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Liposomal L-asparaginase: in vitro evaluation

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Summary

The purpose of this work was the development of liposomal formulations of L-asparaginase (L-ASNase) with the following characteristics: preservation of active enzyme, high entrapment efficiency, prolonged serum half-life and reduced toxicity compared with the free enzyme. Several liposome formulations were developed using simplified dehydration-rehydration vesicles (sDRV) or extruded vesicles (VET). The effect of lipid composition, vesicle size, ionic strength and osmolarity on enzyme encapsulation was investigated. Using a simplified dehydration-rehydration method (sDRV) we were able to achieve encapsulation efficiencies of up to 100% with full preservation (99%) of the specific activity of the encapsulated enzyme. The protein to lipid ratios of the liposomal formulations ranged from 5 to 27 $\mu g/\mu mol$, depending on the lipid composition. Extruded vesicles ranging from 85 to 250 nm in diameter were also tested. The encapsulation efficiency of extruded vesicles was lower than that of large vesicles and the range of preservation of specific activity was dependent on the lipid composition. Lipid combinations of phosphatidylcholine and cholesterol and either stearylamine, phosphatidylinositol or monosialoganglioside resulted in a high encapsulation efficiency (40 and 98% in VET and sDRV, respectively), high stability in saline and human serum (65–90% after 48 h) and considerable preservation of enzymatic activity (74–98%). The liposomal formulations were significantly less toxic than the free enzyme against normal CHO cells. In vivo toxicity, pharmacokinetics, biodistribution and antitumour activity studies are planned with the best formulations described in this paper.

Introduction

L-Asparaginase (L-ASNase; L-asparagine amidohydrolase EC 3.5.1.1) is an enzyme that degrades the amino acid L-asparagine. L-ASNase is an effective antitumor agent against human acute lymphoblastic leukemia (Clarkson et al., 1970). Lymphoblastic leukemia cells have a deficit in L-asparagine synthetase, are absolutely dependent on an exogenous supply of L-asparagine, and

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Abbreviations: P_f and L_f, amounts of final liposome-associated protein and lipid; P_i and L_i, total amounts of initial protein and lipid; P_f/L_f and P_i/L_i, protein to lipid ratios in the final liposome preparation and in the initial mixture, respectively; E.E., encapsulation efficiency; % activity, percentage of specific enzymatic activity preserved in the liposome preparation related to the total initial specific activity before lyophilization; ID_{50}, dose which inhibits 50% of the cellular growth; T_p, phase transition temperature.

susceptible to depletion of the amino acid. The mechanism of action of L-ASNase is based on starvation to death of malignant cells by degradation of its specific substrate, L-asparagine. The intravenous administration of L-ASNase can cause significant side effects in humans, mainly acute allergic reactions ranging from fever, skin rashes to death secondary to anaphylactic shock, thrombosis or hemorrhage (Clarkson et al., 1970; Capizzi et al., 1985).

In the past few years, liposomes have been extensively explored as carriers of common drugs (Lopez-Berestein et al., 1985; Perez-Soler et al., 1986; Gabizon et al., 1989; Vadiei et al., 1989) and, to a smaller extent, of relatively new drugs, namely peptides and proteins (Eppstein and Longenecker, 1988). The increasing availability of pure recombinant proteins, their approval as drugs, and the development of liposome technology capable of preserving protein structure (Gregoriadis and Kirby, 1984; Eppstein and Longenecker, 1988) open new perspectives for the use of liposomes as drug delivery systems for proteins. This approach has already been used with interferons (Eppstein, 1986), IL-1 (Eppstein, 1988), glucose-6-phosphate-dehydrogenase (Ullman et al., 1987), factor VIII (Gregoriadis and Kirby, 1984) and antigens (Gregoriadis et al., 1987).

There are two potential advantages for the use of liposomes as a carrier system for L-ASNase for medical applications. One is the reduction of allergic reactions since antigen determinants of the enzyme may be masked by encapsulation (Gregoriadis and Neerunjun, 1976). A second potential advantage is the prolongation of the blood circulation time of L-ASNase since the enzyme exerts its action by degrading the substrate in the blood and not in tumor tissue.

Liposomes have been explored in the past (Fishman and Citri, 1975; Gregoriadis and Neerunjun, 1976; Gregoriadis and Kirby, 1984) as carriers of L-ASNase, but only with limited success (Fishman and Citri, 1975; Gregoriadis and Neerunjun, 1976), as the entrapment efficiencies of the formulations were low (27–55%) and high doses were needed for optimal antitumour activity (Gregoriadis and Neerunjun, 1976).

In addition to the usual required characteristics, such as high encapsulation efficiency, stability in biological fluids and reduced toxicity, an optimal liposomal formulation of a protein must keep the integrity of the labile protein structure and concomitant activity (Stamp and Juliano, 1979; Cruz et al., 1989).

