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A comparative study of free and liposome-entrapped diethylenetriaminepentaacetic acid used in the treatment of mice loaded with cadmium

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Summary

Liposomes composed of equimolar egg phosphatidylcholine and cholesterol and containing diethylenetriaminepentaacetic acid (DTPA) were prepared by the dehydration-rehydration procedure. Such liposomes retained most of their DTPA content during either storage (for up to 30 days) or incubation in the presence of blood plasma at 37°C. In vivo experiments with mice pretreated with $CdCl_2$ or phosphate-buffered saline (PBS) and subsequently injected intravenously with DTPA-containing liposomes or free DTPA revealed that: (a) liposomal DTPA was cleared from the circulation at a slower rate than the free chelator; (b) liposomal DTPA in Cd-treated mice had a more rapid rate of clearance than in PBS-treated mice; (c) whereas levels of DTPA injected in the free form were practically nil in the liver and spleen of both groups of mice at 24 h, those of liposomal DTPA were considerably higher (15 and 6% in the two tissues, respectively) in normal mice and much higher in cadmium-treated mice (63 and 10%). In separate experiments, the cadmium-chelating action of free and liposomal DTPA injected intravenously was investigated in mice pre-treated with $CdCl_2$. The results showed that although there was no difference in the extent of cadmium reduction in the tissues between the two forms of DTPA, blood levels of the metal were lower for most of the 72 h period after treatment and its urinary excretion higher (during the first 24 h period) in mice injected with the liposomal chelator. It is suggested that treatment of cadmium poisoning with liposomal DTPA would present the kidneys with a lower concentration of cadmium for clearance into the urine over time thereby potentially reducing the nephrotoxicity of the metal.

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Introduction

Administration of chelating agents is generally the most effective treatment for heavy metal poisoning, especially if treatment is given soon after exposure (Cantilena and Klaassen, 1981; Planas-

Bohne and Lehman, 1983). However, in the case of cadmium intoxication where most exposures to the metal occur chronically, the performance of chelating agents has been rather disappointing (May and Bulman, 1983). A variety of chelating agents have been tested as a means to treat cadmium toxicity, with diethylenetriaminepentaacetic acid (DTPA) being the most effective (Cantilena and Klaassen, 1981). Unfortunately, DTPA is excreted very rapidly (over 80% of the dose) in the urine within minutes after intravenous injection (Stather et al., 1983). To compensate for such loss of the chelator, larger doses are administered with resulting toxicity.

Liposomes are known (Gregoriadis, 1988a) to protect agents from inactivation or premature loss before they reach the target organs and to ensure their intracellular delivery, thereby enhancing efficiency. For example, work (Rahman, 1988) on the tissue distribution of free and liposomal EDTA has demonstrated greater uptake and prolonged retention of the latter by tissues. Further, liposomal DTPA was found (Rahman et al., 1973) to be effective in removing polymeric plutonium from animals poisoned with the metal.

Recently, we have carried out studies to investigate a possible role for liposome-entrapped DTPA in the elimination of cadmium from the tissues of cadmium-loaded animals. It was reasoned that liposomal DTPA would mobilize the metal from the tissues in a pattern different from that observed with free DTPA and known to lead to cadmium-induced kidney damage (WHO, 1972; Singhal and Merali, 1979). Our results suggest that, although under the present conditions both free and liposomal DTPA remove cadmium from the tissues to the same extent, the latter does so in a fashion that ensures lower overall levels of the metal in blood. This should present the kidneys with decreased levels of cadmium for clearance over time, thus potentially reducing its nephrotoxicity.

Materials and Methods

The source and grades of egg phosphatidylcholine (PC) and cholesterol have been reported elsewhere (Kirby and Gregoriadis, 1984). Diethylenetriaminepentaacetic acid (DTPA) was from Sigma, London, and diethylenetriaminepenta[1-^{14}C] acetic acid ([^{14}C]DTPA; 1.11 GBq/mmol) from Amersham International, Amersham. Other reagents were of analytical grade.

