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Inoculum Effect with Chloroquine and *Plasmodium falciparum*

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In the studies reported here, we examined the inoculum effect observed with chloroquine and *Plasmodium falciparum*. The 50% effective doses observed with both chloroquine-susceptible and -resistant parasites increased five- to sevenfold from their baseline values as the inoculum was increased from 2×10^5 to 2×10^7 parasitized erythrocytes per ml (parasitemias of 0.1 to 10% with a hematocrit of 2%). Increasing the inoculum also decreased the chloroquine uptake per parasitized erythrocyte 15- to 20-fold with both chloroquine-susceptible and -resistant parasites. However, because of the 100-fold increase in the inoculum, the total amount of chloroquine taken up actually increased sufficiently to reduce the extracellular chloroquine concentration in vitro by 60 to 90%. These studies suggest that a chloroquine uptake of $\geq$2.0 pmol/10^6 parasitized erythrocytes is necessary for chloroquine to inhibit parasite growth. More marked reductions in the amount of chloroquine uptake per parasitized erythrocyte were observed with a hematocrit of 40% using similar parasitemias of 0.1 to 10% (inocula of 4×10^6 to 4×10^8 parasitized erythrocytes per ml). Thin-layer chromatography of [^{3}H]chloroquine taken up by chloroquine-resistant *P. falciparum* revealed no evidence of drug alteration by the parasite. These studies define the mechanism responsible for the inoculum effect observed with chloroquine and *P. falciparum* in vitro.

The term inoculum effect refers to an increase in the amount of drug necessary to inhibit microbial growth (i.e., an apparent decrease in drug activity) with greater numbers of microorganisms per milliliter. This phenomenon has been most thoroughly studied with β-lactams and has been shown to correlate well with the production of β-lactamase active against the β-lactam being studied. For instance, the MIC of cefazolin for facultative gram-negative bacilli increases from 2 to >32 μg/ml and that of penicillin G for *Staphylococcus aureus* from <0.5 to 1,250 μg/ml as the inoculum is increased from 10^3 to 10^7 CFU/ml (24, 26).

Although previous studies of the inoculum effect have concentrated on bacteria in general and on β-lactams in particular, inoculum effects may occur with a much greater variety of microorganisms and antimicrobial agents. In the studies reported here, we examined the antiplasmodial activity of chloroquine against *Plasmodium falciparum* in vitro and defined an inoculum effect with both chloroquine-susceptible and chloroquine-resistant parasites. Because the antiplasmodial activity of chloroquine is associated with its uptake and concentration in the acid vesicles of the parasite (13; D. J. Krogstad, I. Y. Gluzman, and P. H. Schlesinger, submitted for publication), we also measured the amount of [^{3}H]chloroquine taken up by the parasite at different inoculum levels. These studies indicate that the inoculum effect observed with chloroquine and *P. falciparum* in vitro is associated with depletion of the available chloroquine in the extracellular medium.

MATERIALS AND METHODS

Parasite strains and culture conditions. The two *P. falciparum* strains used for these studies were originally provided by Phuc Nguyen-Dinh and C. C. Campbell of the Centers for Disease Control: the chloroquine-resistant Indochina I/CDC strain (18) and the chloroquine-susceptible Haiti 135 strain (25). The parasites were grown in erythrocyte suspensions in vitro with the medium developed by Trager and Jensen (27) with 10% human serum, 0.2% $NaHCO_3$, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Cultures were incubated at 37°C in an atmosphere with 3% oxygen and 3% carbon dioxide (balance nitrogen) in modular incubation chambers (Billups Rothenberg, Inc., Del Mar, Calif.) (19) by gassing for 3 to 5 min with a commercially prepared gas mixture (Linde Division, Union Carbide, New York, N.Y.) whose composition had been confirmed previously by mass spectroscopy. Parasitemias (the percentage of erythrocytes containing parasites) were determined by examination of Giemsa-stained smears.

