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ENERGY DEPENDENCE OF CHLOROQUINE ACCUMULATION AND CHLOROQUINE EFFLUX IN *PLASMODIUM FALCIPARUM*

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Abstract—Chloroquine inhibits the growth of susceptible malaria parasites at low (nanomolar) concentrations because of an energy-requiring drug-concentrating mechanism in the parasite secondary lysosome (food vacuole) which is dependent on the acidification of that vesicle. Chloroquine resistance results from another energy-requiring process: efflux of chloroquine from the resistant parasite with a half-time of 2 min. Chloroquine efflux is inhibited reversibly by the removal of metabolizable substrate (glucose); it is also reduced by the ATPase inhibitor vanadate. These results suggest that chloroquine efflux is an energy-requiring process dependent on the generation and hydrolysis of ATP. Chloroquine efflux cannot be explained by differences in drug accumulation between chloroquine-susceptible and -resistant parasites because the 40–50-fold difference in initial efflux rates between -susceptible and -resistant parasites is unchanged when both parasites contain the same amount of chloroquine. Although chloroquine efflux is phenotypically similar to the efflux of anticancer drugs from multidrug-resistant (*mdr*) mammalian cells, it is not linked to either of the *mdr*-like genes of the parasite.

Worldwide importance of malaria and of chloroquine resistance. Malaria is a public health problem of inestimable importance. Each year there are 2–3 million malaria deaths in sub-Saharan Africa alone and 200–300 million cases cases worldwide [1, 2]. Together with malnutrition and HIV infection, malaria is one of the most important health problems of the developing world.

For more than 40 years, chloroquine was the principal agent used for malaria chemoprophylaxis and treatment because it was both safe and effective [3]. Although it remains safe, chloroquine is no longer effective against many strains of *Plasmodium falciparum* in South America, southeast Asia and Africa [2, 3]. The rising prevalence of chloroquine resistance has increased the morbidity and mortality of malaria [4, 5] and is a critical medical problem for most of the world's population.

P. falciparum. P. falciparum is the most dangerous of the four malaria parasites that infect humans because it can invade red blood cells (RBSs†) of any age to produce overwhelming potentially lethal parasitemias and because it may be chloroquine-resistant. There is virtually no risk of death from the other species that infect humans because they produce more limited parasitemias and can invade only young (*P. vivax* and *P. ovale*) or old (*P. malariae*) red cells [6]. There is no evidence for chloroquine resistance in either *P. ovale* or *P. malariae*. Although there are recent reports of chloroquine resistance in *P. vivax* [7], neither the magnitude or the nature of that resistance are yet clear.

Chloroquine as a diprotic weak base. The partitioning of chloroquine and other diprotic weak bases into acid intracellular vesicles is predicted by their p*K* values, and the ΔpH between their acid vesicles and the extracellular medium [8, 9]. However, neither the extent of chloroquine accumulation by the susceptible parasite [10, 11], or the rapid efflux of chloroquine from the resistant parasite [12] is predicted by these physical–chemical considerations. Thus, the concentration of chloroquine accumulated by the susceptible parasite in excess of that predicted by its properties as a weak base and its efflux by the resistant parasite are unique aspects of the interaction between chloroquine and *P. falciparum*. Both are potentially relevant to the development of new antimalarials.

Rational drug design for the chemoprophylaxis and treatment of malaria. Because of the increasing prevalence of resistance, chloroquine is no longer sufficient for the chemoprophylaxis and treatment of malaria. One potential solution is the development of drugs that act by entirely different mechanisms such as artemisinin [13]. Alternatively, if chloroquine is active at low (nanomolar) concentrations because of a concentrating mechanism in the parasite and is excreted by a different mechanism, it may be possible to design chloroquine analogs that are concentrated effectively in the parasite secondary lysosome but are not excreted by the efflux mechanism. This manuscript addresses that question by determining whether the accumulation of chloroquine by the susceptible parasite and its excretion (efflux) by the resistant parasite are distinct energy-dependent processes.

MATERIALS AND METHODS

Parasite strains and culture system. The chloro-

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† Abbreviations: *mdr*, multidrug resistant; RBC, red blood cell; PRBC, parasitized red blood cell; fmol [^{3}H] chloroquine/10^6 PRBCs, fmol [^{3}H]chloroquine accumulated per 10^6 PRBCs; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FD, fluorescein isothiocyanate linked to dextran.

quine-resistant Indochina I/CDC and chloroquine-susceptible Haiti 135 strains of *P. falciparum* were provided initially by Phuc Nguyen-Dinh and C. C. Campbell of the Centers for Disease Control [14, 15]. They were cloned subsequently by limiting dilution [16].

