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LOCATION OF CHLOROQUINE BINDING SITES IN *PLASMODIUM BERGHEI**

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Abstract—The differential lysis of mouse erythrocytes parasitized with chloroquine-sensitive *Plasmodium berghei* lends insight into which regions of the parasitized cell are responsible for the saturable chloroquine binding processes observed at various drug concentrations. The highest affinity binding sites (affinity constant $\cong 10^8\ M^{-1}$) appear to be associated with the parasite membranes, whereas those with affinity constants of about $10^5\ M^{-1}$ are associated with the cytoplasmic fraction of the parasite.

THE ABILITY of erythrocytes infected with malaria parasites to concentrate chloroquine has recently been correlated with the susceptibility of the parasites to chloroquine therapy. Macomber[1] first suggested this relationship when he found that the ability of mouse erythrocytes parasitized with *P. berghei* to concentrate chloroquine *in vivo* was in the order: chloroquine-sensitive > chloroquine-resistant > normal red blood cells.†

Fitch[2] examined *in vitro* cells parasitized with *P. berghei* and found that the concentration process involved three classes of saturable chloroquine binding sites. These sites have intrinsic association constants with chloroquine of about 10^8, 10^5 and 10^3 M^{-1} at pH 7·2, 22°. Cells parasitized with CS *P. berghei* possessed all three sites, whereas those parasitized with the resistant strain were deficient in the binding sites with the highest affinity. He suggested that chloroquine resistance is due to a decrease in the number or accessibility of the high affinity chloroquine receptor sites. The investigation was extended to *P. falciparum* in monkeys and cells parasitized with resistant strains were similarly found to lack concentrative function.[3]

This paper examines which regions within the parasitized erythrocytes of mice might be responsible for chloroquine binding. The high affinity binding sites ($K_A \cong 10^8$ M^{-1}) appear to be associated with the parasite membranes, whereas those with affinity constants of about $10^5\ M^{-1}$ are associated with the cytoplasmic fraction of the parasite.

MATERIALS AND METHODS

The mice used in this investigation were 5–6 week old males (Walter Reed strain) weighing about 20 g.‡ Initial inocula of CS and CR *P. berghei* were prepared from

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† Chloroquine-sensitive and -resistant variants of the same strain of *P. berghei* will hereafter be referred to as CS and CR *P. berghei*, respectively.

‡ In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

mice obtained from Dr. C. D. Fitch of the Walter Reed Army Institute of Research. Both CS and CR parasites were descendents of the same parent (NYU-2) strain of *P. berghei*.[2]

The materials and methods used to prepare parasitized erythrocytes are according to Fitch,[2] with the following modifications: (1) 1:10 and 1:50 dilutions of CR and CS parasitized whole blood, respectively, were used to prepare inocula. (2) Injections of *d,l*-α-tocopherol were omitted. (3) All blood, immediately after collection in heparinized buffer, was passed through a tightly-packed 2 × 6 cm column of Whatman CF-12 fibrous cellulose[4] to remove white blood cells. (4) Parasite counts were determined as the number of parasites present in at least 500 examined red blood cells, rather than percentage parasitemia. The values were nearly identical from preparation to preparation: confidence limit of the mean (95 per cent) = 522 ± 58 parasites/500 cells. (5) Only hematocrit and wet weight were used to characterize red blood cell pellets, since determinations of total water content and inulin spaces[2] has adequately described the nature of such pellets under the conditions of these experiments.

Procedures for studying the *in vitro* incorporation of chloroquine into intact erythrocytes were also according to Fitch.[2] Plasticware and siliconized glassware were used throughout and chloroquine-3-^{14}C (New England Nuclear), specific activity 1·71 mc/m-mole, was from the batch used by Fitch.

