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Inhibition of Glutathione-dependent Degradation of Heme By Chloroquine and Amodiaquine as a Possible Basis for Their Antimalarial Mode of Action

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ABSTRACT. We propose here a new and detailed model for the antimalarial action of chloroquine (CQ), based on the its ability to inhibit degradation of heme by glutathione. Heme, which is toxic to the malaria parasite, is formed when the intraerythrocytic malaria parasite ingests and digests inside its food vacuole its host cell cytosol, which consists mainly of hemoglobin. The parasite protects itself against the toxicity of heme by polymerizing some of it to insoluble hemozoin (HZ). We show here that in *Plasmodium falciparum* at the trophozoite stage only *ca.* 30% of the heme is converted into hemozoin. We suggest that nonpolymerized heme exits the food vacuole and is subsequently degraded by glutathione, as has been shown before for uninfected erythrocytes. Marginal amounts of free heme could be detected in the membrane fraction of infected cells but nowhere else. It is well established that CQ and amodiaquine (AQ) accumulate in the parasite's food vacuole and inhibit heme polymerization, thereby increasing its efflux out of the food vacuole. We found that these drugs competitively inhibit the degradation of heme by glutathione, thus allowing heme to accumulate in membranes. Incubation of intact infected cells with CQ and AQ results in a marked increase in membrane-associated heme in a dose- and time-dependent manner, and a relationship exists between membrane heme levels and the extent of parasite killing. Heme has been shown to disrupt the barrier properties of membranes and to upset ion homeostasis in CQ-treated malaria-infected cells. In agreement with the predictions of our model, increasing the cellular levels of glutathione leads to increased resistance to CQ, whereas decreasing them results in enhanced sensitivity to the drug. These results insinuate a novel mechanism of drug resistance. BIOCHEM PHARMACOL **56**;10:1305–1313, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *Plasmodium falciparum*; malaria; mode of action; chloroquine; amodiaquine; glutathione

Malaria-infected RBC† are distinguished from normal cells by their high levels of heme: the parasite ingests the cytosol of its host cell which is essentially composed of hemoglobin, and digests it in its acidic food vacuole [1, 2]. Whereas parasite proteases hydrolyze globin to its building blocks [3–6], freed heme must be detoxified because it is lethal to the parasite [7–9]. Free heme has at least three potential fates in the parasitized RBC: 1) it can be sequestered into the insoluble heme polymer HZ [10, 11]; 2) The heme that escapes polymerization can dissolve into, and translocate across, membranes [12–15], reaching the cytosol of the parasite or that of the host cell, where mechanisms exist for its detoxification; or 3) it is possible that some heme exits the parasitized RBC altogether and binds to serum albumin or to hemopexin [16].

Aminoquinoline-containing antimalarials, which are assumed to accumulate in the parasite's food vacuole [17, 18], are able to complex with heme and prevent its polymerization. This has been shown to occur *in vitro*, either in solution [19–21] or in presence of parasite extract, at the acid pH that prevails inside the food vacuole [22–25], as well as in intact infected cells [26, 27]. Heme:drug complex formation does not, however, prevent heme dissolution in phospholipid membranes [14, 15] and its consequent translocation across them. Hence, the toxic effect of heme can be exerted outside of the food vacuole. In the present report we attempt to disclose the molecular details of heme intoxication to resolve the apparent contradiction between that ability of the parasite-infected cell to handle nonpolymerized heme and the involvement of heme in the antimalarial action of CQ and AQ.

MATERIALS AND METHODS

Materials

GSH, RPMI-1640, and AQ dihydrochloride, CDNB, N-acetyl-L-cysteine and BSO were purchased from Sigma Chemical Co. CQ diphosphate was obtained from Serva,

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† *Abbreviations*: AQ, amodiaquine; BSO, L-buthionine-[s,r]-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; CQ, chloroquine; HZ, hemozoin; and RBC, red blood cells.

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and hemin from Porphyrin Products, Logan, UT. [^{3}H]hypoxanthine (43 Ci/mmol) was procured from Amersham. All other chemicals were of the best available grade.

DEGRADATION OF HEME IN SOLUTION, OR WHEN DISSOLVED IN MEMBRANES BY GSH, AND ITS INHIBITION BY DRUGS. Heme and GSH stock solutions were prepared fresh prior to experiment and maintained on ice in the dark until used. Heme (3 μM) and GSH (2 mM) were made in 0.2 M HEPES (pH 7) and incubated at 37°. Heme degradation was observed by measuring the time-dependent decline in absorbance at 400 nm. Drugs were added from stock solutions at 6 μM.

White ghosts were prepared from normal, washed red blood cells by hypotonic lysis in an ice-cold solution of 5 mM Na-phosphate, pH 8 (5P8), and extensive washing in the same buffer. Ghosts (10^8/mL) were incubated with 10 μM heme in 0.2 M HEPES, pH 7, for 7 min at 37°. Membranes were centrifuged (15,000 rpm, 20 min at 4°) and washed once in the same buffer. Heme-loaded ghosts were incubated at 37° in the same buffer ± 2 mM GSH, ± 6 μM of each drug. At different times intervals, aliquots of 0.8 mL were withdrawn, membranes were pelleted at 14,000 rpm for 6 min, and dissolved in 0.8 mL of SDS 1% (w/v) and the absorbance of heme was determined spectrophotometrically at 400 nm.

