LIPOSOMES CONTAINING CHELATING AGENTS

Cellular Penetration and a Possible Mechanism of Metal Removal

YUEH-ERH RAHMAN and BETTY JEAN WRIGHT
From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

ABSTRACT
Electron microscope studies were done on mouse liver, from 5 min to 8 wk after an intravenous injection of liposomes containing ethylenediaminetetraacetic acid (EDTA). Livers of mice receiving an injection of liposomes containing KCl instead of EDTA or an injection of a solution of EDTA were also examined. Liposomes were shown to be phagocytized by hepatocytes as well as by Kupffer cells within minutes after the injection. Initially, there was a close contact between the liposomal membrane and the cellular membrane, followed by an invagination of the latter and the formation of a distinct vesicle surrounding a single liposome or a cluster of several liposomes. No fusion between the liposomal membrane and the cell membrane was observed. Between 15 min and 6 h after liposome injection, the Kupffer cells were found to have an increased number of lysosomes and autophagic vacuoles. Within the latter, morphologically intact liposomes or remnants of liposomes could be seen. At 12 h after injection, a striking increase in macrophages was observed in the liver sinusoids of EDTA-liposome-injected mice, but not in those of KCl-liposome-injected mice. Within the macrophages, remnants of liposomes occasionally could be observed. However, the origin and the physiological role of these cells are unknown. In the hepatocytes, morphological changes were first observed 24 h after injection; there were large numbers of autophagic vacuoles, and some cells showed extensive areas of focal cytoplasmic degeneration. The morphology of the liver cells returned to normal about 7 days after injection. No morphological changes were observed in livers of mice receiving EDTA solution without liposomes. A possible mechanism by which the liposome-encapsulated chelating agents can successfully remove intracellular toxic metals is discussed. The use of liposomes as carriers seems to be a useful tool for intracellular delivery of chelating agents or drugs in general.

For the last 20 yr, the polyaminopolymeric acid-chelating agents, ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA), have been the drugs of choice for therapy of poisoning by various metals. However, the inability of these chelating agents to cross cellular membranes (1, 3) seriously limits their efficacy in removing the intracellular fraction of...
the metals. We have encapsulated these agents within liposomes, which are multilayered lipid spherules made of a dried thin film of a phospholipid (e.g., lecithin) with or without cholesterol. Liposomes containing DTPA, when injected into mice, are readily taken up by tissue cells. These liposomes have been successful in removing various intracellularly deposited toxic metals from mice (e.g., plutonium, lead, and inorganic mercury) (7, 8).

Morphological studies of cells in various tissues after injection of liposomes containing chelating agents were undertaken in order to elucidate: (a) the way liposomes are taken up by cells; (b) the mechanism by which the liposome-transported chelating agent could successfully chelate and remove the intracellularly deposited metals; (c) the possible cellular damage induced by the intracellular release of chelating agents by liposomes. Liposomes were found to be readily taken up by cells in various tissues. The present paper will describe the uptake of liposomes containing chelating agents into liver cells and the cellular responses at subsequent time intervals. A possible mechanism of removal of intracellular metals from liver cells by liposome-encapsulated chelating agents is presented.

**MATERIALS AND METHODS**

**Materials**

Phosphatidylycholine (egg lecithin) was obtained from Sigma Chemical Co., St. Louis, Mo. Cholesterol was acquired from Applied Science Labs., Inc., State College, Pa. Uniformly labeled [14C]ethylenediaminetetraacetic acid ([14C]EDTA), in the form of the sodium salt and at a specific activity of 24 mCi per millimole, was purchased from Amersham/Searle Corp., Arlington Heights, Ill. Nonlabeled EDTA was obtained from Fisher Scientific Co., Fair Lawn, N.J.

**Preparation of EDTA-Liposomes**

Liposomes containing EDTA were prepared as previously described (9). A mixture of phosphatidylycholine (PC) and cholesterol, at a ratio of 3:1 on a weight basis, was used throughout the present study. When PC is partially oxidized or contaminated with free fatty acids, the liposomes obtained tend to aggregate into small clumps. Therefore, PC of high purity is required for satisfactory preparation of liposomes.

**Morphological Identification of EDTA-Liposomes in Mouse Liver After an Injection of Concentrated Liposomes**

As a pilot experiment to identify liposomes within the liver, each of two female CFI mice (Carworth Div., Charles River Breeding Laboratories, Inc., Wilmington, Mass.), 95 days of age and weighing 28.0 g, received a single injection of 1.0 ml of concentrated EDTA-liposomes containing 48 mg of total lipid, via a tail vein. They were killed 10 min after the injection, and liver samples were taken for electron microscope examination.