Lysis of parasitized red blood cells was performed by incubating the cells at 37° for 30 min in 20 times their volume of 0·015% saponin (Matheson, Coleman and Bell, reagent grade) in the isotonic buffer used for incorporation studies on intact erythrocytes.[2] The parasites were collected by centrifugation at 12,100 *g* for 10 min. Light microscopy (1000×) showed the resulting preparation to be intact parasites essentially free of host cells. They are, however, probably "membrane wrapped".[5,6]

Lysis of the parasites was achieved by suspending the saponin pellet in 20 times its volume of distilled water and incubating for 45 min at 23°.[6] The parasite remains were collected by centrifugation at 12,100 *g* for 20 min. Light microscopy indicated that intact erythrocytes and parasites were absent and that only a small number of parasite nuclei remained undisrupted.

The amount of radioactivity in the supernatant fractions was determined by extracting an aliquot with heptane-isoamyl alcohol, using the procedures previously established for the incubation media of intact erythrocytes at equilibrium.[2] Pellets, regardless of their origin, were extracted in identical fashion to pellets of intact parasitized cells. In all cases, recovery of radioactivity exceeded 95 per cent.

Deoxyribonuclease (Worthington Biochemical Corp., recry. 1×) was dissolved in 0·005 M phosphate and 10^{-3} M $MgCl_2$, pH 7·4. The activity of the enzyme under these conditions toward a substrate of DNA isolated from CS *P. berghei* was verified using the ultraviolet spectrophotometric method of Kunitz.[7] Ultraviolet measurements were made using a Cary Model 14 ultraviolet spectrophotometer equipped with a thermostated cell compartment at 23°.

The amount of DNA present in parasitized cells that had been treated with saponin or distilled water (as previously described) was determined by digesting the various pellets in 1N perchloric acid for 1 hr at 70°. The supernatant was then separated by centrifugation and its deoxyribose content determined by the diphenylamine method described by Dische.[8] A standard curve was constructed by analysing known solutions of DNA isolated from *Escherichia coli*.

Binding of chloroquine to macromolecules was determined by incubating solutions of [^{14}C]chloroquine containing the macromolecules for 30 min with sufficient dry Sephadex G-25 Fine (Pharmacia Fine Chemicals) so that the swollen gel volume was at least 25 per cent of the total solution volume. The gel was then allowed to sediment and the external phase was recounted to determine if the concentration of chloroquine had increased. Chloroquine-3-[^{14}C], $5{\cdot}5 \times 10^{-6}$ M, was gel filtered (flow rate = 1 ml/min) on a 0·9-cm diameter column of Sephadex G-25 Fine (Pharmacia) under conditions comparable to those used in the preceding procedure (pH 7·4, $\mu = 0{\cdot}1$) in order to check for binding of the free drug to the gel matrix. Blue Dextran 2000 was employed to determine the external volume and the column effluent was monitored by liquid scintillation spectrometry.

Light microscopy was performed on thin smears made on glass slides and stained with Giemsa stain.

Xylene: dioxane: 2-ethoxyethanol, 1:3:3, counting cocktail[9] was used to count radioactivity on either a Nuclear Chicago Model 724 or Packard Tri Carb Model 3380 liquid scintillation spectrometer.

The binding data were fitted to a hyperbolic equation of the form $y = K_1x/(K_2 + x)$ by the non-linear regression techniques of Tyson[10] using a digital computer. K_1 represents the maximal binding capacity and the reciprocal of K_2 the affinity constant for the interaction. The ordinates of all plots are based on the weight of the intact red blood cells. They are plotted as a function of the equilibrium chloroquine concentration external to the cells prior to lysis. All values of these external (supernatant) concentrations were the average of three independent determinations.

RESULTS AND DISCUSSION

Mouse erythrocytes parasitized with CS *P. berghei* concentrate chloroquine when incubated *in vitro* with $2\text{–}100 \times 10^{-8}$ M chloroquine-3-^{14}C. The distribution of the external and the bound chloroquine concentrations at equilibrium (Fig. 1) is in agreement with the data of Fitch[2] indicating that, under these conditions, chloroquine has interacted predominantly with the high affinity binding sites.