BALANCE SHEET OF HEME DISPOSAL IN INFECTED ERYTHROCYTES. The FCR3 strain of *Plasmodium falciparum* was synchronized and cultivated to the trophozoite stage [28]. Infected cells were separated from uninfected cells as follows: cultures were centrifuged (10,000 rpm, 20 min at room temperature) through two layers of Percoll-wash medium supplemented with 6% (w/v) alanine. The first layer was 60% Percoll (into which all infected cells migrate; ≥90% parasitemia); and the second, 90% Percoll (which contains uninfected cells). Both types of cells were used for the determination of hemoglobin, HZ, and membrane-associated heme, as follows: 1) cells of each layer were suspended in 20 mL of wash medium supplemented with 6% (w/v) alanine; 2) cells were counted in hemacytometer; and 3) the parasitemia was assessed by microscopic inspection of Giemsa-stained, thin blood smears. Cells were spun down and the cell pellet was lysed in ice-cold 5 mM phosphate buffer, pH 8 (5P8), followed by three cycles of freezing in liquid nitrogen and thawing at room temperature. After centrifugation at 14,000 rpm for 5 min, all subsequent steps were done at 4°. Twenty microliters of the lysate were added to 980 μL of Drabkin reagent, the absorbance was read at 540 nm, and the hemoglobin content in μmol/10^{10} cells was calculated from a calibration curve constructed with known amounts of methemoglobin. The pellet was washed three times with 5P8 at 14,000 rpm for 5 min, and dissolved in 1 mL of 2.5% (w/v) SDS. The absorbance of the supernatant (membrane-associated heme) was read at 345 nm (at this wavelength there is no contribution from the traces of hemoglobin that could remain bound to the membrane), and the amount of heme in μmol/10^{10} cells was calculated from a calibration curve constructed with freshly prepared heme dissolved in SDS. The remaining pellet (HZ) was dissolved with 100 μL of 0.2 M NaOH and 900 μL of 2.5% SDS, the absorbance at 400 nm was determined, and the amount of HZ/10^{10} cells was calculated from a calibration curve of heme constructed in the same solvents. Percentage of heme converted to HZ was calculated by dividing the HZ content by the difference between hemoglobin in uninfected cells and hemoglobin in infected cells × 100.

EFFECT OF DRUG TREATMENT ON HZ PRODUCTION AND LEVELS OF MEMBRANE-ASSOCIATED HEME. Synchronized cultures were seeded at the ring stage and allowed to grow for another 20 hr until most parasites reached the trophozoite stage; parasitemia and cell number were determined, and cultures were cultured ± drug for periods of time that are indicated in the Results and Discussion section. One milliliter of culture was washed twice in wash medium (culture medium without plasma, 37°) to remove the drug, and cells were seeded in 24-well culture plates in full culture medium supplemented with 5 μCi/mL of [^{3}H]hypoxanthine. After 4 hr of further cultivation, triplicate samples were transferred into 96-well plates, and parasite-associated radioactivity was determined using the Filtermate/Matrix 96 Direct Beta counter. Inhibition of parasite growth was calculated compared to untreated controls. The remaining cultures were used for the determination of HZ and membrane-associated heme as described above. The same parameters were also determined in infected cells prior to incubation.

MODULATION OF PARASITE SENSITIVITY TO CQ BY AGENTS THAT AFFECT INTRACELLULAR GSH CONCENTRATIONS. Synchronous parasite cultures at the ring stage were grown in 24-well plates in the presence of increasing concentrations of cysteine, N-acetyl cysteine, BSO, and CDNB to alter the cellular level of GSH, respectively. After 24 hr in culture, [^{3}H]hypoxanthine (5 μCi/mL), was added to each well; 18 hr later, triplicate samples were transferred into 96-well plates and parasite-associated radioactivity was determined using the Filtermate/Matrix 96 Direct Beta counter. The IC_{50} was calculated from the extent of inhibition of radioactivity incorporation compared to untreated controls [29]. The effect of CQ was similarly determined in the presence of modifying agents at the following concentrations (equal to the respective IC_{50}): cysteine 15 mM, N-acetyl-cysteine 10 mM, chlorodinitrobenzene 4 μM, BSO 3.5 mM, or in absence of GSH-modifying compounds. Results are shown as mean ± SE of two to six experiments as fold-change in IC_{50}. The effect of GSH-modifying agents on the GSH content of infected cells was tested on gelatin-enriched trophozoite-infected cells, after removal of gelatin and allowing the cultures to recover in culture conditions for 1 hr (cells were counted in hemacytometer and parasitemia was determined by microscopic inspection of Giemsa-