Very little information with respect to the study of these parameters for proteins in general, and specifically for L-ASNase, is currently available.

In this paper, we present a series of systematic studies designed to develop liposomal formulation of L-ASNase with the properties indicated above. By selecting the appropriate incorporation method, the liposome size and lipid composition, and establishing specific incorporation conditions we were able to obtain formulations with enzymatic activity completely preserved and reduced in vitro cytotoxicity. A few formulations have been selected for in vivo biological studies.

Materials and Methods

Materials

Egg phosphatidylcholine (PC), egg phosphatidylinositol (PI), cholesterol (Chol), stearylamine (SA), dicetyl phosphate (DcP), phosphatidylglycerol (PG), dimyristoylphosphatidylcholine (DMPC), monosialoganglioside (GM_1), dimyristoylphosphatidylglycerol (DMPG), dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), sphingomyelin (SM) and L-asparaginase (L-ASNase) were obtained from Sigma Chemical Co. (München, Germany). L-Asparaginase was also purchased from Merck Sharp & Dohme (West Point, PA, U.S.A.). All other reagents were analytical grade.

Preparation of liposomes containing L-ASNase

Two different types of vesicles were prepared: simplified dehydration-rehydration vesicles (sDRV) and extruded vesicles (VET). sDRV were prepared as described previously (Cruz et al., 1989). In brief, the lipid mixtures (15–18 μmol) were dried under a N_2 stream. L-ASNase solution (0.25–0.30 mg/ml for sDRV or 1.70–1.90 mg/ml for VET) was slowly added, with gentle stirring,

to form multilamellar vesicles. The liposome suspension was lyophilized on a Brenda Scientific freeze-drier (25 mTorr for 4 h). The lyophilized powder was then rehydrated with 0.1 ml of 0.3 M mannitol. Mild vortexing of the flask was performed and the final suspension was left at room temperature for 30 min. After this period of time, the volume was brought to 1 ml by adding 0.154 M NaCl. Non-encapsulated enzyme was removed by three cycles of 30-fold dilution with 0.154 M NaCl and centrifugation at $38\,000 \times g$ for 30 min. The liposome pellet was resuspended in 1 ml of 0.154 M NaCl.

VET were prepared by extrusion of non-washed sDRV, containing liposome-free enzyme and liposome-encapsulated enzyme at a total concentration of 1.7–1.9 mg/ml. VET were resuspended in excess volume (10-fold the initial volume) of 0.154 M NaCl and extruded under pressure through policarbonate filters of 800, 200 and 100 nm pore size, sequentially, using an Extruder, obtained from Lipex Biomembranes Inc. (Vancouver, Canada). A final ultracentrifugation step ($184\,000 \times g$ for 90 min) to concentrate the liposome preparation was performed.

Characterization of liposomes containing L-ASNase

The following equations were used to determine the liposome encapsulation parameters: encapsulation efficiency (Cruz et al., 1989; Martins et al., 1990), protein recovery, retention of enzymatic activity and stability.

% encapsulation efficiency

$$= \frac{P_f/L_f \text{ in liposome suspension}}{\text{total } P_i/L_i \text{ in initial mixture}} \times 100$$

% protein recovery

$$= \frac{\text{final liposome associated protein } (P_f)}{\text{total initial protein } (P_i)} \times 100$$

% retention of activity

$$= \frac{\text{specific activity of encapsulated L-ASNase}}{\text{specific activity of free L-ASNase}} \times 100$$

% stability

$$= \frac{\text{L-ASNase activity of pelleted vesicles } (184\,000 \times g \times 90 \text{ min}) \text{ at } x \text{ h}}{\text{total L-ASNase activity at 0 h}} \times 100$$

Protein recovery is the liposome associated protein in the final liposome suspension related to the total protein initially added. This parameter reflects the yield of the process concerning protein.

Encapsulation efficiency (E.E.) is the percentage of the ratio between the final protein to lipid (P/L) ratio and the initial P/L ratio. This parameter, being a ratio between final to inital P/L ratio, is a measure of efficiency of an initial system (lipid + protein) to encapsulate (associate) protein in the final liposomal form.

Retention of activity was expressed as the specific activity of the encapsulated L-ASNase vs that of the same amount of free enzyme.

Stability of liposome encapsulated L-ASNase was assessed by measuring the enzymatic activity in the pelleted vesicles ($184\,000 \times g \times 90$ min centrifugation), at appropriate time points, after incubation either with 0.154 M NaCl at 0°C or diluted 1:2 in human serum at 37°C, related to the enzymatic activity before incubation.

