Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 357-361

FUNCTIONAL DRUG TARGETING TO ERYTHROCYTES IN VIVO USING ANTIBODY BEARING LIPOSOMES AS DRUG VEHICLES[1]

Ajay K. Agrawal, Arun Singhal, and C.M.Gupta[2]

Division of Membrane Biology,
Central Drug Research Institute,
Lucknow-226001, India

Received August 25, 1987

SUMMARY: Covalent attachment of anti-erythrocyte $F(ab')_2$ to the liposome surface has recently been shown to considerably enhance the liposome binding to erythrocytes in vivo. These antibody bearing liposomes have now been found quite effective as vehicles for delivering the antimalarial drug, chloroquine, to erythrocytes in Plasmodium berghei-infected mice. This demonstrates the usefulness of antibody targeted liposomes as carriers for site-specific drug delivery.

Liposomes bearing cell specific ligands on their surface have widely been considered quite useful as vehicles for site-specific delivery of drugs and enzymes in biophase (1,2). Our earlier studies have shown that binding of liposomes to red cells can considerably be enhanced by covalently attaching anti-erythrocyte antibody to their surface (3,4). It has further been demonstrated that at least 20-30% of the cell-bound liposomes delivered their contents to the target cells, presumably via membrane-membrane fusion (4). To examine whether this method of drug delivery would have any functional significance in drug targeting to erythrocytes, we have studied the effect of liposomised chloroquine, an antimalarial agent, on P.berghei infection in mice. Results of these studies indicate that efficacy of chloroquine to control malarial infection is significantly enhanced when delivered in anti-mouse erythrocyte $F(ab')_2$ bearing liposomes.

[1] CDRI Communication No. 4131.

[2] To whom correspondence should be addressed.

Abbreviations: PC, Phosphatidylcholine.

0006-291X/87 $1.50

MATERIALS AND METHODS

Materials: Egg PC, egg [^{14}C] PC and gangliosides were prepared as described earlier (3). Cholesterol was bought from Centron Research Laboratory, Bombay, and used after crystallizing it three times from methanol. Sodium cyanoborohydride and pepsin were purchased from Sigma Chemical Company. Sephadex G-50 and Sepharose 6B were acquired from Pharmacia Fine Chemicals. Chloroquine diphosphate was a kind gift from Walter Reed Army Institute of Research, U.S.A.

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 (350 μmol) by probe sonication (5), and fractionated by centrifugation (6). Free chloroquine from the liposomised drug was removed by gel filtration over Sephadex G-50 (7). The mean outer diameter of these liposomes, as determined by molecular sieve chromatography (5), was about 45 nm.

To test whether chloroquine is entrapped in liposomes or intercalated within the liposomes bilayer, the above liposome preparation was resonicated and gel filtered again over Sephadex G-50. This treatment was found to release over 90% of the initially entrapped drug as free chloroquine in the medium.

Leakage of the entrapped chloroquine was determined by incubating the liposomes with buffer at 37°C for 12 hr, separating free and liposomised drug by gel filtration, and then measuring the amount of chloroquine released in the medium. No leakage of chloroquine from the liposomes was detected under these conditions.

Chloroquine estimation: Chloroquine was estimated by measuring its absorbance at 342 nm. The amount of liposomised drug was determined after lysing the liposomes with Triton X-100 (1%). The chloroquine absorbance at 342 nm was found to remain unaffected by the presence of liposomes and the detergent, and was linear at least upto 100 μg quantity of chloroquine. Since protein absorbance interfered in accurately determining the amount of chloroquine entrapped in antibody bearing liposomes, these amounts were measured prior to the liposome coupling to anti-mouse erythrocyte $F(ab')_2$.

Anti-mouse erythrocyte $F(ab')_2$ bearing liposomes: Anti-mouse erythrocyte antibodies were raised in rabbits and isolated from anti-serum following our published procedure (3). The $F(ab')_2$ fragments from the antibody were prepared, purified, and covalently attached to the liposome surface as described earlier (3). The liposomes were passed through a millipore filter (pore size, 0.45 μm) prior to their use in animal experiments. The protein-to-lipid ratio (3) in the liposomes was about 65 μg protein/μmol lipid P.