TABLE 1. Balance sheet of heme disposal in infected erythrocytes

System	Hemoglobin	Hemozoin	Membrane heme	Hemoglobin digested	% conversion of heme to hemozoin
Experiment No. 1					
Uninfected RBC	17.9	—		—	—
Trophozoite	12.9	1.09	0.17	5.0	22
Experiment No. 2					
Uninfected RBC	18.7	—	—	—	—
Trophozoite	14.9	1.25	0.11	3.8	33

The levels of hemoglobin, hemozoin, and membrane-associated heme have been determined in normal and trophozoite-infected RBC as described in Materials and Methods. Results are expressed as μmols/10^{10} cells for hemoglobin or heme (in hemozoin or membrane-associated). Percentage of heme converted to hemozoin was calculated by dividing the hemozoin content by the difference between hemoglobin in uninfected cells and hemoglobin in infected cells × 100. Results of two independent experiments are depicted.

stained thin blood smears). Uninfected and trophozoite-infected cells were incubated with modifying agents (at the following concentrations: cysteine 15 mM, N-acetyl-Cysteine 10 mM, chlorodinitrobenzene 50 μM, BSO 6 mM) for 2 hr under culture conditions. Cells were then centrifuged (600 *g* for 5 min) and the pellet was resuspended in 5% (w/v) trichloroacetic acid supplemented with 5.3 mM EDTA, and the GSH content was determined in the supernatant according to Beutler [30].

RESULTS AND DISCUSSION

Detoxification of Heme

It is generally assumed that heme polymerization is the mechanism that protects the parasite from this noxious compound that is produced during the digestion of host cell hemoglobin; but to the best of our knowledge, the stoichiometry or the efficacy of this process has not been tested before in *P. falciparum*. This question seems relevant because in *P. berghei*-infected mouse RBC, it was found that only 50% of the heme is converted into HZ [31]. In the present research. we have investigated the fate of heme in *P. falciparum* grown in culture. The hemoglobin heme in noninfected RBC (isolated from the same culture) was estimated and defined as total available heme; hemoglobin heme in the trophozoite-infected RBC was measured to estimate (by difference) the amount of heme ingested and digested. The amount of HZ was determined directly. From these values we found that the polymerization of heme is rather inefficient as it amounts to only some 20–30% (Table 1, column 5). One percent of heme was present in membrane-bound form. No free heme could be detected either inside or outside the infected cell, which is consistent with the full viability of the parasites.

Clearly then, parasites are protected from this potentially noxious compound. What is the mechanism of this protection? A possible candidate could be heme oxygenase. Although heme oxygenase activity has been reported in some species of malaria parasites, such as *Plasmodium berghei* and *P. knowlesi* [32], none could be detected in the most lethal human parasite, *P. falciparum* [11, 33, and our own unpublished observations]. On the other hand, heme can be decomposed by glutathione, as we have recently demonstrated [34]. We have shown that GSH can degrade heme, whether it is free in solution, when it is bound nonspecifically to protein (defatted BSA), when dissolved in erythrocyte membranes, or loaded into intact erythrocytes [34]. It has also been shown in this report that the iron released from degraded heme increased oxidative stress, implying that iron is engaged in the production of oxidative radicals. Heme has been shown before to lyse erythrocytes and, although this phenomenon could not be explained at the time, in agreement with the degrading effect of GSH, this lysis was inhibited by sulfhydryl-containing compounds [7]. In the context of parasite physiology, it is worth mentioning that GSH-dependent heme degradation can also: 1) provide iron to the parasite and concurs with the high levels of iron found in infected cells [35], although alternative or additional mechanisms may exist [36]; and 2) account in part for the high level of the production of reactive oxidative species in infected cells [37] and the consequent high activity of the hexose monophosphate shunt measured in these cells [38], as the iron released from degraded heme could engage in redox cycling thus producing O_2^- which via superoxide dismutase converts to H_2O_2. GSSG is produced both during the degradation of heme and during the reduction of H_2O_2 by glutathione peroxidase. GSSG is reduced back to GSH by glutathione reductase, which uses NADPH as a co-factor. The resulting NADP is recycled through the action of the shunt.

Effects of Antimalarial Drugs on Heme Detoxification

Obviously then, heme will become toxic to the parasite if its polymerization is stopped or its decomposition by GSH is inhibited. As mentioned in the Introduction, aminoquinoline-containing antimalarials accumulate in the parasite's food vacuole and are able to complex with heme at its site of formation and prevent its polymerization. Heme: drug complex formation should not prevent the exit of heme from the food vacuole. The efflux of this additional nonpolymerized heme would not, in itself, explain the toxic effect of the aminoquinolines as it would be decomposed by GSH. There would be enough GSH present to degrade it, as there is no limitation in the recycling or *de novo* synthesis of GSH [39]. The aminoquinolines must also be involved in