Enzymatic activity, lipid and protein determinations

L-ASNase activity in the liposome suspension was determined by the method described by Jayaram et al. (1974) after disruption of liposomes with Triton X-100. Lipid determinations were made using the method described by Fiske and Subbarow (1925) as modified by King (1932). Protein determinations were performed using the method described by Lowry et al. (1951) after disruption of the liposomes with Triton X-100 and SDS. Size distribution studies were carried out using laser light scattering (Zetasizer 3, Malvern, U.K.).

In vitro cytotoxicity against Chinese hamster ovarium (CHO) cells

CHO cells were used to evaluate the in vitro cytotoxicity of free and liposomal L-ASNase. Dif-

ferent concentrations of L-ASNase (ranging from 0.3 to 33 IU) were added to cell cultures and incubated for 18 h at 37°C in a 5% CO_2/95% O_2 humidified atmosphere. Following the incubation period, cell survival was assessed by the quantitation of the coloured decomposition product of MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, by mitochondrial enzymes (Gabizon et al, 1989). The percentage of cell survival was calculated with the following equation:

$$\% \text{ survival} = \frac{A_{570\,\text{nm}}\,(\text{cells incubated with enzyme})}{A_{570\,\text{nm}}\,(\text{control cells})} \times 100$$

ID_{50} for free and encapsulated enzyme was calculated according to the method of Reed and Muench (1938).

Results

Effect of lipid composition on encapsulation of L-ASNase in multilamellar vesicles

Table 1 shows the characteristics of different liposomal formulations (sDRV) containing L-ASNase. The encapsulation efficiency, enzymatic activity, protein to lipid ratio and recovery were found to be highly dependent on the lipid composition used. The presence of cholesterol, the peripheral charge and the phase transition temperature of lipids appeared to influence the parameters mentioned above.

The addition of Chol to PC (prep. 1) and to DMPC (prep. 11) resulted in a decrease of the E.E. and of the specific enzymatic activity (prep. 2, 3 and 12). The addition of negatively charged molecules, such as DcP and DMPG to PC:Chol mixtures and to DMPC increased the E.E. while substantially decreasing the specific activity (cf. prep. 3 with 4 and 5; prep. 11 with 15). The loss of enzymatic activity was proportional to the percentage of DcP to total lipid (prep. 4 and 5). The addition of SA (prep. 6–10) increased the E.E. up to 100%, with only a mild loss of specific activity. The amount of SA was tuned to obtain higher encapsulation parameters (prep. 8 and 9).

Incorporation of L-ASNase in liposomes composed of lipids with a higher T_p (prep. 11–18) indicated that the effect of lipid fluidity on encapsulation parameters was less important than that of peripheral charge. The E.E. and retention of specific enzymatic activity of DMPC and DMPC:Chol liposomes (prep. 11 and 12) are similar to those observed for the corresponding fluid liposomes (prep. 1 and 2). The E.E. was increased by the addition of SA to the lipid mixtures (prep. 13) and the enzymatic activity was greatly reduced by the addition of negatively charged DMPG (prep. 15 and 16). Low E.E. were observed in mixtures of DMPC with DOPC (prep. 14), while specific enzymatic activity was not significantly affected by DOPC. Other high transition temperature lipid mixtures tested (prep. 17 and 18) showed low values for both parameters, which cannot be attributed to any constituent per se, since no further studies were performed.

The influence of SA, PI and GM_1 on the encapsulation efficiency of liposomes containing PC:Chol mixtures was studied, with higher protein concentration (prep. 1–3, Table 2). For similar initial protein and lipid concentrations, the mixtures containing SA showed higher encapsulation parameters, probably due to the larger vesicle size. The use of GM_1 reduced enzyme activity. The lipid combinations resulting in a higher E.E. in sDRV with preservation of specific enzyme activity were PC:Chol:SA (7:2:0.5 and 7:2:0.25) and DMPC:Chol:SA (7:2:0.5) (Table 1).

Effect of P / L ratio and liposome size on encapsulation parameters

Table 2 lists the encapsulation parameters in sDRV and extruded vesicles obtained with an initial protein concentration of 2 mg/ml, 8-times higher than in the experiments shown in Table 1. Comparing preparations of the same lipid composition but different P/L (prep. 9, Table 1 and prep. 1, Table 2) it is evident that a 4-fold increase in the P_i/L_i ratio results in a similar increase of this parameter in the final preparation (P_f/L_f). This final preparation has much

higher intraliposomal enzyme concentration (96 vs 27 μg/μmol of lipid), but the encapsulation efficiency is somewhat reduced (73 vs 90%).

