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Chloroquine Encapsulated in Malaria-Infected Erythrocyte-Specific Antibody-Bearing Liposomes Effectively Controls Chloroquine-Resistant *Plasmodium berghei* Infections in Mice†

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The suitability of liposomes as drug carriers in the treatment of drug-resistant rodent malaria was examined after covalently attaching F(ab′)$_2$ fragments of a mouse monoclonal antibody (MAb), MAb F$_{10}$, raised against the host cell membranes isolated from the *Plasmodium berghei*-infected mouse erythrocytes, to the liposome surface. The antibody-bearing liposomes thus formed specifically recognized the *P. berghei*-infected mouse erythrocytes under both in vitro and in vivo conditions. No such specific binding of the liposomes with the infected cells was observed when MAb F$_{10}$ was replaced by another mouse monoclonal antibody, MAb D$_2$. Upon loading with the antimalarial drug chloroquine, the MAb F$_{10}$-bearing liposomes effectively controlled not only the chloroquine-susceptible but also the chloroquine-resistant *P. berghei* infections in mice. The chloroquine delivered in these liposomes intravenously at a dosage of 5 mg/kg of body weight per day on days 4 and 6 postinfection completely cured the animals (75 to 90%) of chloroquine-resistant *P. berghei* infections. These results indicate that selective homing of chloroquine to malaria-infected erythrocytes may help to cure the chloroquine-resistant malarial infections with low doses of chloroquine.

Malaria is a serious public health problem which affects about 300 million people and accounts for 1 million to 2 million deaths per year worldwide (22). The disease is being controlled by using antimalarial drugs, like chloroquine, to which the malarial parasites are rapidly developing resistance (14). In order to effectively control this disease, it is essential that newer strategies for treating drug-resistant malaria with existing drugs be developed.

Liposomes bearing cell-specific recognition ligands on their surfaces have widely been considered useful as drug carriers in therapy (5). Our earlier studies have shown that encapsulation of the antimalarial drug chloroquine (CHQ) in liposomes bearing antierythrocyte antibody on their surfaces markedly increases its efficacy against both CHQ-susceptible and CHQ-resistant *Plasmodium berghei* infections in mice (1, 2). Also, Peeters et al. (12) have reported that liposomization of CHQ increases not only its maximal tolerable dose but also its efficacy against the CHQ-resistant malarial infections. To further explore the possibility of using liposomized CHQ in the treatment of CHQ-resistant malaria, we examined the antimalarial activity of this drug after its encapsulation in liposomes bearing *P. berghei*-infected mouse erythrocyte-specific antibody on their surfaces. Results of the studies indicated that CHQ-resistant malarial infections can be cured with CHQ by delivering this drug in target-specific liposomes.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (egg PC) and gangliosides were prepared as described earlier (6, 17). Cholesterol was purchased from Centron Research Laboratory, Bombay, India, and was used after crystallizing it three times from methanol. Sodium cyanoborohydride and pepsin were bought from Sigma Chemical Company. Sodium metaperiodate was obtained from Sisco Research Laboratory, Bombay, India. Sephadex G-50 and Sepharose 6B were acquired from Pharmacia Fine Chemicals. [^{125}I]sodium iodide (carrier-free) and [^{14}C]inulin were procured from Amersham, Buckinghamshire, England. Chloroquine diphosphate was a kind gift from Walter Reed Army Institute of Research, Washington, D.C. Normal mouse immunoglobulins (mIg's) were isolated from normal mouse serum.

PAbs. Anti-mouse erythrocyte antibodies (polyclonal antibodies [PAbs]) were raised in rabbits and were isolated from antiserum by following a previously published procedure (17).