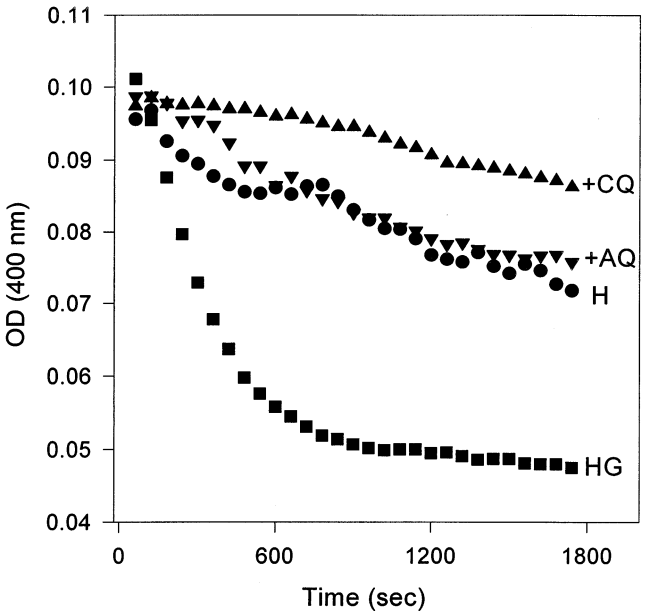

FIG. 1. Heme in solution Degraded by GSH and its inhibition by drugs. The degradation of heme by GSH ± drugs was determined as described in Materials and Methods. Heme alone (H), heme + GSH (HG), heme + GSH + chloroquine (CQ), and heme + GSH + amodiaquine (AQ).

inhibiting the degradation of the 70–80% of heme that escapes polymerization into HZ.

What is the evidence that the aminoquinolines can indeed interfere with GSH-dependent heme degradation? The results shown in Fig. 1 (filled squares) confirm our previous observations that, in the presence of GSH, heme in solution (as well as in the presence of defatted BSA at 1:10 protein:heme molar ratio, data not shown) is rapidly degraded, as can be seen from the decline in the absorbance of heme [34]. Absorbance does not decline to zero, as the degradation product also absorbs, to some extent, at 400 nm. We now show that low concentrations of CQ (filled triangles) or AQ (filled inverted triangles) totally inhibit GSH-dependent degradation of heme, presumably by complexing with it.

Heme is an amphipathic compound and as such, has a tendency to dissolve in cell membranes. However, membrane-associated heme is not protected from degradation by GSH. In the presence of GSH, the decline of membrane-associated heme is again faster than that of free heme (Fig. 2). The rate of degradation of membrane-associated heme in the presence of GSH is considerably slower than that of heme in solution, probably due to the limited access of the polar GSH to membrane-associated heme. However, CQ and AQ are able totally to inhibit GSH-dependent degradation of this heme. We have observed that mefloquine also inhibits the GSH-dependent degradation of heme in solution, but not when heme is dissolved in the membrane (manuscript in preparation). This would suggest that the degradation of heme does not occur following its dissociation from the membrane into the aqueous medium.

Following on these findings in solution and in ghost membranes, we have investigated directly the effect of drugs on membrane-associated heme in drug-treated malaria-infected RBC. In preliminary experiments, either uninfected or infected cells were incubated with increasing concentrations of heme, and their membranes were assayed for the presence of heme. In both cases there was a good correlation between the concentration of heme in the incubation medium and the amounts of membrane-associated heme (data not shown). RBC infected with mid-term trophozoites and uninfected RBC from the same culture were separated from control and drug-treated cultures by Percoll gradient centrifugation. HZ and membrane-associated heme were determined for both cell fractions, and the cytotoxic effect of the drugs was evaluated. Results are shown in Table 2. Comparing the levels of HZ at the beginning of the experiment (t = 0) and at the end of 4 hr of incubation (in the absence of drug), shows that HZ production continued unabated in the absence of the drug. However, drug treatment for 4 hr resulted in decreased HZ content compared with untreated controls, and more than a doubling in the levels of membrane-associated heme. The same treatment irreversibly inhibited parasite growth to a very large extent, as previously reported [40]. Inhibition of parasite growth with sulfathiazole, a non-aminoquinoline drug that acts by inhibiting de novo pyrimidine synthesis, did not affect membrane-associated heme. While these results were obtained at supra-pharmacological drug concentrations, the results depicted in Fig. 3 indicate that the effect is dose-dependent. One sees that, unless the level of membrane-associated heme exceeds a critical level above

