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INCREASED STIMULATION OF ALKALINE PHOSPHATASE BY SMALL DOSES OF COLCHICINE ENTRAPPED IN LIPOSOMES

A BIOCHEMICAL TEST TO DETECT EFFECTIVE LIPOSOME–HEPATOCYTE INTERACTION

JORGE CERBON,* MERCEDES NORIEGA and MARCOS ROJKIND

Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del IPN, 07000 México, D.F., México

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Abstract—The subcutaneous administration of colchicine encapsulated in small unilamellar vesicles reduced the initial toxicity peak and maintained for several days an adequate level of the drug in the liver. Colchicine is an excellent marker for effective liposome–hepatocyte interaction since it fulfills the following criteria: (a) When taken up by the hepatocytes within liposomes, it is active and induces the synthesis of alkaline-phosphatase two to three times over control values. The injection of at least ten times more free colchicine is necessary to attain a similar induction. (b) If released from extracellular liposomes, colchicine is cleared rapidly from the circulation. The results show that liposomes, in spite of their reduced aqueous compartment (~ 1.0 μl/μmole of lipid), can achieve clinical utility when administered subcutaneously because of their efficient interaction with parenchymal cells and their continual arrival from the injection site.

The potential usefulness of liposomes as carriers of therapeutic agents has been reviewed recently [1–3]. The major uptake of liposomes *in vivo* by liver and spleen [4] and the enhanced rate of leakage of the entrapped solutes in plasma [5] may restrict their successful application as drug carriers in therapy. Also, the retention and uptake of circulating liposomes larger than 1000 Å in diameter by cells of the reticulum endothelium system (RES) and blood monocytes are major obstacles to experimental efforts to target liposomes to other cell types [6]. Effective contact of liposomes with cells should be an essential prerequisite *in vivo* if liposomes, as carriers for drugs, are to achieve widespread clinical utility. [^{14}C]Inulin has been utilized and found to provide a valid index of the presence of intact liposomes [7]. It does not, however, distinguish between liposomes taken up by the cells and those that remain extracellular, either adsorbed to organ parenchymal or endothelial cells or trapped mechanically in capillary beds. Also, it will be necessary to be able to administer them by traditional routes: intramuscular (i.m.), subcutaneous (s.c.), and oral. With few exceptions, the great majority of studies with drugs have been performed utilizing intravenous administration and large vesicles. In the present work we decided to utilize small vesicles because they are cleared less rapidly than large ones [8] and can reach cells other than those of the RES. The vesicles were introduced by s.c. injection because, although liposome uptake by phagocytic tissue will presumably occur, the mononuclear phagocyte system would not be expected to dominate liposome disposition to the extent observed when liposomes are injected i.v. The drug of choice was colchicine, because when administered *in vivo* it increases the activity of alkaline phosphatase in the liver of rats [9] and the increment is dose and time dependent [10]. These properties allow one to know, not only that the drug is present, but that it is acting upon hepatocytes. It is known that long-term colchicine treatment causes a reduction in some of the clinical manifestations of liver cirrhosis and induces the synthesis of alkaline phosphatase in patients [11].

The present results show that colchicine is an excellent probe for efficient liposome–hepatocyte interaction and suggest that drugs administered parenterally in small vesicles can achieve clinical utility. The entrapped drug was as active as ten times more free colchicine in its capacity to induce alkaline phosphatase activity.

MATERIALS AND METHODS

Materials and animals. All the experiments were performed with adult Wistar rats weighing 180–200 g. Egg phosphatidylcholine (PC) was prepared as described [12] and cholesterol was purchased from Sigma (St. Louis, MO). Tritium-labeled colchicine [ring C, *methoxy*-^{3}H] (19.6 Ci/mmole) was obtained from New England Nuclear (Boston, MA). Radio-

* Address all correspondence to: Dr Jorge Cerbon, Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 14–740, 07000 México, D.F., México.

active colchicine was at least 95% pure as indicated by thin-layer chromatography. Other chemicals were reagent grade and were used as purchased. Aquasol solution was used as scintillation liquid.

