Chloroquine-Resistant *Plasmodium falciparum*: Effect of Substrate on Chloroquine and Amodiaquin Accumulation

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Glucose stimulates the high-affinity processes of chloroquine and amodiaquin accumulation in owl monkey erythrocytes infected with a chloroquine-susceptible strain of *Plasmodium falciparum*. Although these erythrocytes have greater ability to accumulate amodiaquin than chloroquine, glucose has relatively less effect on amodiaquin accumulation than on chloroquine accumulation. In contrast to these findings with chloroquine-susceptible *P. falciparum*, glucose stimulates amodiaquin but not chloroquine accumulation in erythrocytes infected with chloroquine-resistant *P. falciparum*. This lack of function of a substrate-dependent component of chloroquine accumulation distinguishes chloroquine-resistant from chloroquine-susceptible *P. falciparum*.

Erythrocytes infected with malaria parasites develop a novel process for the accumulation of chloroquine and amodiaquin with high affinity (1-4), and the operation of this process apparently determines the degree of susceptibility of the parasites to treatment with chloroquine and related drugs (1-4). Most of the molecular details of the process remain to be elucidated, but we have evidence that specificity is conferred by a drug receptor that recognizes 4-aminoquinoline derivatives such as chloroquine (3, 5). The function of this receptor is deficient in erythrocytes infected with chloroquine-resistant (CR) *Plasmodium berghei* (1) or CR *Plasmodium falciparum* (2, 4). In addition, we have found recently that substrate utilization is required for chloroquine accumulation by the high-affinity process in mouse erythrocytes infected with *P. berghei*. Either glucose or glycerol is effective as a substrate (5). Chloroquine accumulation by the high-affinity process in erythrocytes infected with a CR variant of *P. berghei* is less responsive to glucose and glycerol (5).

Insofar as the processes have been studied, the process of chloroquine accumulation by owl monkey erythrocytes infected with *P. falciparum* is qualitatively similar to that of erythrocytes infected with *P. berghei*. There are quantitative differences in these two models, however. For example, the ability of the high-affinity process to accumulate chloroquine and the degree of resistance to chloroquine are much greater in the *P. berghei* model (1, 7). Because of these and other differences (7), conclusions drawn from studies of the *P. berghei* model must be verified experimentally in a *P. falciparum* model before extrapolating them to man. Accordingly, the present studies were undertaken to evaluate the effects of substrates on chloroquine and amodiaquin accumulation by owl monkey erythrocytes infected with chloroquine-susceptible (CS) or with CR *P. falciparum*.

MATERIALS AND METHODS

Owl monkeys (*Aotus trivirgatus*) were provided by L. H. Schmidt of the Southern Research Institute, Birmingham, Ala. Some of them were infected either with the CS Malayan Camp CH/Q strain or with the CR Vietnam Oak Knoll strain of *P. falciparum*. Details of the maintenance of the monkeys and of the characteristics of these two strains of parasites in the owl monkey have been published (8). The parasitemia of the blood specimen studied and the type of infection are given for individual monkeys in Table 1. Two of the monkeys, 6659 and 6854, served as donors of uninfected erythrocytes several weeks before they were inoculated to provide erythrocytes infected with CS *P. falciparum*.

[14C]amodiaquin, [4-[(7-chloro-4-quinolyl)amino]- \( \alpha \)-[(diethylamino)-o-cresol]-\( \alpha \)-14C] dihydrochloride dihydrate, was provided by A. J. Glazko of Parke, Davis and Co., Ann Arbor, Mich. This preparation of [14C]amodiaquin was from the same lot that was used in an earlier study (4). It was labeled in the methylene bridge (4), and it had a specific activity of 1.21 mCi/mmol. [3-14C]chloroquine (specific activity, 1.71 mCi/mmol) with the label in the quinoline ring was purchased from New England Nuclear,
Boston, Mass. Both the absence of accumulation, arterial blood and the glycerol or a Sorvall GLC-1 centrifuge, the washed water: for centrifuged were or the

ity activity from the end coat as

incubation, was demonstrated both in comparable results.