Susceptibility testing. Susceptibility testing was performed by a modification of the methods developed by Desjardins et al. (7) and Pfaller and Krogstad (19) and was based on the ability of chloroquine to inhibit uptake of [^{3}H]hypoxanthine by the parasite. Previous studies have shown that estimates of drug activity obtained with this method are similar to those obtained by quantitative parasite counts (23). In this protocol, synchronous cultures containing 6- to 12-h ring stage parasites (0.6 ml of a 2% [vol/vol] erythrocyte suspension with parasitemias of 0.1 to 10%, 2×10^5 to 2×10^7 parasitized erythrocytes per ml) (14, 20) in culture medium are added to the wells of a tissue culture plate (Linbro, Hamden, Conn.) and incubated for 24 h after replacing the initial (drug-free) medium with medium containing the desired final chloroquine concentrations. The next day (at +24 h), the culture medium is aspirated from over the settled erythrocyte layer and replaced with fresh medium containing 1.33 times the desired final chloroquine concentration. After mixing, 150-μl samples of these suspensions are added to 50 μl of medium containing 0.5 μCi of [^{3}H]hypoxanthine (1.0 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) without

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chloroquine in the wells of a microtiter plate (Linbro). This produces the desired final chloroquine concentrations and 0.5 μCi of [^{3}H]hypoxanthine in each microtiter well. After an additional 12 h of incubation (at +36 h), the contents of the microtiter wells (late trophozoite and schizont stage parasites) are harvested onto 11-mm-diameter glass fiber filter paper disks (Reeve-Angel 934 AH; Whatman, Inc., Clifton, N.J.) with a cell harvester (Titertek Cell Harvester; Flow Laboratories, McLean, Va.). The filter papers are then dried, placed in glass scintillation vials (Research Products International, Mt. Prospect, Ill.) with 8 ml of counting fluid (Ready Solv MP; Beckman, Fullerton, Calif.), and counted in a liquid scintillation counter (LS 8000; Beckman). The effect of chloroquine on parasite growth was estimated by its ability to inhibit [^{3}H]hypoxanthine uptake relative to that of untreated (control) parasitized erythrocyte suspensions (0% inhibition). Similar suspensions of unparasitized erythrocytes were included in each experiment to control for the [^{3}H]hypoxanthine uptake of unparasitized erythrocytes, which was less than 1% of the uptake of the parasitized (untreated) erythrocyte suspensions. The effect of chloroquine on parasite growth was quantitated by calculating the 50% effective dose (ED_{50}) (using linear regression and the method of Litchfield and Wilcoxon) (4, 15). The percent inhibition of [^{3}H]hypoxanthine uptake was related to the $\log_{10}$ of the chloroquine concentration, since the effect of chloroquine and other antimalarial agents on parasite growth is a semilogarithmic function over the active range of drug concentrations (23), as are the effects of other antimicrobial agents (11).

Measurement of [^{3}H]chloroquine uptake. In these experiments, four parts of culture medium containing parasitized erythrocytes (at a hematocrit of 2.5%) were mixed with one part of medium containing [^{3}H]chloroquine. The [^{3}H]chloroquine-containing medium had 5 nM [^{3}H]chloroquine (51 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and either 45 or 500 nM unlabeled chloroquine. Thus, the final mixtures contained a 2% (vol/vol) suspension of parasitized and unparasitized erythrocytes, 1 nM [^{3}H]chloroquine, and total chloroquine concentrations of either 10 or 100 nM.

These suspensions were then incubated at 37°C for either 2 or 4 h, based on previous experiments which showed that [^{3}H]chloroquine uptake reached steady state in 1 to 2 h with the Haiti 135 strain and in 3 to 4 h with Indochina I (Krogstad et al., submitted. Chloroquine uptake was determined by subtraction after measuring the chloroquine remaining in the medium by counting 50-μl portions of the supernatant after centrifugation for 30 s at 2,000 × *g* and room temperature. This protocol was based on previous studies which established that more than 95% of the counts could be accounted for by adding those in the erythrocyte pellet to the counts remaining in the supernatant (Krogstad et al., submitted). Each experiment included controls with a 2% (vol/vol) suspension of unparasitized erythrocytes. To calculate the [^{3}H]chloroquine uptake due to the parasite, the [^{3}H]chloroquine uptake of the unparasitized erythrocyte controls (~0.01 pmol/10^6 unparasitized erythrocytes) was subtracted from the [^{3}H]chloroquine uptake of the parasitized erythrocyte suspensions.

