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Hemozoin production by *Plasmodium falciparum*: variation with strain and exposure to chloroquine

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Synchronized *Plasmodium falciparum* parasites were grown in erythrocytic culture for measurement of malaria pigment (hemozoin) production using a simple method based on the insolubility of β-hematin, the principal pigment of hemozoin. In the last 44 h of the life cycle, one strain (chloroquine-susceptible) incorporated an average of 960 pmol of ferriprotoporphyrin IX (FP) from hemoglobin into β-hematin per 10^6 parasitized erythrocytes. By comparison, another strain (chloroquine-resistant) incorporated 515 pmol of FP into β-hematin. When exposed to 25 ng of chloroquine per ml of culture medium, chloroquine-susceptible *P. falciparum* incorporated 240 pmol of FP into β-hematin per 10^6 parasitized erythrocytes in one intraerythrocytic life cycle. In contrast chloroquine-resistant *P. falciparum* exposed to 100 ng of chloroquine per ml incorporated 630 pmol of FP into β-hematin. Thus, chloroquine inhibits hemozoin production in chloroquine-susceptible *P. falciparum* but not in chloroquine-resistant *P. falciparum*. On the contrary, sublethal concentrations of chloroquine partially reverse a deficiency of hemozoin production in chloroquine-resistant *P. falciparum*. These results indicate that the adaptation responsible for chloroquine resistance in *P. falciparum* prevents the accumulation of toxic FP by preventing chloroquine from uncoupling the processes of hemoglobin degradation and hemozoin production.

Introduction

With the recent discovery of heme polymerase, an enzyme which catalyzes the polymerization of ferriprotoporphyrin IX (FP) to form β-hematin [1,2], there is a resurgence of interest in the FP hypothesis, which proposes that chloroquine acts as an antimalarial drug by interfering with malaria pigment (hemozoin) production thus causing toxic concentrations of FP to accumulate [3]. Apparently, the toxic form of FP normally is kept low because heme polymerase converts it to β-hematin.

β-Hematin is the principal pigment of hemozoin [4,5]. It is virtually insoluble in water or 2.5% aqueous SDS at physiologic pH values and below; it has the typical spectrum of aggregated FP when suspended in SDS, i.e., a broad Soret band and an absorption maximum at 645–655 nm; and it is readily soluble in dilute solutions of sodium hydroxide [4]. The insolubility at physiologic pH is due to the presence of poorly dissociable bonds involving the ferric iron of one FP molecule and the carboxyl group of a propionate sidechain of another FP molecule [5].

Chloroquine inhibits the activity of heme polymerase in vitro [1] and down-regulates it in vivo [2]. Since heme polymerase may be a key target for chloroquine and related antimalarial drugs, we consider it important to obtain quantitative information about intracellular hemozoin production and the effect of chloroquine.

Materials and Methods

Materials

The parasites studied were the chloroquine-susceptible HB-3 and the chloroquine-resistant Indochina I/CDC strains of *P. falciparum*. The initial inocula of these parasites were supplied respectively by Daniel E. Goldberg and Donald J. Krogstad, both of the Washington University School of Medicine, St. Louis, MO, USA. Human erythrocytes and serum, both blood group O, rhesus factor positive, were obtained from our institution's blood bank. RPMI 1640 powder, Hepes, and hypoxanthine were from GIBCO Labs., Grand Island, NY; glucose and sodium bicarbonate were from Fisher Scientific Company, Fair Lawn, New Jersey; and chloroquine diphosphate and gentamicin sulfate were

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from Sigma, St. Louis, MO. The medium, including supplementation with Hepes, sodium bicarbonate and serum, was prepared according to Jensen and Trager [6]. Each liter was supplemented with 2 g of glucose, 50 mg of hypoxanthine, and 19.2 mg of gentamicin sulfate (1.62 mg/ml of doubly distilled water).

Parasite culture

The malaria parasites were grown essentially as described by Jensen and Trager [6], with incubation in a gas mixture containing 3% O_2, 3% CO_2, and 94% N_2.