Preparation of liposomes

DTPA-containing liposomes were prepared by a method which is based on the induction of fusion of preformed vesicles by means of dehydration and controlled rehydration, gives high solute entrapment values and is operationally simple (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987, 1990). Briefly, in a typical experiment, 1.0 ml small unilamellar vesicles (SUV) made (Kirby et al., 1980) in distilled water from equimolar PC (16.2 μmol) and cholesterol, were mixed with an equal volume of DTPA (50 μmol in water) to which [^{14}C]DTPA [$(1.2–10.1) \times 10^6$ dpm] had been added. Following dehydration and controlled (Kirby and Gregoriadis, 1984) rehydration to form DTPA-containing dehydration-rehydration vesicles (DRV), the suspension (2.0 ml) was diluted to 8 ml with PBS (0.44 mM sodium phosphate, 2.7 mM potassium chloride and 0.14 M sodium chloride, pH 7.4) and centrifuged at $10\,000 \times g$ for 45 min at 4°C to separate the entrapped from non-entrapped DTPA. The pellet was washed twice with PBS, suspended in the same buffer (2 ml) and assayed for ^{14}C radioactivity (Kirby and Gregoriadis, 1984). DTPA entrapment in DRV liposomes was expressed as a percentage of the solute used.

DTPA retention by DRV liposomes on storage or incubation with mouse plasma

Aliquots (0.2 ml; 1.2×10^5 dpm) of DRV liposomes containing DTPA and tracer DTPA were stored at 4°C for 1, 7 and 30 days. At the end of each of the time intervals, the liposomal suspensions were centrifuged and the pellets obtained washed as above. ^{14}C radioactivity was then assayed in suspended pellets and combined supernatants to determine DTPA retention by liposomes. DTPA-containing DRV (0.4 ml; 2.4×10^5 dpm and 3.24 μmol phospholipid) were also incubated with 5 volumes of fresh T.O. mouse (see

below) plasma pre-heated at 37°C, for up to 3 h at the same temperature. Aliquots withdrawn at time intervals were suitably diluted with PBS, centrifuged as above and ^{14}C radioactivity was assayed in both the washed pellets and the pooled supernatants to determine DTPA retention by liposomes. In a parallel experiment, DTPA-containing DRV were exposed to PBS under conditions identical to those used for incubation with plasma.

Animal experiments

In studies of DTPA clearance from the circulation and uptake by tissues outbred mice (T.O. strain, Clinical Research Centre, Harrow, Middlesex, U.K.) weighing 20–25 g were randomly divided into two groups of 30 each. One of the groups were injected intraperitoneally with $CdCl_2$ (0.01 mmol per kg body weight) and this treatment was repeated 2 days later. The other (control) group were injected twice with PBS as above. 48 h after the second cadmium and PBS injections, each of the two groups were divided randomly into two subgroups of 15 mice each. Two of the subgroups treated with Cd and PBS, respectively, were injected into the tail vein with 0.2 ml free DTPA in PBS (0.05 mmol per kg body weight) mixed with [^{14}C]DTPA (3×10^5 dpm) and the remaining two subgroups with 0.2 ml liposome-entrapped DTPA (typically 0.05 mmol DTPA and 64.8 μmol phospholipid per kg body weight) spiked with 3×10^5 dpm [^{14}C]DTPA. All animals were bled from the tail vein at 15 min, 1 h and 3 h after DTPA injections and 50 μl of freshly drawn blood were poured into tubes containing 1 ml PBS. Following centrifugation of the samples at $1000 \times g$ for 15 min to remove the blood cells, ^{14}C radioactivity was assayed in the supernatants. Concentration of DTPA in the total blood volume was estimated as described elsewhere (Kirby et al., 1980) and expressed as a percentage of the injected dose. Groups of five mice from all four subgroups were subsequently killed 24, 48 and 72 h after the injection of DTPA and liver, kidney and spleen removed and homogenized in 5, 3 and 2.5 ml water, respectively. Tissue homogenates (1.0 ml) in duplicates were then assayed (Gregoriadis and Ryman, 1972) for ^{14}C radioactivity and the results expressed as a percentage of the injected dose per whole organ.