When these cells are lysed with saponin, chloroquine remains bound to the membrane-wrapped parasites and almost no chloroquine is released into the medium (Fig. 1). Furthermore, if the parasitized erythrocytes are first lysed with saponin and then incubated with [^{14}C]chloroquine, the same degree of chloroquine uptake as for the intact parasitized erythrocytes is observed (Table 1). Thus, at these low concentrations of chloroquine, neither hemoglobin nor other erythrocyte contents seem to be involved in the binding process.

When CS parasitized erythrocytes are equilibrated with the same low concentrations of drug used above and then lysed sequentially with saponin and distilled water, the majority of the chloroquine stays with the membranous pellet obtained by centrifugation at 12,100 *g* for 20 min (Fig. 2). This indicates that high affinity binding is associated with an insoluble portion of the parasite itself, the red blood cell membrane, or both. When the above lysis is performed using 10^{-3} M $MgCl_2$ in place of the distilled water the results are identical to those obtained in the absence of $MgCl_2$.

The hyperbolic equation parameters listed in Table 2 show that the intact cells and the water lysis pellet have nearly the same affinity constant (about 5×10^7 M^{-1}) for

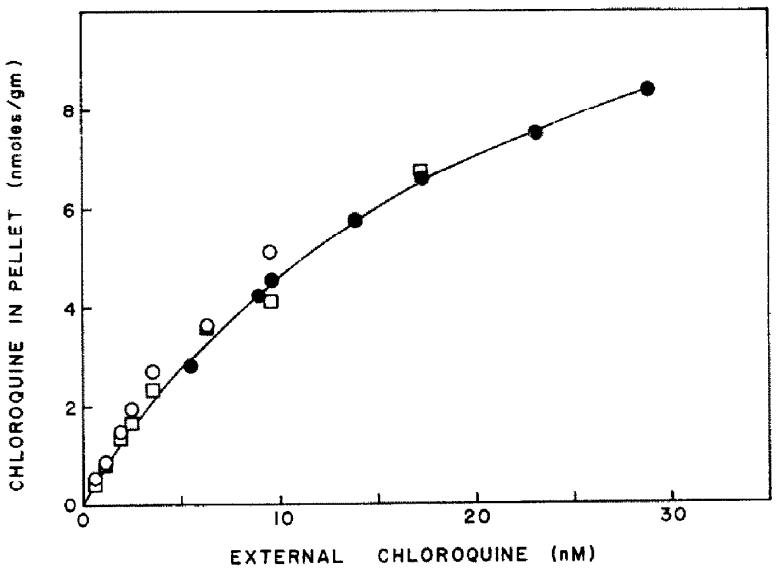

FIG. 1. The effect of saponin lysis on high affinity chloroquine binding in CS *P. berghei*. The equilibrium distribution of chloroquine between the cells (before and after lysis) and the medium external to these cells. Each point represents a separate incubation. Two preparations of intact cells prior to lysis, ○, ●; the cells represented by unfilled circles after incubation in 20 times their volume of saponin (0·015%, 37°, 30 min), □.