P. berghei infection: P.berghei parasite was obtained from National Institute of Communicable Diseases, New Delhi, and maintained in Swiss male mice (average weight, 25 g) through serial blood passage.

Drug treatment: Chloroquine in free or liposomised form (0.2 ml) was given intravenously to mice 3 days after infecting them with P.berghei. The animals were kept under observation to record parasitaemia and mortality. Parasitaemia was determined by counting 10^3 red cells in a thin blood smear stained with Giemsa, and expressed as number of parasitized cells/100 erythrocytes. The animals given only Tris-buffered saline (0.2 ml) were used as controls, and referred to as untreated animals. The parasitaemia level on the day of chloroquine treatment was 1-2%, excepting one experiment where the level was 2-4%.

RESULTS AND DISCUSSION

The antimalarial drug chloroquine was entrapped in liposomes consisting of PC, cholesterol and gangliosides. Free drug from the liposomised chloroquine was removed by gel filtration. Most of the liposomised chloroquine resided in the liposomes internal aqueous phase rather than the phospholipid bilayer, as resonication of liposomes was found to release over 90% of the entrapped drung in the external aqueous medium. These liposomes were covalently coupled to anti-mouse erythrocyte $F(ab')_2$, and used in subsequent experiments after filtering them through a millipore filter (pore size, 0.45 μm).

Chloroquine is a very effective antimalarial drug when given orally at 20 mg/kg dose for three consecutive days. Since liposomes are knwon to be ineffective by oral route (8), we analysed the efficacy of liposomised chloroquine after its single intravenous administration to mice infected with P.berghei. The efficacy was determined from the ratio of the mean parasitaemia in drug-treated mice to the mean parasitaemia in untreated mice. These ratios were calculated at various intervals (e.g. day 1, day 2, day 3, etc.) after the drug administration, and compared to ascertain the relative efficacy.

A marked difference between the efficacies of free chloroquine and the chloroquine entrapped in anti-mouse erythrocyte $F(ab')_2$ bearing liposomes was observed on day 1 after the drug treatment (Fig. 1). Chloroquine entrapped in antibody targeted liposomes was at least 5-10 times more effective as compared to free chloroquine in controlling P.berghei infection in mice. The enhanced drug efficacy observed here is mainly due to targeted delivery of chloroquine to erythrocytes in vivo, since the antimalarial activity of chloroquine delivered in antibody-free liposomes was similar to that of the free drug (Fig. 1).

These results indicate that ability of chloroquine to suppress parasitaemias in P.berghei-infected mice is considerably enhanced by its encapsulation in anti-mouse erythrocyte $F(ab')_2$ bearing liposomes. this suppression

