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Chloroquine delivery to erythrocytes in *Plasmodium berghei*-infected mice using antibody-bearing liposomes as drug vehicles*

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Abstract. Suitability of anti-erythrocyte $F_{(ab')2}$-bearing liposomes as vehicles for chloroquine in the treatment of chloroquine resistant *Plasmodium berghei* infections in mice has been examined. Free chloroquine or chloroquine encapsulated in antibody-free liposomes failed to show much effect on the resistant infections, but the same doses of this drug after being encapsulated in antibody-bearing liposomes exhibited a significant inhibitory effect on this infection. These results indicate that chloroquine delivery in antibody targeted liposomes may help in the successful treatment of the chloroquine resistant malarial infections.

Keywords. Drug targeting; liposomes; antibody; erythrocyte; malaria; drug-resistant infection; mice.

1. Introduction

Antibody-bearing liposomes are useful as carriers in drug targeting to specific cells in experimental animals (Agrawal *et al* 1987; Bankert *et al* 1989; Hospenthal *et al* 1989; Hughes *et al* 1989). We have previously shown (Singhal and Gupta 1986) that liposome binding to erythrocytes can be markedly increased by covalently attaching anti-erythrocyte $F_{(ab')2}$ to the liposome surface. Also, it has been demonstrated that these liposomes could serve a useful purpose in drug homing to erythrocytes during malarial infections (Agrawal *et al* 1987). To further evaluate the usefulness of these liposomes as drug homing devices in malaria, we have now studied the efficacy of the liposomized chloroquine (chq) against both chq-sensitive and chq-resistant *Plasmodium berghei* infections in mice.

2. Materials and methods

2.1 *Materials*

Egg phosphatidylcholine (PC), egg [^{14}C] PC and gangliosides were prepared as described earlier (Singhal *et al* 1986). Chloroquine diphosphate, sodium cyanoborohydride and pepsin were purchased from the Sigma Chemical Company

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Abbreviations used: Chq, Chloroquine; PC, phosphatidylcholine.

St. Louis, Mo, USA. Na [^{125}I] (carrier-free) was obtained from the Bhabha Atomic Research Centre, Bombay.

2.2 *Liposomes*

Liposomes were prepared from egg PC (20 μmol), cholesterol (20 μmol) and gangliosides (4 μmol) in 0·8 ml of borate-buffered saline (10 mM borate, 60 Mm NaCl, pH 8·4) containing chloroquine diphosphate (350 μmol) by sonication (Kumar and Gupta 1985) and fractionated by centrifugation (Kumar and Gupta 1983). Free chq from drug entrapped liposomes was removed by gel filtration over Sephadex G-50 (Gupta and Bali 1981). The mean outer diameter of these liposomes, as determined by molecular sieve chromatography (Kumar and Gupta 1985) was about 45 nm. The amount of chq entrapped in liposomes was about 170 ± 3 μg/μ mol lipid P.

2.3 *Chq estimation*

Chq was estimated by measuring its absorbance at 342 nm as described earlier (Agrawal *et al* 1987).

2.4 *Anti-mouse erythrocyte $F_{(ab')2}$ bearing liposomes*

Anti-mouse erythrocyte antibodies were raised in rabbits and isolated from anti-serum following the procedure of Singhal *et al* (1986). The $F_{(ab')2}$ fragments from the antibody were prepared, purified and covalently attached to the liposome surface as described earlier (Singhal *et al* 1986). The liposomes were passed through a millipore filter (pore size, 0·22 μm) prior to their use in animal experiments. The protein-to-lipid ratio (Singhal *et al* 1986) in the liposomes was about 90 μg protein/μmol lipid P.

2.5 *Animals*

Randomly bred Swiss mice were obtained from the animal house of our institute. Male mice (8–10 weeks old) of 20 ± 2 g weight were used. The animals were kept in plastic cages and given a diet of pellets (Hindustan Lever Limited) and water *ad libitum.*

2.6 *Parasites*

P. berghei parasites were obtained from the National Institute of Communicable Diseases, New Delhi, and maintained in the Swiss mice through serial blood passage. The strain was fully sensitive to chq; the ED 90 being 15 mg/kg × 4 days (i.p.). Parasitaemia was determined by counting 10^3 red cells in thin blood smear stained with Giemsa, and expressed as number of parasitized cells/100 erythrocytes.