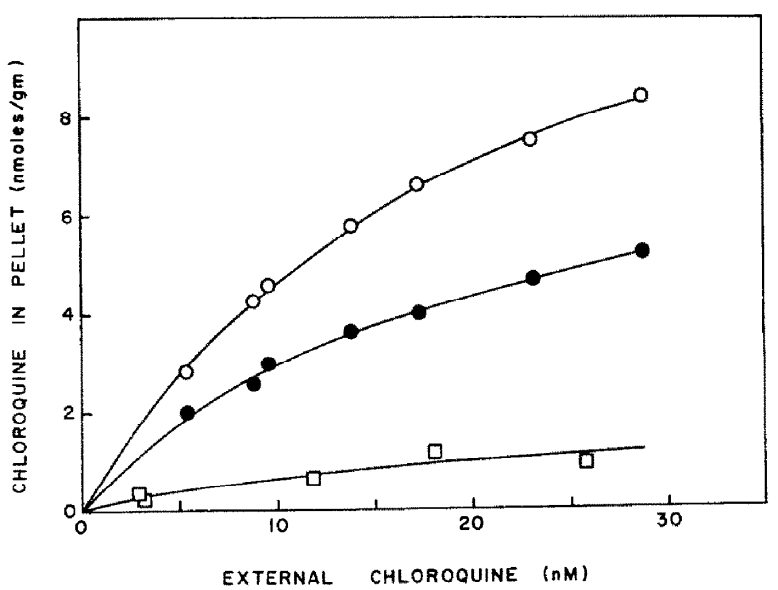

FIG. 2. The effect of sequential saponin and distilled water lyses on high affinity chloroquine binding in erythrocytes parasitized with *P. berghei*. The equilibrium distribution of chloroquine between the cells (before and after lysis) and the medium external to these cells. Each point represents a separate incubation. Intact cells prior to lysis, ○; cells from the same preparation after incubation in 20 times their volume of saponin (0·015%, 37°, 30 min) followed by distilled water (23°, 45 min), ●; cells parasitized with CR *P. berghei* after saponin and distilled water lyses, □.

TABLE 1. EFFECT OF SAPONIN LYSIS ON HIGH-AFFINITY CHLOROQUINE-BINDING IN CS *P. berghei*

Total concentration chloroquine in system (μM)	Nanomoles chloroquine present in pellet/g intact cells		
	No treatment	Saponin after* addition of drug	Saponin before* addition of drug
0·211	5·13	4·07	4·33
0·105	2·70	2·27	2·21
0·063	1·48	1·30	1·30

* 37°, 30 min, 0·015% saponin. Basis for pellet is wet weight of original intact red blood cells.

the drug, but the maximal binding capacity of the parasite membranes is only 60–65 per cent that of the intact parasitized cells. Some of this reduction in binding capacity is accountable by the losses encountered in the centrifugation procedure.

DNA determinations on the various pellets show that the parasites obtained by lysis of the cells with saponin contain about 4×10^{-14} g DNA/parasite and only 10–15 per cent of this DNA is lost during lysis with 10^{-3} $MgCl_2$. This indicates that the parasite DNA is bound to the membranous fragments of the parasite or that the hypotonic lysis procedure does not rupture as many parasite nuclei as the examination by light microscopy seems to indicate. Both of these factors probably contribute to the large percentage of DNA found in the lysis pellet. Treatment with deoxyribonuclease in 10^{-3} M $MgCl_2$ (about 10^{-14} g enzyme/parasite) reduces the amount of deoxyribose associated with the pellet by 50–60 per cent, but does *not* solubilize chloroquine from the water-lysed pellet (Table 3).

While these results do not rule out a role for DNA in high affinity chloroquine binding, the apparent intrinsic association constants (Table 2) are several orders of magnitude higher than the apparent constants of 10^3–10^5 M^{-1} which have been reported for the binding of chloroquine to purified DNA *in vitro*.[11,12,13] Moreover,

TABLE 2. COMPUTER-GENERATED PARAMETERS FOR THE HYPERBOLIC EQUATION $y = K_1x/(K_2 + x)$

Run	High affinity binding intact cells		Water-lysis pellet	
	K_1 (nmoles/g)	K_2 (nM)	K_1 (nmoles/g)	K_2 (nM)
1	20·4	26·7	9·40	18·4
2	13·6	19·4	—	—
3	14·7	22·0	8·58	19·2
4	—	—	7·87	18·9
1, 2, 3, combined*	13·6	17·9	—	—
1, 3, 4, combined*	—	—	7·95	16·6

* Curve fitted to the aggregate values from three runs.