Parasites were grown *in vitro* in suspensions of human RBCs using the medium developed by Trager and Jensen [17] containing RPMI-1640, 25 mM Hepes (pH 7.4), 0.2 g/dL $NaHCO_3$ and 10% human serum [17]. Cultures were incubated at 37° in modular incubation chambers (Billups-Rothenberg, Inc., Del Mar, CA, U.S.A.) in an atmosphere containing 3% O_2, 3% CO_2 and 94% N_2 by gassing for 3 min. Synchronous cultures were produced by treatment with 5% sorbitol at intervals of 48 or 96 hr [17, 18].

Measurement of chloroquine accumulation. Suspensions of PRBCs were incubated at 37° for 60 min in RPMI-1640 culture medium containing 25 mM Hepes (pH 7.4), 1–5 nM [^{3}H]chloroquine (15.3 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) and 0.1% human serum, but no $NaHCO_3$. Aliquots of the reaction mixture were then centrifuged through silicon oil in 400-μL microfuge tubes to obtain the cell pellet, leaving the aqueous medium with the remaining [^{3}H]chloroquine in the upper layer. The cell pellet was removed by cutting the microfuge tube with a razor blade through the silicon oil layer and was placed in a larger (1.8 mL) microfuge tube, digested with 75 μL of Protosol™:ethanol (1:2) for 60 min at 58°, bleached with 25 μL of 30% H_2O_2, and acidified with 25 μL of 1 N HCl. Scintillation counting was performed by placing the 1.8-mL microfuge tube containing the tip of the 400-μL microfuge tube and the digested cell pellet in a 20-mL scintillation vial, adding 8 mL of Universol Cocktail (ICN Radiochemicals, Irvine, CA, U.S.A.) and counting with an LS8000 liquid scintillation counter (Beckman, Fullerton, CA, U.S.A.). Results were calculated as fmol [^{3}H]chloroquine/10^6 PRBCs. Accumulation experiments were performed under non-depleting conditions: the parasitized red cell suspensions were diluted so that the [^{3}H]chloroquine accumulated by the cell pellet was $\leq$10% of the [^{3}H] chloroquine available.

Measurement of chloroquine efflux. After incubation with [^{3}H]chloroquine for 60 min at 37°, the PRBC suspensions were centrifuged and resuspended (at zero time) in an equal volume of the same medium without [^{3}H]chloroquine. At each subsequent time point, aliquots of the cell suspension were harvested as described above. Results were calculated relative to the amount of chloroquine present at zero time (percent of [^{3}H]chloroquine remaining in the cell pellet). Initial efflux half-times were calculated using the least squares method to estimate the initial slope with a high degree of confidence ($r \geq 0.98$) after logarithmic transformation of the data [12].

Use of media with limited metabolizable carbohydrate substrate. Because PRBCs generate ATP by the metabolism of glucose to lactate [19], the limitation of metabolizable carbohydrate substrates is a simple, potentially reversible way to identify ATP-dependent processes. To accomplish this, RPMI-1640 medium without glucose was used with 10 mM glucose, 10 mM fructose, 10 mM fucose or no added carbohydrate (~5 μM glucose from the 0.1% human serum in the medium). These studies permitted comparison of the effects of two metabolizable carbohydrates (glucose, fructose) with those of a non-metabolizable carbohydrate (fucose) [20].

Inhibition of ATPase activity with vanadate. If the energy produced by metabolism of carbohydrate substrates is converted into ATP and hydrolysed by an ATPase, similar effects should also be produced by the addition of an ATPase inhibitor such as vanadate [21]. In contrast to the effects of adding or removing carbohydrates, the effects of adding vanadate may be irreversible.

Measurement of acid vesicle (food vacuole) pH. To measure acid vesicle (food vacuole) pH, unparasitized RBCs were subjected to osmotic shock using a medium containing FD and then resealed by the addition of hypertonic medium to restore the osmolality to normal [22]. Synchronous cultures were prepared by inoculating these FD-labeled RBCs with synchronous cultures of late trophozoite stage parasites. FD in the RBC cytoplasm was removed by RBC lysis with digitonin (200 μg/mL) prior to spectrofluorimetry. Vesicle pH was then measured by using the F(490/450) ratio (Aminco-Bowman Model 500 Spectrofluorimeter, Silver Spring, MD, U.S.A.). pH changes occurred within 3–5 min after adding chloroquine or NH_4Cl and were reversible on their removal.