Synchronized culture

For quantitative measurement of hemozoin production, synchronized parasite cultures were used [7]. Five ml of 1–6 day old cultures with 3%–10% ring forms were centrifuged in 15 ml plastic tubes for 5 minutes at $530 \times g$ to obtain a pellet of 0.3–0.5 ml. This pellet was resuspended in 7 ml of a solution of 5% sorbitol dissolved in doubly distilled water (weight per volume) and incubated at room temperature for 5 min before centrifuging again at $530 \times g$ for 5 min. The erythrocyte pellets were then washed 4 times by resuspending them in 7 ml of culture medium and centrifuging at $530 \times g$ for 5 min each time. At the end of each wash any brown film on top of the red pellet was carefully pipetted off with the supernatant fluid. This procedure removed hemozoin debris from the synchronized culture. Giemsa-stained smears of these synchronized and washed cultures revealed that approx. 99% of the parasites were at the ring stage of development. In some preparations, there were a few trophozoites, usually young, some of which contained microscopically detectable hemozoin.

Quantitative measurement of hemozoin production by P. falciparum grown with and without chloroquine

After synchronization of the parasites, the erythrocytes were suspended in culture medium at a density of between 10^8 and 10^9 cells per ml. Aliquots of 7 ml of the suspension were placed in culture flasks, and seven ml of culture medium containing $2\times$ the required concentration of chloroquine were added to the flasks. For example, when a final concentration of 25 ng of chloroquine per ml was required, 7 ml of 50 ng of chloroquine (as the base) per ml medium were mixed with 7 ml of the cell suspension. For control cultures, 7 ml of medium without chloroquine were added to the cell suspension. The flasks were then sealed in a chamber which was subsequently flushed for 5 min with the gas mixture for incubation and placed at 37°C at time 0. After 24 h of incubation, the culture in each flask was agitated gently but thoroughly to suspend the cells.

After mixing, each suspension was transferred to a separate sterile test tube. From there, 2 ml aliquots from each culture were transferred into six sterile, high-speed Oak Ridge centrifuge tubes with screw caps. One aliquot was used to obtain cell counts and prepare smears for determination of parasitemia and two of the aliquots were processed for β-hematin as described below. To continue the culture, the three remaining aliquots from each culture were centrifuged at $530 \times g$ for 5 min, and 1.8 ml of the supernatant were pipetted off and replaced with 2 ml of fresh medium, which contained the appropriate final concentration of chloroquine. For example, spent medium from a culture containing 25 ng of chloroquine was replaced with fresh medium containing 25 ng of chloroquine per ml. These tubes were gently agitated to resuspend the cells, placed back in the chamber, gassed, and incubated for an additional 20 h to bring the total growth period to 44 h.

Measurement of β-hematin

Two ml of parasite cultures were centrifuged at 4°C for 30 min at $27000 \times g$. The supernatant fluid was discarded and the cells were hemolyzed by adding 10 ml of cold 5 mM sodium phosphate solution (pH 7.6) to the tubes and shaking them vigorously by hand. The tubes were then kept on ice for 10 min before being centrifuged again for 30 min at $27000 \times g$. The supernatant fluid was discarded, and the pellet containing the hemozoin was washed once by resuspending it in 10 ml of 20 mM Tris-buffered solution, pH 7.2, and centrifuging at 4°C for 30 min at $27000 \times g$. The washed pellet was suspended in 2 ml of 2.5% SDS buffered with 25 mM Tris to pH 7.8 and left overnight (16 h) at room temperature. At this point it was possible to obtain the typical absorption spectrum of hemozoin (Fig. 1, left panel, spectrum a).

To measure the amount of FP in the β-hematin of hemozoin, the insoluble material was pelleted from the SDS suspension by centrifugation at $27000 \times g$ for 60 min. The supernatant fluid was replaced with 1.8 ml of fresh SDS/Tris-buffered solution, and 0.2 ml of 1 N sodium hydroxide was added to achieve a final concentration of 0.1 N. Next, the tube was sealed, vigorously vortexed, and shaken by hand. After allowing 2 h at room temperature for hydrolysis of the β-hematin, FP was measured spectrophotometrically [8]. Using a similar method but a lower concentration of sodium hydroxide (0.02 N) for hydrolysis, Chou and Fitch [2] accounted for 99% of the FP in parasitized mouse erythrocytes.

Calculation of hemozoin content of parasitized erythrocytes

After quantitative measurement of FP in the β-hematin of hemozoin, the amount per 10^6 parasitized erythrocytes was calculated based on the number of erythrocytes in the culture and the% parasitemia obtained after growing the synchronized culture for 24 h.