Measurement of chloroquine uptake at a hematocrit of 40%. To estimate the potential chloroquine uptake of parasitized erythrocytes in vivo, we used parasitemias similar to those tested above (from 0.1 to 10%) with a hematocrit of 40% (4 × 10^6 to 4 × 10^8 parasitized erythrocytes per ml). In these experiments, the total chloroquine concentrations tested were chosen to reflect those achieved in vivo with either chemoprophylaxis (100 nM) or treatment (300 and 1,000 nM) (1, 3, 10, 16).

TLC studies of chloroquine. Chloroquine was extracted from culture medium by a modification of the method of Essien and Afamefuna (8). In this procedure, 1 volume of medium and 1 volume of NH_4OH (1.0 N) are mixed with 2 volumes of $CHCl_3$, vortexed at high speed for 2 min, and separated by centrifugation for 2 min at 11,000 × *g* and room temperature. The chloroform-containing supernatant is aspirated, and the procedure is repeated a total of three times. Each set of extracts (from an individual sample) is evaporated under nitrogen with an N-EVAP analytical evaporation apparatus (Organomation Associates, South Berlin, Mass.) and dissolved in 100 μl of methanol for thin-layer chromatography (TLC). TLC was performed with Silica Gel G-coated plastic sheets (Polygram Sil G; Brinkmann, Westbury, N.Y.) prepared by running with acetone and activated by heating to 75°C for 1 h in a drying over prior to use. After chromatography with a mixture of benzene-methanol-diethylamine (0.75:0.15:0.10), each lane is divided into segments (with R_f values from 0.00 to 1.00), scraped from the TLC sheet into glass test tubes, and extracted three times with 1.0 ml of $CHCl_3$-CH_3OH (1:1). These extracts are combined in scintillation vials, evaporated to dryness, and counted. Control experiments demonstrated (i) complete extraction of chloroquine and mono- and didesethyl chloroquine from the Silica Gel G TLC sheets with chloroform-methanol, as assessed by repeat TLC, and (ii) >95% recovery of [^{3}H]chloroquine counts with this technique.

Based on previous studies, which demonstrated that NH_4Cl released ≥90% of [^{3}H]chloroquine from parasitized erythrocytes within 3 to 5 min (Krogstad et al., submitted), a protocol was developed to release [^{3}H]chloroquine from parasitized erythrocytes into the culture medium for TLC. Parasitized erythrocytes were initially suspended in culture medium with 10 nM [^{3}H]chloroquine for 1 h at 37°C. They were then centrifuged and suspended in culture medium with 50 mM NH_4Cl (without chloroquine) to release the [^{3}H]chloroquine that had been taken up into the culture medium so that TLC could be performed without the confounding effects of hemolysis on the TLC system.

RESULTS

Effect of inoculum size on chloroquine susceptibility in vitro. With parasitemias from 0.1 to 10% and a hematocrit of 2% (inocula from 2 × 10^5 to 2 × 10^7 parasitized erythrocytes per ml), the ED_{50}s observed with both chloroquine-susceptible and -resistant parasites increased approximately five- to sevenfold from their baseline values (Table 1). This effect was most apparent with suspensions containing ≥5 × 10^6 parasitized erythrocytes per ml.

Effect of inoculum size on chloroquine uptake. The amount of chloroquine taken up per 10^6 parasitized erythrocytes was substantially reduced at higher inocula with both chloroquine-susceptible and -resistant parasites (Table 2). This phenomenon was more marked at an external chloroquine concentration of 10 nM with the chloroquine-susceptible Haiti strain and at a 100 nM chloroquine concentration with the resistant Indochina strain. Based on the susceptibility testing results described above (Table 1), these data suggest that a chloroquine uptake of ≥2.0 pmol/10^6 parasitized erythrocytes is necessary for chloroquine to inhibit parasite growth: 2 to 3 pmol/10^6 parasitized erythrocytes with chloroquine-susceptible parasites and 10 to 20 pmol/10^6 parasitized erythrocytes with resistant parasites (Table 2). The