Genetic studies of chloroquine resistance. The chloroquine-resistant Dd2 clone of the Indochina III strain and the chloroquine-susceptible HB3 clone of the Honduras strain were maintained in culture and used to produce gametocytes. Anopheline mosquitoes fed on these clones were used to infect a chimpanzee from which parasites were cloned and identified using a series of DNA probes to characterize restriction fragment length polymorphisms [23]. The probe used to identify the parasite's *mdr*-like sequences was synthesized based on the mammalian (mouse) *mdr* sequence adjusted for the preferential codon usage of *P. falciparum* [23].

RESULTS

Chloroquine accumulation by P. falciparum

Measurement of acid vesicle buffering capacity. To determine whether the effects of chloroquine on acid vesicle pH in the parasite can be accounted for entirely by its properties as a diprotic weak base, we measured the buffering capacities of mammalian and parasite acid vesicles with NH_4Cl and chloroquine. These experiments revealed that a base load in the form of NH_4Cl was handled equally well by both cell types (buffering capacities of 0.1 and 0.2). In contrast, a base load in the form of chloroquine was handled 600–800-fold less well by the parasite vesicle (Table 1 [10]). The comparable buffering capacities observed with NH_4Cl suggest that the proton pumps of the two cell types are fundamentally similar. Potential explanations for the discrepancies in buffering capacity between NH_4Cl and chloroquine

Table 1. Buffering capacities of parasite and mammalian acid vesicles

	NH_4Cl	Chloroquine
Foreskin fibroblasts	0.2	0.2
P. falciparum	0.1	0.0001

Buffering capacities are moles of H^+ for a vesicle pH change of 1 pH unit [10].

Table 2. Intravesicular accumulation of chloroquine

Cell type	Chloroquine concentation*	
	Observed	Predicted
J774.2 Macrophages	0.11	0.141
P. falciparum	68.7	0.141

* Observed intravesicular chloroquine concentration based on NH_4Cl-inhibitable [^{3}H]chloroquine accumulation [10, 22]. Predicted intravesicular chloroquine concentration based on the Henderson–Hasselbalch equation, the pK values of chloroquine, and the pH of parasite and mammalian acid vesicles [10].

with the parasite include: (1) physical disruption of the parasite vesicle by chloroquine, (2) a chloroquine-concentrating mechanism in the parasite vesicle, and (3) inhibition of the parasite vesicle proton pump by chloroquine. The first explanation is unlikely because the increment in vesicle pH produced by chloroquine was rapidly reversed by the removal of that chloroquine from the medium [10, 22]. This should not have been the case if the vesicle had been disrupted physically. The second explanation was tested experimentally and is supported by the results of those experiments (see below). The third explanation is considered in the Discussion.

Measurement of chloroquine accumulation by plasmodia and mammalian cells. Using [^{3}H]-chloroquine, we compared the intravesicular chloroquine accumulation of *P. falciparum* and mammalian cells. These results demonstrate that the chloroquine accumulation observed with mammalian cells is virtually identical to that predicted by the Henderson–Hasselbalch equation (Table 2). In contrast, the vesicles of the susceptible parasite accumulate 600–800-fold more chloroquine than predicted. This discrepancy is consistent with the reduced buffering capacity observed with chloroquine and suggests that the parasite has a chloroquine-concentrating mechanism.

Evidence for an active chloroquine-concentrating mechanism in the parasite vesicle. Two lines of evidence suggest that the parasite has an active chloroquine-concentrating mechanism in its food vacuole (secondary lysosome). The first line of evidence is based on vesicles reconstituted after disruption of the parasite by nitrogen cavitation. These reconstituted vesicles accumulate [^{3}H]-chloroquine in a specific (inhibition by unlabeled chloroquine), time- and ATP-dependent fashion with an apparent K_m of 21 μM for ATP [23]. This activity is not found in unparasitized RBCs and is not supported by nucleotide triphosphates other than ATP. The second line of evidence is based on a subcellular food vacuole preparation developed by Goldberg *et al.* [25] to study the aspartic protease responsible for the proteolysis of hemoglobin internalized in the food vacuole. Using a modification of that preparation, we have found that its chloroquine accumulation is also ATP-dependent. The maximal accumulation observed with both preparations is approximately 7 pmol chloroquine/μg protein and is similar in both chloroquine-susceptible and -resistant parasites.

