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Chloroquine Uptake by *Plasmodium falciparum*-Infected Human Erythrocytes During In Vitro Culture and Its Relationship to Chloroquine Resistance

FRANÇOISE VERDIER,[1]* JACQUES LE BRAS,[2] FRANÇOISE CLAVIER,[1] ISABELLE HATIN,[2] AND MARIE-CLAUDE BLAYO[1]

Institut National de la Santé et de la Recherche Médicale, Unité 13,[1] and Institut de Médecine et d'Epidémiologie Tropicales,[2] Hôpital Claude Bernard, 75944 Paris Cédex 19, France

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Chloroquine uptake by *Plasmodium falciparum*-infected human erythrocytes (RBC) was studied in vitro before and during culture by measuring the chloroquine gradient between the cells and medium (C/M) by high-pressure liquid chromatography. The C/M values were 5.9 ± 2.7 (n = 23) for uninfected RBC, 13 to 34 for six chloroquine-susceptible isolates (concentration required to inhibit 50% of parasite growth, <100 nmol/liter) in partially infected RBC (parasitemia from 0.3 to 5%) (n = 28), and 8.4 to 4.9 for four chloroquine-resistant isolates (concentration required to inhibit 50% of parasite growth, 320 to 1,500 nmol/liter) in partially infected RBC (parasitemia from 0.4 to 5%) (n = 26). Two isolates were studied before and after adaptation to continuous culture. C/M was found to decrease (34.2 to 2.1 and 19.3 to 4.9), whereas the concentration required to inhibit 50% of parasite growth increased (35 to 1,400 and 54 to 1,500 nmol/liter), thus indicating the acquisition of chloroquine resistance. These results demonstrate that chloroquine uptake decreased in RBC in which the infective strain, initially susceptible, became resistant in culture and imply that the drug is bound to ferriprotoporphyrin IX to a lesser extent or that a parasite protein competes with ferriprotoporphyrin IX to a greater extent. We suggest that genotypic modifications in the mechanism of chloroquine uptake might occur in the parasite.

The rapid activity of antimalarial drugs is related to their high binding capacity to infected erythrocytes (RBC). The high accumulation of chloroquine in *Plasmodium berghei*-infected RBC was first demonstrated by Macomber (14). This was confirmed by Fitch (4), who showed that the accumulation was decreased when the *P. berghei* strains were chloroquine resistant. Later, Fitch confirmed the decrease in chloroquine uptake by *Aotus trivirgatus* RBC infected with chloroquine-resistant *Plasmodium falciparum* (5).

The difference in chloroquine uptake might be due to lower penetration across infected membranes of RBC (variations in permeability), a difference in binding to the intraerythrocytic receptor ferriprotoporphyrin IX (FP), to which chloroquine is bound with high affinity (3, 8), or both.

In our laboratory, we maintain long-term continuous cultures of *P. falciparum* strains isolated from humans. We have shown that initially chloroquine-susceptible *P. falciparum* isolates become resistant after adaptation to culture (13). The next step was to study chloroquine uptake by RBC infected with a *P. falciparum* strain when it was initially chloroquine susceptible and when it became chloroquine resistant after adaptation to culture. This was the aim of the present work.

MATERIALS AND METHODS

Eight uncloned *P. falciparum* isolates were obtained from malarial patients treated at Claude Bernard Hospital in Paris. Six isolates from West Africa were susceptible to chloroquine in vivo and in vitro as shown by the concentration required to inhibit 50% of parasite growth (IC_{50}) of less than 100 nmol/liter (12). The other two isolates (from patients from Thailand and Tanzania) were resistant to chloroquine in vivo and in vitro (IC_{50}, 670 and 320 nmol/liter, respectively).

Isolates (uncultured specimens) were identified by a chronological number for each patient; strains (cultured for over 5 weeks) were identified by FCM (for *falciparum*) and a chronological number, also. Both identifications were followed by the country of origin.

The isolates were cultivated in RPMI 1640 with 25 mmol of $NaHCO_3$ per liter, 25 mmol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) per liter, and 10% pooled normal fresh human serum in an atmosphere of 5% O_2–6% CO_2–89% N_2 by the petri dish method of Trager and Jensen. The details are described elsewhere (11).