For similar P_i/L_i ratios, the extrusion of sDRV resulted in a marked reduction of E.E., enzyme recovery and P_f/L_f ratio, directly related to the reduction of the vesicle size. Specific enzymatic activity was not affected by filtration. For similar initial enzyme concentration, the E.E. for extruded vesicles composed of PC:Chol:SA was 52% for 200 nm (prep. 4) and 38% for 100 nm size (prep. 5), as compared to 73% without the extrusion step (prep. 1). The decrease in these parameters is proportional to the reduction of

TABLE 1

Effect of lipid composition on encapsulation parameters of L-asparaginase in liposomes (sDRV)

Preparation no.	Lipid composition (molar ratio)	P_i/L_i (μg/μmol)	P_f/L_f (μg/μmol)	E.E. (%)	Recovery (%)	Activity (%)
1	PC	20.9 ± 1.6	8.2 ± 2.9	39.0 ± 2.8	30.0 ± 4.2	89.5 ± 7.8
2	PC:Chol (4:1)	20.6 ± 0.4	6.9 ± 0.7	37.3 ± 9.2	32.1 ± 5.5	80.6 ± 12.0
3	PC:Chol (1:1)	22.1 ± 2.1	6.9 ± 0.6	31.2 ± 0.4	28.3 ± 0.5	76.7 ± 5.5
4	PC:Chol:DcP (7:2:1)	21.3 ± 0.8	15.2 ± 1.3	70.0 ± 3.6	45.3 ± 7.2	32.8 ± 9.5
5	PC:Chol:DcP (7:1:2)	21.9 ± 4.5	14.5 ± 1.3	68.0 ± 14.1	38.0 ± 8.9	26.7 ± 17.6
6	PC:Chol:SA (7:2:2)	26.1 ± 2.3	12.8 ± 0.8	49.5 ± 7.8	34.3 ± 8.1	97.0 ± 3.6
7	PC.Chol:SA (7:2:1)	31.3 ± 0.4	26.0 ± 1.3	83.0 ± 2.8	44.0 ± 8.5	88.0 ± 0.0
8	PC:Chol:SA (7:2:0.5)	23.7 ± 4.9	25.7 ± 1.7	100.0 ± 1.0	53.5 ± 3.5	99.5 ± 0.7
9	PC:Chol:SA (7:2:0.25)	30.5 ± 0.7	27.2 ± 1.4	89.5 ± 6.4	77.4 ± 1.8	99.7 ± 0.3
10	PC:Chol:SA (7:2:0.125)	24.3 ± 0.6	22.0 ± 1.3	90.5 ± 7.8	70.3 ± 5.7	69.0 ± 2.8
11	DMPC	20.2 ± 0.8	9.0 ± 0.4	44.5 ± 3.5	38.0 ± 6.2	94.7 ± 2.5
12	DMPC:Chol (4:1)	16.4 ± 2.6	4.6 ± 1.2	28.0 ± 5.3	23.3 ± 7.4	82.5 ± 9.7
13	DMPC:Chol:SA (7:2:0.5)	18.1 ± 1.4	15.7 ± 0.8	86.7 ± 6.8	70.3 ± 10.2	81.3 ± 9.0
14	DMPC:DOPC (7:3)	20.4 ± 7.6	7.6 ± 2.3	38.3 ± 5.4	35.8 ± 4.3	86.6 ± 9.0
15	DMPC:DMPG (7:2:1)	17.4 ± 2.4	16.5 ± 2.4	90.0 ± 1.4	55.3 ± 12.9	50.5 ± 0.7
16	DMPC:DMPG:SA (7:2:1)	17.9 ± 0.4	15.1 ± 1.0	84.5 ± 7.8	50.7 ± 9.5	36.7 ± 3.2
17	SM:Chol:PI (10:5:1)	25.6 ± 0.6	5.8 ± 1.6	24.0 ± 4.5	16.3 ± 3.0	28.3 ± 5.7
18	DSPC:Chol:PI (10:5:1)	27.9 ± 11.5	6.6 ± 0.9	25.0 ± 7.1	18.5 ± 0.7	29.5 ± 2.1

[a] Initial protein concentration: 0.25–0.30 mg/ml.

[b] Initial lipid concentration: 14.5–17.0 μmol/ml.

[c] P_i, L_i, P_f and L_f were determined at least in triplicate.