Although the parasitemia at 0 hour was determined, it was not used for calculation of hemozoin production because preliminary experiments showed that not all the ring forms continued to grow after synchronization, particularly when chloroquine was added. The parasitemia after 44 h growth also was not used for calculation because most of the surviving parasites had already completed multiplication, and their offspring had reinvaded other erythrocytes in the culture but had not contributed to hemozoin production. In these studies β-hematin was used as a surrogate for hemozoin since it is the principal if not the only pigment in hemozoin [4,5].

Results

In preliminary experiments we found that hemolysis of normal erythrocytes in cold 5 mM sodium phosphate solution at a ratio of about 1:20 followed by a wash with Tris-buffered solution consistently produced membrane preparations with minimal hemoglobin contamination. When the membranes were suspended in 2.5% SDS overnight and centrifuged at a high speed, little or no pellet was obtained, indicating that the membranes had been solubilized. In contrast, when parasitized erythrocytes were hemolyzed and treated similarly, a dark pellet was obtained after centrifugation. This pellet contained all of the β-hematin of hemozoin.

The absorption spectrum of a suspension of a crude hemozoin preparation from *P. falciparum* is shown in Fig. 1 (spectrum a, left panel). It is essentially identical to that reported for hemozoin of *P. berghei* [4]. It has a band with an absorption maximum at 650–665 nm which is characteristic of β-hematin [4] and which does not change with purification (spectrum b, left panel). It also has a broad Soret band with a superimposed absorption maximum at 400 nm (spectrum a, left panel). The FP responsible for the superimposed peak at 400 nm was soluble in SDS and did not sediment with centrifugation (spectrum c). As expected [4], the typical hemozoin spectrum was lost when the pH of the hemozoin suspensions were raised with sodium hydroxide (Fig. 1, right panel), and it was replaced by an absorption spectrum characteristic of hematin in SDS which has a narrow Soret band at 400 nm and an absorption maximum at 600 nm. The difference between the absorbance of samples a and b (Fig. 1) represents non-hemozoin FP, including hemichrome and hemoglobin which may have been bound to the cell membranes.

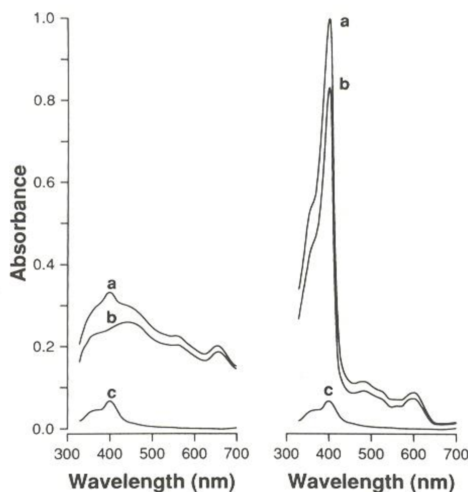

Fig. 1. Spectral characteristics of hemozoin of *P. falciparum*. Left panel: Spectra a and b are, respectively, suspensions of crude and purified hemozoin in 2.5% SDS buffered with 25 mM Tris to pH 7.8. Spectrum c is from the supernatant obtained after centrifuging the crude hemozoin suspension at 27000× *g* for 60 min. Right panel: To hydrolyse the hemozoin to FP, sodium hydroxide was added at a final concentration of 0.1 N to samples a, b, and c. The hemozoin used here was from chloroquine-resistant *P. falciparum*. Hemozoin preparations from chloroquine-susceptible parasites gave similar results.

Spectrum c of Fig. 1 (both panels) is from the supernatant fluid obtained after centrifuging the crude suspension of hemozoin. Spectrum c did not change when sodium hydroxide was added to the solution, indicating that it was nonpolymerized FP. All of the β-hematin had been removed by centrifugation.

Quantity of hemozoin produced by intraerythrocytic P. falciparum in one intraerythrocytic life cycle.

The asexual form of *P. falciparum* has a 48-h life cycle in erythrocytes. It begins with invasion of an erythrocyte by a merozoite and ends with the release of new merozoites from the infected erythrocyte. During the 48 h of growth and multiplication in the erythrocyte, the parasite develops through a ring stage during the first 20–24 h, a trophozoite stage during the next 12–18 h, and a schizont stage during the remaining period [9]. Erythrocytes containing trophozoites or schizonts are susceptible to hemolysis by sorbitol, whereas erythrocytes containing ring forms are not [7]. In experiments not shown here, we found erythrocytes containing ring forms to resist lysis by sorbitol during the first 14–24 h of an infection.