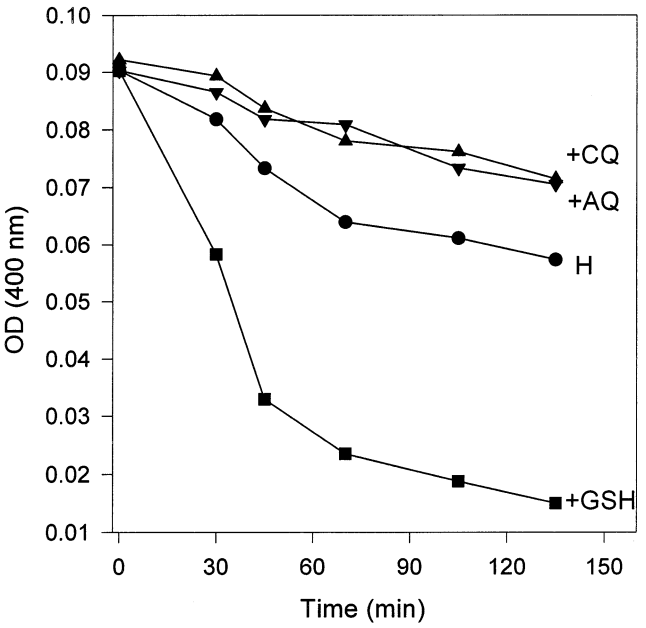

FIG. 2. Membrane-associated heme degraded by GSH and its inhibition by drug. The degradation of membrane-associated heme by GSH ± drugs was determined as described in Materials and Methods. H, heme alone; +GSH, heme + GSH; CQ, heme + GSH + chloroquine; AQ, heme + GSH + amodiaquine

TABLE 2. Effect of drug treatment on hemozoin production and levels of membrane-associated heme

System	Hemozoin	Membrane heme	Total heme	% growth inhibition
Experiment No. 1				
Trophozoite t = 0	1.09	0.17	14.16	—
no drug	1.93	0.13	13.76	—
+ CQ	0.94	0.33	13.54	66.4
+ AQ	1.16	0.43	15.90	68.5
Experiment No. 2				
Trophozoite t = 0	1.25	0.11	16.25	—
no drug	2.09	0.20	15.04	—
+ CQ	1.52	0.39	17.68	82.3
+ AQ	1.49	0.41	18.31	74.8
+ ST	2.47	0.16	18.71	81.7

Hemozoin and membrane-associated heme were determined before (Trophozoite t = 0) and after cultivation ± 10 μM chloroquine (CQ), amodiaquine (AQ), or sulfathiazole (ST) for 4 hr. The viability of the parasites was determined by their ability to incorporate [^{3}H]hypoxanthine after the drugs have been removed. Results of two independent experiments are depicted. Data (given in μmols heme/10^{10} cells) indicate that, in absence of drug, hemozoin continued to be produced; while in presence of drug, it was almost totally inhibited. In the presence of drug there was more than doubling of membrane-associated heme levels.

the equable concentration of untreated infected cells, this accumulation does not result in parasite killing. At CQ concentrations of ≥0.1 μM, i.e. well within the therapeutic range of this drug, membrane-associated heme reaches critical levels as it consorts with parasite killing. Similar results were obtained with AQ (data not shown). In Fig. 4, we show the effects of CQ and AQ on the time-dependence of heme accumulation in the membranes of infected cells and parasite killing. Here again one sees a strong correlation between these tow observable parameters and that AQ is substantially more effective than CQ at short times of incubation. Results depicted in Fig. 5 indicate a hyperbolic relationship between membrane-associated heme and parasite killing, implying a causative effect.

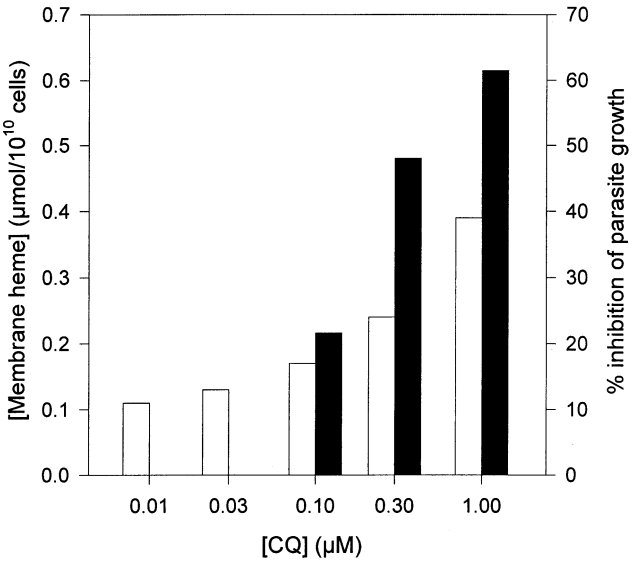

FIG. 3. Dose-dependent effect of chloroquine on membrane-associated heme and parasite killing. Infected cells were incubated for 4 hr with increasing concentrations of chloroquine and subsequently analyzed for membrane-associated heme (empty columns; left ordinate) and for parasite killing (gray columns; right ordinate).

The Cytotoxic Effect of Heme

How, then, does heme kill the parasite? That CQ and AQ both inhibit HZ production and increase membrane-associated heme has been reported in the *P. berghei* mouse model [23, 26]. High levels of heme have been shown to destabilize the barrier properties of phospholipid membranes, increasing their permeability to cations [41]. Consistent with this, it has been reported that heme and heme:CQ complexes induce potassium leaks in normal erythrocytes [7]. Although the experimental methods used in this investigation could not inform whether heme accumulates in parasite membranes or in that of the host or in both, it has been reported that [K^+] decreases and [Na^+] increases in the parasite compartment of CQ-treated trophozoite-infected RBC [42], suggesting that the permeabilizing effect of heme has been exerted on the parasite membrane. We could expect, therefore, that a depletion of parasite potassium concentration would inhibit glycolysis (the activity of one of the key glycolytic enzymes, pyruvate kinase, strongly depends on [K^+]) and the synthesis of DNA, as has indeed been observed [43]. Even if the drug is removed after it has caused these effects, the parasite will not be able to restore the necessary cellular [K^+] as it can not produce the ATP needed to fuel the cation pumps. This mechanism explains the cytotoxicity (the irreversible effect) of CQ and AQ [40].