**Injection of EDTA-Liposomes for Electron Microscope Studies on the Cellular Uptake and Subsequent Fate of Liposomes**

A total of 18 female CFI mice were used. Each mouse received a single intravenous injection of liposomes containing about 90,000 cpm of [14C]EDTA, with a total dose of EDTA at 30 mg/kg and in a vol of 0.4 ml as previously described (9). Two mice were killed at each of the following time intervals after the liposome injection: 5 min, 15 min, 1 h, 6 h, 12 h, 1 day, 3 days. A single mouse was killed at 7 days, 2.5 wk, 4 wk, and 8 wk. Samples from the liver were taken for electron microscope examination.

**Injection of KCl-Liposomes and Nonliposome-encapsulated EDTA for Electron Microscope Studies**

Two groups of six mice each were used. One group received a single intravenous injection of liposomes containing KCl instead of EDTA, and the other group a single intravenous injection of EDTA solution. The amount of lipid in the liposomes, or the dosage of EDTA, received by these mice was the same as that received by the EDTA-liposome-treated mice. A single mouse from each group was killed at the following time intervals after injection: 15 min, 6 h, 12 h, 24 h, 1 wk, and 2 wk. Samples from the liver were taken for electron microscope examination.

**Preparation of Tissues for Transmission Electron Microscopy**

Small pieces of tissue were fixed for 4-6 h in phosphate-buffered paraformaldehyde-glutaraldehyde (4) at pH 7.4, washed overnight in phosphate buffer, and postfixed in phosphate-buffered 1% osmium tetroxide for 1 h. Some samples were fixed directly in phosphate-buffered 1% osmium tetroxide for 1 h. After osmium fixation, the tissues were rinsed with phosphate buffer, dehydrated in graded alcohols, and infiltrated and embedded in Epon (5). Sections were cut with a DuPont diamond knife (DuPont Instruments, Wilmington, Del.)

---

1 M. W. Rosenthal, Y.-E. Rahman, E. S. Moretti, and E. A. Cerny. Submitted for publication.
on a Huxley ultramicrotome, double-stained with 2% uranyl acetate for 1 h and then lead citrate (12) for 2 min, and examined in a JEOLCO JEM-7A electron microscope.

RESULTS

Liver Morphology of Mice After Receiving Nonliposome-encapsulated EDTA

Between 15 min and 2 wk after a single intravenous injection of EDTA solution, the livers of mice show no morphological changes.

Morphological Identification of Liposomes Injected into Mice

The morphology of liposomes can be easily demonstrated in the mouse liver after an injection of highly concentrated EDTA-liposomes (Fig. 1). The liposomes appear as bodies formed of multiple bilayers of lipids. The lipid bilayers have a rather dense appearance under the electron microscope. The irregular shape of injected liposomes differs from the usual spheroid shape of liposomes suspended in solution. 10 min after the injection of such a large amount of liposomes, most of the liposomes are still seen in the liver sinusoids. However, some are within the space of Disse among the microvilli of the hepatocyte (Fig. 2) and some are more deeply lodged within the infoldings of the cellular membrane (Figs. 3, 4).

Uptake of Liposomes by Liver Cells and Their Subsequent Fate

Liposomes appear in both hepatocytes and Kupffer cells within a short time after either EDTA-liposome or KCl-liposome injection. Figs. 5 and 6 show EDTA-liposomes within liver parenchymal cells 15 min after injection. In both figures the liposome is located near the sinusoidal margin of the cell. Liposomes are also seen at this time in the Kupffer cells lining the liver sinusoid (Figs. 7, 8). More than one liposome are usually found in the cytoplasm of the Kupffer cells. These liposomes are clustered together in large vacuolar areas.

The liver shows no significant morphological changes from 15 min to 6 h after injection, except for a decrease in the number of liposomes found in the sinusoids and a concomitant increase in number of liposomes in both hepatocytes and Kupffer cells. 12 h after EDTA-liposome injection, however, numerous macrophages appear filling the sinusoids (Figs. 9, 10). The cytoplasm of these macrophages contains numerous granules. However, no such mobilization of macrophages can be observed in the liver sinusoids of mice receiving KCl-liposomes (Fig. 11), even though liposome-like vesicles can be found in Kupffer cells and hepatocytes of these livers (Figs. 11, 12). In the livers of EDTA-liposome-injected mice, engorged Kupffer cells exhibiting the same kind of vacuolar areas containing liposomes as found at earlier times bulge into the sinusoidal lumen. At 12 h, the Kupffer cell cytoplasm also shows an increase in lysosomes and residual bodies. The hepatocytes show no differences in structure from earlier times.