Sorbitol-resistant ring forms of *P. falciparum* contained only small amounts of hemozoin; the mean values in pmol of FP/10^6 parasitized erythrocytes were 50 for chloroquine-susceptible and 27 for -resistant parasites. Some of the hemozoin in these preparations could be attributed to contamination by trophozoites.

During development of ring forms to trophozoites (24 h) and schizonts (44 h), chloroquine-resistant parasites produced only about half as much β-hematin as chloroquine-susceptible parasites ($P < 0.0005$ at both trophozoite and schizont stages, Student's *t*-test). The chloroquine-susceptible strain of *P. falciparum* incorporated an average of 750 ± 140 (mean ± S.D.) pmol of hemoglobin FP into the β-hematin of hemozoin per

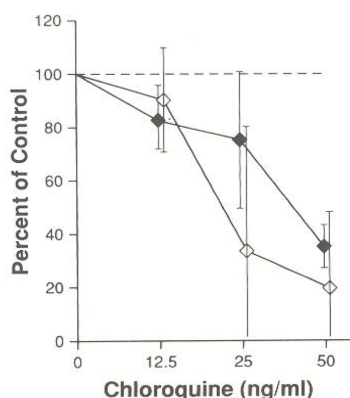

Fig. 2. Chloroquine-susceptible *P. falciparum*: survival and production of hemozoin with and without chloroquine. The parasites were synchronized by sorbitol lysis at the ring stage before culturing at 37°C for 24 h with and without chloroquine. Open symbols represent β-hematin; closed symbols represent parasitemia. The means $\pm$ SD for three experiments are shown. The control parasitemias ranged between 2% and 3%. The control β-hematin concentrations ranged between 660 and 960 pmol FP per 10^6 parasitized erythrocytes.

10^6 parasitized erythrocytes, whereas the chloroquine-resistant strain incorporated 350 ± 140 pmol of FP into β-hematin per 10^6 parasitized erythrocytes during the first 24 h after synchronization. When the parasites were permitted to complete their intraerythrocytic development and multiplication by culturing the synchronized ring forms for a total of 44 h, chloroquine-susceptible *P. falciparum* incorporated an average of 960 $\pm$ 118 pmol of FP into β-hematin per 10^6 parasitized erythrocytes, whereas the chloroquine-resistant strain incorporated 515 ± 150 pmol of FP into β-hematin per 10^6 parasitized erythrocytes.

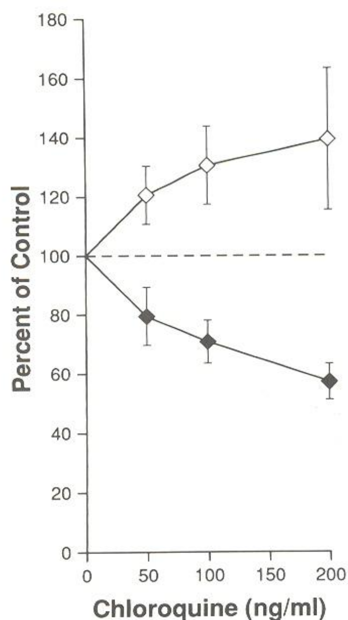

Fig. 3. Chloroquine-resistant *P. falciparum*: survival and production of hemozoin with and without chloroquine. The parasites were synchronized by sorbitol lysis at the ring stage before culturing at 37°C for 24 h with and without chloroquine. The means $\pm$ SD for three experiments (except with 50 ng of chloroquine per ml there were 4 experiments) are shown. All the concentrations of chloroquine were not tested at the same time, but each experiment had its own control culture, and calculations were based on that culture. Open symbols represent β-hematin; closed symbols represent parasitemia. The control parasitemias ranged between 3% and 8%. The control β-hematin concentrations ranged between 160 and 520 pmol FP per 10^6 parasitized erythrocytes.

