Chloroquine Sensitivity and Pigment Formation in Rodent Malaria* (33596)

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Chloroquine is the therapeutic agent of choice for the treatment of malarial infections. The recent recognition of the existence of malarial parasites which are refractory to this treatment, in particular strains of *Plasmodium falciparum* and *Plasmodium vivax*, has led to intensive search for the mechanism of drug resistance in malaria. The experimental induction of chloroquine resistance in malaria parasites requires prolonged exposure to the drug, with gradually increasing doses, until a maximal host-tolerated dose is achieved in the presence of which the malaria parasite continues to grow and multiply (1–4). Morphological studies of the parasites during drug exposure show clumping of pigment within minutes and degeneration of some parasites (5, 6). The parasites which survive contain little or no detectable pigment. Pigment formation in subsequent passages of the parasite in the presence of chloroquine remains depressed. When the drug is discontinued some strains are reported to revert to their previous, normal sensitivity very quickly and to form pigment again (7), while other strains maintain resistance for some weeks before reverting and forming detectable amounts of pigment (2, 7, 8). The absence or depression of pigment formation has therefore been generally associated with chloroquine resistance.

In vitro studies have shown that chloroquine binds with precursors of malaria pigment (11, 12), which led some investigators to suggest that the formation of such complexes might offer an explanation of the mechanism of chloroquine action and the evolution of chloroquine resistance (8, 11, 12). According to Peters (8, 13), Schueler and Cantrell (12), and Cohen *et al.* (11), the drug, selectively bound to pigment precursors, is retained by the parasite resulting in toxic effects, while parasites which form little or no pigment would not accumulate toxic quantities of the drug. The fact that chloroquine resistant parasites do indeed accumulate decidely less drug than sensitive parasites was subsequently demonstrated by Macomber *et al.* (14).

Recently a naturally occurring, chloroquine resistant strain of rodent malaria *P. berghei yoeli*, has been isolated (9). The strain meets the criteria of drug resistance as defined by a standard test (9, 10). The erythrocytic stages of this parasite contain considerable quantities of pigment while, in contrast, strains with experimentally induced drug resistance either lack or exhibit significantly less pigment (1–3, 8). The availability of this new strain prompted a further investigation of the relationship of pigment formation to cholorquine sensitivity.

Materials and Methods. Young mice were inoculated with *P. berghei yoeli*, the naturally resistant strain which forms pigment, and *P. berghei* CR, a strain in which maximal resistance had been induced and which forms little detectable pigment. The results were compared with the highly sensitive strain of *P. berghei* NYU-2 which forms large quantities of pigment, in regard to: (i) actual ability of the parasites to grow over an extended period of time in the presence of chloroquine, (ii) uptake of chloroquine-^{14}C, and (iii) presence or absence of pigment before, during, and after the experimental period. These strains were further compared with the behavior of *P. berghei* NYU-2 passed continuously in a different host, the rat.

The test strains were maintained by repeated blood passages in young mice or rats. *P. berghei yoeli* was obtained from Dr. R.

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Ward, through the courtesy of Dr. Killick-Kendrick, London School of Tropical Medicine. *P. berghei* NYU-2 has been maintained for over 4 years in the Institute by mouse to mouse blood passages. The resistant strain, *P. berghei* CR, was developed by Dr. P. Macomber in 1964 by continuous exposure of the sensitive *P. berghei* NYU-2 to increasing doses of chloroquine. The strain was passed regularly from mid-1964 until June 1967 at which time samples were frozen for storage. The strain was reactivated in January 1968 and has subsequently been maintained under constant exposure to chloroquine.

Malarial infection was induced in mice or rats by intraperitoneal injections of parasitized red cells. Each animal receiving chloroquine was given an intraperitoneal injection once daily. Mice infected with *P. berghei* NYU-2 received drug only on 4 successive days. Mice infected with either strain of chloroquine-resistant organisms received daily injections until the animal expired, spontaneous cur occurred or the experiment was terminated. Procedures followed in the determination of drug accumulation in parasitized red blood cells were those of Macomber *et al.* (14).