TABLE 1. Effect of inoculum size on the chloroquine susceptibility of *P. falciparum* in vitro

% Parasitemia of inoculum (no. of parasitized erythrocytes/ml)	Chloroquine ED_{50} (nM)[a]	
	Haiti 135 (susceptible)	Indochina I (resistant)
0.1 (2×10^5)	2.4	42.1
0.2 (4×10^5)	2.8	47.0
0.5 (1×10^6)	6.6	50.2
1.0 (2×10^6)	6.8	55.0
2.5 (5×10^6)	9.3	72.6
5.0 (1×10^7)	11.0	116.0
10.0 (2×10^7)	14.6	192.0

[a] Each well contained a 2% (vol/vol) suspension of erythrocytes with parasitemias from 0.1 to 10.0%. The ED_{50} was determined by inhibition of [^{3}H]hypoxanthine uptake (see text for details).

amount of chloroquine taken up by parasitized erythrocytes in these experiments was sufficient to lower the total extracellular chloroquine concentration from 10 to 1.1 to 2.8 nM and from 100 to 9.9 to 39 nM with chloroquine-susceptible and -resistant parasites, respectively. The most marked reductions in the extracellular chloroquine concentration were observed with 2×10^7 parasitized erythrocytes per ml and the smallest changes with 2×10^5 parasitized erythrocytes per ml.

Chloroquine uptake at a hematocrit of 40%. With parasitemias of 0.1 to 10% and a 40% hematocrit (4×10^6 to 4×10^8 parasitized erythrocytes per ml), the amount of chloroquine taken up per parasitized erythrocyte varied with the inoculum used at total external chloroquine concentrations of 100, 300, and 1,000 nM (Fig. 1). These results suggest that this effect may be more important with chloroquine-resistant strains such as Indochina I. With this strain, total chloroquine uptakes of $\geq$2.0 pmol/10^6 parasitized erythrocytes were achieved only at 300 and 1,000 nM chloroquine with 4×10^7 parasitized erythrocytes per ml (at a parasitemia of 1%) and not at all with $>4 \times 10^7$ parasitized erythrocytes per ml. The inoculum effect was observed with both the chloroquine-susceptible Haiti strain and the resistant Indochina strain. Even with the chloroquine-susceptible Haiti strain, $\geq$2.0 pmol of chloroquine uptake per 10^6 parasitized erythrocytes was observed only at 1,000 nM chloroquine with $>4 \times 10^7$ parasitized erythrocytes per ml.

TLC studies of chloroquine. The [^{3}H]chloroquine peak was consistently found at an R_f of 0.85 in methanol alone (not shown) and in extracts of culture medium (Fig. 2). This peak also coincided with that of cold (unlabeled) chloroquine in the same solvent system, as demonstrated with I_2 (2). Material extracted from parasitized erythrocytes revealed no additional peaks consistent with chloroquine metabolites. Specifically, no ^{3}H peaks were observed at the positions of

TABLE 2. Effect of inoculum size on the chloroquine uptake of *P. falciparum* in vitro[a]

% Parasitemia of inoculum (no. of parasitized erythrocytes/ml)	Chloroquine uptake (pmol/10^6 parasitized erythrocytes)			
	Haiti 135 (susceptible)		Indochina I (resistant)	
	10 nM	100 nM	10 nM	100 nM
0.1 (2×10^5)	20.35	29.10	2.00	70.75
0.2 (4×10^5)	16.80	23.60	1.55	38.03
0.5 (1×10^6)	11.13	26.92	1.49	27.11
1.0 (2×10^6)	6.57	21.75	1.64	17.53
2.5 (5×10^6)	2.98	24.64	1.26	18.75
5.0 (1×10^7)	1.55	15.63	0.84	10.23
10.0 (2×10^7)	0.80	8.07	0.62	5.54

[a] Each well contained a 2% (vol/vol) suspension of parasitized and unparasitized erythrocytes with parasitemias from 0.1 to 10%. The final extracellular chloroquine concentrations observed were 1.1 and 9.9 nM with the Haiti 135 strain and 2.8 and 39 nM with the Indochina I strain for solutions which initially had chloroquine concentrations of 10 and 100 nM, respectively.