A 5% suspension of infected RBC (0.7 ml) was incubated in 16-mm-diameter wells with the drug. Chloroquine concentrations varied from 100 to 4,000 nmol/liter. The contact time between chloroquine and the suspension of RBC was 28 and 47 h at 37°C.

After incubation, RBC were removed from the supernatant by centrifuging at 2,000 × g for 15 min without washing to avoid chloroquine extraction. The two fractions, cells (C) and medium (M), were frozen in plastic tubes at −30°C until the chloroquine assay was done. The fractions were then thawed, diluted in distilled water, sonicated, and alkalinized with 1 N NaOH. Chloroquine was assayed by high-pressure liquid chromatography (HPLC) after extraction by dichloromethane at pH 11 (18). The organic phase was then reextracted with 0.1 N HCl, and the hydrochloric extract was injected on a μBondapak C_{18} reversed-phase column. The UV absorbance was detected at 254 nm.

Standard chloroquine curves were plotted for normal RBC and for culture medium. The RBC were lysed and diluted as described above.

* Corresponding author.

TABLE 1. Ratio of intracellular to extracellular chloroquine concentration (C/M) in normal RBC and in RBC infected with chloroquine-susceptible and chloroquine-resistant *P. falciparum*

Sample	Day of culture	IC_{50} (nmol/liter)	Parasitemia (%)	C/M (mean ± SD [*n*])
Normal RBC				5.9 ± 2.7 (23)
RBC infected with chloroquine-susceptible *P. falciparum*				
Patient no. 1 (Tanzania)	1	50	0.5	27.1 ± 8.0 (13)
Patient no. 2 (Mali)	1	65	1.0	13.4 ± 4.0 (2)
Patient no. 3 (Gabon)	1	42	0.3	26.5 ± 6.5 (3)
Patient no. 4 (Mauritania)	1	37	0.45	19.0 ± 5.2 (3)
FCM18 (Mauritania)	1	54	0.4	19.3 ± 1.6 (3)
FCM20 (Senegal)	1	35	5.0	34.2 ± 6.7 (4)
RBC infected with chloroquine-resistant *P. falciparum*				
FCM6 (Thailand)	322	980	0.6	8.0 ± 1.8 (14)
FCM18 (Mauritania)	70	1,500	0.4	4.9 ± 1.3 (5)
FCM20 (Senegal)	43	500	5.0	8.4 ± 3.3 (5)
Patient no. 5 (Tanzania)	1	320	1.5	6.2 ± 3.1 (4)

We carried out two control tests to ensure that all of the chloroquine had been recovered from RBC after extraction.

(i) A sample of RBC was overloaded with 1,000 nmol of chloroquine per liter. The chloroquine was measured by HPLC as described above after incubation at 37°C for 1 h, with shaking every 10 min.

(ii) We compared the chloroquine concentration gradient (C/M) determined by HPLC with that determined by a radioisotopic method with [^{3}H]chloroquine (2,300 nmol/liter) ([^{3}H] exchange was done by the Commissariat à l'Energie Atomique, Saclay, France). The isotopic measurements were done with an LKB 1218 Rackbeta liquid scintillation counter. The fractions (C and M) were treated as follows before counting. (i) Culture medium was bleached with an equal volume of 33% hydrogen peroxide solution and added to 4 ml of Insta-Gel (Packard Instrument S.A., Rungis, France). (ii) The RBC were dissolved with Soluene 350 (Packard) and isopropanol (1:1 dilution), bleached with 33% hydrogen peroxide solution, and then added to 4 ml of Insta-Gel with 0.5 N HCl (9:1 dilution).

RESULTS

The results of the control tests were as follows. (i) Chloroquine was recovered from the RBC ($n = 3$) at a 100% rate (1,008 ± 21 nmol/liter). (ii) Chloroquine concentration gradients (C/M) obtained by HPLC and the radioisotopic method, expressed as the mean plus or minus standard deviation of measurements done on three wells, were as follows, respectively: 2.8 ± 0.4 and 2.3 ± 0.3 for uninfected RBC; and 2.9 ± 0.7 and 3.2 ± 0.3 for a culture in which 6.4% of RBC were infected with a chloroquine-resistant strain.

Table 1 shows the C/M gradients obtained at two contact times (28 and 47 h) and at various chloroquine concentrations between 200 and 4,000 nmol/liter for normal RBC and for RBC infected with chloroquine-susceptible (patient no. 1 [Tanzania]) or chloroquine-resistant (FCM6 [Thailand]) *P. falciparum*.