Preparation of liposomes. Liposomes containing colchicine were prepared largely according to methods previously described [8]. Briefly, a solution of 500 mg of lipids dissolved in chloroform (phosphatidylcholine and cholesterol, 2:1 molar ratio) was evaporated under nitrogen into the wall of a glass tube, followed by the addition of 10 ml of buffer (isotonic phosphate-buffered saline, pH 7.2) containing [^{3}H]colchicine to be incorporated by the liposomes. Sonic dispersion was carried out with a Branson sonifier (B-12, 60 W) under nitrogen atmosphere for 60 periods of 3 min each, with a 1-cm diameter tip. Small unilamellar vesicles containing colchicine were separated from free drug by filtration on a Biogel A-5 m column. The void volume fraction contained all the lipid plus encapsulated drug, whereas the free drug was retarded by the column. Drug-containing liposomes were further processed by centrifugation at 145,000 *g* at 4° per 15 min in order to remove large liposomes and by Amicon filtration to concentrate the sample. Two percent of the total drug added was entrapped.

Clearance and disposition studies. Colchicine, free or encapsulated in liposomes, was given to rats by subcutaneous (s.c.) injection into the outer part of the left foot. In the cases where free drug was used, 10 μg of the drug was injected per animal, whereas usually 5 μg of encapsulated colchicine was used. The specific activity of the injected drug was 2 μCi/10 μg. For determination of the elimination rate from the blood, serum samples were taken at 30–60 min post-injection and periodically thereafter. Serum levels of tritium were determined and expressed as nmoles/ml. The tissue disposition of the administered drug was determined from 1 up to 70 hr post-injection. The following tissues were taken: spleen, kidney, heart, lung, liver and brain. Samples were homogenized with 3 vol. of phosphate-buffered saline and 0.14 ml of the homogenate was mixed with 12.5 ml of aquasol and counted in a Packard scintillation counter (Mod. 577) with automatic external standarization. Tritiated water (^3H_2O) was utilized also as an internal standard.

Identification of colchicine in the liver. Approximately 2 ml of liver homogenate was mixed with 20 ml of 10% sodium dodecyl sulfate (SDS) and 6.0 ml of chloroform. After vigorous shaking, the emulsion was separated by centrifugation at 3500 rpm for 15 min at 4°. The organic layer containing colchicine was evaporated to dryness, redissolved in a small amount of chloroform, and applied onto a silica thin-layer plate. Chromatography was performed using absolute methanol as solvent [13]. A standard of colchicine was always utilized to facilitate the location of labeled colchicine in the sample. The position of colchicine was determined by its u.v. absorption. Bands 0.25 cm wide were scraped from the origin to the front of the solvent, added to vials that contained Aquasol, and counted. In some cases, a saturating amount of sodium sulfate was added to break the emulsion, and the process was repeated up to four times. The chloroform extracts were mixed and evaporated to dryness under a stream of nitrogen. The sample was dissolved in a small amount of solvent containing cold colchicine, run in thin-layer chromatography, and counted as described.

Separation of parenchymal and non-parenchymal cells. Parenchymal cells were isolated after perfusion of the liver with collagenase, and non-parenchymal cells were isolated after perfusion with pronase. Cell separation and determination of label uptake were done according to Ref. 14.

Data analysis. For comparison of the total amount of drug retention in tissues after administration of free or encapsulated colchicine, the following analysis was used. Measured values of tritium (disintegrations per minute) were converted into nanograms of drug. By expressing the results in terms of ng/g of tissue the data are independent of organ size.

Alkaline phosphatase activity. Liver samples were homogenized with 4 vol. of 0.25 M sucrose and extracted with *n*-butanol as described by Morton [15]. In brief, *n*-butanol was added to the liver homogenate up to a final concentration of 18% (by volume), and the sample was stirred vigorously with a magnetic stirrer for 30 min at 4°. After centrifugation at 22,500 *g* for 30 min at 4°, the aqueous layer was removed carefully and used for measuring the enzyme activity as follows. The assay mixture (1 ml) contained 10 mM *p*-nitrophenyl phosphate, 100 mM glycine, 1 mM magnesium chloride, and 1 μM zinc sulfate, adjusted to pH 10.5 with sodium hydroxide. The reaction was initiated by the addition of 0.1 ml of enzyme and was terminated after incubation for 30 min at 37° by the addition of 10 ml of 0.02 M NaOH. The formation of *p*-nitrophenol was estimated spectrophotometrically at 410 nm. Background color was subtracted after bleaching the *p*-nitrophenol color with 0.1 ml of 12 N HCl. One unit of enzyme activity (I.U.) was defined as the formation of 1 nmole of *p*-nitrophenol per min at 37°. Results were expressed as units of enzyme activity per g of fresh liver ± S.D. Optimum recovery of enzymatic activity was obtained when butanol extraction was repeated two more times. The results are the sum of the activities determined in the three aqueous layers obtained after butanol extraction. Protein was estimated by the method of Bradford [16] using seroalbumin as standard.