The Sensitivity to CQ Is Modulated by Cellular GSH

Our suggestion that GSH is involved in the mechanism of action of CQ and AQ, implies that modulation of the intracellular levels of GSH [44] should affect drug sensitivity. In conformity with this prediction, we found that incubation of infected cells with L-cysteine and N-acetylcysteine increased intracellular GSH levels (due to increased *de novo* synthesis), whereas incubation with CDNB (which conjugates GSH) and BSO (which inhibits γ-Glu-Cys synthetase) decreased them (Fig. 6). The parasites

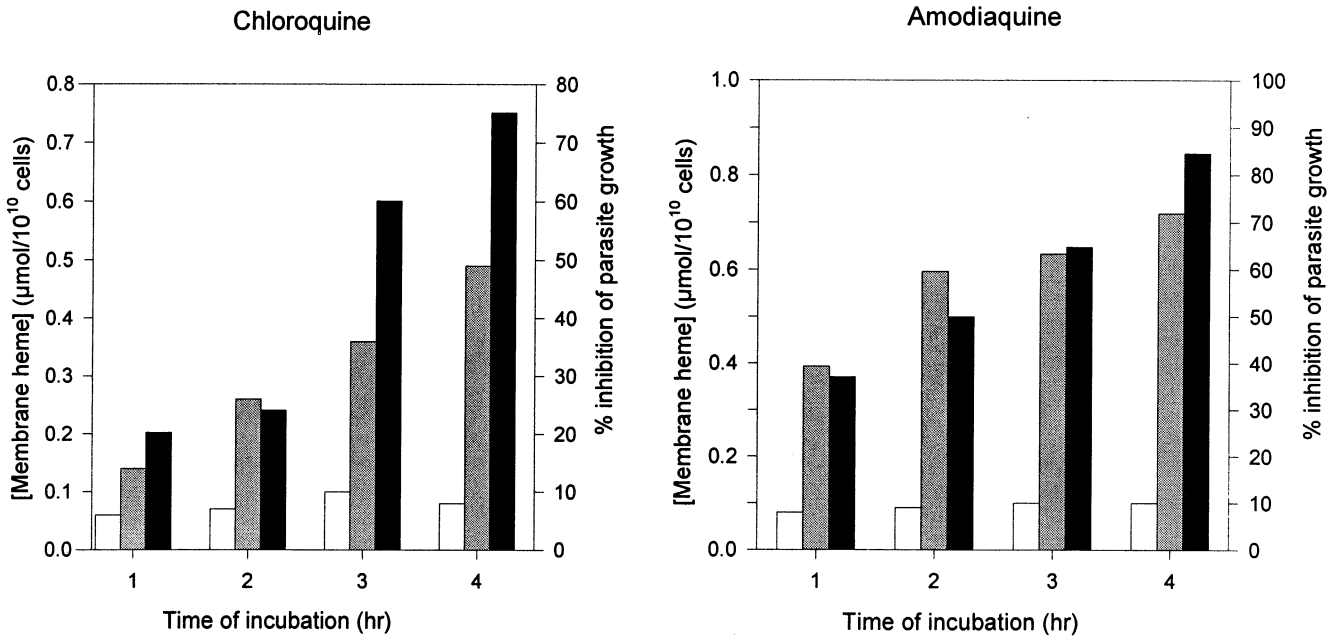

FIG. 4. Time-dependence of heme accumulation in the membrane fraction of infected cells and parasite killing as affected by chloroquine and amodiaquine. Infected cells were incubated ±1 μM chloroquine. Aliquots were taken at different time intervals and analyzed for membrane-associated heme (gray columns, left ordinate) and for inhibition of parasite growth (black columns, right ordinate). The levels of membrane associated heme in control cells are depicted by the white columns, left ordinate).

became more resistant to CQ when L-cysteine or N-acetylcysteine was present in the incubation medium and more sensitive when CDNB or BSO was present instead. In fact,

