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Efflux of Chloroquine from *Plasmodium falciparum*: Mechanism of Chloroquine Resistance

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Chloroquine-resistant *Plasmodium falciparum* accumulate significantly less chloroquine than susceptible parasites, and this is thought to be the basis of their resistance. However, the reason for the lower accumulation of chloroquine was unknown. The resistant parasite has now been found to release chloroquine 40 to 50 times more rapidly than the susceptible parasite, although their initial rates of chloroquine accumulation are the same. Verapamil and two other calcium channel blockers, as well as vinblastine and daunomycin, each slowed the release and increased the accumulation of chloroquine by resistant (but not susceptible) *Plasmodium falciparum*. These results suggest that a higher rate of chloroquine release explains the lower chloroquine accumulation, and thus the resistance observed in resistant *Plasmodium falciparum*.

MALARIA IS A DISEASE OF IMmense importance, with an estimated 200 to 300 million cases and 2 million deaths each year (*1*). Chloroquine, the drug most widely used for the treatment of malaria, is effective against three of the four malaria species that infect humans. However, the species which poses the greatest risk of complications and death (*Plasmodium falciparum*) is often resistant to treatment with chloroquine (*2*). Chloroquine-resistant *P. falciparum* is now established in Southeast Asia and South America (*3*), and has recently spread westward across Africa from Kenya and Tanzania to Nigeria (*4*).

Beginning with the studies of Fitch (*5*), a number of investigators have shown that chloroquine-resistant *P. falciparum* accumulate significantly less chloroquine than do susceptible parasites. Our studies (*6*) and those of Yayon *et al.* (*7*) indicate that chloroquine is concentrated in the acid vesicular compartment of the parasite. Although the amount of chloroquine accumulated by resistant parasites is substantially less than that accumulated by susceptible parasites (*6*), the reason for this difference has been unknown.

Recent studies suggest that the accumulation of chloroquine in parasite acid vesicles inhibits parasite growth by raising the *p*H of the acid vesicle (*6, 7*). In mammalian cells, chloroquine enters and leaves the cell by passive diffusion and is concentrated as a weak base in the acid vesicle by the *p*H gradient across the vesicle membrane (*8*). In contrast, both the susceptible and resistant parasite accumulate more chloroquine in their vesicles than can be explained by its properties as a weak base (*6, 9*). We examined the kinetics of chloroquine accumulation and release by the parasite to determine whether they could account for the differences in chloroquine accumulation associated with susceptibility and resistance.

Parasites were grown in vitro in suspensions of O-positive red cells, with RPMI 1640; 25 m*M* Hepes, and $NaHCO_3$ at 0.2 g/100 ml, in a culture system devised by Trager and Jensen (*10*). The cultures were exposed to an atmosphere of 3% O_2, 3% CO_2, and 94% nitrogen in modular incubation chambers (Linde Division, Union Carbide, New York) and then maintained at 37°C (*11*). The Haiti 135 and Indochina I/CDC strains of *P. falciparum* were used because of their known susceptibility and resistance to chloroquine (median effective doses of 3 to 6 and 50 to 60 n*M*, respectively) (*12*).

[^{3}H]Chloroquine accumulation and release were calculated in two ways: (i) from the residual radioactivity of the culture medium supernatant after centrifugation of the parasitized red cell suspension through silicon oil (*6, 13*), and (ii) from the radioactivity of the pellet after alkali digestion of the cell pellet (*6*). Preliminary experiments showed excellent agreement between the two methods, and demonstrated that >95%