Since there was no significant difference between the C/M mean values plus or minus standard deviation at 28 and 47 h (6.5 ± 3.3 [$n = 12$] and 5.2 ± 1.9 [$n = 11$] for normal RBC; 9.3 ± 2 [$n = 5$] and 7.2 ± 1.1, [$n = 9$] for chloroquine-resistant infected RBC; and 26.5 ± 7 [$n = 13$] and 27.1 ± 8 [$n = 13$] for chloroquine-susceptible infected RBC) and no significant correlation between C/M values and the chloroquine concentrations between 200 and 4,000 nmol/liter, the mean of the total C/M values was used for the group of normal RBC and for each isolate or strain for the two groups of infected RBC.

In the group of chloroquine-susceptible *P. falciparum*, all IC_{50}s were below 100 nmol/liter. The measurements were done within 2 days of blood collection, i.e., before beginning the culture (day 1). The C/M values varied between 13.4 and 34.2 for parasitemias from 0.3 to 5%, with chloroquine concentrations between 200 and 4,000 nmol/liter in the isolate from patient no. 1 (Tanzania) and two different concentrations (100 to 400 nmol/liter) for the other isolates or strains.

In the group of chloroquine-resistant *P. falciparum*, all IC_{50}s were above 100 nmol/liter (320 to 1,500 nmol/liter). The measurements were done after adaptation to culture (day 43 to 322), except for one which was done on an initially chloroquine-resistant isolate (day 1). The C/M values varied from 4.9 to 8 for parasitemias between 0.4 and 5%, with chloroquine concentrations between 200 and 4,000 nmol/liter for FCM6 (Thailand) and at least three different concentrations (200 to 4,000 nmol/liter) for the other isolates or strains.

Figure 1 shows the C/M values in RBC infected with *P. falciparum* before and after adaptation to culture. At day 1, both strains (FCM18 and FCM20) were chloroquine susceptible (IC_{50}, 54 and 35 nmol/liter, respectively). At the same time, the corresponding C/M values were 19.3 and 34.2 for parasitemias of 0.4 and 5%, with chloroquine concentrations from 100 to 400 nmol/liter with chloroquine-susceptible *P. falciparum* and 200 to 4,000 nmol/liter with chloroquine-resistant *P. falciparum*. After adaptation to culture, the IC_{50} and C/M values were 1,500 nmol/liter and 4.9, respectively, for FCM18 on day 70. For FCM20, the IC_{50} and C/M values were 500 nmol/liter and 8.4, respectively, on day 43 and 1,400 nmol/liter and 2.1, respectively, on day 68.

The C/M values were compared in normal RBC, in RBC infected with a chloroquine-susceptible strain, and in RBC infected with a chloroquine-resistant strain.

There was a significant difference in the mean C/M values plus or minus standard deviation between (i) normal RBC (5.9 ± 2.7 [$n = 23$]) and RBC infected with a chloroquine-susceptible isolate from patient no. 1 (Tanzania) (27.1 ± 8.0 [$n = 13$]) ($P < 0.001$); (ii) normal RBC (5.9 ± 2.7 [$n = 23$]) and RBC infected with a chloroquine-resistant strain, FCM6

(Thailand) (8.0 ± 1.8 [n = 14]) ($P < 0.02$); and (iii) RBC infected with a chloroquine-susceptible isolate from patient no. 1 (Tanzania) and with a chloroquine-resistant strain (FCM6 [Thailand]) at similar parasitemias (0.5 and 0.6%, respectively) ($P < 0.001$).

DISCUSSION

The results of the two control tests done to verify the chloroquine extraction from the RBC show that all of the drug is extracted with our method. The fact that chloroquine has a high affinity for its receptor ($K_d = 10^{-8}$ M) (4) does not prevent total recovery of intracellular chloroquine under these conditions. Chloroquine uptake by RBC is known to be rapid (less than 30 min) (5). However, we chose contact times of 28 and 47 h (Table 1) to correspond to the in vitro activity tests. Thus, there was no significant difference between the C/M values for these contact times.