In a separate experiment, 24 T.O. mice were injected twice with $CdCl_2$ as already described. 48 h after the second injection the mice were randomly divided into three groups of eight each and injected into the tail vein as above with 0.2 ml of free DTPA in PBS, liposome-entrapped DTPA and PBS, respectively. All animals were kept in metabolic cages for the collection of 24 h urine and faeces for up to 3 days. The mice were bled from the tail vein at 90 min, and 5, 24, 48 and 72 h after DTPA injection and collected blood stored for the assay of cadmium. Following the last collection of blood on day 3, animals were killed and liver, kidney, lungs and spleen collected and washed with deionized water. Determination of cadmium in blood and urine was carried out after appropriate dilutions. To determine cadmium in tissues (0.2 g) and homogenized faeces (0.5 g), samples were digested in a mixture of HNO_3, $HClO_4$ and H_2SO_4 (6:1:1 v/v) by controlled heating on a hot plate until white fumes of acid ceased. The digests were suitably diluted with deionized water and assayed for cadmium in a flameless Perkin Elmer atomic absorption spectrophotometer.

Results and Discussion

Entrapment of DTPA and its retention by liposomes

Preliminary experiments on the entrapment of DTPA in DRV liposomes showed that using concentrations of 10–50 μmol DTPA for a fixed amount of liposomal phospholipid (16.2 μmol), entrapment efficiency was correspondingly reduced from 30 to 23% of the chelator used (results not shown). Nonetheless, because the absolute amount of entrapped DTPA was greatest (10.3–11.8 μmol) when the highest (50 μmol) concentration was used, the latter concentration was adopted in all encapsulation experiments in the study. Although all liposomal formulations were used immediately after their preparation, it appeared that DTPA leakage from the vesicles on storage at 4°C was minimal. For instance,

93–95% of the entrapped DTPA in two liposomal formulations was found associated with the vesicles after 1, 7 and 30 days storage (results not shown).

Prior to intravenous injection of liposomal DTPA into mice, it was necessary to establish that the chelator would remain associated with the carrier during its circulation in the blood. In previous work (Kirby et al., 1980; Senior et al., 1985; Gregoriadis, 1988b) it was shown that solute leakage from both small unilamellar and large multilamellar liposomes in the presence of blood plasma and in vivo was minimal when vesicles were composed of equimolar phospholipid and cholesterol. It was, therefore, anticipated that the lipid composition (equimolar PC and cholesterol) used for the preparation of liposomal DTPA would ensure reduced leakage of DTPA from the vesicles in the presence of plasma. Indeed, the data in Table 1 indicate that after exposure to plasma at 37°C for 1 h, more than 90% of the chelator is retained by the carrier and that retention is only modestly reduced to about 79% after 3 h of exposure. Since in subsequent experiments of this study the bulk (over 90%) of the DRV liposomes were expected (Senior et al., 1986) to be cleared from the circulation of intravenously treated mice in less than 1 h after injection, investigation of the effect of plasma on DTPA retention for longer periods of time was not carried out.

TABLE 1

Retention of DTPA by liposomes in the presence of plasma

[^{14}C]DTPA-containing DRV were mixed with 5 volumes of mouse blood plasma and incubated at 37°C for up to 3 h. At time intervals, samples were assayed for liposome-associated and free ^{14}C radioactivity (see Materials and Methods). Values (% of radioactivity added to plasma) are shown for both liposome-retained and released DTPA to demonstrate nearly quantitative recovery of the label.

Incubation time (h)	DTPA retained (% of total)	DTPA released (% of total)
0	91.9	8.1
0.5	90.6	9.5
1.0	80.5	19.5
3.0	79.2	20.3
3.0	94.3 [a]	5.0 [a]

[a] [^{14}C]DTPA-containing DRV were incubated with 5 volumes of PBS.