TABLE 3. THE EFFECT OF SALT AND DEOXYRIBONUCLEASE ON CHLOROQUINE-BINDING IN CS *P. Berghei*

Treatment	Percentage of chloroquine remaining in water-lysed parasite pellet (nmoles/g intact parasitized cells)
None	100
Water wash	96
KCl wash, 0·1 M	34
KCl wash, 0·3 M	27
KCl wash, 0·6 M	22†
Deoxyribonuclease*	99†

* 5 μg/ml, 23°, 45 min, 10^{-3} M Mg^{2+}, $\mu = 0{\cdot}013$.
† Two determinations.

treatment of the membranous pellet with deoxyribonuclease does not reduce bound chloroquine in proportion to the reduction in DNA content. If DNA associated with the membrane pellet is responsible for the high affinity binding, it would have to behave differently from purified DNA in both these properties. There is, however, no data to indicate whether or not deoxyribonuclease is effective against DNA containing intercalated chloroquine.

Normal red cell membranes are extremely ineffective in binding chloroquine at low drug concentrations. Only 0·31 nmoles of chloroquine/g intact erythrocytes are retained at an external chloroquine concentration of 156 nM. This value is less than 5 per cent of that obtained for the CS parasite membranes at comparable drug concentrations and argues against the red cell membrane playing a major role in high affinity binding. Structural alterations in the red cell membrane have been observed following infection,[15] and one might speculate that erythrocyte membranes thus altered possess an enhanced binding capacity. The data, however, are most consistent with the interpretation that the high affinity chloroquine binding sites are associated with membranous remains of the parasite.

A single washing of the water lysis pellet with 0·6 M KCl removes over twenty times more drug than that obtained with distilled water wash. The amount of drug removed by the salt solution is a function of its concentration and increases with increasing ionic strength (Table 2). Sephadex G-25 was utilized to determine whether or not the chloroquine thus released by the KCl solution was still bound to macromolecules. Free chloroquine itself does not adhere to the gel: under the conditions of pH, temperature and ionic strength used in the wash procedure, the drug has a partition coefficient (K_D) of 1·1 on a column of Sephadex G-25.[14] When unswollen (dry) Sephadex is added to the above KCl wash solutions, an increase in drug concentration should be observed in the external phase if the chloroquine is bound to molecules of molecular weight greater than 5000 since it would be unable to accompany water molecules into the gel matrix. However, no change in the concentration of chloroquine is observed, suggesting that chloroquine has been dissociated from its binding sites by the salt solution. Alternatively, if the KCl solution has dissociated the complex of chloroquine and its binding sites from the membranous pellet, the binding molecules must be sufficiently small so as to enter the Sephadex matrix.

Two preparations of mouse erythrocytes parasitized with CR *P. berghei* were also examined at the chloroquine concentrations used above to observe high affinity binding in CS *P. berghei.* The maximal binding capacity of the intact cells on *in vitro* incubation with chloroquine is about 50 per cent of that observed with CS parasites, a value greater than that observed by Fitch.[2] The effect of differential lysis on the cells parasitized with CR *P. berghei* is qualitatively similar to that for the CS *P. berghei* system. Saponin lysis again does not release chloroquine and combined saponin and water lysis releases about 40 per cent of the drug. As shown in Fig. 2, this results in water lysis pellets containing less than 25 per cent the amount of chloroquine observed in their CS counterparts. While it has not been definitely established that the same binding sites are responsible for chloroquine binding by both the CR and CS parasites in this concentration range, the amount of chloroquine bound by CR *P. berghei* represents an estimate of the maximum possible contribution of the high affinity sites. One can, therefore, conclude that if the high affinity binding sites on the CS parasite membranes are indeed present in the resistant strain, they must be either less numerous or less efficient.

In order to determine the location of those binding sites which have affinity constants of about 10^5 M^{-1}, similar experiments were conducted on the effects of differential lysis on the equilibrium distribution of the drug. Erythrocytes parasitized with CS *P. berghei* are equilibrated with isotonic buffer solutions containing 1–40 mM chloroquine. The resulting equilibrium distributions of the drug between the cells and medium (Fig. 3) confirm the observations of Fitch[2] that chloroquine interacts predominantly with a saturable middle affinity site at these chloroquine concentrations.