MAbs. BALB/c mice were immunized intraperitoneally (i.p.) at 3-week intervals with the erythrocyte membranes isolated from about 1.5×10^7 mouse erythrocytes infected predominantly with the trophozoite stage of *P. berghei*. Isolation of membranes from infected cells was carried out essentially by our previously published procedure (8). After four to six immunizations, spleen cells were fused with PAI-0, a myeloma cell line (19), by using polyethylene glycol (4). Hybridoma supernatants were screened by immunofluorescence assay with infected erythrocytes as described earlier (13). After cloning by limiting dilutions, two different clones, F$_{10}$ and D$_2$, were selected on the basis of their Western blotting (immunoblotting) patterns by using erythrocyte membranes isolated from infected cells for ascitic fluid generation. MAbs F$_{10}$ and D$_2$ were purified from the ascitic fluids by affinity chromatography with protein A-Sepharose at pH 8.0. These antibodies were of the IgG1 type as determined by enzyme-linked immunosorbent assay with isotype-specific antibodies (Sigma Chemical Company). The purity of each MAb was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

F(ab′)$_2$ fragments and their radioiodination. F(ab′)$_2$ fragments of antibodies were prepared (17) and radioiodinated (9) by previously published procedures.

Liposomes. Egg PC (20 μmol), cholesterol (20 μmol), and gangliosides (4 μmol) were dissolved in a chloroform-methanol mixture in a thick glass tube, and the solvents were removed under a slow jet of N$_2$ to give a thin lipid film on the wall of the tube. The traces of solvents were removed by leaving the tube overnight under vacuo. It was dispersed in 0.8 ml of borate-buffered saline (10 mM borate, 60 mM NaCl [pH 8.4]) containing CHQ (350 μmol). The dispersion was sonicated with a probe-type sonicator for 30 min under an N$_2$ atmosphere at 4°C. The sonicated preparation was centrifuged in a Beckman L 5-65B ultracentrifuge (SW 50.1 rotor) at $105{,}000 \times g$ for 1 h at 5°C to effect the removal of titanium particles as well as the poorly dispersed lipids. Only the top two-thirds of the supernatant was used in further studies. Free CHQ from liposomized CHQ was separated by gel filtration over Sephadex G-50. The mean outer diameter of these liposomes, as determined by negative staining electron microscopy, was about 60 ± 10 nm. The liposomes used for in vitro or in vivo binding

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with erythrocytes were also prepared as described above, except that in place of CHQ [^{14}C]inulin was used in the dispersion buffer.

Covalent coupling of F(ab′)$_2$ with liposomes. ^{125}I-labelled (or unlabelled) F(ab′)$_2$ fragments from PAbs, MAb F_{10}, MAb D_2, and mIg were covalently attached to liposomes as reported earlier (17). Briefly, the liposomes containing CHQ were subjected to periodate oxidation for generating aldehyde groups from gangliosides. After removing the excess periodate, the oxidized liposomes were reacted with the free amino group(s) of F(ab′)$_2$ fragments, and the labile Schiff's base thus formed was stabilized by reducing it with sodium cyanoborohydride. The protein-to-lipid ratio in the immunoliposomes obtained by this procedure was about 80 μg/μmol of lipid P.

CHQ estimation. CHQ was estimated as described earlier (1) by measuring the A_{342}. The amount of CHQ associated with liposomes was about 140 ± 3 μg of CHQ/μmol of lipid P.

Animals. Randomly bred 8- to 10-week-old male Swiss or BALB/c mice (weight, 20 ± 2 g) were used in the study. The animals were given pellet diet (Hindustan Lever Ltd., Bombay, India) and water ad libitum.

Parasites. *P. berghei* parasites were obtained from the National Institute of Communicable Diseases, New Delhi, India, and were maintained in the Swiss mice through serial blood passage. The strain was fully susceptible to CHQ, with the 90% effective dose being 15 mg/kg of body weight given consecutively for 4 days (i.p.). Parasitemia was determined by counting 10^3 erythrocytes in thin blood smears stained with Giemsa and was expressed as the number of parasitized cells per 100 erythrocytes.

Development of CHQ resistance. CHQ resistance was developed by the relapse technique (21). Randomly bred Swiss mice were infected with about 10^7 *P. berghei*-infected erythrocytes, and on the same day a single dose (60 mg/kg, i.p.) of CHQ was administered. After the animals developed about 2% parasitemia, the infected blood from these animals was transfused into healthy animals, which were also given CHQ (60 mg/kg, i.p.) simultaneously. The operation described above was repeated several times until the organisms causing the infection were rendered resistant to CHQ (50 mg/kg given consecutively for 4 days, i.p.).