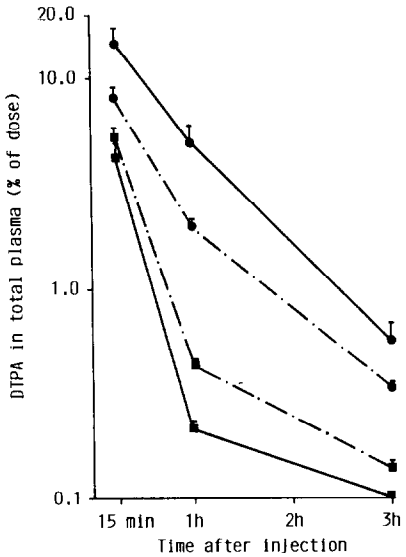

Fig. 1. Clearance of DTPA from the blood of injected mice. PBS (normal)- or cadmium-treated mice were injected intravenously with free or liposome-entrapped [^{14}C]DTPA. Animals (five in each of the four groups) were bled at time intervals after injection and blood plasma assayed for ^{14}C radioactivity. Values (means + SE) are expressed as % of the injected dose per total blood. For other details see Materials and Methods. (■) Free DTPA, (●) liposomal DTPA, (———) normal mice, (· · ·) cadmium-treated mice.

Clearance of liposomal DTPA from the circulation and uptake by tissues after intravenous injection

It has long been established (Gregoriadis, 1988b) that liposomes which retain their solute contents quantitatively during their circulation in the blood of intravenously injected animals alter the pharmacokinetics of the solutes with the latter acquiring both the carrier's rate of clearance and tissue distribution. The liposomes used in the present study were multilamellar so as to ensure (Gregoriadis and Ryman, 1972) rapid deposition of the chelator in tissues and of a lipid composition (equimolar phospholipid and cholesterol) which (as indeed shown in Table 1) would promote DTPA retention by the carrier in the presence of blood plasma.

Fig. 1 shows that, as expected, DTPA entrapped in DRV was cleared from the circulation rapidly with over 80% of the dose removed in 15 min and over 90% in 1 h. These values are similar to those determined elsewhere (Senior et al., 1985) using carboxyfluorescein as a marker solute for liposomes. Interestingly, clearance of liposomal DTPA administered into cadmium-loaded mice was significantly more rapid than that seen in normal animals (Fig. 1). As is the case with other solutes (Senior et al., 1986), some of the liposome-associated DTPA may be loosely adsorbed onto the vesicles' surface and, therefore, be accessible to cadmium present in the plasma of mice treated with the metal (see Fig. 5). It is thus conceivable that cadmium would be chelated by such DTPA with the complex being removed into the urine. Clearance of free DTPA in both intact and cadmium-loaded mice was much more rapid with over 99% of the dose removed in 1 h, presumably (Stather et al., 1983) through the kidney into the urine.

DTPA tissue distribution, as judged by radioactivity data, is shown in Figs 2 and 3. 24 h after liposomal DTPA injection (by which time circulating blood is expected (Fig. 1) to be devoid of the chelator), the level of DTPA in the whole liver of normal mice is 15% of the injected dose (Fig. 2). However, the corresponding value in cadmium-loaded mice is 63% of the dose (Fig. 3). As the level of DTPA in the liver (also in other tissues, see Figs 2 and 3) declined gradually during the subsequent 48 h, it is reasonable to assume that DTPA levels in both control and cadmium-treated mice were considerably higher at time intervals earlier than 24 h. Indeed, it is very likely that uptake of liposomal DTPA by the tissue of normal animals soon after injection was at least as high as that seen in cadmium-treated mice: In previous work with EDTA (Gregoriadis, 1980) and other solutes (e.g. Gregoriadis et al., 1977) entrapped in multilamellar liposomes, liver was found to take up most (about 60–80%) of the injected solute during the first 15 min or so following intravenous injection. Subsequently, and for solutes which do not exhibit affinity for, or are degraded in the liver, the level of the solute diminished rapidly to reach low levels (down to