TABLE 2

Effect of liposome size on encapsulation efficiency

Preparation no.	Lipid composition type	Diameter (nm)	P_i/L_i (μg/μmol)	P_f/L_f (μg/μmol)	E.E. (%)	Recovery (%)	Activity (%)
1	PC:Chol:SA sDRV	1249.2 ± 248.6	130.8 ± 6.2	95.0 ± 9.8	72.5 ± 7.2	60.6 ± 7.3	99.4 ± 0.9
2	PC:Chol:PI sDRV	698.8 ± 244.5	96.0 ± 4.2	66.0 ± 11.3	69.0 ± 14.1	45.0 ± 2.8	98.6 ± 0.9
3	PC:Chol:GM_1 sDRV	501.2 ± 125.7	125.5 ± 26.4	116	72	40	86
4	PC:Chol:SA VET_{200}	254.8 ± 150.3	86.0 ± 11.3	44.0 ± 4.2	51.5 ± 2.1	30.5 ± 0.8	98.2 ± 2.6
5	PC:Chol:SA VET_{100}	85.1 ± 38.6	115.8 ± 17.6	44.2 ± 8.8	38.4 ± 5.7	26.2 ± 3.6	96.2 ± 4.1
6	PC:Chol:PI VET_{200}	158.9 ± 17.0	109.5 ± 10.6	33.0 ± 4.2	30.5 ± 0.8	20.5 ± 6.4	92.0 ± 0.5
7	PC:Chol:PI VET_{100}	142.4 ± 23.1	115.9 ± 13.1	31.5 ± 8.8	27.6 ± 8.1	19.7 ± 4.3	88.9 ± 8.7
8	PC:Chol:GM_1 VET_{200}	–	97.0 ± 4.2	43.5 ± 0.8	55.0 ± 14.1	33.0 ± 9.9	73.5 ± 3.6
9	PC:Chol:GM_1 VET_{100}	141.8 ± 21.5	125.5 ± 26.4	33.8 ± 13.8	26.4 ± 7.6	19.3 ± 4.6	83.6 ± 4.5

[a] Lipid composition: PC:Chol:SA (7:2:0.25); PC:Chol:PI (10:5:1): PC:Chol:GM_1 (10:5:1).
[b] Initial protein concentration: 1.7–1.9 mg/ml.
[c] Initial lipid concentration: 15.5–17.5 μmol/ml.
[d] L_i, P_i, L_f and P_f were determined at least in triplicate with the exception of prep. 3.

vesicle size after extrusion, which is in the order of 4–6-fold, for 100 nm pore size (prep. 1 and 5; 2 and 7; 3 and 9).

Effect of ionic strength and osmolarity on encapsulation parameters

All encapsulation parameters decreased upon increasing the ionic strength of the aqueous solution used for reconstitution, as shown in Table 3. The most significant changes occurred in the retention of activity, from 82 to 45%, for PC:Chol and from 79 to 53% for PC:Chol:SA, using water and saline as rehydration solution, respectively. The use of 300 mM mannitol instead of deionized water as rehydration solution led to an improved recovery as a result of an increase in

TABLE 3

Effect of ionic strength and osmolarity on encapsulation parameters

Lipid composition	Rehydration medium	P_f/L_f (μg/μmol)	E.E. (%)	Activity (%)
PC:Chol	water	6.7 ± 2.6	31.7 ± 12.6	82.0 ± 12.2
	0.154 M NaCl	3.9 ± 1.5	29.6 ± 17.2	45.0 ± 11.4
	0.300 M mannitol	5.7 ± 1.2	38.0 ± 10.2	86.7 ± 11.0
PC:Chol:SA	water	14.2 ± 1.8	65.2 ± 10.0	79.0 ± 17.4
	0.154 M NaCl	8.0 ± 1.4	41.6 ± 19.4	53.0 ± 14.0
	0.300 M mannitol	12.1 ± 2.4	59.3 ± 11.5	56.3 ± 21.0

[a] Type of vesicles: sDRV.
[b] Lipid composition: PC:Chol (1:1); PC:Chol:SA (7:2:0.25).
[c] L_i, P_i, L_f and P_f were determined at least in triplicate.

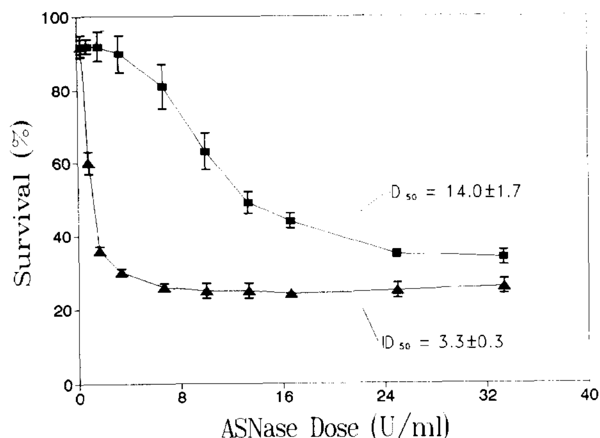

Fig. 1. In vitro cytotoxicity of free (▲) and liposomal (■) L-ASNase against CHO cells. Liposomes (sDRV) composed of PC:Chol:SA (7:2:0.25) were used. The ID_{50} for free enzyme was 3.3 ± 0.3 U/ml and for liposomal enzyme 14.0 ± 1.7 U/ml.