Liposome binding to erythrocytes in vitro. Blood (5 ml) from *P. berghei*-infected and healthy uninfected BALB/c mice was drawn in heparinized glass tubes containing phosphate-buffered saline (10 mM NaCl [pH 7.4]). It was passed through a CF-11 column (5 ml) to remove leukocytes. Infected and normal erythrocytes were separated by Ficoll-Conray gradient. The gradient was prepared by mixing Conray 420 (33% [wt/vol] solution in water on the basis of the sodium iothalamate content) to a 9% solution of Ficoll 400 in normal saline until the density of the mixture was 1.08 g/ml. A total of 1 ml of the cell suspension (50% hematocrit) was carefully layered on the top of 2 ml of gradient in a glass tube (5 ml). It was centrifuged at 300 × *g* for 10 min at 20 ± 2°C. Infected cells were aspirated from the top and washed several times with Tris-buffered saline (TBS; 10 mM Tris containing 150 mM NaCl [pH 7.4]). The infected erythrocyte preparation thus obtained was contaminated with ≤3% normal erythrocytes.

Erythrocytes (about 65 × 10^6 cells) were incubated with liposomes (100 to 500 nmol of lipid P), doubly radiolabelled by attaching ^{125}I-labelled F(ab′)$_2$ fragments to the liposome surface, and entrapping [^{14}C]inulin in the aqueous compartment in sucrose-supplemented TBS (750 μl) at 37°C for 30 min. After completing the incubation, the cells were pelleted and washed extensively with ice-cold, sucrose-supplemented TBS. The amount of cell-associated ^{125}I was determined without disrupting the cells, whereas for measuring ^{14}C, the cells were lysed with Triton X-100. The amounts of measured ^{14}C were duly corrected for quenching by hemoglobin.

Distribution of liposomes in tissue. Doubly radiolabelled liposomes (0.75 to 1.25 μmol of lipid P, 250 μl) were injected into the tail veins of healthy and *P. berghei*-infected (50 to 60% parasitemia) BALB/c mice. Animals were sacrificed immediately after drawing about 1 ml blood at 30 min postinjection, and the various organs (liver, spleen, lung, kidney, and heart) were removed, washed, blotted, and weighed. A 10% (wt/vol) homogenate of these organs was prepared in sucrose-supplemented TBS. The homogenate was centrifuged at 5,000 × *g*, and measured aliquots of the supernatant were used for determining the radioactivity. The amounts of radioactivity associated with plasma and erythrocytes were determined as follows.

Blood (1 ml) was centrifuged, and the volumes of the plasma and cell pellet thus separated were measured. Plasma was collected in a glass tube, and the cell pellet was washed with TBS. The radioactivity in known aliquots of cells and plasma was determined by counting the ^{14}C and ^{125}I. The amounts of radioactivity associated with plasma and cells (predominantly erythrocytes) in vivo were calculated by assuming that the total blood volume was 8% of the body weight (18).

Drug treatment. Several preparations of CHQ-containing liposomes were evaluated for their efficacies. Swiss mice (6 to 10 animals per group) were injected on day zero with about 10^6 erythrocytes infected with CHQ-susceptible or CHQ-resistant *P. berghei* strains. The treatment was started on day 4 postinfection when the parasitemia in the animals reached 0.1 to 0.5%. The animals were observed on various days for parasitemia. The percent suppression of parasitemia in CHQ-treated animals was calculated by comparing the parasitemia in these animals with that in animals treated under identical conditions with buffer.

Statistical analysis. Statistical significance (*P* value) was ascertained by performing *t* tests on both the parasitemia and survival data by using Sigma plot statistics software package (Sigmaplot Scientific Graphing System).