Initial rate of chloroquine accumulation. If the mechanism responsible for chloroquine accumulation was altered in the resistant parasite, one would expect different initial rates of chloroquine accumulation. However, the initial rates of chloroquine accumulation are indistinguishable between the two parasites [12]. This result provides a third line of evidence suggesting that the chloroquine accumulation mechanism is fundamentally similar in chloroquine-susceptible and -resistant *P. falciparum*.

Energy dependence of chloroquine accumulation. Because energy is required to maintain the proton gradient across the acid vesicle, maneuvers that inhibit the production or the hydrolysis of ATP should also decrease chloroquine accumulation. This hypothesis is supported by the experimental results. Either the removal of metabolizable carbohydrate substrate (glucose, Table 3) or the addition of vanadate (not shown) inhibits chloroquine accumulation by the susceptible parasite. The effects of glucose are not related to the Na^+/glucose transporter of the RBC because similar effects were observed with fructose.

Chloroquine efflux from P. falciparum

Initial efflux half-time. There is a 40–50-fold difference in initial efflux half-time between chloroquine-susceptible and -resistant parasites (2 vs $\geq$50–75 min) [12]. The magnitude of this difference is consistent with the differences in their IC_{50} values and steady-state chloroquine accumulations [11, 12].

Effect of initial chloroquine content (concentration). Chloroquine efflux was studied in the chloroquine-resistant Indochina I and chloroquine-susceptible Haiti 135 clones of *P. falciparum*. In the initial 60-min incubation with [^{3}H]chloroquine, the extracellular chloroquine concentrations were adjusted so that the final amounts of chloroquine accumulated would be equal in order to test whether the difference in efflux half-time resulted simply from a difference in initial chloroquine content. The resistant parasite was exposed to increased external chloroquine concentrations so that both parasites contained 175–180 fmol [^{3}H]chloroquine/10^6 PRBCs at zero time. The results of this experiment indicate that, even under these conditions, the initial rate of chloroquine efflux was 40–50-fold more rapid from the resistant parasite and show that this difference in efflux rate is not the result of a difference in initial chloroquine content.

Chloroquine efflux as an active process. To further

Table 3. Effect of glucose on chloroquine accumulation

	Chloroquine accumulation (fmol chloroquine/10^6 PRBCs)	
	10 mM Glucose	≈5 μM Glucose
Chloroquine-susceptible *P. falciparum* (Haiti 135)	1291	151
Chloroquine-resistant *P. falciparum* (Indochina I)	87	172

Table 4. Effect of a raised baseline vesicle pH on chloroquine accumulation and efflux in *P. falciparum*

	Chloroquine accumulation (fmol/10^6 PRBCs)	Initial efflux half-time (min)
Chloroquine-susceptible *P. falciparum* (Haiti 135)	9649	≥60
Chloroquine-susceptible *P. falciparum* + 20 mM NH_4Cl	597	≥60
Chloroquine-resistant *P. falciparum* (Indochina I)	257	2

test whether efflux is an active process, efflux was measured after the removal of metabolizable carbohydrates such as glucose or fructose from the test medium, and after the addition of vanadate. The results indicate that efflux is an active process. Both the removal of metabolizable carbohydrates (glucose, Table 3) and the addition of vanadate (not shown) *increased* chloroquine accumulation by the resistant parasite and *prolonged* the efflux half-time. The opposite effects were observed with the chloroquine-susceptible parasite for which chloroquine accumulation is dependent on the proton gradient in the acid vesicle [11, 22]. Thus the results observed with the resistant parasite cannot be explained by defective acidification of its food vacuole (a raised baseline vesicle pH in the resistant parasite).

Alterations in baseline vesicle pH of the resistant parasite. A raised baseline vesicle pH in the resistant parasite should decrease chloroquine accumulation and has, therefore, been suggested as a potential explanation for chloroquine resistance in *P. falciparum* [26, 27]. However, a raised baseline vesicle pH should not produce active chloroquine efflux. This hypothesis was tested by using NH_4Cl to raise vesicle pH in the susceptible parasite. The results indicate that raising vesicle pH with NH_4Cl reduces chloroquine accumulation but does not produce chloroquine efflux (Table 4).