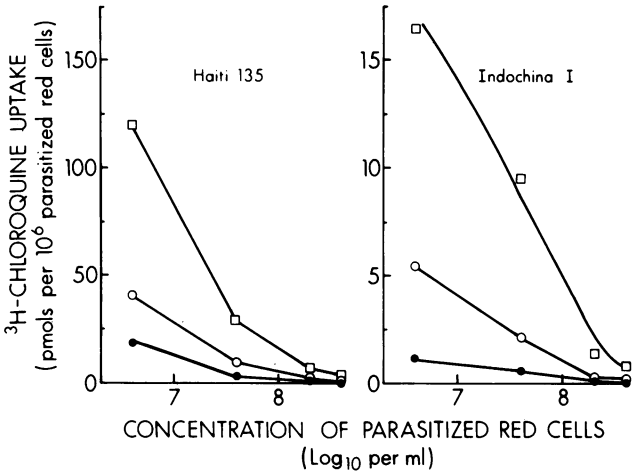

FIG. 1. Effect of inoculum size on chloroquine uptake by the Haiti 135 and Indochina I strains of *P. falciparum*. Results are shown for total external chloroquine concentrations of 1 μM (□), 300 nM (○), and 100 nM (●). These experiments were performed with a 40% (vol/vol) cell suspension. Note that the scale of the ordinate for the Indochina I strain is 10-fold less than that for the Haiti 135 strain because of the reduced chloroquine uptake observed with chloroquine-resistant parasites (Krogstad et al., submitted).

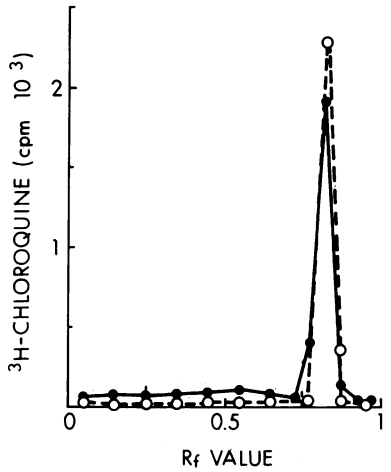

FIG. 2. Effect of parasite uptake and release of [^{3}H]chloroquine on migration in TLC plates. The ^{3}H peak observed with material extracted from parasitized erythrocytes (●) was identical in position to that observed with [^{3}H]chloroquine in the culture medium (○) and with larger amounts of unlabeled chloroquine (10 μg) visualized with I_2 (not shown). In this experiment, parasitized erythrocytes were suspended in medium with 50 mM NH_4Cl at a hematocrit of 0.5% (7.5×10^6 parasitized erythrocytes per ml) after incubation with 10 nM [^{3}H]chloroquine for 1 h to release the [^{3}H]chloroquine that had been taken up into the medium (see text for details). The position of the [^{3}H]chloroquine peak (R_f, 0.85) was distinctly different from those of the unlabelled mono- and didesethyl derivatives of chloroquine (R_fs of 0.72 and 0.45, respectively), which were also visualized with I_2.

either the mono- or didesethyl derivatives of chloroquine (R_f of 0.72 and 0.45, respectively).

DISCUSSION

The studies presented here define an inoculum effect with chloroquine and *P. falciparum* consistent with the previous suggestions of Ponnudurai et al. (21) and Rieckmann (22) and indicate that this effect occurs with both chloroquine-susceptible and -resistant parasites. They demonstrate that an inoculum effect may be produced by mechanisms other than drug-inactivating enzymes. Studies from a number of laboratories, including ours, suggest that chloroquine resistance is associated with decreased drug uptake (9; Krogstad et al., submitted). The TLC studies presented here suggest that resistant parasites do not alter chloroquine chemically, in contrast to the mechanisms that have been established for resistance to β-lactams and aminoglycosides in bacteria (6, 24, 28). However, these studies could not detect such reactions if they modified less than 15% of the drug taken up by the parasite (sensitivity of approximately 85%).