RESULTS

Two MAbs (D_2 and F_{10}), which recognized *P. berghei*-infected mouse erythrocytes, were covalently attached to the surfaces of [^{14}C]inulin-containing liposomes after removing their Fc portion by pepsin treatment and labelling the resulting F(ab′)$_2$ fragments with ^{125}I. The antibody-bearing liposomes (MAb D_2-Lip and MAb F_{10}-Lip) thus obtained were interacted with normal and *P. berghei*-infected mouse erythrocytes in vitro in order to assess their binding specificities. The binding was determined by measuring the cell-associated ^{125}I and ^{14}C. Liposomes bearing F(ab′)$_2$ of normal mIg on their surfaces (mIg-Lip) were used as controls. The results given in Fig. 1 indicate that MAb D_2-Lip could bind to both normal and infected erythrocytes. Unlike this finding, no such nonspecific binding was observed with MAb F_{10}-Lip. These liposomes recognized only the infected cells but not the normal erythrocytes. The maximum binding of MAb F_{10}-Lip observed with *P. berghei*-infected erythrocytes was about 16%, in comparison with ≤3% binding observed with normal cells.

To further confirm the specificity of MAb F_{10}-Lip binding with infected erythrocytes, the liposome preparations described above were administered intravenously to healthy and *P. berghei*-infected (50 to 60% parasitemia) BALB/c mice, and their distributions in various tissues were determined. Figure 2 shows that, unlike MAb D_2-Lip, MAb F_{10}-Lip did not significantly bind to the erythrocytes in healthy animals, but it readily recognized these cells in *P. berghei*-infected mice. These results thus clearly established that MAb F_{10}-Lip recognized specifically the infected erythrocytes.

The suitability of MAb F_{10}-Lip as a drug carrier in the treatment of malarial infections was examined by loading these liposomes with CHQ and then evaluating the efficacies of the CHQ-loaded liposomes against *P. berghei* infections in Swiss mice. Table 1 shows that the efficacy of CHQ against CHQ-susceptible *P. berghei* infections was considerably increased ($P < 0.001$) by delivering this drug in antibody-bearing liposomes. The best results were observed when CHQ was delivered at a dose of 5.0 mg/kg. At this dose, CHQ encapsulated in MAb F_{10}-Lip effectively controlled not only the CHQ-susceptible but also the CHQ-resistant *P. berghei* infections (Table 2).

The experiments described above were carried out by administering only one dose of CHQ to the infected mice. To examine whether increasing the number of CHQ doses would completely cure the treated animals of malarial infections, we delivered to the CHQ-resistant *P. berghei*-infected mice two doses of liposomized CHQ on days 4 and 6 postinfection. Table 3 shows that in the MAb F_{10}-Lip–CHQ-treated group, 75 to 90% of the animals survived the CHQ-resistant *P. berghei* infections and no parasites were detectable in their blood even on day 30 posttreatment. Unlike this observation, only 40 to 50% of the animals in the MAb D_2-Lip–CHQ-treated group, which were treated under identical conditions, survived.

DISCUSSION

The present study demonstrates that grafting of MAb F_{10} on the liposome surface enables the liposomes to selectively recognize the *P. berghei*-infected mouse erythrocytes under both in vitro and in vivo conditions. Another MAb, MAb D_2, although it recognized the infected erythrocytes as well as MAb F_{10} did, at least under in vitro conditions, was not very specific to these cells because it also readily recognized the normal