Study of isolated parasite vesicles. As noted above, we have examined two types of parasite vesicle preparation for their ability to accumulate chloroquine [23, 24]. Although both preparations accumulate similar amounts of chloroquine in a specific and ATP-dependent fashion, neither vesicle preparation demonstrates efflux. Comparisons between preparations from susceptible and resistant parasite clones reveal only modest differences in chloroquine accumulation. Thus, these data suggest that the parasite vesicle is the site of the active accumulation responsible for chloroquine action but is not the site of the efflux responsible for chloroquine resistance.

Genetic studies of chloroquine resistance

Because the malaria parasite completes its life cycle by passing through the mosquito and the chimpanzee, it was possible to perform a cross between chloroquine-susceptible and -resistant *P. falciparum* clones in order to study the genetic basis of chloroquine resistance. The results of this cross indicate that chloroquine resistance is not linked to either of the *mdr*-like genes of the parasite (which are located on chromosomes 5 and 14) [23], but is linked to a locus of approximately 400 kb on chromosome 7 [28]. The effects of removing metabolizable carbohydrates or adding vanadate on chloroquine accumulation with clones derived from this cross are similar to those obtained with the cloned natural isolates.

DISCUSSION

Chloroquine accumulation

Chloroquine accumulation in chloroquine-susceptible and -resistant parasites. The chloroquine accumulation of susceptible parasites is 600–800-fold greater than predicted by the properties of chloroquine as a diprotic weak base [11]. The energy-dependent mechanism responsible for this excess chloroquine accumulation permits the action of chloroquine against the parasite at concentrations that have no detectable effects on mammalian cells [9, 22].

Three lines of evidence suggest that this mechanism is similar in chloroquine-susceptible and -resistant parasites: (1) vesicles reconstituted from -susceptible or -resistant parasites disrupted by nitrogen cavitation accumulate similar amounts of chloroquine [22]; (2) vesicles isolated on density gradients from -susceptible or -resistant parasites that have not been disrupted by nitrogen cavitation [24] also accumulate similar amounts of chloroquine, and; (3) initial rates of chloroquine accumulation are indistinguishable between -susceptible and -resistant intact parasitized red cells [12].

As noted above, it is possible that chloroquine has a direct inhibitory effect on the parasite vesicle proton pump. However, that effect, if it exists, is a minor factor because the excess accumulation of chloroquine by the parasite vesicle accounts quantitatively for the discrepancy between the buffering capacities observed with NH_4Cl and chloroquine.

Chloroquine efflux.

Active efflux as the basis of chloroquine resistance. In addition to energy-dependent efflux [12], two alternative hypotheses have been proposed to explain the reduced chloroquine accumulation associated with chloroquine resistance: a raised baseline vesicle pH in the resistant parasite, and a passive efflux process that is more rapid in the resistant parasite because of its reduced chloroquine accumulation [26, 27].

A raised baseline vesicle pH would reduce chloroquine accumulation. However, it would not increase the rate of chloroquine efflux (Table 4). It is also inconsistent with our previous observations which suggest that baseline vesicle pH is similar in chloroquine-susceptible and -resistant parasites [22]. The minmal differences observed in those studies suggest that baseline vesicle pH may actually be slightly higher in the *susceptible* parasite. Finally, a significant change in baseline vesicle pH is unlikely because it would require a new complement of proteolytic enzymes which retained their activity at that elevated pH [25].

Genetics of chloroquine resistance

One hypothesis to explain chloroquine resistance is an *mdr*-like gene in the parasite. However, the results obtained with the cross [23, 28] indicate that resistance is not linked to either of the parasite's *mdr*-like genes. Thus, this explanation is no longer likely.

Conclusions

The action of chloroquine against the malaria parasite is consistent with inhibition of the proteolysis of hemoglobin. The specificity of chloroquine action against the parasite is consistent with an active accumulation mechanism in the parasite acid vesicle (secondary lysosome, food vacuole) which is dependent on the hydrogen ion gradient across that vesicle.

Chloroquine resistance is an active process in the resistant parasite. There is no other apparent explanation for the paradoxical increase in chloroquine accumulation observed with the removal of glucose or the addition of vandate (Table 3). The efflux responsible for chloroquine resistance is phenotypically similar to the efflux observed in mammalian cells with multidrug resistance, but is not linked to either of the two parasite genes with known *mdr*-like sequences. The genetic basis of this resistance remains to be determined.

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