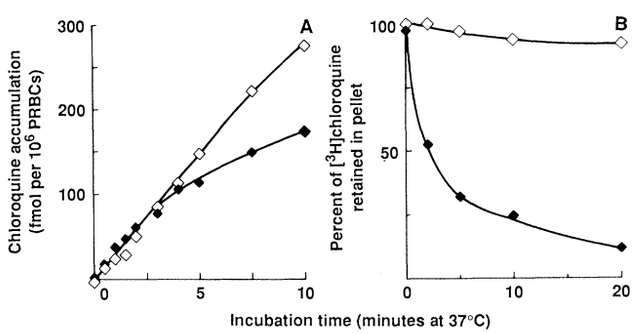

Fig. 1. Chloroquine accumulation and release by parasitized red cells. (**A**) The initial rates of chloroquine accumulation observed with susceptible (Haiti 135, open diamonds) and resistant (Indochina I, filled diamonds) *P. falciparum* were indistinguishable (28.6 ± 1.5 versus 29.1 ± 3.8 fmol per 10^6 parasitized red cells for the first 4 minutes). In these experiments suspensions of parasitized red cells (2×10^6 per milliliter) were suspended in culture medium containing 1 n*M* [^{3}H]chloroquine at zero time. Linear regression was used to calculate the rate of chloroquine accumulation for both parasites. PBRCs, parasitized red cells. (**B**) Chloroquine was released more rapidly from the resistant parasite than from the susceptible parasite. Similar values for the initial release $t_{1/2}$ were obtained when culture medium containing 1 n*M* [^{3}H]chloroquine was used with both parasites for the initial 60-minute incubation with [^{3}H]chloroquine (which produced different chloroquine accumulations) or different concentrations of [^{3}H]chloroquine (0.1 and 1.0 n*M*, respectively, for susceptible and resistant *P. falciparum*) to produce similar accumulations of chloroquine (174 to 180 fmol per 10^6 parasitized red cells) (B). Four separate experiments were performed in each case (A and B).

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Table 1. Effects of calcium blockers, vinblastine, and daunomycin on the release of chloroquine from resistant *Plasmodium falciparum*. Suspensions of the chloroquine-resistant Indochina I strain of *P. falciparum* (at a density of 2×10^6 parasitized red cells per milliliter) (*6, 12*) were incubated in culture medium at 37°C for 60 minutes with 1 n*M* [^{3}H]chloroquine. Before chloroquine release was measured, the parasitized red cells were washed twice in medium containing 1 n*M* unlabeled chloroquine and suspended for 30 minutes at 37°C in medium with 1 n*M* unlabeled chloroquine and the agent being tested. Because daunomycin required a longer preincubation time, it was added during the initial 60-minute exposure to 1 n*M* [^{3}H]chloroquine. The initial $t_{1/2}$ of chloroquine release observed with the susceptible Haiti 135 strain was ≥85 minutes.

Agent	Initial $t_{1/2}$ (minutes)
Verapamil (3.1 μ*M*)	23.0 ± 3.8
Diltiazem (10 μ*M*)	13.7 ± 0.7
TMB-8 (10 μ*M*)	8.6 ± 2.3
Vinblastine (3.1 μ*M*)	12.8 ± 4.6
Daunomycin (10 μ*M*)	13.9 ± 2.0
None (controls)	2.2 ± 0.4

of the ^{3}H that disappeared from the medium during chloroquine accumulation could be accounted for in the parasitized red cell pellet. The extracellular [^{3}H]chloroquine concentration used (1 n*M*) does not alter parasite vesicle *p*H (*6, 9*).

To measure chloroquine accumulation, we exposed suspensions of parasitized red cells to 1 n*M* [^{3}H]chloroquine. To minimize chloroquine depletion from the medium (*12*), we used parasite densities that reduced the amount of [^{3}H]chloroquine in the medium by ≤15%. As in previous studies (*5, 6*), susceptible parasites accumulated significantly more chloroquine than resistant parasites. However, the initial rates of chloroquine accumulation observed with susceptible and resistant parasites were indistinguishable (28.6 ± 1.5 versus 29.1 ± 3.8 fmol per 10^6 parasitized red cells per minute) (Fig. 1A).

To measure chloroquine release, we exposed suspensions of parasitized red cells to 1 n*M* [^{3}H]chloroquine for 1 hour in culture medium. The parasitized cells were then washed twice in medium containing 1 n*M* unlabeled chloroquine and suspended in the same medium without chloroquine. We had previously shown that [^{3}H]chloroquine released from parasitized red cells is chemically unaltered as defined by thin-layer chromatography (*12*). The time necessary to release one-half the chloroquine present at zero time (the initial release $t_{1/2}$) was calculated by linear regression to relate the logarithm of the amount of drug remaining to time. Chloroquine was released rapidly from resistant parasites ($t_{1/2}$, 2.2 ± 0.4 minutes), but slowly from susceptible parasites ($t_{1/2}$, ≥85 minutes) (Fig. 1B). The rapid rate of chloroquine release observed with resistant parasites did not result from the lack of chloroquine in the medium because similar release rates were observed using medium with ($t_{1/2}$, 1.9 minutes) or without 1 n*M* unlabeled chloroquine ($t_{1.2}$, 2.1 minutes). These results indicate that resistant parasites have a 40- to 50-fold more rapid release of chloroquine than susceptible parasites.