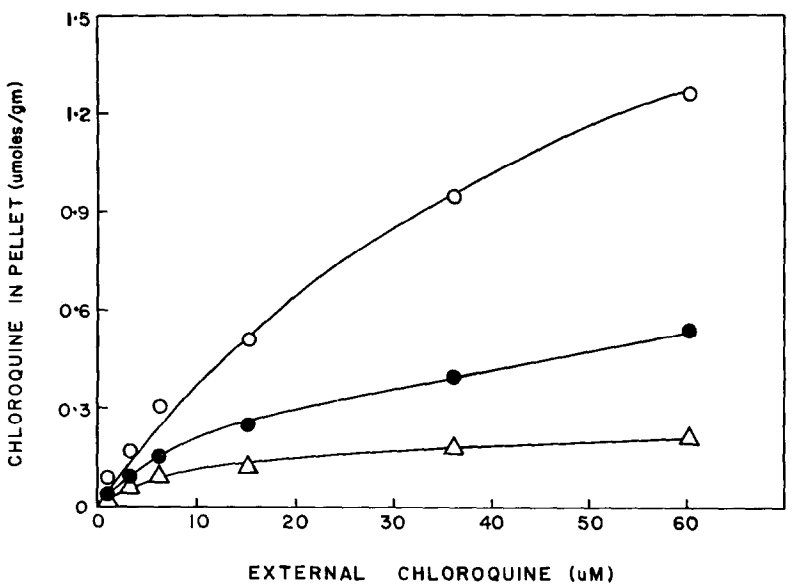

FIG. 3. The effect of lysis on middle affinity chloroquine binding in erythrocytes parasitized with CS *P. berghei.* The equilibrium distribution of chloroquine between the cells (before and after lysis) and medium external to these cells. Each point represents a separate incubation, but all points were obtained from the same preparation of blood. Intact cells prior to lysis, ○; cells after lysis in 20 times their volume of saponin (0·015%, 37°, 30 min), ●; cells after sequential lysis in saponin and distilled water (23°, 45 min), △.

Saponin lysis of these cells releases about half of the chloroquine originally present (Fig. 3). This is far greater than the 10–15 per cent released by this procedure at the low chloroquine concentrations where high affinity binding alone is operative. The contribution of the red blood cell contents towards binding cannot be determined from these data alone since saponin results also in the release of parasite cytoplasm and the lysis of some parasites.[5,6] Such an occurrence does not affect the high affinity sites (Fig. 1), presumably because of their membranous nature, but can account for a fraction of the chloroquine released by saponin at these higher drug concentrations.

Saponin lysis combined with a subsequent water lysis of the parasites releases about 85 per cent of the original drug into the medium: less than 15 per cent sediments with the parasite membranes after centrifugation at 12,100 *g* for 20 min. The maximal binding capacity (K_1) for the intact cells is 1·80 μmoles/g while that of the water lysis pellet is only 0·263 μmoles/g. The dissociation constants (K_2) of chloroquine from these two pellets also differ. A value of 41·5 μM is obtained for the intact cells while that for the water lysis pellet is 15·3 μM, probably reflecting the fact that both high and middle affinity binding are operative in this concentration range and one of the sites is being removed.

This lysis also does not release appreciable DNA (see above). This finding and the above data, together with the fact that intact normal erythrocytes show a clear lack of these middle affinity sites,[2] indicate that the more likely location of the middle affinity site is within the parasite cytoplasm.

It appears, therefore, that distinctly separable, subcellular components are responsible for the processes by which mouse erythrocytes parasitized with CS *P. berghei* concentrate chloroquine. High affinity binding appears to be associated with the parasite membranes and middle affinity binding with the parasite cytoplasm. The lowest affinity process ($K_A = 10^3\ M^{-1}$) presumably is in the erythrocyte itself since it is already present in unparasitized cells.[2] The altered concentrative function, and possibly the receptor itself, observed for high affinity binding of the chloroquine resistant parasites can, therefore, probably best be studied by investigating the binding of chloroquine to the parasite membranes.

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