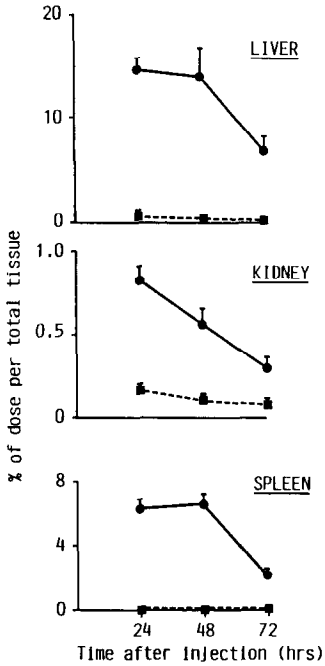

Fig. 2. Tissue distribution of DTPA injected into mice. PBS (normal)-treated mice were injected intravenously with free or liposome-entrapped [^{14}C]DTPA. Animals were killed at time intervals (five in each of the groups at each time interval) and tissues assayed for ^{14}C radioactivity. Values (means + SE) are expressed as % of the injected dose per total organ. For other details see Materials and Methods. (■) Free DTPA, (●) liposomal DTPA.

about 10% of the dose) by 24 h. With solutes which bind to tissue components or are not degradable and are too large to escape from the lysosomal apparatus (where liposomes transfer their contents through endocytosis; Gregoriadis and Ryman, 1972), these persisted in the liver, for several days or longer (Dapergolas et al., 1976; Gregoriadis et al., 1977). It is, therefore, conceivable that much of the liposomal DTPA injected into cadmium-loaded mice binds to the metal present (see Fig. 4) in the liver and its excretion is delayed.

Similar differences in DTPA retention 24 h after injection were also observed for the kidney and spleen of normal and cadmium-treated mice (cf. Figs 2 and 3). In contrast, levels of DTPA in the liver and spleen of mice treated with the free chelator were, at 24 h, practically nil, regardless

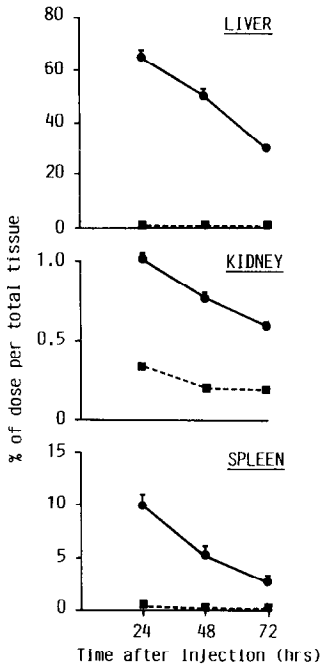

Fig. 3. Tissue distribution of DTPA injected into mice. Cadmium-treated mice were injected intravenously with free or liposome-entrapped [^{14}C]DTPA. For other details see legend to Fig. 2 and Materials and Methods.

of the presence or absence of cadmium in the tissues. There were, on the other hand, small amounts of DTPA in the kidney of both groups of animals, with those loaded with cadmium having more of the chelator (cf. Figs 2 and 3). It is likely that cadmium in the kidney (Fig. 3) binds to the DTPA as it passes into the urine.

The cadmium-chelating action of DTPA in cadmium-loaded mice

Having established that injected liposomes deliver DTPA to the liver, spleen and kidney, the chelating action of the liposomal DTPA in such mice was investigated and compared with that of free DTPA. The results in Fig. 4 show that 72 h after its injection, liposomal DTPA removed as much cadmium from the liver and spleen as did free DTPA. Thus, in the case of liver, for instance, where cadmium content at 72 h was 34.2 μg (PBS-treated control mice), this was reduced to 27.1 and 25.0 μg per g tissue, respectively. There was no change in the concentration of cadmium in the kidney with either form of the chelator and only liposomal DTPA removed a modest amount of cadmium from the lungs.

A different pattern of DTPA action was seen, however, in the blood (Fig. 5). Levels of cadmium throughout the 72 h period following injection of the chelator were generally lower in animals treated with the liposomal DTPA than in those injected with the free DTPA. Interestingly, excretion of cadmium into the urine during the first 24 h was twice as high in animals treated with the liposomal DTPA than those injected with the free chelator (Fig. 6). DTPA-induced excretion of cadmium into the faeces (biliary excretion) was also observed (Fig. 7) for some of the time intervals. It was significantly higher for liposomal