Because Martin *et al.* (*14*) showed that the combination of verapamil and chloroquine inhibits the growth of chloroquine-resistant parasites, we examined the effects of verapamil and other agents on the accumulation and release of chloroquine by *P. falciparum*.

Verapamil, two other calcium channel blockers [diltiazem and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8)], vinblastine, and daunomycin each enhanced the accumulation of chloroquine by the resistant (but not the susceptible) parasite (Fig. 2, A to D). Neither dantrolene sodium nor the calcium ionophore A23187 altered the accumulation of chloroquine by the resistant parasite. Verapamil, diltiazem, TMB-8, vinblastine, and daunomycin each inhibited the release of chloroquine from the resistant parasite (Table 1). None of these compounds affected the release of chloroquine from the susceptible parasite. These results suggest that verapamil produces synergism with chloroquine against resistant parasites (*14*) by inhibiting release and thus enhancing chloroquine accumulation.

Because previous studies suggest that the biologically significant accumulation of chloroquine within the parasite is in the acid vesicle (*6, 7, 9*) and requires an acid vesicle *p*H (*6, 7*), we examined the effect of raising intravesicular *p*H with NH_4Cl on the abilities of these drugs to enhance the accumulation of chloroquine by the resistant parasite. Raising parasite vesicle *p*H by ≥1.0 *p*H unit blunted the effect of these compounds by >50%, suggesting that their locus of action was in the acid vesicular compartment.

Our results indicate that the initial rates of chloroquine accumulation are the same in susceptible and resistant parasites (Fig. 1A). The most striking difference between the susceptible and resistant parasites is the rapid release of chloroquine by resistant parasites (Fig. 1B). The 43-fold lower steady-state accumulation of chloroquine by resistant parasites (234 versus 10,027 fmol per 10^6 parasitized red cells) is consistent with their 40- to 50-fold greater rate of chloroquine release. These data suggest that the critical difference between susceptible and resistant *P. falciparum* is the rate at which they release chloroquine.

The ability of verapamil to inhibit the release of chloroquine from the resistant parasite (Table 1) is strikingly similar to its ability to inhibit the release of anticancer agents from mammalian cancer cells with the multidrug resistance phenotype (*15*). In those cells, verapamil enhances the efficacy of anticancer drugs by inhibiting their release and thus increasing their intracellular

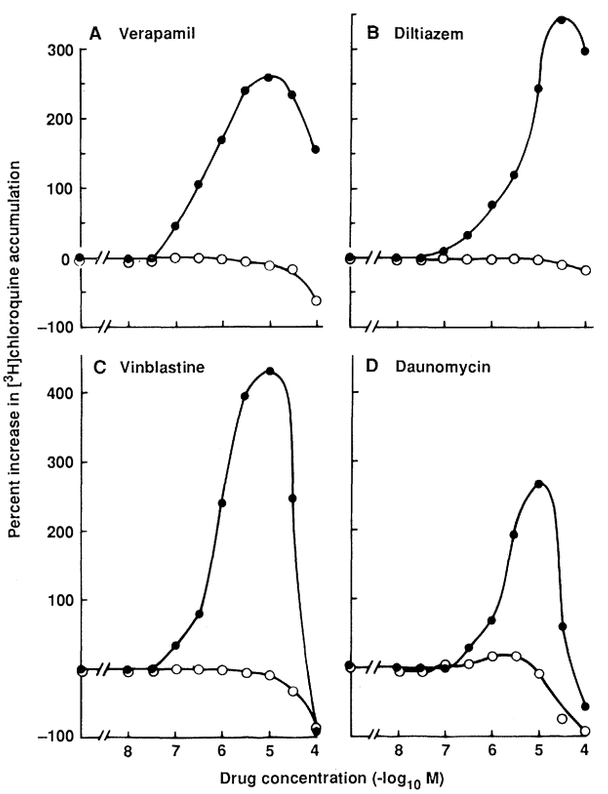

Fig. 2. Effects of calcium channel blockers, vinblastine, and daunomycin on [^{3}H]chloroquine accumulation. Each of these agents enhanced the accumulation of chloroquine by the resistant Indochina I strain of *P. falciparum* (filled circles), but not the susceptible Haiti 135 strain (open circles). Suspensions of parasitized red cells (2×10^6 per milliliter) were exposed to 1 n*M* [^{3}H]chloroquine for 60 minutes at 37°C with varying concentrations of these agents. Although the steady-state accumulation of chloroquine by the susceptible parasite (10,027 fmol per 10^6 parasitized red cells) was substantially greater than that of the resistant parasites (234 fmol per 10^6 parasitized red cells) (*6, 9*), the results are presented as percentage increase relative to baseline and thus demonstrate greater changes with the resistant parasite. Three or more separate experiments were performed with each agent tested.

accumulation. These results suggest that chloroquine resistance in *P. falciparum* and multidrug resistance in mammalian cells may be due to the same (or to a similar) mechanism.