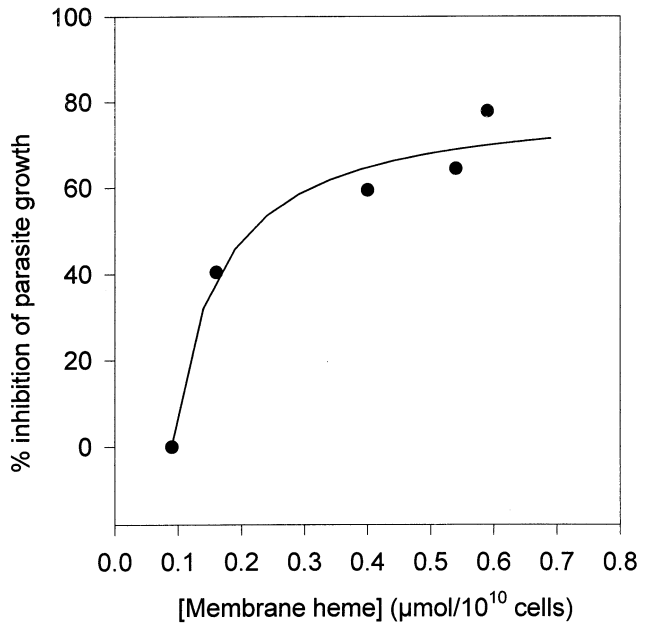

FIG. 5. Membrane-associated heme and parasite killing relationship. Infected cells were exposed to 10 μM chloroquine, and aliquots were taken at various time intervals and tested for membrane-associated heme and for parasite viability as described in Materials and Methods. Inhibition of parasite growth was plotted against the concentration of membrane heme. The continuous line is the best fit of the data to the Michaelis-Menten equation, yielding a K_m of 0.16 μmol heme/10^{10} cells.

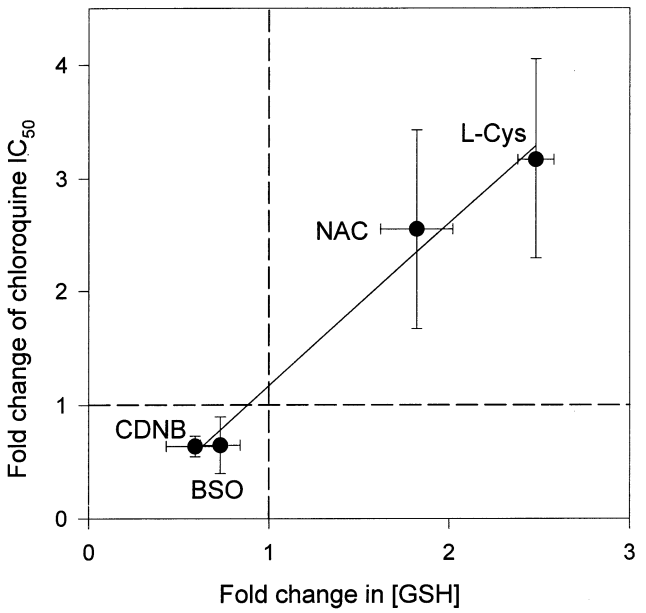

FIG. 6. Cellular GSH levels and sensitivity to chloroquine relationship. The IC_{50} of chloroquine in the absence or the presence of GSH-modifying agents and the effect of these agents on GSH levels in infected cells were determined as described in Materials and Methods. Results are shown as mean ± SE of three to six experiments as fold-change in IC_{50} and fold-change in GSH content compared to controls, which are depicted by the broken lines. The fold-change values were used rather than the actual values of the two parameters to normalize for variability in culture conditions and behavior. A linear correlation exists between fold-change in IC_{50} and fold-change in GSH content, r = 0.991.

