncomplicated malaria. Using both in vivo and in vitro drug-sensitivity tests we compared the prevalence of drug-resistant malaria between severe malarial anaemia (SA) and non-anaemic malaria (NAM) patients. Assessment of treatment outcome using the WHO in vivo criteria showed no significant difference in parasite resistance between the two groups. The mean parasite clearance time was also comparable. Treatment failures of about 14 per cent and 12 per cent were observed between SA and NAM patients respectively. The in vitro drug susceptibility test showed overall mean IC50 values of $0.41\times10^{-6}\text{mol/l}$ and $0.32\times10^{-6}\text{mol/l}$ blood for SA and NAM groups respectively. Geometric mean pre-treatment blood levels of chloroquine did not differ much between the two groups. Findings from this study could not therefore implicate drug-resistant parasites in the pathogenesis of severe malarial anaemia.

Introduction
Severe malarial anaemia is an important and a common life threatening complication of Plasmodium falciparum infection, especially among children [1, 2]. The pathogenesis of severe anaemia in malaria infection is complex and not well understood. In addition to direct destruction of erythrocytes by parasites during schizogony and premature removal of both parasitized and non-parasitized erythrocytes, reversible suppression of erythropoiesis is reported to play a major role in its pathogenesis [3–5]. Kurtzhals and his colleagues demonstrated that the presence of P. falciparum causes suppression of the bone marrow’s response to erythropoietin which was rapidly reversed after clearance of parasites [5]. Infections with drug-resistant strains of P. falciparum are likely to prolong inhibition to the bone marrow’s response to erythropoietin and thus increase the risk of development of anaemia from hitherto uncomplicated malaria. We therefore hypothesized that severe malarial anaemia should be associated with drug-resistant strains of P. falciparum.

To test the hypothesis, we compared the sensitivity of P. falciparum to chloroquine in vivo between malaria patients with Hb ≤ 5.0 and those with Hb ≥ 8.0 using the 14-day WHO in vivo method.
with a slight modification [6]. The modification being, the extension of the age limit from 60 to 120 months. The parasitological outcome was preferentially chosen to the clinical outcome as the end point of treatment to enable proper evaluation of the role resistant parasites play in the pathogenesis of severe malarial anaemia. Since the WHO in vivo method is limited by its inability to distinguish between recrudescence and re-infection, genotyping of both pre- and post-treatment recurrent samples were done in order to identify genuinely recrudescence malaria [7, 8]. Susceptibility of P. falciparum isolates to chloroquine in vitro was also carried out on randomly selected patient’s blood samples from both groups. The in vitro assessment allows for almost complete exclusion of host-related factors, such as drug failure or host immunity and also provides a more objective insight into inherent drug sensitivity than does the in vivo assay. Pre-hospital treatment blood levels of chloroquine were determined for all patients participating in the study.

Materials and methods

Study population and site
Children up to 120 months of age, presented to the Department of Child Health, Korle Bu Teaching Hospital in Accra were screened for inclusion in the study. The criteria for inclusion were the presence of asexual P. falciparum parasites and an axillary temperature \( \geq 37.5^\circ \text{C} \) on day of recruitment or measured within the first 24 h afterwards. Children with any disease other than malaria or with a positive sickling test were excluded. Furthermore, the patients should have either severe malarial anaemia (SA) based on reported definition [5] or have \( \text{Hb} \geq 8.0 \text{ g/dl} \) measured on day of recruitment in the presence of P. falciparum. Briefly, patients with SA had \( \text{Hb} \leq 5.0 \text{ g/dl} \), full consciousness and exclusion of other possible causes of anaemia. A project physician examined each patient thoroughly to ensure there was no other cause for the severe anaemia such as malnutrition and helminth infestations among others. Parents or guardians of children signed an informed consent for participation of their children in the study. The study was approved by the ethical and protocol review committee of the University of Ghana Medical School. Clinical monitoring of patients on admission was done daily till discharged and those who failed treatment with chloroquine were given alternative antimalarials.

Drug treatment
Each patient was treated with chloroquine according to the standard regimen in use at the Department of Child Health at the time, i.e. a course of 25 mg per kg body weight given over three days. Supportive treatment, which included blood transfusion, was given as clinically indicated.

Blood sampling and follow-up of patients
On day of enrolment, whole blood was drawn from each patient for haematological, biochemical and in vitro drug susceptibility tests. Blood was also stored for pre-treatment chloroquine level measurement. Thick and thin Giemsa stained blood films for each patient were examined daily till parasite clearance then again on day 7 and 14. The in vivo sensitivity of the parasites to chloroquine was defined in accordance with reported WHO criteria [6].

Molecular analysis
Blood blot filter paper sample labelled ‘pre-treatment’ (P) was prepared from patient’s sample on day of recruitment and a second sample denoted ‘after treatment sample’ (A) was taken upon re-occurrence of the disease. DNA was extracted from the papers using reported method and amplified by nested PCR with primers specific for genes encoding two highly polymorphic P. falciparum antigens, the merozoite surface proteins MSP1 and MSP2 [9]. The pre-treatment and after treatment samples from the same patient were run in adjacent lanes on agarose gel for ease of comparison.