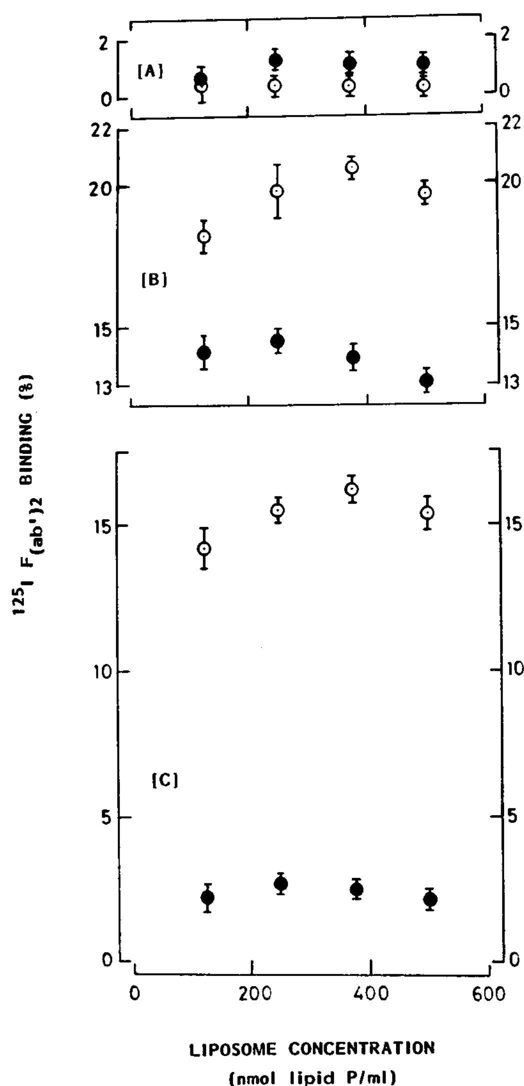

FIG. 1. Binding of liposomes to erythrocytes in vitro expressed as a percentage of the total ^{14}C or ^{125}I associated with 65 × 10^6 erythrocytes. The ^{14}C binding is not shown here for the sake of clarity. (A) mIg-Lip; (B) MAb D_2-Lip; (C) MAb F_{10}-Lip. The open and closed circles represent binding with *P. berghei* infected and normal uninfected mouse erythrocytes, respectively. Values are means ± standard deviations of three determinations.

mouse erythrocytes. From these results it would appear that MAb F_{10} perhaps recognizes some surface antigen which is unique to the *P. berghei*-infected erythrocytes, whereas MAb D_2 may possibly be binding some surface determinant which could be common to both the normal and infected cells.

Earlier studies have shown that the intracellular malarial parasite modifies the membrane protein composition in the host erythrocytes (15). On one hand, it structurally alters the native erythrocyte membrane proteins (16), while on the other, it inserts some new proteins in the plasma membrane of the host cell (7, 11). It is therefore tempting to speculate that MAb F_{10} might have been directed against some new protein(s) of parasite origin, whereas MAb D_2 could be recognizing some modified erythrocyte component in infected cells.

The present study shows that the antimalarial activity of CHQ is markedly increased by encapsulating this drug in MAb

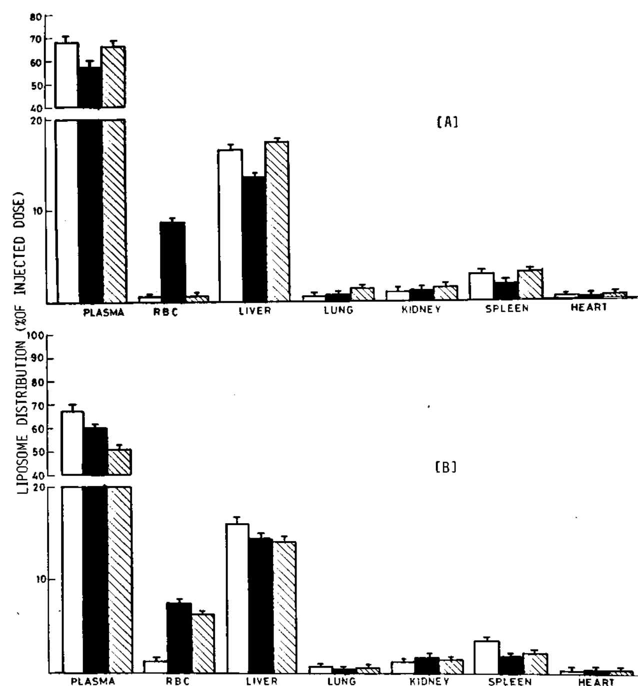

FIG. 2. Distribution of liposomes in various organs and tissues. Liposomes containing ^{125}I-labelled F(ab′)$_2$ on their surfaces and [^{14}C]inulin in their aqueous compartments were injected separately to healthy (A) and *P. berghei*-infected (B) mice, and their distributions in various tissues were determined 30 min after the injection. Only the ^{125}I distributions are shown for the sake of clarity. Values are means ± standard deviations for three animals. Open bars, mIg-Lip; solid bars, MAb D_2-Lip; shaded bars, MAb F_{10}-Lip; RBC, erythrocytes.