Table 1. Chloroquine and amodiaquin accumulation in the absence of substrate

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Strain of P. falciparum</th>
<th>Parasitemia</th>
<th>Chloroquine accumulation</th>
<th>Amodiaquin accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Medium (nM)</td>
<td>Pellet (μmol/kg)</td>
</tr>
<tr>
<td>6879</td>
<td>None</td>
<td>None</td>
<td>121</td>
<td>1.35</td>
</tr>
<tr>
<td>6854</td>
<td>None</td>
<td>None</td>
<td>168</td>
<td>1.13</td>
</tr>
<tr>
<td>6985</td>
<td>None</td>
<td>None</td>
<td>134</td>
<td>1.67</td>
</tr>
<tr>
<td>7193</td>
<td>None</td>
<td>None</td>
<td>178</td>
<td>1.10</td>
</tr>
<tr>
<td>6659</td>
<td>None</td>
<td>None</td>
<td>159</td>
<td>1.24</td>
</tr>
<tr>
<td>6856</td>
<td>CR Oak Knoll</td>
<td>2040</td>
<td>141</td>
<td>1.34</td>
</tr>
<tr>
<td>6911</td>
<td>CR Oak Knoll</td>
<td>2120</td>
<td>118</td>
<td>1.54</td>
</tr>
<tr>
<td>6961</td>
<td>CR Oak Knoll</td>
<td>2040</td>
<td>112</td>
<td>1.88</td>
</tr>
<tr>
<td>7174</td>
<td>CR Oak Knoll</td>
<td>2520</td>
<td>115</td>
<td>2.24</td>
</tr>
<tr>
<td>6921</td>
<td>CS Camp CH/Q</td>
<td>2900</td>
<td>106</td>
<td>1.87</td>
</tr>
<tr>
<td>6861</td>
<td>CS Camp CH/Q</td>
<td>5000</td>
<td></td>
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<td>CS Camp CH/Q</td>
<td>4100</td>
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<td>147</td>
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<tr>
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<td>CS Camp CH/Q</td>
<td>1910</td>
<td>144</td>
<td>1.55</td>
</tr>
<tr>
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<td>118</td>
<td>1.71</td>
</tr>
<tr>
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<td>CS Camp CH/Q</td>
<td>3950</td>
<td>84.2</td>
<td>2.20</td>
</tr>
<tr>
<td>6659</td>
<td>CS Camp CH/Q</td>
<td>4120</td>
<td>71.6</td>
<td>2.18</td>
</tr>
</tbody>
</table>

* Suspensions of erythrocytes with hematocrits of approximately 5% were incubated for 1 h under room air at 25 C and pH 7.4 in the buffered salt solution described in the text. The initial concentration of chloroquine was 214 nM, and the initial concentration of amodiaquin was 426 nM. Parasitemia, assessed by examination of Giemsa-stained blood films, is expressed as the number of parasites per 10,000 erythrocytes. The distribution ratio was calculated by dividing the concentration of drug in erythrocyte pellet, expressed in nanomoles per kilogram, by the amount of drug in the medium, expressed in nanomoles per liter.

Degradation products of chloroquine were sought using the same methods as those described previously for mouse erythrocytes infected with *P. berghei* NYU-2 (1). To search for radioactive degradation products of [14C]amodiaquin after incubation with infected owl monkey erythrocytes in vitro, erythrocyte pellets were exhaustively eluted with the buffered salt solution containing 4 mM nonradioactive chloroquine. More than 90% of the radioactivity calculated to be present in erythrocyte pellets was recovered in the eluate, which subsequently was adjusted to pH 9 with 50% (wt/vol) K2HPO4 and extracted with an equal volume of chloroform. More than 85% of the radioactivity in the eluate was recovered by this single chloroform extraction. After evaporating the chloroform under an air stream, the residue was mixed with nonradioactive amodiaquin, and this mixture was dissolved quantitatively in acetone for preparative thin-layer chromatography on 0.25-mm silica gel plates (pre-coated, Quanta-gram thin-layer plates purchased from Arthur H. Thomas Co., Philadelphia, Pa.) with an ethyl acetate-isopropanol-water (79:15:6) solvent system. All of the radioactivity migrated with amodiaquin as a single band in this system, and it was quantitatively recovered by scraping the band into an extraction tube and eluting three times with 5 ml of chloroform-methanol-ammonia (20%) (75:25:2). Finally, the partially purified radioactive material was subjected to thin-layer chromatography on 0.25-mm