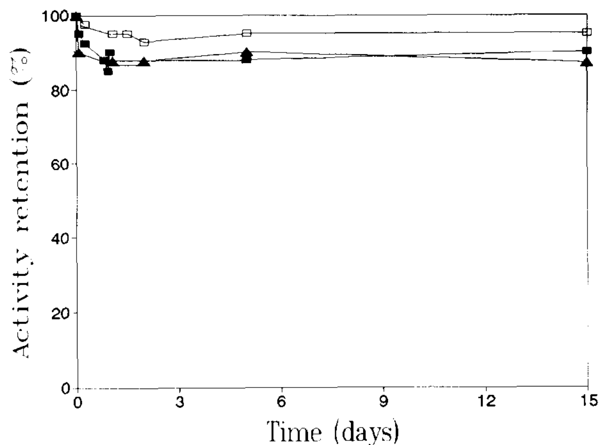

Fig. 2. Stability of liposomes (sDRV) in saline at 4°C. The retention of enzymatic activity with incubation time was assessed for liposomes of different compositions: PC:Chol (1:1) (■); PC:Chol:SA (7:2:0.25) (▲) and DMPC:Chol:SA (7:2:0.5) (□).

the volume of the pellet after centrifugation. Although no significant changes in the encapsulation parameters were observed, rehydration in the presence of mannitol instead of water is recommended, since it maintains a physiological intraliposomal osmolarity.

In vitro cytotoxicity against CHO cells

Fig. 1 shows the results of cell survival after incubation of CHO cells with free and liposomal L-ASNase. Liposomal L-ASNase was significantly less cytotoxic than free L-ASNase (ID_{50}: 14.0 ± 1.7 vs 3.3 ± 0.3 U/ml). Normal cells are able to synthesize asparagine in sufficient quantities to survive, but are damaged by the rapid depletion of asparagine secondary to treatment with L-ASNase, since they need time to recover from enzyme injury (Celle et al., 1973). This occurs to a smaller extent when L-ASNase is entrapped in liposomes, as the enzyme is released slowly from the vesicles.

Stability of liposomes containing L-ASNase in normal saline

The stability in saline of selected preparations with high E.E. and retention of activity was investigated (Fig. 2).

The most stable sDRV preparations were those composed of PC:Chol:SA and DMPC:Chol:SA with activity retention values of 87 and 95%, respectively.

Stability of liposomes containing L-ASNase in human serum

The stability in human serum of selected formulations (sDRV and VET_{100}) is shown in Fig. 3.

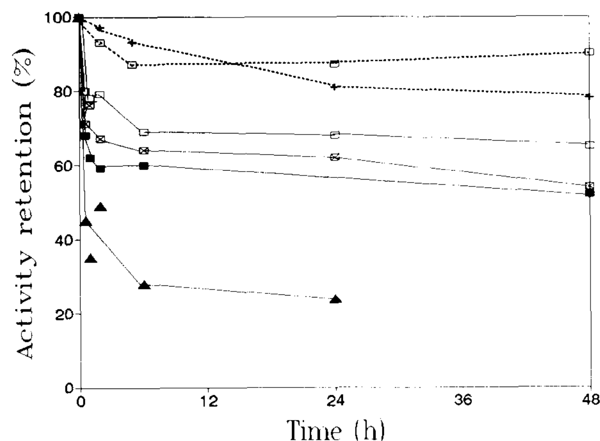

Fig. 3. Stability of liposomes (sDRV and VET) in human serum at 37°C. The retention of activity after appropriate time periods was assessed for liposomes of different lipid compositions: sDRV (full line) – PC:Chol (1:1) (■); PC:Chol:SA (7:2:0.25) (□); DMPC (▲); DMPC:Chol:SA (7:2:0.5) (⊠); VET_{100} (dotted line) – PC:Chol:SA (7:2:0. 25) (□); PC:Chol:PI (10:5:1) (+).

For sDRV formulations at 48 h, the activity retention was 65 and 55% for PC:Chol:SA and DMPC:Chol:SA, respectively. VET_{100} preparations, composed of PC:Chol:SA and PC:Chol:PI, were highly stable, with 80–90% of the initial enzyme activity retained.

Discussion

The use of liposomes as carriers of L-ASNase has been studied by other investigators in the past (Fishman and Citri, 1975; Gregoriadis and Neerunjun, 1976; Ohsawa et al., 1985; Michailin et al., 1986). However, the preparations described were suboptimal from the galenic or pharmacological points of view. Previous studies lack appropriate characterization of the L-ASNase liposomal formulations, since systematic studies of the parameters affecting encapsulation as well as in vitro and in vivo behaviour of the formulations were not carried out.