The liver examined 24 h after injection of the mice with EDTA-liposomes shows the first morphological change in the hepatocytes. There are large patches of autophagic vacuoles with increased numbers of lysosome-like dense bodies (Fig. 13). Liposomes can still be identified within these areas of degeneration. Single liposomes within a vacuolar enclosure are still found in the hepatocytes, but are closer to the region of the bile canaliculus and lysosomes (for example, the single liposome within a vacuole seen in Fig. 14). The Kupffer cells are the same as at earlier times. Their cytoplasm contains liposomes in association with autophagic vacuoles (Figs. 15, 16). No striking morphological changes are seen in hepatocytes of mice receiving KCl-liposomes 24 h earlier. However, vacuoles with liposome-like inclusions can still be observed in some of the Kupffer cells.

The liver has returned to a relatively normal appearance at 1 wk after injection of EDTA-liposomes (Fig. 17). However, some Kupffer cells still exhibit large vacuolar areas and a concentration of liposomal remnants (Fig. 18). By 2.5 wk, the liver morphology appears entirely normal (Figs. 19, 20).

DISCUSSION

We have demonstrated by electron microscope studies that liposomes containing the chelating agent EDTA rapidly enter liver cells by phagocytosis. The penetration of liposomes into tissue cells within minutes after the liposome injection is also supported by our previous kinetic studies showing their rapid disappearance from the blood (9). We have reported also that mice receiving [14C]EDTA encapsulated within liposomes show a higher concentration and longer retention of the [14C]-radioac-
FIGURE 1 Liposomes (L) can be identified in the sinusoid of the liver within 10 min after an injection of 1.0 ml of packed EDTA-liposomes. Length of bar equals 1 μm in all figures unless otherwise stated. × 12,000.

FIGURE 2 The liver sinusoid is occupied by an erythrocyte and a liposome (L center). Smaller, flattened liposomes (L) can be identified among the microvilli within the space of Disse. × 10,000.

FIGURES 3 and 4 Higher magnifications of the margin of the hepatocyte show a liposome (L) within an invagination of the hepatic cellular membrane. × 36,000.
Figures 5 and 6  Liposomes (L) are shown in the cytoplasm of the hepatocyte within a vacuolar space near the sinusoidal margin 15 min after injection of EDTA-liposomes. Fig. 5. × 32,000. Fig. 6. × 19,000.

Figures 7 and 8  15 min after injection of EDTA-liposomes (L), the Kupffer cell cytoplasm is filled with large vacuolar areas which contain liposomes. The Kupffer cell seems engorged and occupies almost the entire sinusoid. Fig. 7. × 8,200. Fig. 8. × 12,500.
FIGURES 9 and 10  12 h after injection of EDTA-liposomes, the sinusoid is filled with macrophages and engorged Kupffer cells. The montage (Fig. 9) shows a macrophage filled with dense granules. Most of the figure is occupied by a Kupffer cell with large vacuolar areas containing liposomes. The sinusoid (Fig. 10) contains parts of four macrophages. Fig. 9, × 11,000. Fig. 10, × 9,000.
FIGURES 11 and 12. 12 h after injection of KCl-liposomes, a large portion of the sinusoid is occupied by a Kupffer cell, within which vacuolar areas containing liposome-like remnants are seen (Fig. 11). A single liposome surrounded by vacuolar space is seen in the hepatocyte (Fig. 12). No macrophages are observed in the sinusoids (Figs. 11 and 12). Figs. 11 and 12. × 10,750.
FIGURES 13, 14, 15, and 16 24 h after injection of EDTA-liposomes, the liver shows several morphological changes. The hepatocytes have large areas of cytoplasmic degeneration containing remnants of liposomes (Fig. 13). Single liposomes enclosed in a vacuolar area are found in the region of the bile canaliculus (Fig. 14). The Kupffer cell cytoplasm contains large autophagic vacuoles and liposomes in close association with these vacuoles (Figs. 15 and 16). Figs. 13 and 14, × 12,500. Fig. 15, × 9,000. Fig. 16, × 12,500.
FIGURES 17 and 18 1 wk after injection of EDTA-liposomes, the liver parenchymal cell returns to a normal appearance (Fig. 17). The Kupffer cell still has areas with large autophagic vacuoles containing remnants of liposomes (Fig. 18). Fig. 17, x 12,500. Fig. 19, x 10,500.