RESULTS AND DISCUSSION

The fate of small doses of colchicine (5 μg/200 g body wt) injected subcutaneously entrapped in liposomes consisting of phosphatidylcholine (PC) and cholesterol (CHOL) in a 2:1 molar ratio was investigated. All vesicle preparations had an entrapped volume of 1.0 μl/μmole of lipid, based on [^{3}H]colchicine encapsulation. Vesicles with a diameter of 50 nm show an entrapped volume of about 1.2 μl/μmole [17]. Therefore, our preparations consisted primarily of vesicles not larger than 50 nm. Because of its solubility in water, it seemed likely that colchicine would be in the internal aqueous compartment of the liposomes, although NMR studies (unpublished) indicate that colchicine may also intercalate into the hydrocarbon region of the liposome membrane. Once prepared, the liposomes

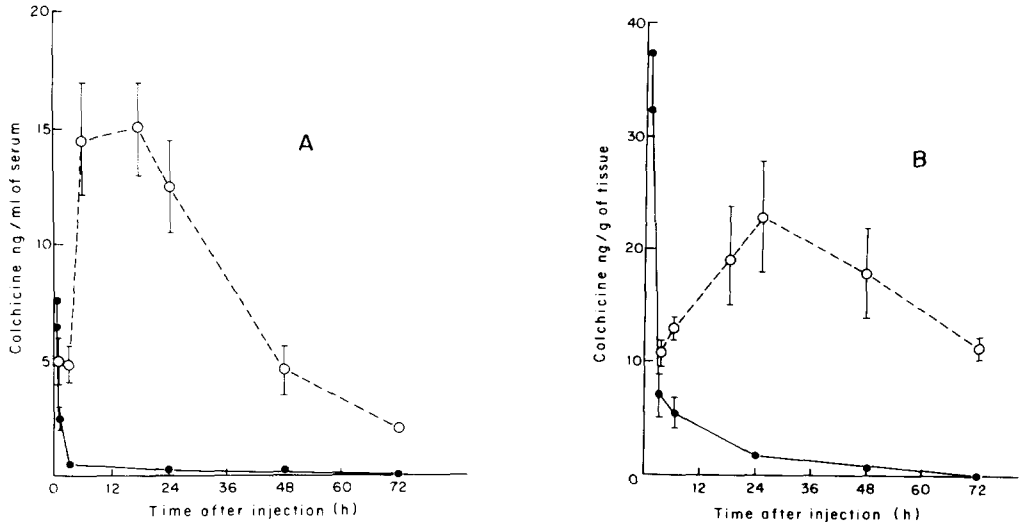

Fig. 1. Time course of the uptake of colchicine after subcutaneous injection of 5 μg of ^{3}H-labeled colchicine per animal (Wistar rats). At various times after injection, the amount of radioactivity associated with serum (A) or liver (B) was determined. Mean values ± S.D. of four animals are given. Key: (●—●) free; and (○—○) vesicle encapsulated.

were used within 24 hr, the time in which only 4% of the drug was lost (data not shown). Previous *in vitro* experiments have shown that colchicine remains firmly associated with liposomes, even in the presence of blood elements [8].

Disposition of free and encapsulated drug after s.c. injection. Figure 1A shows that, 6 hr after s.c. injection of free colchicine, the label was almost completely removed from the blood, whereas when injected entrapped in liposomes, there was a substantial rise in activity in the blood from 3 to 6 hr. This time course is indicative of a transport of vesicle-associated ^{3}H-label from the site of injection to the blood. This level of drug was maintained for at least 18 hr more due to a sustained release of drug from the subcutaneous depot and then leveled off slowly. After 72 hr, the level of drug was still higher than that observed at 3 hr after injection of the free drug. A similar behaviour was observed with the liver uptake (Fig. 1B). After s.c. injection of free colchicine, the label was removed from the liver very fast in the first 3 hr and disappeared after 24–48 hr. When the drug was injected s.c. but entrapped in liposomes, the proportion of liver-associated drug rose two times from 3 to 24 hr and then leveled off very slowly, returning after 72 hr to values similar to those found during the first 3 hr. This time course suggests a very effective mechanism of capture of vesicle-associated drug by the liver, favored by the slow rate of blood clearance. An almost identical behavior has been observed with small (30–60 nm) liposomes composed of PC and equimolar cholesterol and containing quenched carboxyfluorescein and ^{111}In-labeled bleomycin after footpad injection [18]. From the values of latent carboxyfluorescein in the plasma, PC-small liposomes entered the blood circulation intact with 3.7 ± 0.3% of the dose per g of liver remaining at 48 hr post-injection. This value is very similar to the one observed for colchicine (~4%). To know if there is a real intracellular uptake of vesicles by this organ, colchicine uptake by parenchymal cells and non-parenchymal cells was determined. When the level of entrapped drug was at its maximum value (at 24 hr), the results showed that 85–90% of the liver-associated label was in the parenchymal cells, while an average of 15% was recovered in the non-parenchymal cells (17,070 ± 1,500 dmp/10^{7} PC vs 3,210 ± 300 dpm/10^{7} NPC, mean ± S.D. of four animals). Similar results have been obtained for neutral cholesterol/sphingomyelin small vesicles (1:1 molar ratio) injected intravenously [14]. Contrary to these results, large vesicles composed of PC/CHOL are cleared from the circulation of the rat by the RES [6].