Effect of chloroquine on hemozoin production

At 12.5 ng of chloroquine per ml, the lowest concentration tested, chloroquine had little or no effect on parasitemia or hemozoin production in cultures of chloroquine-susceptible *P. falciparum* (Fig. 2). At 25 ng of chloroquine per ml, there was considerable variability in parasite counts and the reduction in parasitemia was not statistically significant, but there was a dramatic reduction in hemozoin production ($P < 0.02$ by the 2-tailed paired t-test).

As expected, higher concentrations of chloroquine were required to noticeably affect chloroquine-resistant *P. falciparum* (Fig. 3). For example, 100 ng of chloroquine per ml of culture medium were required to reduce parasitemia ($P < 0.025$ by the 2-tailed paired *t*-test). Surprisingly, at this concentration of chloroquine, hemozoin production by surviving parasites increased ($P < 0.01$ by the 2-tailed paired *t*-test).

Discussion

The simple method we have used permits accurate and reproducible measurement of the quantity of hemozoin produced at any stage of development of intraerythrocytic malaria parasites. This method opens the door to quantitative study of intracellular production of hemozoin and the effects of drugs. In the absence of drugs, we found only small amounts of hemozoin in ring forms which were resistant to sorbitol lysis. Nevertheless, the quantity of hemozoin in chloroquine-susceptible parasites was reproducibly higher than in chloroquine-resistant parasites. After incubation of synchronized ring forms for 24 or 44 h, during which time the parasites developed into trophozoites and schizonts, this difference was magnified. In this regard the present strain of chloroquine-resistant *P. falciparum* is similar to chloroquine-resistant *P. berghei* [10], i.e., hemozoin production is deficient in both.

Because the FP molecules released by the parasite from hemoglobin are polymerized into β-hematin and stored in hemozoin, quantitation of β-hematin permits a reasonable estimate of intracellular hemoglobin degradation. Our measurements indicate that in one intraerythrocytic life cycle chloroquine-susceptible *P. falciparum* releases 50–55% of the approx. 1800 pmol of hemoglobin FP available in 10^6 parasitized human erythrocytes, whereas the chloroquine-resistant strain releases 25–30%. The latter result agrees with Roth et

al. [11], who found that the chloroquine-resistant [12] FCR-3 strain of *P. falciparum* degrades 25% of the host-cell hemoglobin. In bird and monkey malarias, hemoglobin degradation has been estimated to be 25–30% and 80% respectively [13,14].

We may now ask how hemoglobin degradation might make malaria parasites susceptible to chloroquine. In the absence of any evidence of a primary effect of chloroquine on hemoglobin digestion, it is reasonable to suppose that the primary effect is to inhibit or down-regulate heme polymerase. Reducing the activity of heme polymerase would, in effect, uncouple the process of FP production (hemoglobin degradation) from the process of hemozoin production. Consequently, toxic, nonhemozoin FP would accumulate after chloroquine exposure, as has been demonstrated for *P. berghei* in vitro [2]. Then, as previously suggested [1,15], accumulation of this toxic form of FP would inhibit hemoglobin digestion in intact parasitized erythrocytes, possibly by inhibiting proteases, as it does in vitro [15]. Inhibition of hemoglobin digestion would, in turn, cause the previously described accumulation of hemoglobin in *P. falciparum* parasites exposed to chloroquine [16,17]. This hypothetical explanation of the mode of action of chloroquine is consistent with the FP hypothesis [3] and all currently available information.

To explain chloroquine resistance in *P. falciparum*, attention may be focused on the apparent failure of chloroquine to uncouple hemoglobin degradation from hemozoin production. Indeed the observed increase in hemozoin production in response to sublethal concentrations of chloroquine means that hemoglobin degradation also was increased. We conclude, therefore, that hemoglobin degradation in chloroquine-resistant *P. falciparum* remains tightly coupled to hemozoin production despite exposure to chloroquine. Otherwise, toxic amounts of FP would have accumulated, and the parasites would not have been resistant to chloroquine. Tight coupling could persist despite chloroquine exposure (a) if chloroquine accumulation by the parasite is insufficient to inhibit or down-regulate heme polymerase, (b) if the amount of heme polymerase is so large that it would still have ample capacity to make β-hematin even after maximal inhibition or down-regulation, or (c) if chloroquine is ineffective against the heme polymerase of chloroquine-resistant *P. falciparum*. The first possibility, insufficient accumulation of chloroquine, currently has experimental support [18,19]. Further investigation of the other two possibilities is needed.

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