In vitro test
An in vitro assessment of the susceptibilities of P. falciparum isolates was performed using a modification of the WHO micro-technique [10]. The modification involves the use of inhibition of radiolabeled hypoxanthine incorporation by parasite to demonstrate drug effect. Venous blood collected randomly from patients was processed by previously described methods [11, 12]. The test was performed in a 96-well microtitre plate pre-dosed with final concentrations of chloroquine in the range of 1–64 pmol (0.2–12.8 \( \mu \text{mol/l} \) blood). The culture was incubated at \( 37^\circ \text{C} \) and 1 \( \mu \text{Ci} \) of \(^3\text{H}\)-hypoxanthine was added to each well after 18–24 h. Incubation was continued for a further 24 h after which the cells were harvested onto a glass fibre filter paper. Incorporation of \(^3\text{H}\)-hypoxanthine by the parasites was determined with a scintillation counter. The concentrations of chloroquine achieving 50 per cent (IC50) inhibition were then determined from a regression analysis of log-dose/response curve.

Blood chloroquine level measurement
Whole blood levels of chloroquine were measured using the high performance liquid chromatography (HPLC) method described previously [13].

Statistical analysis
The data was initially entered in Microsoft Excel and re-organised into Sigmastat program. The mean,
Table 1
Characteristics of SA and NAM patients

<table>
<thead>
<tr>
<th>Patient group</th>
<th>SA</th>
<th>NAM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>160</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Day-0 parasite density</td>
<td>14894</td>
<td>22250</td>
<td>0.7513</td>
</tr>
<tr>
<td>Day-0 Hb (g/dl)</td>
<td>4.0 (0.7)</td>
<td>9.4 (1.4867)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Temp. on enrolment (°C)</td>
<td>37.8 (1.04)</td>
<td>37.7 (1.0003)</td>
<td>0.9983</td>
</tr>
<tr>
<td>Duration of fever (days)</td>
<td>2</td>
<td>2</td>
<td>0.9999</td>
</tr>
<tr>
<td>Rate of parasite clearance (days)</td>
<td>2.6</td>
<td>2.9</td>
<td>0.9306</td>
</tr>
</tbody>
</table>

1Median; 2Geometric mean; 3Mean (standard deviation); n.a., not applicable.

Table 2
In vivo treatment outcome according to malaria group (SA and NAM)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>SA</th>
<th>NAM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>160</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>138 (86.2%)</td>
<td>70 (87.5%)</td>
<td>0.9905</td>
</tr>
<tr>
<td>Resistant</td>
<td>22 (13.8%)</td>
<td>10 (12.5%)</td>
<td>0.9375</td>
</tr>
<tr>
<td>RI</td>
<td>16 (10.0%)</td>
<td>7 (8.75%)</td>
<td>0.9152</td>
</tr>
<tr>
<td>RII</td>
<td>6 (3.8%)</td>
<td>2 (2.5%)</td>
<td>0.7409</td>
</tr>
<tr>
<td>RIII</td>
<td>0 (0%)</td>
<td>1 (1.25%)</td>
<td>0.1016</td>
</tr>
</tbody>
</table>

Figures in parenthesis are percentages of total number in the patient group.

Results

Overall, 160 severe malarial anaemia and 80 non-anaemic malaria patients took part in the in vivo study. Symptom duration and body temperatures on day of presentation were similar in both groups (Table 1). Haemoglobin levels of 4.0 and 9.4 g/dl were measured for SA and NAM respectively. The geometric mean day-0 parasitaemia was 14894/μl for SA and 22250/μl for NAM. The mean parasite clearance time was estimated as 2.6 days for the SA group and 2.9 days for the NAM group. Treatment outcome of the in vivo study is shown in Table 2 and Fig. 1. Using the WHO criteria, 138 (86 per cent) of the SA patients had parasites that were sensitive to chloroquine whilst 10.0 per cent and 4 per cent showed resistance at RI and RII levels respectively. There was no RIII level of resistance observed in this group. Seventy (87.5 per cent) of the NAM patients had chloroquine sensitive parasites. Of the NAM patients with resistant parasites, seven (8.75 per cent), two (2.5 per cent) and one (1.25 per cent) had parasites that were resistant at RI, RII and RIII levels, respectively. No significant difference in parasite’s sensitivity to chloroquine was therefore observed between the two groups. Overall IC_{50} values of $0.41 \times 10^{-6}$ mol/l blood and $0.32 \times 10^{-6}$ mol/l blood were estimated for SA and NAM patients respectively (Table 4).

Geometric mean pre-treatment blood chloroquine levels were 58.7 ng/ml and 62.9 ng/ml for SA and NAM patients respectively (Table 3). The chloroquine to desethylchloroquine ratio was estimated as 3.4 for SA and 2.7 for NAM patients.