In this manuscript, we describe improved liposomal formulations of L-ASNase, both using multilamellar vesicles and extruded vesicles. The encapsulation of L-ASNase in sDRV is the highest ever reported in the literature. We obtained enzyme recovery values of up to 77% while values of 28–48% have been reported for similar parameters of multilamellar vesicles (Fishman and Citri, 1975; Gregoriadis and Neerunjun, 1976) and of 60% for large vesicles (Michailin et al., 1986). In addition, the recovery of L-ASNase is much higher than that reported for albumin and factor VIII using similar methods (30–40%) (Gregoriadis and Kirby, 1984). There is no comparison available of P/L (final/initial) ratios that could give further information on the process. The retention of enzymatic activity (up to 99.7%) is comparable to or higher than that reported previously (Fishman and Citri, 1975; Michailin et al., 1986). Also, there is no comparison available for the encapsulation of L-ASNase in extruded vesicles. An equivalent method (with DRV as starting material and size reduction by microfluidization instead of extrusion) was used for encapsulation of small molecules and peptides but not for macromolecules (Gregoriadis et al., 1990). The efficiencies of both methods are similar, with recoveries of up to 33% (present work) and 42% (tetanus toxoid; Gregoriadis et al., 1990) and similar final solute/lipid ratios of up to 44 $\mu g/\mu mol$ (present work) and 46 $\mu g/\mu mol$ (Gregoriadis et al., 1990), for similar initial solute/lipid ratios, respectively of 97–116 $\mu g/\mu mol$ (present work) and 109 $\mu g/\mu mol$ (Gregoriadis et al., 1990).

Although the encapsulation parameters of extruded vesicles are inferior to those of sDRV, they offer the advantage of a longer circulation time in the blood. Extruded vesicles show a high retention of specific enzymatic activity (73–99%), as compared to that reported for enzyme adsorbed to the external surface of small preformed vesicles (55%) (Michailin et al., 1986). To compensate for the relatively low E.E. of VET and to achieve high intraliposomal L-ASNase concentrations, we increased the initial P/L ratio by 4-fold and extruded the sDRV without previous separation of the non-encapsulated protein. With this method we were able to obtain a concentration of L-ASNase in VET_{100} 2-fold higher than in sDRV of the same lipid composition. The observation that higher initial P/L ratios enhance the incorporation of the enzyme in the liposomes is in agreement with the studies on albumin previously mentioned (Cruz et al., 1989).

It would be expected (Senior, 1987) that L-ASNase encapsulated in small size liposomes prepared with soybean phospholipids (Michailin et al., 1986) would remain for a longer period in circulation than encapsulated in sDRV as described here. The greater stability of sDRV, prepared with selected lipids, stresses the importance of lipid composition on stability. Besides the importance of lipid composition, the size is a crucial factor for stability. The stability in human plasma of sDRV was further increased (from 65 to 95%) after size reduction by extrusion. The finding that encapsulation parameters and stability in biological fluids increase with charge might be due to the widening of the aqueous space between the lipidic bilayers (Gruner, 1987). Besides, the presence of SA in liposomes should favour the establishment of electrostactic interactions with the enzyme, which is negatively charged at the working pH (pI 4.99). However, this is not

the major effect ruling out L-ASNase encapsulation as it is not proportional to the percentage of SA present in the total lipid mixture. The inadequacy of some negatively charged vesicles (containing DcP and PG) as carriers for L-ASNase is due to inhibition of enzymatic activity and not to encapsulation problems. Other negatively charged lipids such as PI and GM_1 result in formulations with high E.E. without significantly reducing enzymatic activity. The influence of these components in prolonging the circulation time has already been reported (Gabizon and Papahadjopoulos, 1988; Allen et al., 1989) for other biological agents. With respect to the encapsulation of L-ASNase, no systematic studies on the influence of lipids or charge have been reported in previous work (Fishman and Citri, 1975; Gregoriadis and Neerunjun, 1976; Ohsawa et al., 1985; Michailin et al., 1986). The encapsulation parameters and stability of vesicles composed of mixtures of PI and lipids with high T_p, known to result in vesicles with high stability (Allen et al., 1989), could not be studied due to technical difficulties of performing tests in the presence of high detergent concentrations needed for solubilization.

Although it is probably important to avoid the presence of osmotic gradients after in vivo administration of liposomes, it is surprising how little attention has been given to the effects of solute osmolarity on protein encapsulation. We found that the use of an isotonic solution such as 0.154 M NaCl resulted in a decrease of the E.E. for all lipid compositions tested, probably due to competition of the ionic species for the polar heads of the lipids. The observed loss of enzyme activity may be due to the presence of Na^+ that acts as a L-ASNase inhibitor. By contrast, the use of a mannitol solution to rehydrate the liposomes did not alter the encapsulation parameters. These results are in agreement with previous work on the effect of osmolarity on protein encapsulation (Cruz et al., 1989). For this reason, and the fact that a suspension of liposomes in mannitol solution can readily be centrifuged, we conclude that this is the best way to maintain iso-osmolarity.