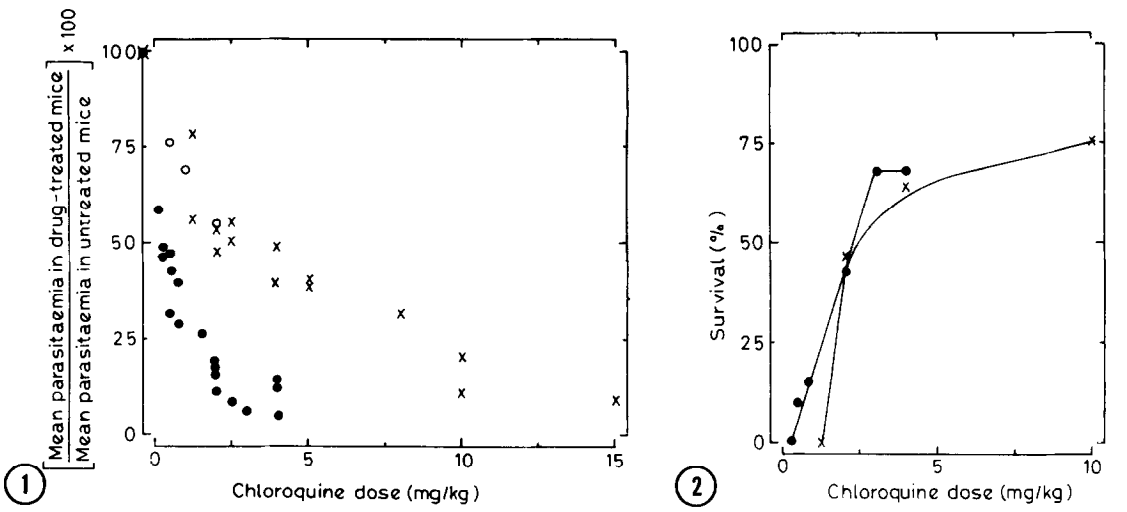

Fig. 1 Efficacy of free and liposomised chloroquine in controlling parasitaemias in P.berghei-infected mice. x, free chloroquine; o, chloroquine entrapped in antiboy-free liposomes; •, chloroquine entrapped in anti-mouse erythrocyte $F(ab')_2$ bearing liposomes. Chloroquine was administered intravenously on day 3 after the infection, and parasitaemia measured after 24 hr of the treatment. Each point is a mean of 4-6 animals. The number of points shown at a given chloroquine dose denotes the number of experiments carried out at that dose.

Fig. 2 Effect of chloroquine treatment on survival of P.berghei-infected mice on day 8 after the infection. x, free chloroquine; •, chloroquine entrapped in anti-mouse erythrocyte $F(ab')_2$ bearing liposomes. All the infected animals in untreated group died by day 8 after the infection. The number of animals for each chloroquine dose was 10-15.

though not significant on day 2 (or 3) after the drug administration, was quite marked on day 1 after the treatment. However, only marginal differences were observed in the numbers of animals which survived in free chloroquine and antibody bearing liposome-encapsulated chloroquine treated groups (Fig.2).

Several studies have shown that liposomes coupled to cell-specific antibody are very effective as vehicles for delivering bioactive substances to specific cells in vitro (9-12). The present study further extends the scope of this drug delivery method by demonstrating its usefulness in delivering drugs to target cells in vivo. It may be noted that inspite of the facts that only 15-20% of the injected dose of liposomes bind to the erythrocytes (4) and of these cell-bound liposomes, only 20-30% deliver their contents to the target cell (4), the chloroquine entrapped in antibody bearing liposomes is at least 5-10 times more effective than the free chloroquine in controlling malarial infection in animals. We, therefore, conclude that antibody

bearing liposomes could prove highly useful as carriers for targeting of drugs/enzymes to specific cells *in vivo*.

REFERENCES

1. Schneider, M. (1985) in: Drug Targeting (Buri, P. and Gumma, A. eds) pp. 119-134, Elsevier, Amsterdam, New York.
2. Alving, C.R. (1986) Parasit. Today 2, 101-107.
3. Singhal, A., Bali, A. and Gupta, C.M. (1986) Biochim. Biophys. Acta 880, 72-77.
4. Singhal, A. and Gupta, C.M. (1986) FEBS Lett. 201, 321-326.
5. Kumar, A. and Gupta, C.M. (1985) Biochemistry 24, 5157-5163.
6. Singhal, A., Bali, A., Jain, R.K. and Gupta, C.M. (1984) FEBS Lett. 178, 109-113.
7. Gupta, C.M. and Bali, A. (1981) Biochim. Biophys. Acta 663, 506-515.
8. Gregoriadis, G. (1985) Trends Biotechnol. 3, 235-241.
9. Huang, A., Kennel, S.J. and Huang, L. (1983). J. Biol. Chem. 258, 14034-14040.
10. Matthay, K.K., Heath,T.D. and Papahadjopoulos, D. (1984) Cancer Res. 44, 1880-1886.
11. Matthay, K.K., Heath, T.D., Badger, C.C., Bernstein, I.D. and Papahadjopoulos, D. (1986) Cancer Res. 46, 4904-4910.
12. Yemul, S., Berger, C., Estabrook, A., Suarez, S., Edelson, R. and Bayley,H. (1987) Proc. Natl. Acad. Sci. USA 84, 246-250.