For measurement of amodiaquin and chloroquine accumulation, arterial blood was obtained in heparinized syringes. The erythrocytes were washed twice to remove plasma constituents and as much of theuffy coat as possible, by using a buffered salt solution with the following composition in mmols per liter of water: NaCl, 68; KCl, 4.8; MgSO4, 1.2; Na2HPO4, 50; and the pH was adjusted to 7.4 with HCl. Subsequently, the washed erythrocytes were incubated in the absence of substrate or in the presence of glucose or glyceral in the buffered salt solution containing amodiaquin or chloroquine labeled with 14C. At the end of incubation, the erythrocyte suspensions were centrifuged for 5 min at 5,000 rpm in the SPX rotor in a Sorvall GLC-1 centrifuge, after which the media were removed to leave pellets with hematocrits in excess of 90%.

The uptake of the labeled compounds was evaluated either by measuring the disappearance of radioactivity from the medium (1,4) or, in the case of chloroquine uptake, by the appearance of radioactivity in the erythrocyte pellet (1). Control experiments demonstrated that all of the radioactivity added at the beginning of incubation could be recovered at the end of incubation and that both methods yield comparable results. Counting errors were less than 5% in both cases.
silica gel plates with the ethyl acetate-isopropanol-water solvent system or with a methanol-triethylamine (40/1) solvent system. To verify that degradation products would be detected if they were present, the same methods were applied to mouse erythrocytes infected with *P. berghei* NYU-2 and exposed to [14C]amodiaquin in vivo. In this case degradation products were easily detected, as expected (6).

**RESULTS**

No radioactive degradation products were found after incubating infected erythrocytes in vitro either with [14C]chloroquine or with [14C]amodiaquin. In both cases all of the radioactivity extracted from erythrocyte pellets co-chromatographed with the authentic compounds. The results of thin-layer chromatography of chloroquine were identical to those obtained previously with *P. berghei* NYU-2 (1). For amodiaquin, the *R*<sub>f</sub> values of several different preparations ranged from 0.32 to 0.38 with the ethyl acetate-isopropanol-water solvent system and from 0.77 to 0.78 with the methanol-triethylamine solvent system. Because of these findings, the radioactivity measured in the present studies was considered to represent either chloroquine or amodiaquin, and the concentrations of these compounds in erythrocyte pellets and media were calculated from measurements of radioactivity.

In addition to giving the strain of parasite with which each monkey was infected and the degree of parasitemia, Table 1 gives the results of studies of chloroquine and amodiaquin accumulation in the absence of substrate. These data are shown to confirm the previously reported variation in results from one monkey to the next (2, 4), since the method of presentation used in Fig. 1 and 3 was chosen to minimize the effect of this variation. It is noteworthy that infection of erythrocytes with malaria parasites had relatively little effect on chloroquine or amodiaquin accumulation in the absence of substrate in comparison to the effect observed when glucose is supplied as a substrate (2, 4).

Figure 1 shows the effect of glucose on chloroquine accumulation by various preparations of erythrocytes. Chloroquine accumulation by erythrocytes infected with CS *P. falciparum* was stimulated by 1 mM glucose. This concentration of glucose had no effect on uninfected erythrocytes or on erythrocytes infected with CR *P. falciparum*. A high concentration of glucose, 86 mM, was less effective than the 1 mM concentration in stimulating chloroquine accumulation by erythrocytes infected with CS *P. falciparum*, and it inhibited chloroquine accumulation by uninfected erythrocytes and by erythrocytes infected with CR *P. falciparum*. In contrast to the findings in the *P. berghei* model, 1 mM glycerol failed to stimulate chloroquine accumulation by any of the three types of erythrocyte preparations (experiments not shown). Possibly, both CS and CR *P. falciparum* lack glycerol kinase and cannot initiate the metabolism of glycerol.

Figure 2 shows chloroquine accumulation as a function of the chloroquine concentration in the medium after an hour of incubation. In agreement with the observations presented in Fig. 1, 1 mM glucose had no effect on chloroquine accumulation by uninfected erythrocytes, but it stimulated chloroquine accumulation by erythrocytes infected with CS *P. falciparum*. The shapes of the curves depicting chloroquine accumulation were not noticeably affected by glucose. Only the capacity to accumulate chloroquine appeared to increase.