2.7 *Development of chq-resistance*

Chq-resistance was developed by the relapse technique, as described earlier (Warhurst and Folwell 1968). Mice were infected with about 10^7 *P, berghei*-infected

erythrocytes and on the same day, a single dose (60mg/kg, i.p.) of chq was administered. After the animals developed about 2% parasitaemia, the infected blood from these animals was transfused into healthy animals which were also given chq (60 mg/kg, i.p.) simultaneously. The above operation was repeated several times till the infection was rendered resistant to chq (50 mg/kg× 4 days, i.p.).

The chq-resistant *P. berghei* strain used in this study was developed about 4 years ago by us using the above technique, and has since been regularly maintained in mice under constant drug pressure. The strain retained its chq-resistant character for about a year even after withdrawing the drug pressure.

2.8 *Drug treatment*

Swiss mice (4–5 animals/group) were infected on day zero with about 10^6 erythrocytes infected with chq-sensitive or chq-resistant *P. berghei* strains. These animals were given a single intravenous dose of chq, chq loaded in non-targeted liposomes (free of $F_{(ab')2}$) or chq encapsulated in targeted liposomes (bearing $F_{(ab')2}$) on day 4 after the infection when parasitaemia was 0·01–0·10%. Parasitaemia was determined regularly from day 5. The per cent suppression of parasitaemia in animals treated with drug-laden liposomes was calculated by comparing the parasitaemias in these animals with those treated with an identical dose of free chq or buffer.

3. Results

Liposomes were formed from PC, cholesterol and gangliosides both in the presence and absence of chq by sonication and fractionated by ultracentrifugation. Most of the chq (over 90%) loaded in liposomes were found to reside in the liposomes internal aqueous phase rather than the lipid bilayer. Targeted liposomes were prepared by covalently attaching anti-mouse erythrocyte $F_{(ab')2}$ to the non-targeted liposomes surface. Mice infected with chq-sensitive or chq-resistant *P. berghei* strains were given only one dose of free or liposomized chq by intravenous route on day 4 after the infection. This administration of liposomes did not induce hemolysis, as judged by the measurement of haemoglobin levels in plasma of the injected animals (data not shown).

Erythrocyte surface structure and properties are altered during malarial infection (Howard 1982). It may thus be argued that the targeted liposome preparation used in this study could have an altered erythrocyte binding in infected mice as compared to the normal animals. To examine this possibility, we have determined the tissue distributions of liposomes before and after infecting the animals with *P. berghei.* Figure 1 shows that this distribution was not much affected by the infection, suggesting that the erythrocyte binding capacity of targeted liposomes was not influenced by infecting the animals with *P. berghei* (0·01–0·10% parasitaemia).

Efficacy of chq against malarial infections was examined at its various doses in mice infected with chq-sensitive *P. berghei.* Free and liposomized chq were administered to the separate groups of infected animals, and the efficacy of this treatment was determined by comparing parasitaemias in these animals with those treated with saline. Table 1 shows that the efficacy of chq against malarial infection