Colchicine induction of alkaline phosphatase activity. It is known that *in vivo* administration of 50–100 μg of colchicine/200 g body weight to rats increases the activity of hepatic alkaline phosphatase two to three times above normal values [9] and that the effect is dose and time dependent [10]. The dose of colchicine employed for the treatment of experimental liver cirrhosis in rats (10 μg/200 g of body weight per day [19]) is at least ten times smaller than that used to increase the alkaline phosphatase activity [9] and twenty times smaller than that used to impair secretory functions in rat liver [10, 20]. Because there is an effective uptake of the drug by the liver when administered entrapped in liposomes and the drug remains in the organ for long periods of time, its possible effect on the induction of alkaline phosphatase activity was determined. It can be seen (Fig. 2) that the s.c. injection of 10 μg of colchicine entrapped in liposomes can increase up to four times the alkaline phosphatase activity over that of controls (injected with isotonic saline). The s.c. injection of free colchicine (10 μg daily during 5 consecutive

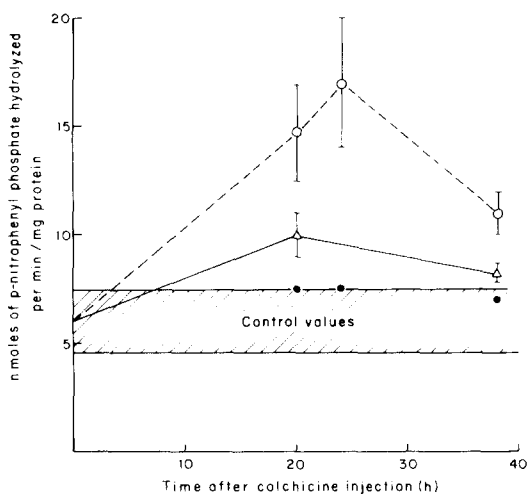

Fig. 2. Changes in the activity of alkaline phosphatase in homogenates of rat livers as a function of time after the administration of colchicine. Rats were injected subcutaneously with 10 μg of colchicine/200 g body weight. Liver homogenates were prepared from three rats for each experiment at the times indicated, and alkaline phosphatase was determined. Each point represents the average of the results obtained from two experiments ± S.D. The control values are from three experiments. Key: (○—○) vesicle encapsulated; (●—●) free; and (△—△) free, during 5 consecutive days.

days) induced the alkaline phosphatase activity two times over that of control rats, whereas with the s.c. injection of a single dose of 10 μg of colchicine no significant increment was found. The maximum activity was observed at 20–24 hr, in agreement with previous reports [9, 10].

The effect of very large doses of free colchicine (100 μg to 1 mg/200 g body wt) is maintained only during 7 hr suggesting that, during this time, the drug within the cells may be gradually metabolized into an inactive form [20]. We found that, at 24–48 hr after the injection of colchicine entrapped in liposomes, 50% of the total liver radioactivity could be extracted by chloroform and comigrated with colchicine in thin-layer chromatography. Control experiments in which [^{3}H]colchicine was added to liver homogenates and extracted with the same procedures gave 60–70% recovery. The results showed that the continual arrival of small amounts of liposome-entrapped colchicine was as effective as a single dose of ten times more free colchicine, for increasing the alkaline phosphatase activity of the rat liver. Furthermore, the induction of enzymatic activity allows one to distinguish liposomes that are taken up by the cells from those that remain extracellularly. The daily administration of small doses of colchicine to rats during several days also causes an increment in the activity of this enzyme. Patients under prolonged colchicine treatment for liver cirrhosis show a similar behavior [11]. The tissue distribution of colchicine entrapped in liposomes following s.c. administration is shown in Table 1. It can be seen that a small percentage of drug concentrated in the liver, plasma, kidney and spleen, whereas very small amounts were recovered in other tissues. Other workers found an important local retention of liposome label after s.c. injection [21].