Summary of the molecular analysis of pre- and after- treatment samples taken from patients with recurring malaria is shown in Table 5. Of the 34 pairs of samples analysed for both groups, only two had new parasite (reinfection) whilst three had a mixture of both original and new parasites. Novel parasites had alleles different from that of the original parasites. A representative picture of the PCR fragments separated on 2 per cent agarose gel is shown in Fig. 2.

Discussion

Observations made in this study do not support the assertion that drug-resistant *P. falciparum* contributes importantly to the development of anaemia in malaria infection. There was no significant difference in the prevalence of resistant parasites in vivo between SA and NAM patients. In vitro drug susceptibility tests, which are devoid of the influence of the patient’s immunity, could not also establish significant differences in IC_{50} values between both categories of patients.

Proposed mechanisms of the pathogenesis of severe malarial anaemia include erythrocyte lysis and phagocytosis, increased sequestration of...
parasitized erythrocytes and their destruction by the immune system as well as reversible suppression of the bone marrow’s response to erythropoiesis [2, 3, 5]. None of these, however, adequately explain the severity and extent of the anaemia developed during the infection [14].

The erythropoietic changes reported by Kurtzhals and his colleagues, which became the basis for this study could probably be related to host’s immunological response to ‘parasite materials’ rather than the presence of resistant parasites 15, 16, 17. Individuals may thus be prone to develop anaemia to different degrees, depending on their immune status.

The similarities observed in the in vivo treatment outcome between the two groups in our study couldn’t have been influenced by previously ingested chloroquine since the pre-treatment blood levels of the drug was similar among both groups. However, the values of the Chloroquine/desethylchloroquine ratio for the SA and NAM groups revealed a quite recent uptake of the drug in the latter. Whole blood chloroquine levels were measured after the clinical

parasites resistance to chloroquine among severe malarial anaemia (SA) and non-anaemic malaria (NAM) patients.

Table 3
Pre-treatment blood concentrations of chloroquine and desethylchloroquine measured for SA and NAM using HPLC method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clinical group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA</td>
<td>NAM</td>
</tr>
<tr>
<td>Chloroquine level¹ (ng/ml)</td>
<td>58.7</td>
<td>62.9</td>
</tr>
<tr>
<td>Desethylchloroquine level¹ (ng/ml)</td>
<td>15.2</td>
<td>27.4</td>
</tr>
<tr>
<td>*CQ/DCQ ratio</td>
<td>3.4</td>
<td>2.7</td>
</tr>
</tbody>
</table>

¹Geometric Mean *CQ = chloroquine, DCQ = desethylchloroquine.

Table 4
Outcome of in vitro test of the sensitivity of P. falciparum to chloroquine among SA and NAM patients

<table>
<thead>
<tr>
<th>% Inhibition conc.</th>
<th>Treatment group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA (n = 17)</td>
<td>NAM (n = 14)</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>0.41×10⁻⁶ mol/l</td>
<td>0.32×10⁻⁶ mol/l</td>
</tr>
<tr>
<td>IC₉₀</td>
<td>5.4×10⁻⁶ mol/l</td>
<td>3.2×10⁻⁶ mol/l</td>
</tr>
</tbody>
</table>
study and, thus could not have influenced clinical decisions.

Over 95 per cent of all patients with recurring malaria in this study had genuinely recrudescent parasites. Adjustment in the data was appropriately made to reflect the outcome of the molecular analysis. Novel parasites from post-treatment sample could originate from several sources. Firstly it could be infection from new mosquito bite. Interestingly, the patients with the novel parasites were all on admission in the teaching hospital for at least 7 days. This means if the re-infection was through sporozoites inoculated by mosquitoes then the bite could have been received whilst on admission. This situation is alarming and calls for drastic measures to rid all hospitals of vectors of diseases. It is also possible that the novel parasites were derived from resistant parasites, which were sequestered at the time the initial blood sample was taken.

An important observation in this study is the presence of an appreciable level of parasites resistant to chloroquine. The recrudescent parasites, which were confirmed by molecular analysis to be genuine recrudescence of the original parasites, showed levels of resistance of 13.8 per cent for SA patients and 12.5 per cent for the NAM patients. This level of resistance compares well with results from previous studies in Ghana, which showed resistance to chloroquine of between 10–45 per cent in vivo [18, 19]. Reasons for the presence of drug-resistant *P. falciparum* among the study groups and in particularly in Ghana include the use of sub-curative doses of chloroquine for the treatment of ‘fever’. This was confirmed by the measurement of significant levels of pre-hospital treatment blood levels of chloroquine in this study and by others [20]. The indiscriminate use of the drug increases the drug tolerance level of the parasites and thus the evolution of strains with reduced susceptibility to the drug.

In view of the increasing level of parasite resistance, Ghana has recently changed its first-line antimalarial drug from chloroquine to a combination of amodiaquine and artesunate. Artemisinin-based combination therapies have been shown to improve treatment efficacy and to delay drug resistance [21–23]. The change is therefore important since prompt treatment of acute malaria is necessary in order to avoid the occurrence of anaemia.

**References**