The inhibition of chloroquine release observed with calcium channel blockers (verapamil, diltiazem, and TMB-8) (Table 1) suggests that alterations in intracellular calcium may be necessary for the release of chloroquine from the resistant parasite. The mechanism of daunomycin action is not clear but may be related to its ability to act as a calcium antagonist in some systems (*16*). The effects of vinblastine (a known inhibitor of microtubular function) (*17*) suggest that cytoskeletal proteins may be involved in the release of chloroquine from the resistant parasite. Alternatively, these compounds may inhibit chloroquine efflux by competing with chloroquine for binding to a carrier analogous (or identical) to the P-170 glycoprotein of the multidrug-resistant cancer cell (*18*).

However, even in the presence of these drugs, the chloroquine release observed with the resistant parasite was greater than observed with the susceptible parasite (Table 1). This resulted in a greater chloroquine accumulation in the susceptible than in the resistant parasite (Fig. 2) (*6, 7, 12*).

Although there may be more than one mechanism of chloroquine resistance in *P. falciparum*, verapamil reduces the inhibitory concentrations of chloroquine for resistant isolates from West Africa (*19*) and we have observed the rapid efflux phenomenon in a resistant isolate from South America ($t_{1/2}$, 1.9 minutes). These results indicate that the rapid efflux resistance phenotype is present in each of the three continents with chloroquine resistance.

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Diel Periodicity of Photosynthesis in Polar Phytoplankton: Influence on Primary Production

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In the Southern Ocean, primary production estimated from seasonal chemical and geochemical changes is two to four times greater than the value calculated from carbon-14 uptake. Since carbon uptake had typically been measured only during midday incubations, the influence of diel periodicity of photosynthesis on daily productions was not considered. Phytoplankton from McMurdo Sound, Antarctica, exhibited distinct, but seasonally variable diel patterns of light-saturated and light-limited photosynthesis. Maximum photosynthetic capacity occurred about noon in early September, and its occurrence progressively shifted to about midnight by late October. This shift was accompanied by a concomitant phase shift in the occurrence of minimum photosynthetic capacity from midnight to midday. Daily production estimated from time-of-day corrected photosynthetic characteristics and from 24-hour incubations was 2.5 to 4 times greater than that predicted from 6-hour midday incubations. If similar diel periodicity in photosynthesis occurs in other polar oceans, primary production would be significantly higher than previously estimated from carbon-14 uptake measurements.

THE SOUTHERN OCEAN SUPPORTS large populations of zooplankton, marine mammals, and seabirds (*1*) and is the site of approximately 80% of global biogenic silica sedimentation (*2, 3*). Despite this indirect evidence suggesting high primary production (*4–6*), direct measurements of production are low and similar to those of tropical and subtropical oligotrophic oceans (*7–10*). Primary production in the Antarctic is generally measured only during the austral summer and is often based on single, midday measurements (*7, 10*); thus the influence of diel periodicity of photosynthesis on estimates of daily production has not been considered (*11–13*).

Diel periodicity in photosynthesis is a common characteristic of temperate and tropical phytoplankton (*12–14*), and temporal changes in photosynthetic characteristics can significantly influence estimates of areal production (*12, 15*). Polar phytoplankton are subject to a unique photic regime where 4-month periods of continual darkness or light are separated by 2-month transition periods when the photoperiod changes by about 20 minutes a day. Thus the diel patterns of photosynthesis of phytoplankton in polar regions could differ from those of phytoplankton in temperate and tropical regions that evolved under regularly alternating periods of light and dark (*16*). Departures of maximum photosynthetic capacity from the midday period could account for part of the discrepancy between direct [carbon-14 (^{14}C)] and indirect estimates of primary production.

We have found that phytoplankton from McMurdo Sound, Antarctica, show diel periodicity in rates of both light-limited and light-saturated photosynthesis. Maximum photosynthetic capacity, P_{max} (*17*), shifted from about noon in early and mid-September to midnight by late October. Throughout the austral summer, minimum photosynthetic capacity, P_{min} (*17*), occurred midday and the P_{max}:P_{min} ratio was about 15. Primary production estimated from time-of-

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