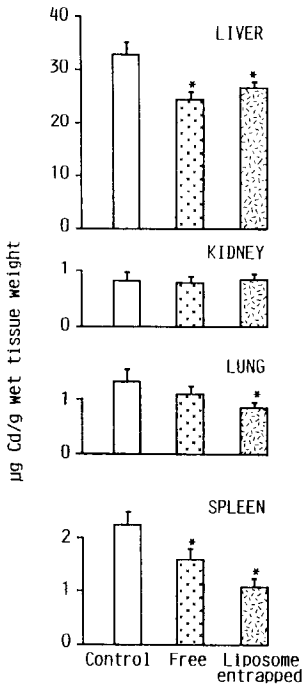

Fig. 4. Cadmium concentration in the tissues of mice after treatment with DTPA. Cadmium-loaded mice were injected intravenously with PBS (control), free DTPA or liposome-entrapped DTPA. Animals (6–8 in each of the three groups) were killed 72 h later and cadmium measured in the liver, kidney, lungs and spleen. Values (means + SE) are expressed as μg Cd per g wet tissue. * Denotes values significantly ($p < 0.001$–0.05, Student's t-test) different from control values. For other details see Materials and Methods.

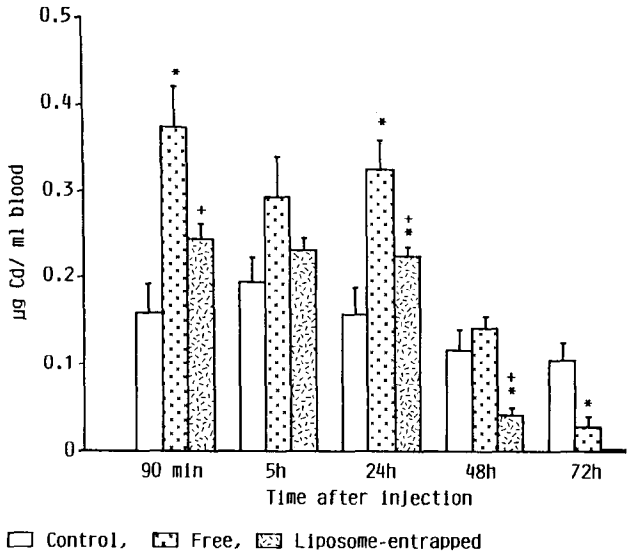

Fig. 5. Cadmium concentration in the blood of mice after treatment with DTPA. Cadmium-loaded mice were injected with DTPA as in the legend to Fig. 4. Animals (6–8 in each of the three groups) were bled at time intervals after DTPA injection and cadmium measured in the blood. Values are expressed as μg Cd per ml blood. For explanation of bars and other details see Fig. 4 and Materials and Methods. * Denotes values significantly ($p < 0.01$; Student's t-test) different from control values. + Denotes values significantly ($p < 0.05$; Student's t-test) different from values for mice treated with free DTPA.

DTPA (compared with free DTPA) only during the third day.

Previous studies (Stather et al., 1976, 1983) have shown that a variety of chelators (including EDTA and DTPA), injected intravenously, are excreted rapidly and almost wholly into the urine with very little of the chelators being taken up by the liver and other tissues. Yet, free DTPA in the present study was clearly successful in mobilising some of the cadmium deposited in the liver and spleen. On the other hand, liposomal DTPA, in spite of its high concentration in the liver, was not more effective in removing cadmium. Work (Cantilena and Klaassen, 1981, 1982b) has al-

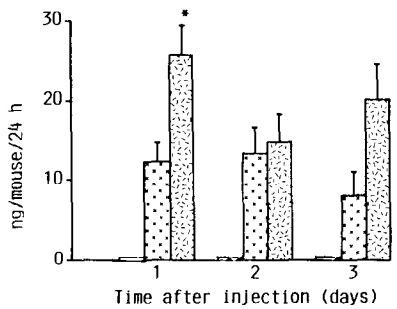

Fig. 6. Urinary excretion of cadmium in mice injected with DTPA. Cadmium-loaded mice (five in each group) were injected with PBS (control), free DTPA or liposome-entrapped DTPA. 24 h urine was collected for up to 72 h and assayed for cadmium. Values are expressed as ng cadmium per 24 h urine. * Denotes values significantly ($p < 0.05$; Student's t-test) different from values for mice treated with free DTPA. For explanation of bars and other details see Fig. 4 and Materials and Methods.