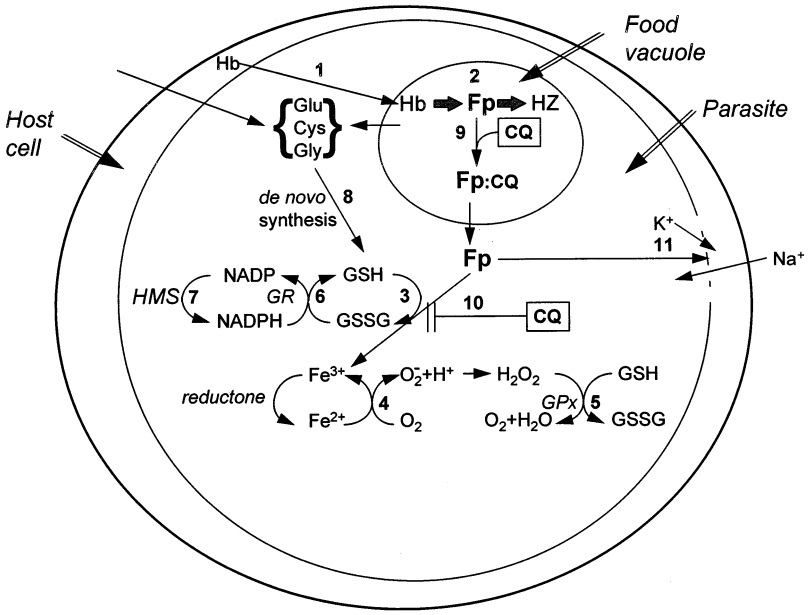

FIG. 7. The GSH-dependent mechanism of heme detoxification and the antimalarial mode of action of CQ and AQ. Hemoglobin (Hb; 1) is ingested from the host cell [1] and is digested inside the parasite's food vacuole, and heme (FP) is polymerized to HZ (2). Nonpolymerized heme exits the food vacuole into the parasite's cytosol, where it is degraded by GSH (3). In this process, GSH is oxidized to glutathione disulfide [GSSG]. The released iron can enter into redox cycling, thus producing superoxide ([GRAPHIC]), which is dismutated to H_2O_2 (4). The latter is reduced by catalase and glutathione peroxidase (GPx) while oxidizing GSH to GSSG (5). GSSG is reduced back to GSH by glutathione reductase (GR) using NADPH as a reducing cofactor (6). The resulting NADP is reduced back to NADPH by the hexose monophosphate shunt (HMS; 7). GSH can also be synthesized de novo from Glu, Cys and Gly (8) provided either from digested globin or from outside the cell. CQ or AQ accumulate to high levels inside the acid food vacuole (9), where they complex with heme and prevent its polymerization. Free- or complexed-heme exits the food vacuole where its degradation by GSH is inhibited competitively by the drugs (10). Heme thus accumulates in the membranes of infected cells and permeabilizes them to cations, thereby disturbing cation homeostasis in the parasite, (11) leading to parasite death.

a direct correlation has been observed between the fold change in GSH levels and the fold change in the IC_{50} of CQ, suggesting a causative connection. Relevant to these results is the demonstration that mouse cells, infected with a *P. berghei* strain that was selected for CQ resistance under drug pressure, contain higher levels of GSH than their sensitive counterparts, and that co-treatment with BSO (which inhibits GSH synthesis) increased sensitivity to CQ [45]. As there was a parallel increase in the levels of glutathione transferase, these authors suggested that resistance may be due to the formation of a nontoxic adduct between CQ and GSH. However, no adduct formation with CQ could be found in cultures of resistant and sensitive strains of *P. falciparum* that were exposed to this drug for 16 hr [46], attesting that resistance can not be assigned to drug detoxification. It was also found that H_2O_2 increased the susceptibility of *P. falciparum* to CQ [47]. Such potentiation would be consistent with the reduction of GSH concentration due to a greater demand for the detoxification of H_2O_2 by glutathione peroxidase.

Conclusions and Ramifications

Our model of the antimalarial mode of action of CQ and AQ is depicted in Fig. 7. The parasite digests host cell hemoglobin inside its food vacuole. Noxious heme is either polymerized to HZ *in situ* or degraded by GSH when it exits the vacuole. The ability of CQ and AQ to inhibit GSH-mediated heme degradation, as demonstrated in this work, provides a new clue to the mechanism of the cytotoxic activity of these antimalarial drugs: the drugs inhibit heme polymerization and thus increase the flux of heme out of the food vacuole. This would be inconsequential to parasite viability (because its cellular GSH can degrade the noxious heme) unless the drugs were able to inhibit GSH-dependent heme degradation, as shown here. These concerted events allow heme to reach critical levels in the membranes of the infected cell, infringe their barrier properties, and disturb ion homeostasis in the parasite, which leads to death. Although it has previously been suggested that this latter process mediates the cytotoxicity of antimalarial drugs [48], we here show that the inhibition by drugs of GSH-dependent heme detoxification is the crucial element in the mechanism of the cytotoxic action of CQ and AQ. Many of the present results, however, could be also explained by recent findings showing that antimalarial drugs inhibit the catalase and peroxidase activities of heme [49]. According to this mechanism H_2O_2, which is produced during the digestion of hemoglobin, should be detoxified by heme, and when this process is inhibited in the presence of drugs, the parasite would be killed by excessive oxidative stress. Such stress would be obviously counteracted by increased levels of cellular GSH. This attractive hypothesis, however, has not been tested in drug-treated malaria-infected cells; it contradicts the demonstration that CQ actually decreases oxidative stress [38] as would be predicted from the inhibition of GSH-mediated heme degradation (when this process is inhibited there is less GSH recycling and less iron available for the generation of oxidative stress). The fact that membrane-associated heme

continues to increase with time of incubation with drug (Fig. 4), suggests that most if not all nonpolymerized heme promptly exits the food vacuole; otherwise, it should have inhibited the proteolytic digestion of ingested hemoglobin [50] and the eventual release of heme. Finally, given the high permeability of membranes to H_2O_2 and the high catalase [38] and glutathione peroxidase [51] activities of the parasite, it is questionable whether the catalase activity of heme is quantitatively important at all.

The present results also invoke additional or alternative modes of drug resistance that may involve the metabolism of glutathione and antioxidant defense mechanisms, and hence, provide novel approaches for the reversal of drug resistance. It should be emphasized that, whereas the inhibition of heme polymerization would depend on the concentration of CQ inside the food vacuole, the GSH-dependent degradation will be inhibited by cytosolic drug concentrations that are presumably equal to the extracellular concentration. In the first case, drug resistance may result from reduced drug accumulation; in the second case, drug resistance may result from increased levels of GSH. Both modes of resistance may act cooperatively. These issues are presently being investigated in our laboratory.

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