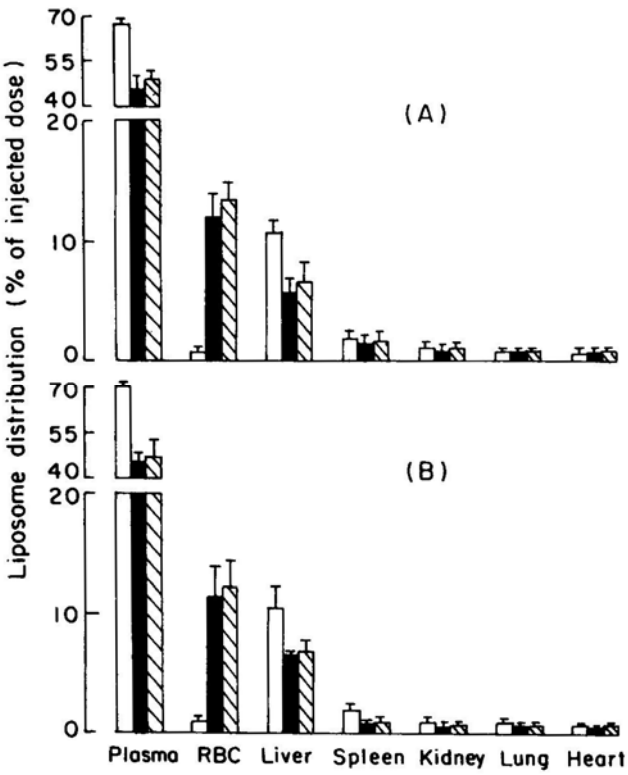

Figure 1. Distribution of liposomes in various tissues. Values shown are mean of 4 animals ± SD. (**A**) Normal mice. (**B**) *P. berghei*-infected mice. Tissue distributions were determined 15 min after injecting the liposomes, essentially as described earlier (Singhal and Gupta 1986). The non-targeted liposomes were radio-labelled by incorporating traces of egg [^{14}C] PC in their bilayers, while the 'targeted liposomes besides having egg [^{14}C] PC in their bilayers also contained [^{125}I] labelled $F_{(ab')2}$ on their surface. (□), Non-targeted liposomal [^{14}C] ; (■), targeted liposomal [^{14}C] ; (▩), targeted liposomal [^{125}I].

Table 1. Efficacy of chq against chq-sensitive *P. berghei* infection in mice after encapsulation in targeted liposomes.

		Efficacy on day 6		Efficacy on day 11	
Treatment	Dose (mg/kg)	Parasitaemia inhibition (%)	Survival	Parasitaemia inhibition (%)	Survival
Free Chq	5	64·6 ± 5·4	9/10	43·4 ± 7·7	5/10
	2·5	43·1 ± 3·0	8/10	31·5 ± 2·7	3/10
Liposomized Chq	5	95·2 ± 0·8	10/10	83·0 ± 2·7	9/10
	2·5	87·3 ± 3·9	9/9	62·3 ± 5·1	7/9

Treatments were given on day 4 after the infection. Values are mean ± SE.

was considerably increased by delivering it in targeted liposomes; both the per cent survival and per cent parasitaemia suppression were high in the liposomized chq-treated animals as compared to those treated with free chq. This result is in agreement with earlier studies (Agrawal *et al* 1987).

To ascertain whether this method of chq delivery could be effective in controlling the chq-resistant malarial infections also, we evaluated the efficacy of free and liposomized chq in mice which were infected with chq-resistant *P. berghei.* The parasites were made resistant to chq as described earlier in §2. The chq resistance was ascertained after treating the infected animals with chq. While this drug failed to eliminate the resistant infections even at 50mg/kg×4 days (i.p) dose, it completely cured the chq-sensitive infections at a much lower dose (15 mg/kg× 4 days, i.p.).

Table 2 shows that parasitaemias in chq-resistant *P. berghei*-infected mice after treatment with free chq were similar to those observed in saline or drug-free liposome-treated animals. However, the same doses of chq were very effective in controlling the chq-resistant infections when delivered in targeted liposomes (table 2). A 5 mg/kg dose of the liposomized chq appeared optimal, as the antimalarial effect did not significantly improve by increasing the drug dose (data not shown). This dose (5 mg/kg) of the liposomized chq was very effective in suppressing the parasitaemias at least up to day 12 after the infection (figure 2). Also, it considerably prolonged the survival time of the treated animals, when compared to the animals which were given chq in free form (figure 3),

4. Discussion

This study demonstrates that efficacy of chq against the malarial infection is markedly increased by delivering this drug in anti-erythrocyte $F_{(ab')2}$-bearing liposomes. The liposomized chq is effective in controlling not only the chq-sensitive infections but also the infections that were resistant to free chq. The latter effect of the liposomized chq appears to be related to the ability of our liposomes to concentrate the drug in erythrocyte, as chq encapsulated in nontargeted liposomes failed to exhibit any effect against the resistant infections (table 2).

Although the targeted liposomes used in this study were derived by covalently attaching anti-mouse erythrocyte $F_{(ab')2}$ to the liposome surface, their ability to specifically recognize erythrocytes *in vivo* was not altered by the malarial infection (figure 1). This may be attributed to the wider specificity of our antibodies, the low degree of infection or both.

Table 2. Efficacy of chq against chq-resistant *P. berghei* infection in mice after encapsulation in targeted liposomes.