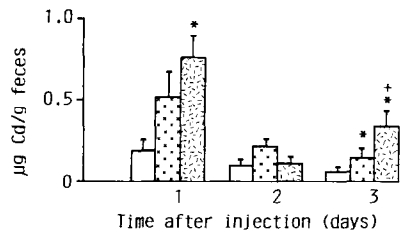

Fig. 7. Faecal excretion of cadmium in mice injected with DTPA. Cadmium-loaded mice (five in each group) were injected with PBS (control), free DTPA or liposome-entrapped DTPA. 24 h faeces were collected for up to 72 h and assayed for cadmium. Values are expressed as μg cadmium per g faeces. * Denotes values significantly ($p < 0.01$; Student's t-test) different from control values. + Denotes values significantly ($p < 0.01$–0.05; Student's t-test) different from values for mice treated with free DTPA.

ready shown that free DTPA is effective in removing cadmium from the liver, spleen, kidney and brain of cadmium-loaded mice only if given immediately after cadmium. The failure of DTPA to chelate effectively cadmium and to remove it from the tissues at later time intervals has been attributed by the same authors to cadmium-mediated metallothionein induction which, for intraperitoneally given cadmium (as in the present study), reaches a maximum after 36–48 h (Probst et al., 1977). Cantilena and Klaassen (1982a) have proposed that, as a result of metallothionein induction, cadmium is transferred to a deeper pharmacokinetic pool to become less available for interaction with chelators. On the basis of our data, it appears that although interaction of liposomal DTPA with cadmium does take place (as demonstrated by increased concentration of DTPA in the liver of cadmium-loaded mice; Fig. 3), removal of the metal from the liver and other tissues is certainly not better than with free DTPA (Fig. 4). However, in spite of the apparent inability of liposomal DTPA to remove more cadmium from the tissues than free DTPA (an observation also made by Stather et al. (1976) in mice contaminated with monomeric plutonium), the former does so in a fashion which leads to lower overall levels of metal in blood (Fig. 5). This would present the kidneys with a lower concentration of cadmium for clearance into the urine over time, thereby potentially reducing its nephrotoxicity. It is of interest that Cantilena and Klaassen (1982b) have found that repeated administration of DTPA to cadmium-loaded mice (after induction of metallothionein) increases the urinary excretion of the metal. However, chronic administration of DTPA has been shown to be fatal to dogs (Taylor et al., 1974) and it may be that injection of the chelator in the liposome form (perhaps less frequently) will reduce such toxicity.

Acknowledgements

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References

Cantilena, L.R. Jr and Klaassen, C.D., Comparison of the effectiveness of several chelators after single administration on the toxicity, excretion and distribution of cadmium, *Toxicol. Appl. Pharmacol.*, 58 (1981) 452–460.

Cantilena, L.R. Jr and Klaassen, C.D., Decreased effectiveness of chelation therapy with time after acute cadmium poisoning. *Toxicol. Appl. Pharmacol.*, 63 (1982a) 173–180.

Cantilena, L.R. Jr and Klaassen, C.D., The effect of repeated administration of several chelators on the distribution and excretion of cadmium. *Toxicol. Appl. Pharmacol.*, 66 (1982b) 361–367.

Dapergolas, G., Neerunjun, E.D. and Gregoriadis, G., Penetration of target areas in the rat by liposome-associated bleomycin, glucose oxidase and insulin. *FEBS Lett.*, 63 (1976) 235–239.

Gregoriadis, G., The liposome drug-carrier concept: its development and future. In Gregoriadis, G. and Allison, A.C. (Eds), *Liposomes in Biological Systems*, Wiley, Chichester, 1980, pp. 25–86.

Gregoriadis, G., *Liposomes as Drug Carriers: Recent Trends and Progress*, Wiley, Chichester, 1988a.