FIGURES 19 and 20 At 2.5 wk postinjection, the liver exhibits an entirely normal morphology. The hepatocytes show no areas of degeneration, and no liposomes or their remnants are found. The lumen of the sinusoid contains no free liposomes, the macrophages have disappeared, and the Kupffer cells have returned to normal and are no longer engorged. Fig. 19, x 5,000. Fig. 20, x 6,250.
tivity in various tissues than do mice receiving nonencapsulated [14C]EDTA solution (9). By combining results of the kinetic studies and the present morphological studies, we can reasonably assume that the chelating agent encapsulated within liposomes has been successfully transported across the cellular membrane.

The injection into mice of the chelating agent DTPA in liposome-encapsulated form has been shown to remove a fraction of the metal plutonium from the liver that was not available to nonencapsulated DTPA therapy (8). This fraction of the metal is presumably localized inside the liver cells. Thus, chelating agents injected in encapsulated form were not only transported across the cellular membrane, but they were released inside the cell where they successfully chelated the metal and removed it from the cells.

We have demonstrated here that liposomes containing chelating agents are rapidly taken up by both Kupffer and parenchymal cells of the liver, but we have not attempted to determine the number of liposomes in the two cell types. We plan to approach this problem by using radioactively labeled liposomes and subsequent cellular fractionation of the liver to separate the hepatocytes from Kupffer cells.

As phosphatidylcholine and cholesterol are both normal constituents of all cellular and intracellular membranes, the determination of the fate of the injected liposomes within the cells is a most difficult problem. We have labeled liposomes with [14C]cholesterol and attempted to use autoradiographic techniques to study their metabolism within cells. This approach was unproductive, due to the extremely rapid exchange of the [14C]cholesterol incorporated in the liposomal lipid bilayer with the cholesterol of intracellular membranes. About 30% of the liposomal 14C-radioactivity was found outside the labeled liposomes as early as 15 min after the liposome injection. In vitro exchange of cholesterol and phospholipids between liposomes and intracellular particles such as mitochondria has also been reported recently (2).

Our previous studies showed that about 40% of liposome-encapsulated [14C]EDTA was taken up by the liver, and remained in the liver up to 12 h before a significant decrease could be observed (9). This long retention of [14C]EDTA in the liver is probably due to the slow release of the chelating agent from within the liposomes. This retention and slow release would provide the cells with a continuous high intracellular level of chelating agent compared to the levels after injection of the nonencapsulated EDTA (9). Therefore, the chelating capacity of liposome-encapsulated chelating agents for the intracellular metal deposits is markedly improved.

After administration of liposome-encapsulated DTPA to mice injected with plutonium, the urinary excretion of plutonium is significantly increased over that of mice receiving the conventional, nonencapsulated DTPA therapy, while there is no difference in the fecal excretion of plutonium after the two forms of therapy (8). The increased plutonium excretion through the urine rather than the feces could be due to the high level of DTPA maintained over a prolonged period within the liver cells by the liposomes. This maintained level was sufficient to break up the large molecules of polymeric plutonium into smaller particles, capable of crossing the cell membrane into the liver sinusoids, and thereby gaining access to the kidneys. Future studies on the molecular form of plutonium chelates in the liver and the urine will be necessary to confirm such a possibility.

The origin of the numerous macrophages that appeared in the liver sinusoids of mice receiving [14C]EDTA liposomes is not known. These macrophages appear rather specifically at 12 h after the liposome injection, and are not observed in mice receiving KCl-liposomes. For this rather puzzling phenomenon, we would like to offer the following hypothesis: after the injection of liposomes containing chelating agents, soluble metal chelates are released from liver cells into the sinusoids. These metal chelates may induce either a mobilization of macrophages from other tissues to the liver sinusoids, or a transformation of the abundant endothelial cells of the liver sinusoids into cells with macrophage appearance. The macrophages observed at 12 h after the liposomal [14C]EDTA injection might play a crucial role in the reduction of [14C]EDTA levels in the liver which began 12 h after the liposome injection (9).