Treatment	Chq dose (mg/kg)	Parasitaemia on day 8 (%)	Survival on day 13
Saline	—	2·9 ± 0·8	0/4
Drug-free non-targeted liposomes	—	2·5 ± 0·4	0/4
Drug-free targeted liposomes	—	2·0 ± 0·2	1/4
Chq-laden non-targeted liposomes	5·0	2·5 ± 0·5	1/4
Chq-laden targeted liposomes	2·5	1·1 ± 0·2	2/4
	5·0	0·25 ± 0·04	3/4
Free Chq	2·5	2·1 ± 0·3	0/4
	5·0	1·9 ± 0·9	0/4

Treatments were given on day 4 after the infection. Values are mean ± SE

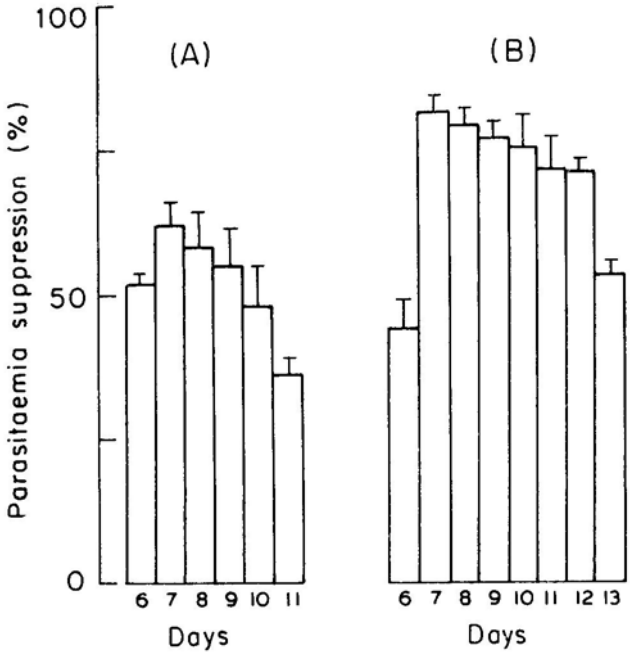

Figure 2. Efficacy of chq-laden targeted liposomes against chq-resistant *P. berghei* infection on various days after the treatment. The treatments were given on day 4 after the infection. Parasitaemia suppression was calculated by using the parasitaemias in free chq-treated animals as the control values (**A**) chq at 2·5 mg/kg dose. (**B**) chq at 5 mg/kg dose. Values are means ± SE. The number of animals used in each group was ten.

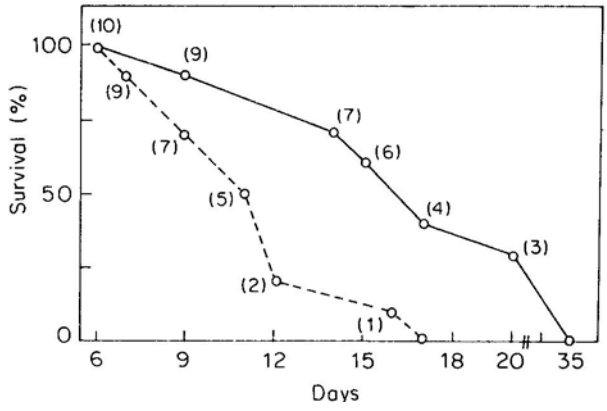

Figure 3. Effect of chq treatment before (broken line) and after (solid line) encapsulating the drug in targeted liposomes, on long-term survival of animals infected with chq-resistant *P. berghei.* The treatments (5 mg/kg dose of chq) were given on day 4 after the infection. Numbers in parentheses denote the number of animals surviving on a given day after the infection.

Peeters *et al* (1989) who recently studied the effect of liposomized chq on the chq-resistant *P. berghei* infections showed that encapsulation of chq in liposomes (non-targeted) increased not only the maximal tolerable dose from 0·8 to 10 mg chq/animal when given intraperitoneally but also the drug effectivity against the infections. A dose of 8 mg chq/mouse/day for three consecutive days was found to be the most effective. Although we have given only a single intravenous dose (5 mg/kg) of chq in targeted liposomes, which was much smaller than that used by these workers (2–8 mg/mouse), the antiparasitic effect observed by us against the chq-resistant infections was still quite significant. This finding clearly suggests that chq delivery in targeted liposomes could be successful in curing the chq-resistant infections, using low drug doses.

The demonstrated biologic response of chq-laden targeted liposomes against the chq-resistant malarial infections is not optimal, and may further be improved by using an infected erythrocyte-specific antibody rather than the nonspecific antibody, as used in this study. Also, the response may increase by changing the route of administration, and increasing the dose number. Finally, the intracellular concentration of chq in erythrocytes infected with chq-resistant *P. berghei* strain may be increased by encapsulating an appropriate Ca^{2+}-channel inhibitor along with chq in targeted liposomes, which in turn should increase the antimalarial activity (Martin *et al* 1987).

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