TABLE 1. Efficacy of CHQ against CHQ-susceptible *P. berghei* infections in mice after encapsulating CHQ in liposomes

Treatment and comparison group[a]	Dose (mg/kg)	% Parasitemia on day 10 posttreatment[b]:
Saline		19.50 ± 0.50 (2)
Free CHQ	5.0	7.90 ± 2.90 (5)
mIg-Lip–CHQ	5.0	6.44 ± 1.80 (5)
PAb-Lip–CHQ	5.0	0.65 ± 0.30 (6)
	2.5	2.50 ± 1.20 (6)
	1.0	4.33 ± 1.50 (6)
MAb D_2-Lip–CHQ	5.0	0.68 ± 0.25 (6)
	2.5	1.80 ± 1.20 (6)
	1.0	4.50 ± 1.70 (6)
MAb F_{10}-Lip–CHQ	5.0	0.44 ± 0.25 (6)
	2.5	1.40 ± 0.98 (6)
	1.0	4.02 ± 1.20 (6)
P value		
Free CHQ vs PAb-Lip–CHQ	5.0	<0.001
Free CHQ vs MAb D_2-Lip–CHQ	5.0	<0.001
Free CHQ vs MAb F_{10}-Lip–CHQ	5.0	<0.001
PAb-Lip–CHQ vs MAb D_2-Lip–CHQ	2.5	>0.1
PAb-Lip–CHQ vs MAb F_{10}-Lip–CHQ	2.5	<0.1
MAb D_2-Lip–CHQ vs MAb F_{10}-Lip–CHQ	2.5	>0.1

[a] Treatment was given intravenously only once on day 4 after the infection. Each group consisted of six animals.

[b] Values are means ± standard deviations. Figures given in parentheses denote the numbers of animals surviving on day 10.

TABLE 2. Efficacy of CHQ against CHQ-resistant *P. berghei* infections in mice after its encapsulation in liposomes

Treatment and comparison group[a]	% Parasitemia on the following day posttreatment[b]:		
	6	8	10
Saline	1.57 ± 0.38 (5)	4.33 ± 0.90 (3)	All dead
Free CHQ	1.70 ± 0.87 (5)	4.20 ± 1.80 (3)	All dead
PAb-Lip–CHQ	1.36 ± 0.34 (6)	3.40 ± 0.75 (5)	6.80 ± 1.20 (2)
MAb D_2-Lip–CHQ	1.20 ± 0.39 (6)	3.23 ± 0.73 (6)	4.56 ± 1.40 (3)
MAb F_{10}-Lip–CHQ	1.12 ± 0.20 (6)	1.58 ± 0.26 (6)	2.58 ± 1.10 (6)
P value			
Free CHQ vs PAb-Lip–CHQ	>0.1	>0.1	
Free CHQ vs MAb D_2-Lip–CHQ	>0.1	>0.1	
Free CHQ vs MAb F_{10}-Lip–CHQ	>0.1	<0.05	
PAb-Lip–CHQ vs MAb D_2-Lip–CHQ	>0.1	>0.1	>0.1
PAb-Lip–CHQ vs MAb F_{10}-Lip–CHQ	>0.1	<0.001	<0.01
MAb D_2-Lip–CHQ vs MAb F_{10}-Lip–CHQ	>0.1	<0.001	<0.1

[a] Treatment with CHQ (5 mg/kg) was given intravenously only once on day 4 after the infection. Each group consisted of seven animals.
[b] Values are means ± standard deviations. Figures given in parentheses denote the numbers of animals surviving on that particular day.