Because the relationship between in vitro test results (e.g., the chloroquine ED_{50}) and in vivo outcome is imprecise in malaria, the most appropriate inoculum for in vitro susceptibility testing cannot be unequivocally defined at this time. However, on the basis of the data presented here (Table 1), parasitemias of 0.5 to 1.0% with a hematocrit of 2% may be most reasonable with the [^{3}H]hypoxanthine test system (1×10^6 to 2×10^6 parasitized erythrocytes per ml). These parasitemias are high enough to permit the use of Giemsa-stained smears in setting up experiments and appear to below enough (based on the protocol used here) to avoid the pronounced inoculum effect observed with $\geq 1 \times 10^7$ to 2×10^7 parasitized erythrocytes per ml.

One additional important aspect of the chloroquine uptake experiments is the reduced concentration of chloroquine left in the medium after exposure to as few as 2×10^6 parasitized erythrocytes per ml (Table 2). With the chloroquine-susceptible Haiti strain, 90% of the extracellular chloroquine was removed by the parasite at an initial extracellular chloroquine concentration of 10 nM. Even the resistant Indochina strain lowered the extracellular chloroquine concentration by 60 to 70%. Because our previous studies have shown that extracellular chloroquine concentrations ≤1 nM do not increase vesicle pH or inhibit parasite growth even with chloroquine-susceptible parasites (13; Krogstad et al., submitted), these results suggest that the chloroquine depletion of the extracellular medium observed in these experiments caused the inoculum effect described in Table 1. Despite the higher chloroquine concentrations achieved in plasma with chemoprophylaxis (100 nM) or treatment (300 to 1,000 nM) (1, 3, 10, 16), inoculum-dependent phenomena may occur in vivo because the absolute concentrations of parasitized erythrocytes are substantially greater (approximately a 20-fold increase with a hematocrit of 40% versus 2%).

The experiments performed with a hematocrit of 40% indicate that higher absolute parasite counts can significantly decrease the amount of chloroquine taken up per 10^6 parasitized erythrocytes in vitro as a result of chloroquine depletion. With external chloroquine concentrations of 1,000 nM, the chloroquine uptake per cell observed with the resistant Indochina strain was low at parasitemias of 5 to 10% (<2.0 pmol/10^6 parasitized erythrocytes). This finding suggests that treating infections caused by such strains with chloroquine may not be effective and is consistent with clinical experience. Although a similar pattern was also observed with the chloroquine-susceptible Haiti strain, its chloroquine uptake was >2.0 pmol/10^6 parasitized erythrocytes with 1,000 nM chloroquine, consistent with the efficacy of chloroquine in treating infections caused by this strain.

Despite these correlations, it is essential to be cautious in drawing conclusions about the potential in vivo significance of in vitro studies such as the ones reported here. Many factors which are important in vivo can be reproduced or tested in vitro either not at all or only with great difficulty. In malaria, such factors include the total drug distribution volume (beyond that in the intravascular compartment), drug half-life and metabolism, the role of cell-mediated and humoral immunity, and the mechanical role of the spleen in removing parasitized (less deformable) erythrocytes (5; D. J. Krogstad, S. P. Sutera, C. W. Boylan, and I. Y. Gluzman, submitted for publication). Nevertheless, the findings reported here indicate that the ability of the parasite to take up chloroquine is great enough to result in chloroquine depletion (of the extracellular medium) in vitro (Table 2, Fig. 1); they suggest that it may also deplete the extracellular plasma chloroquine concentration in vivo at high parasitemia levels. We recognize that this is an unproven hypothesis. However, if this speculation is correct, the role of heroic measures such as erythrocyte exchange transfusion (17) may be, in part, to reduce the parasite concentration so that standard therapeutic doses of chloroquine will yield chloroquine levels in plasma high enough to inhibit parasite growth even after the chloroquine uptake resulting from incubation with large numbers of parasitized erythrocytes in vivo.

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