In the livers of mice receiving nonencapsulated EDTA solutions, no morphological changes were observed. Vacuole formation in kidney of rats receiving injections of EDTA solution has been reported by Schwartz and co-workers (10, 11). They suggested that EDTA-induced vacuologenesis is an indication of the induction of pinocytosis by the chelate. However, the toxic doses of EDTA used in their studies (1.0 and 2.5 g EDTA per kg) may have been responsible for the formation of
vacuoles. Further, with radioactively labeled EDTA, they demonstrated less than 0.1% of the total injected radioactivity in cells of the kidney cortex (11). Low levels of EDTA in kidneys have also been shown in our work (9). Only at early time intervals after injections, correlated with the early and rapid EDTA urinary excretion (9), are high concentrations of EDTA found in kidneys. The tissue disposition of liposome-encapsulated EDTA, however, is strikingly different from that of nonencapsulated EDTA (9). The highest therapeutic doses of chelating agents used in our studies (7, 8) were 100 mg (EDTA or DTPA) per kg, and under such experimental conditions no vacuologenesis was observed in liver, lungs, and spleen, the tissues we have examined so far.

While this paper was in preparation, a study on the interaction of cationic liposomes containing horseradish peroxidase with cells in culture was reported by Magee et al. (6). They reported that "liposomes containing horseradish peroxidase were adsorbed by HeLa cells at least 300 times as effectively as was free horseradish peroxidase". However, the way by which liposomes were taken up by HeLa cells was not shown in their electron micrographs. The authors concluded that "many particles did not gain access to the interior of the cells, many were phagocytized, and some enzyme was transferred to the cell membrane, perhaps as a result of fusion of the liposomal membrane with the cell membrane." In our present study, liposomes were taken up into cells by way of phagocytosis, and no fusion of the liposomal membrane with the cell membrane was observed. Fusion of liposomal membrane with cell membranes can be detected by electron microscopy, where contact between the two membranes can be observed, and no space should be found between these two membranes.

In conclusion, we have demonstrated that liposomes containing the chelating agent EDTA are phagocytized by liver cells within minutes of their injection. Both parenchymal and Kupffer cells are capable of taking up liposomes. The chelating agents introduced into liver cells could therefore explain the higher efficiency of liposome-encapsulated chelating agent compared to the nonencapsulated form in removing intracellular heavy metals. Thus the use of liposomes as carriers to introduce chelating agents (or other drugs) into cells seems to be a useful tool for intracellular delivery of drugs. The lipid spherules also have the advantage of being completely "biodegradable" and in addition, as they are not protein in nature, immunological reactions are unlikely to occur.

We thank Dr. M. W. Rosenthal, Ms. E. A. Cerny, Ms. E. S. Moretti, and Mr. G. T. Chubb for consultation and valuable assistance.

This work was supported by the United States Atomic Energy Commission.

Received for publication 3 September 1974, and in revised form 23 December 1974.

REFERENCES

1. CATSCH, A. 1964. Radioactive metal mobilization in medicine. Charles C. Thomas Publisher, Springfield, Ill. 59.

2. EHNHOLM, C., and D. B. ZILVERSMIT. 1973. Exchange of various phospholipids and of cholesterol between liposomes in the presence of highly purified phospholipid exchange protein. J. Biol. Chem. 248:1719-1724.

3. FOREMAN, H. 1960. The pharmacology of some useful chelating agents. In Metal-binding in Medicine. M. J. Seven and L. A. Johnson, editors. J. B. Lippincott Co., Philadelphia, Pa. 82.

4. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27:137 A (Abstr.).

5. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409-414.

6. MAGEE, W. E., C. G. GOFF, J. SCHOKNECHT, M. D. SMITH, and K. CHERIAN. 1974. The interaction of cationic liposomes containing entrapped horseradish peroxidase with cells in culture. J. Cell Biol. 63:492-504.

7. RAHMAN, Y. E., and M. W. ROSENTHAL. 1973. A new approach to the therapy of metal poisoning: liposome encapsulation of chelating agents. Radiat. Res. 55:516-517.

8. RAHMAN, Y. E., M. W. ROSENTHAL, and E. A. CERNY. 1973. Intracellular plutonium/removal by liposome-encapsulated chelating agent. Science (Wash. D.C.) 180:300-302.

9. RAHMAN, Y. E., M. W. ROSENTHAL, E. A. CERNY, and E. S. MORETTI. 1974. Preparation and prolonged tissue retention of liposome-encapsulated chelating agents. J. Lab. Clin. Med. 83:640-647.

10. SCHWARTZ, S. L., J. R. HAYES, R. S. IDE, C. B. JOHNSON, and P. D. DOOLAN. 1966. Studies of the nephrotoxicity of ethylenediaminetetraacetic acid. Biochem. Pharmacol. 15:377-389.

11. SCHWARTZ, S. L., C. B. JOHNSON, and P. D. DOOLAN. 1970. Study of the mechanism of renal vacuologenesis induced in rat by ethylenediaminetetraacetic acid. Mol. Pharmacol. 6:54-60.

12. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.