F_{10}-Lip. MAb F_{10}-Lip–CHQ controlled efficiently not only the CHQ-susceptible *P. berghei* infections but also the infections that were otherwise resistant to the CHQ treatment. The high therapeutic efficacy of liposomized CHQ observed here could primarily be attributed to the following factors: (i) the high degree of specificity of MAb F_{10}-Lip to the malaria-infected erythrocytes, (ii) the specific binding of MAb F_{10}-Lip possibly to free parasites, and (iii) the efficient internalization of the cell-bound MAb F_{10}-Lip. This finds strong support from our present observations that (i) in spite of the similar extents of bindings of MAb D_2-Lip and MAb F_{10}-Lip to the infected erythrocytes, MAb D_2-Lip–CHQ showed considerably lower levels of antimalarial activity in comparison with those of MAb F_{10}-Lip–CHQ and (ii) MAb F_{10}-Lip alone, without CHQ, did not exhibit the antimalarial activity (unpublished data).

It is interesting to observe that the CHQ-resistant malarial infection can be cured with CHQ by encapsulating it in target-specific liposomes. Because one of the reasons for parasite resistance to CHQ has been attributed to the enhanced efflux of CHQ from the resistant parasites, preventing intracellular

TABLE 3. Efficacy of CHQ against CHQ-resistant *P. berghei* infections in mice after encapsulating it in liposomes

Treatment and comparison group[a]	% Parasitemia on the following day posttreatment[b]:		Survival on day 30 post treatment (no. of surviving mice/total no. tested)
	8	30	
Saline	4.16 ± 1.03	All dead	0/8
	4.28 ± 0.62	All dead	0/8
	5.90 ± 2.30	All dead	0/10
Free CHQ	4.50 ± 0.95	All dead	0/8
	6.30 ± 1.94	All dead	0/8
	5.30 ± 0.92	All dead	0/10
PAb-Lip–CHQ	2.80 ± 0.62	0.00	3/8
	2.75 ± 1.40	0.00	4/8
	2.15 ± 0.69	0.05 ± 0.08	4/10
MAb D_2-Lip–CHQ	1.58 ± 0.43	0.00	4/8
	1.67 ± 0.48	0.00	4/8
	1.80 ± 0.71	0.02 ± 0.04	4/10
MAb F_{10}-Lip–CHQ	0.97 ± 0.19	0.00	7/8
	0.81 ± 0.26	0.00	6/8
	1.15 ± 0.25	0.00	8/10
P-value			
PAb-Lip–CHQ vs MAb D_2-Lip–CHQ	<0.01		>0.1[c]
	>0.05		
	>0.05		
PAb-Lip–CHQ vs MAb F_{10}-Lip–CHQ	<0.001		<0.01
	<0.01		
	<0.01		
MAb D_2-Lip–CHQ vs MAb F_{10}-Lip–CHQ	<0.05		<0.01
	<0.01		
	<0.05		

[a] Treatments with CHQ (5 mg/kg/day) were given intravenously on days 4 and 6 after infection. Three independent experiments were carried out for each treatment group.
[b] Values are means ± standard deviations.
[c] *P* values for survival data were calculated by pooling the data from all three experiments.

concentrations from achieving toxic levels (3, 10, 20), we infer that the CHQ delivered through MAb F_{10}-Lip could be reaching the infected cells in quantities sufficient to kill the intracellular parasite. This could indeed be the case, since delivery of CHQ in MAb F_{10}-Lip should help to concentrate the drug in infected cells not only by the selective recognition of these cells but also by the ability of liposomes to deliver several drug molecules at a time to the target cells.

Peeters et al. (12) have shown that encapsulation of CHQ in liposomes (nontargeted) increases not only the maximal tolerable dose from 0.8 to 10 mg of CHQ per animal when given i.p. but also the effectiveness of the drug against both the CHQ-susceptible and CHQ-resistant *P. berghei* infections. A dose of 8 mg per mouse per day for 3 consecutive days was found to be the most effective, which was at least 80 times greater (assuming that the average weight of a mouse is about 20 g) than the CHQ dose used in the present study. From these findings we conclude that the therapeutic efficacy of CHQ can be markedly increased by delivering this drug through target-specific liposomes.

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