Gregoriadis, G., Fate of injected liposomes: Observations on entrapped solute retention, vesicle clearance and tissue distribution in vivo. In: Gregoriadis, G. (Ed.), *Liposomes as Drug Carriers: Recent Trends and Progress*, Wiley, Chichester, 1988b, pp. 3–18.

Gregoriadis, G. and Ryman, B.E., Lysosomal localization of fructofuranosidase-containing liposomes injected into rats. *Biochem. J.*, 129 (1972) 123–133.

Gregoriadis, G., Neerunjun, E.D. and Hunt, R., Fate of a liposome-associated agent injected into normal and tumour-bearing rodents. Attempts to improve localization in tumour tissues. *Life Sci.*, 21 (1977) 357–370.

Gregoriadis, G., Davis, D. and Davies, A., Liposomes as immmunological adjuvants in vaccines: Antigen incorporation studies. *Vaccine*, 5 (1987) 145–151.

Gregoriadis, G., Da Silva, H. and Florence, A.T., A procedure for the efficient entrapment of drugs in dehydration-rehydration liposomes (DRVs). *Int. J. Pharm.*, 65 (1990) 235–242.

Kirby, C. and Gregoriadis, G., Dehydration-rehydration vesicles (DRV): a new method for high yield entrapment in liposomes. *Biotechnology*, 2 (1984) 979–984.

Kirby, C., Clarke, J. and Gregoriadis, G., Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *Biochem. J.*, 186 (1980) 591–598.

May, P.M. and Bulman, R.A., The present state of chelating agents in medicine. In: Ellis, G.P. and West, G.B. (Eds), *Progress in Medicinal Chemistry*, Elsevier, Amsterdam, 1983, pp. 225–236.

Planas-Bohne, F. and Lehman, M., Influence of chelating agents on the distribution and excretion of cadmium in rats. *Toxicol. Appl. Pharmacol.*, 67 (1983) 408–416.

Probst, G.S., Bousquet, W.F. and Miya, T.S., Kinetics of cadmium-induced hepatic and renal metallothionein syn-

thesis in the mouse. *Toxicol. Appl. Pharmacol.*, 39 (1977) 51–60.

Rahman, Y.E., Use of liposomes in metal poisonings and metal storage disease. In: Gregoriadis, G. (Ed.), *Liposomes as Drug Carriers: Recent Trends and Progress*, Wiley, Chichester, 1988, pp. 485–495.

Rahman, Y.E., Rosenthal, M.W. and Cerny, E.A., Intracellular plutonium: removal by liposome-encapsulating chelating agent. *Science*, 180 (1973) 300–302.

Senior, J., Crawley, J.C.W. and Gregoriadis, G., Tissue distribution of lipsomes exhibiting long half-lives in the circulation after intravenous injection. *Biochim. Biophys. Acta*, 839 (1985) 1–8.

Senior, J., Waters, J.A. and Gregoriadis, G., Antibody-coated liposomes: the role of non-specific antibody adsorption. *FEBS Lett.*, 196 (1986) 54–58.

Singhal, R.L. and Merali, Z., Biochemical toxicity of cadmium. In: Mannear, J.H. (Ed.), *Cadmium Toxicity*, Dekker, New York, 1979, pp. 61–111.

Stather, J.W., Smith, H., James, A.C. and Rodwell, P., The experimental use of aerosol and liposomal forms of Ca-DTPA as a treatment for plutonium contamination. In: *Diagnosis and Treatment of Incorporated Radionuclides*, STI/PUB/411, Atomic Energy Agency, Vienna, 1976, pp. 387–400.

Stather, J.W., Smith, H., Bailey, M.R., Birchall, A., Bulman, R.A. and Crawley, F.E.H., The retention of ^{14}C-DTPA in human volunteers after inhalation or intravenous injection. *Health Phys.*, 44 (1983) 45–52.

Taylor, G.N., Williams, J.L., Roberts, L., Atherton, D.R. and Shabestari, L., Increased toxicity of Na_3-CaDTPA when given by a protracted administration. *Health Phys.*, 27 (1974) 285–288.

WHO Technical Rep. Ser., 505 (1972).