RBC infected with chloroquine-susceptible *P. falciparum* accumulate more chloroquine than RBC infected with chloroquine-resistant strains (Table 1). In addition, the evolution of a strain during the weeks of culture at a constant parasitemia level is indicated by the acquisition of chloroquine resistance (IC_{50} increase) and a decrease of chloroquine uptake to a level similar to that of uninfected RBC. The plasmodial behavior might be modified by the in vitro conditions; clonal selection might occur during culture. Despite this, the low C/M gradient in both infected RBC after long-term culture and RBC infected with a naturally chloroquine-resistant strain has demonstrated that chloroquine uptake is greater in chloroquine-susceptible strains than in resistant ones. This may involve the penetration of chloroquine across the membranes of the RBC, the biochemical pathways of the parasite itself, or both. Indeed, various alterations of infected membranes have been recently described by several authors. For example, the normal membrane, which is impermeable to substrates such as sorbitol, becomes permeable when infected (9); electron transport is modified (16, 17); the components of the surface membrane are altered (10); and the structure itself is modified (presence of knobs) (1). However, the relationship between these modifications and chloroquine resistance is still not clearly understood.

The degradation of hemoglobin is a major source of amino acid for the parasite. This catabolism results in the formation of the malarial pigment in which FP is trapped. On account of its soluble heme binders, the parasite forms a nontoxic complex with FP (2) which is then stored in its food vacuole. Chloroquine acts by diverting FP complexes with soluble parasitic products into a toxic FP-chloroquine complex which impairs the ability of the parasite and the host RBC to maintain cation gradients. The parasite dies because of the ionic changes or because of outright lysis (6).

FP production is increased by the digestive processes of the parasite (19). In a study on *P. berghei*, Mahoney and Eaton (15) showed that chloroquine-susceptible and -resistant strains had different capacities of degrading hemoglobin (i.e., producing FP). They related this phenomenon to a modification in protease activity. In recent work, however, Yayon et al. (20) demonstrated that both chloroquine-susceptible and -resistant strains of *P. berghei* digested hemoglobin and concluded that chloroquine resistance cannot be related to a lack of FP production. FP produced by chloroquine-resistant parasites is unable to bind chloroquine with high affinity. This might be due to an increase in the intra-erythrocytic heme binders of the parasite and an increase of the FP sequestration phenomenon (6, 7).

In our work, the decrease of chloroquine uptake at low constant parasitemia levels by *P. falciparum*-infected human RBC during long-term culture (i.e., after the acquisition of chloroquine resistance) supports the hypothesis that FP produced by chloroquine-resistant parasites does not bind chloroquine to a great extent. In the *P. berghei* models, parasitemia was usually high (50 to 80%), lysis of infected and uninfected RBC was probably considerable, and the chloroquine fixation was partly extra-erythrocytic. In our *P. falciparum* model on human RBC, the low parasitemia was similar to that in malarial patients. The lysis of the RBC was very low, and the extracellular chloroquine fixation by oxidized derivatives of hemoglobin was unlikely.

The similar behavior regarding chloroquine uptake by human RBC, whether infected with a naturally resistant strain of *P. falciparum* or with an initially chloroquine-susceptible strain which became resistant without drug pressure during culture, might suggest that the parasite undergoes genotypic modifications. These results have encouraged us to compare the behavior of other amino-4-quinolines in relation to drug uptake. We are studying these questions in our current research.

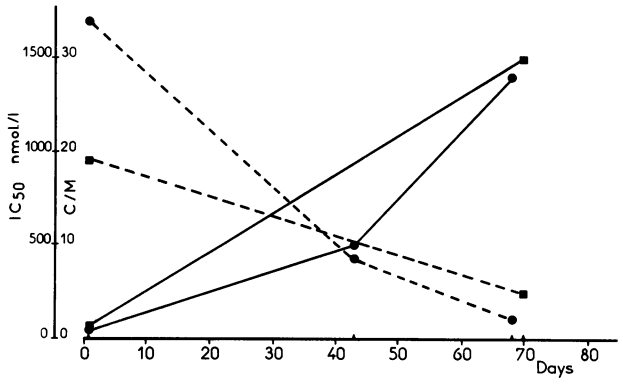

FIG. 1. Variations of C/M values (broken lines) and IC_{50}s (solid lines) before and after adaptation to in vitro culture. [Isolates from two *P. falciparum* strains with parasitemia of 0.4% for FCM18 (●) and 5% for FCM20 (■).]

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