With free colchicine, it was obvious that the rate of uptake was faster in the first hour (Fig. 1, A and B). This initial high concentration may be toxic. The results are compatible with the view that liposomes administered subcutaneously may serve as a depot from which sustained release of entrapped drug may take place [2, 18]. The type of vesicle that we used seems particularly useful for this purpose because, in spite of its low trapping efficiency, it has a slow rate of clearance from the blood and a suitable interaction with cells other than those of the RES. Information is needed to examine their safety when administered repeatedly at frequent intervals over periods of several weeks or months. Although contrary to other lipids (glycolipids, cardiolipin and phosphatidylserine), phosphatidylcholine and cholesterol are non-antigenic [22]. The use of colchicine and colchicine derivatives as antimitotic agents in cancer therapy is being limited because of their toxicity [23]. Colchicine, as other antitumor agents, has a rather short biological half-life due to both rapid excretion and rapid metabolic inactivation [24]. As described in this paper (Fig. 1, A and B, and Table

Table 1. Radioactivity distribution 24 hr after subcutaneous injection of [^{3}H]colchicine

Organ	% Injected label		(ng/g Drug* tissue)	
	Free	Entrapped	Free	Entrapped
Liver	0.42 ± 0.06	4.56 ± 1.00	2.6 ± 0.3	28.5 ± 4.0
Spleen	0.06 ± 0.1	1.36 ± 0.04	0.6 ± 0.08	13.6 ± 2.0
Kidney	0.14 ± 0.01	0.23 ± 0.05	4.1 ± 0.5	6.7 ± 0.7
Brain	0.02 ± 0.005	0.02 ± 0.002	0.5 ± 0.05	0.5 ± 0.06
Heart	0.02 ± 0.005	0.03 ± 0.007	1.2 ± 0.15	2.0 ± 0.25
Lung	0.08 ± 0.01	0.06 ± 0.01	3.6 ± 0.5	2.7 ± 0.3
Serum	0.10 ± 0.05	1.27 ± 0.15	1.25 ± 0.15	15.85 ± 2.0

Animals were killed 24 hr after injection of radiolabeled free or liposome-encapsulated drug, and organ samples were removed and processed as described in Materials and Methods. The data represent the means ± S.D. for three to five animals.

* Measured values of Tritium (dis/min) were converted into nanograms of drug. By expressing the results in ng/g of tissue, the data are independent of organ size.

1), liposome encapsulation prolonged the plasma life-time and increased tissue retention of colchicine. These effects may be especially important for drugs that have to act during an important period of time in the cell cycle. It has been proposed that liposome encapsulation may allow the establishment of an adequate treatment without the necessity of continuous drug infusion [25]. It is known that encapsulation of amphotericin B in liposomes markedly reduces its toxicity including nephrotoxicity, while causing minimal changes in its therapeutic potency [26]. Thus, it is possible to administer larger doses of encapsulated than of free drug and obtain superior action. In the present study we show that it is also possible to administer smaller doses and obtain a higher activity. Administration of small liposomes can also cause blockade of RES clearance, but the onset of blockade is delayed until a substantial fraction of liposomes have been removed from the circulation. For blocking the RES clearance, a loading dose of small liposomes (25 mg PC/12.5 mg CHOL per rat) has been used per intravenous route [6]. The dose we used was similar (25 mg PC/6.5 mg CHOL per rat) but the route used (s.c.) was different. Contrary to the rapid accumulation of SUVs when injected intravenously [6], there is a continuous arrival of small amounts of drug entrapped in liposomes when injected s.c. with only 8% found in the liver at the 24-hr time period examined.

An average of only 15% of the drug was recovered in the non-parenchymal cells at this time period. Therefore, under these conditions, a blockade of the RES is unlikely to occur. It has been reported that up to 72% of the lipid can be found in the skin 24 hr after s.c. injection of small liposomes [21]. Experiments are in progress to test the effect of colchicine entrapped in small liposomes on the development of experimental liver cirrhosis and liver cancer.

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