Results and Discussion. The results of the drug uptake studies are summarized in Table I. In our hands, *P. berghei yoeli* shows considerably less resistance than *P. berghei* CR. With comparable doses of chloroquine, *P. berghei yoeli* attains only up to 10% parasitemia while *P. berghei* CR never falls below 50% and in the invariably fatal infection reaches terminally a level of 90% parasitemia of all red cells present. *P. berghei yoeli* continues to grow and multiply at a low degree of parasitemia in the presence of the drug in doses which would promptly eradicate infection by the sensitive strain. The infection with *P. berghei yoeli* is self-limiting, at 14–21 days following first appearance of parasites in the peripheral blood smear, independent of chloroquine administration. *P. berghei* NYU-2 infection which is invariably fatal in the untreated mouse, is cured after only 4 days of minimal drug therapy. The accumulation of chloroquine-^{14}C differs markedly between the three strains. The highly sensitive strain accumulates the drug readily whereas the maximally resistant strain accumulates less than one-third the amount, but accumulates significantly more than the uninfected red cells. *P. berghei yoeli* shows an intermediate accumulation of drug between the sensitive and the maximally resistant strain. *P. berghei* NYU-2 in the rat accumulates chloroquine in a manner comparable

TABLE I. *In Vivo* Uptake of Chloroquine-^{14}C by Different Strains of Malaria Parasites.[a]

Strain	Dose of chloroquine base (mg/kg)	Chloroquine-^{14}C uptake (μg/ml of erythrocytes[b])
1. *P. berghei* NYU-2 in mice	5 (in four doses cures)	105 (90–126)
2. *P. berghei yoeli* in mice	45 (2–10% parasitemia generally persists until spontaneous cure)	64[c] (52– 76) 70[d] (50– 78)
3. *P. berghei* CR in mice	45 (parasites continue to grow and multiply to parasitemia >50%)	36[c] (27– 44) 33[d] (28– 39)
4. *P. berghei* NYU in rat	Responds to 60–80	96 (87–120)
Control RBCs in mouse and rat	—	8 (5– 10)

[a] Each animal was inoculated with approximately 4 × 10^6 parasitized RBCs. Strains 1, 2, and 3 were passed continuously in mice; strain 4 was adapted to the rat.

[b] Each animal was injected i.p. with chloroquine, 25 mg/kg; containing 50 μC. Samples were taken 4 hr later and processed as described by Macomber *et al.* (14). All values are corrected to represent the same level of parasitized red cells. Values represent averages of six or more determinations. Numbers in parentheses represent range of determinations.

[c] Parasites maintained under constant chloroquine pressure.

[d] Parasites tested after single injection of chloroquine-^{14}C.

to that of the same sensitive strain in the mouse but responds favorably only to doses of chloroquine at 60–80 mg/kg per rat, which is a significantly greater resistance to chloroquine than the same strains shows in the mouse. The fact that a shift in hosts results in a significant alteration of drug sensitivity has also been shown by Trager *et al.* (15). These investigators found that on passage of a maximally chloroquine resistant strain of *P. berghei* from the mouse into hamsters drug resistance was lost. The parasites in the hamster exhibited less pigment than the parent sensitive strain. In both drug-resistant strains tested by us in the mouse, a single dose of chloroquine-^{14}C is accumulated by the infected red cells in approximately the same amount as in cells continuously exposed to chloroquine. Both *P. berghei* NYU-2 and *P. berghei yoeli* show clumping of pigment within minutes of drug exposure. As treatment proceeds, surviving *P. berghei yoeli* parasites appear relatively free of pigment. If therapy is discontinued, pigment formation resumes within 4–5 days as the infection progresses to a plateau after which the plasmodia disappear as previously noted. The *P. berghei* CR strain maintained under constant exposure to chloroquine (45 mg/kg per mouse) contains little detectable pigment and does not appear to produce increased amounts of pigment following passage in the absence of the drug for periods up to 4 weeks. Drug resistance persists at the maximum level, i.e., doses of chloroquine toxic to the host will not eradicate the parasite.