The results of studies of amodiaquin accumu-
of amodiaquin in the medium. The shapes of the curves depicting amodiaquin accumulation as a function of external concentration appear unaffected.

**DISCUSSION**

A distinguishing characteristic of CR *P. falciparum* is demonstrated by the findings summarized in Fig. 1 and 2. Namely, chloroquine accumulation with high affinity by erythrocytes infected with CR *P. falciparum* is totally unresponsive to glucose. This is the first observation of total lack of function of a substrate-dependent component in the process of chloroquine accumulation in erythrocytes infected with malaria parasites. By contrast, only a partial deficiency in function of a similar substrate-dependent component occurs in erythrocytes infected with CR *P. berghei* (5).

Glucose also stimulates the accumulation of amodiaquin by infected erythrocytes. Nevertheless, there are major differences in the handling of amodiaquin and chloroquine. As previously reported, the amount of amodiaquin accumu-

![Fig. 2. Effect of glucose on chloroquine accumulation by erythrocytes infected with CS *P. falciparum*. The conditions of this study are the same as those described in Table 1 except that a series of chloroquine concentrations was studied and one set of incubation mixtures contained glucose in an initial concentration of 1 mM (○), whereas no substrate was added to another set (□). The concentration of chloroquine in the media (external chloroquine) at the end of incubation are shown on the abscissa. Top panel, erythrocytes from monkey 7024 infected with CS *P. falciparum*; middle panel, erythrocytes from monkey 7024 infected with CS *P. falciparum*; bottom panel, erythrocytes from monkey 6659 prior to infection.](http://aac.asm.org/)

![Fig. 3. Effect of glucose on amodiaquin accumulation. The conditions of this study are the same as those described in Table 1 except that in some of the incubation mixtures glucose in an initial concentration of 1 mM (striped bars) or 86 mM (stippled bars) was substituted iso-osmotically for NaCl. Two other concentrations of glucose were studied but the data are not shown: 0.1 mM glucose had no effect on any of the erythrocyte preparations and the effect of 10 mM glucose was indistinguishable from the effect of 1 mM glucose. The numbers underneath the sets of bars identify the monkeys from which the specimens of blood were obtained.](http://aac.asm.org/)
in the *P. falciparum* model, the two compounds are closely related structurally (4), and amodiaquin competitively inhibits chloroquine accumulation in the *P. berghei* model (3), indicating in this model at least that the two compounds interact with the same receptor. If chloroquine and amodiaquin in fact are recognized by the same receptor and accumulated by the same process in the *P. falciparum* model, it could be argued that the receptor is more accessible to amodiaquin than to chloroquine (4) and that extra energy is required to make the receptor accessible to chloroquine. These arguments in turn raise the possibility that chloroquine resistance is due to a failure of the parasite to make a drug receptor accessible to chloroquine, although the receptor is present and can interact with amodiaquin.

Turning now to the effects of high glucose concentrations, it should be mentioned that 86 mM glucose was used in earlier studies of chloroquine and amodiaquin accumulation (1–4). Fortunately, the differences between CS and CR *P. falciparum* were still observed in the early studies since they are only partially obscured by the inhibitory effect of 86 mM glucose. It is apparent, however, that such unphysiologically high concentrations of glucose should not be used in future studies of chloroquine and amodiaquin accumulation.

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**LITERATURE CITED**


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**Fig. 4. Inhibition of amodiaquin accumulation by high concentrations of glucose.** The conditions of this study were the same as those described in Table 1 except that a series of amodiaquin concentrations was studied and glucose in an initial concentration of 1 mM (open circles) or 86 mM (closed circles) was substituted iso-osmotically for NaCl. The concentrations of amodiaquin in the media (external amodiaquin) at the end of incubation are shown on the abscissa. Top panel, erythrocytes from monkey 6921 infected with CS *P. falciparum*; middle panel, erythrocytes from monkey 7174 infected with CR *P. falciparum*; bottom panel, erythrocytes from monkey 6854 prior to infection.