The formation of pigment and the accumulation of drug by the various strains of *P. berghei* may well be influenced by the age of the red cell invaded. Peters (13) as well as others have observed that drug resistant parasites invade younger red cells than the drug-sensitive ones, suggesting that resistant parasites require certain nutrients "that only immature red cells can supply." This hypothesis has not been proven so far, although the fact of preferential invasion of reticulocytes is not disputed. Our light and electron microscopic observations confirm that the resistant parasites, indeed, invade young red cells. This phenomenon may be related to differences in the growth rate (rapidity of development of parasitemia) which, in mice is considerably slower for the resistant strains than for the sensitive NYU-2 strain. *P. berghei* NYU-2 passed repeatedly in the rat preferentially invades young red cells, has a growth rate comparable to the resistant strains in the mouse, forms distinctly less pigment than the same parasite in the mouse, and displays an increased tolerance to chloroquine. *P. berghei* NYU-2 in the rat behaves like the resistant strains in mice with one important exception: It readily accumulates chloroquine-^{14}C. This would indicate that the generally held concept that drug resistance is inversely related to drug uptake is not universally valid. It also appears that the amount of pigment formed does not correlate with drug sensitivity as originally stated. Pigment formation is definitely affected in parasites growing in the presence of chloroquine, but a direct relationship between pigment formation and drug sensitivity has yet to be established. The amount of pigment formed by the parasite does not correlate with chloroquine uptake.

Summary. The degree of selective uptake of chloroquine by *P. berghei yoeli* and *P. berghei* CR in the mouse is inversely related to the level of resistance demonstrable and does not depend on previous or concurrent exposure to the drug. Chloroquine sensitive *P. berghei* NYU-2 when passed into the rat, exhibits an increased tolerance to chloroquine, behaving in this respect like *P. berghei yoeli* in the mouse. However, it accumulates chloroquine-^{14}C to an essentially identical degree as the same strain in the mouse, which is extremely sensitive to chloroquine. The decrease or disappearance of pigment in *P. berghei* NYU-2 when passed into the rat, ite growth in the presence of chloroquine and may not have any direct relationship to the mechanism(s) of resistance.

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Melanocyte-Stimulating Activity Following Adrenalectomy in Deermice* (35597)

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Melanin deposition is inhibited to some degree by feeding black or brown rats a diet free of vitamin B; an effect that is reversible by adrenalectomy but not when adrenalectomized animals are provided with desoxycorticosterone acetate (1–3). A profound darkening of coat color follows adrenalectomy in prairie deermice (*Peromyscus maniculatus bairdii*) without prior dietary deficiency (4). This species of deermouse, whose normal adult coloration may be described as a brownish back and a white to light gray belly, turns dark gray or black within 3–10 weeks after removal of the adrenal glands. The purposes of the present experiments were twofold: (a) to determine the effect of hypophysectomy with or without adrenalectomy on coat color, and (b) to study the development of melanocyte-stimulating (M-S) activity in the plasma of adrenalectomized animals.

Materials and Methods. Adult male deermice were either adrenalectomized, hypophysectomized, or subjected to both types of operations. A single dose of 80 μg cortisone acetate (i.p., in saline) was given immediately following adrenalectomy and such animals were maintained thereafter on 1% NaCl drinking water. Hypophysectomized and hypophysectomized–adrenalectomized animals were maintained on a solution of 1% NaCl and 5% glucose. Reflectometer readings were obtained as indices of belly fur color for all animals that survived 7 weeks or longer (20 Adx, 6 Hypox, and 5 Adx-Hypox) and compared to similar readings obtained from 20 sham-operated and 80 intact males of the same approximate age. Reflectometer observations were made as averages of 3 readings on the lower half of the belly of conscious animals. The reflectometer (model 610, Photovolt Corporation) utilized a tristimulus filter and its meter was set to read 80 against a gray cardboard working standard (which, in turn showed a reading of 58 when the meter was adjusted to 100 against the white enamel standard provided with this machine).

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