The liposomal formulations of L-ASNase described are significantly less toxic than free L-ASNase against normal CHO cells, in agreement with other authors (Fishman and Citri, 1975). The reduction in L-ASNase cytotoxicity secondary to liposome encapsulation might be due to a slower release of L-ASNase and slower substrate depletion, allowing cells to adapt and synthesize asparagine. A similar mechanism of slow release is expected in vivo, allowing the maintenance of drug concentration in the blood. In conclusion, as a result of screening different liposomal formulations of L-ASNase, we have been able to select formulations with high E.E., good in vitro stability and in vitro toxicity lower than that of the free enzyme. Theoretically, the high intraliposomal concentration of enzyme and stability in plasma of the selected formulations should result in an increase of circulating enzyme and enhanced therapeutic activity, since the target is a circulating substrate. The manufacturing method is extremely simple and easy to scale up. The main steps are characterized by: formation of MLV in the presence of an aqueous solution of L-ASNase; these vesicles are lyophilized without neglecting the non-encapsulated enzyme; mannitol is used in the rehydration step; high dilution of sDRV is used before filtration; non-washed sDRV are used as starting material to prepare VET; no freeze-thaw steps are involved; the vesicle size is approx. 100 nm and the preferred lipid compositions are PC:Chol:SA; PC:Chol:PI and PC:Chol:GM_1.

The procedures and experimental conditions developed for L-ASNase can be extrapolated for other protein agents, since they result in high intraliposomal protein concentrations, high stability and preservation of protein activity. In previous studies, using ultrasonication to encapsulate enzymes into small liposomes (SUV), damaging of enzymatic activity has been observed (data not shown). Since no adsorption of L-ASNase was observed at the outer surface of the liposomes (data not shown), we conclude that there is no significant interaction between the lipids and the enzyme and, therefore, the entrapment of L-ASNase mainly occurs as a result of a passive mechanism. Therefore, under the above described encapsulation conditions, a similar behaviour in entrapment, comparable characteristics of liposomes, and similar stability on storage

and in biological fluids are expected irrespective of the enzyme used for liposome entrapment. Biological activity studies are in progress to clarify the in vivo behaviour and mechanism of action, and to evaluate the therapeutic potential of the above selected liposomal formulations of L-ASNase.

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References

Allen, T.M., Hansen, C. and Rutledge, J., Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta,* 981 (1989) 27–35.

Capizzi, R.L., Powell, B.L., Cooper, M.R., Stuart, J.J., Muss, H.B., Richards II, F., Jackson, D.V., White, D.R., Spurr, C.L., Zekan, P.J., Cruz, J.M. and Craig, J.B., Sequential high-dose Ara-C and asparaginase in the therapy of previously treated and untreated patients with acute leukemia. *Semin. Oncol.,* 12 (1985) 105–113.

Celle, G., Dodero, M. and Pannacciulli, I., The liver damaging effect of L-asparaginase: an experimental study of chronic toxicity. *Eur. J. Cancer,* 9 (1973) 55–57.

Clarkson, B., Krakoff, I., Burchenal, J., Karnofsky, D., Golbey, R., Dowling, M., Oettgen, H. and Lipton, A., Clinical results of treatment with *E. coli* L-asparaginase in adults with leukemia, lymphoma, and solid tumors. *Cancer,* 25 (1970) 279–305.

Cruz, M.E.M., Corvo, M.L., Jorge, J.S. and Lopes, F., Liposomes as carrier systems for proteins: factors affecting protein encapsulation. In Lopez-Berestein, G. and Fidler, I. (Eds), *UCLA Symposia on Molecular and Cellular Biology: Liposomes in the Therapy of Infectious Diseases and Cancer,* A.R. Liss, New York, 1989, pp. 417–426.

Eppstein, D.A., Medical utility of interferons: approaches to increasing therapeutic efficacy. *Pharm. Int.,* 7 (1986) 195–199.

Eppstein, D.A., Pragmatic approaches to delivery of peptides and proteins as drugs. In Gregoriadis G. (Ed.), *NATO ASI Series A: Life Sciences, Vol. 155, Targeting of Drugs: Anatomical and Physiological Considerations,* Plenum, New York, 1988, pp. 189–202.

Eppstein, D.A. and Longenecker, J.P., Alternative delivery systems for peptides and proteins as drugs. *CRC Crit. Rev. Ther. D. Sys.,* 5 (1988) 99–139.

Fishman, Y. and Citri, N. L-Asparaginase entrapped in liposomes: preparation and properties. *FEBS Lett.,* 60 (1975) 17–20.

Fiske, C.H. and Subbarow, Y., The colorimetric determination of phosphorus. J. *Biol. Chem.,* 66 (1925) 375–400.

Gabizon, A. and Papahadjopoulos, D., Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA,* 85 (1988) 6949–6953.

Gabizon, A., Sulkes, A., Peretz, T., Druckmann, S., Goren, D., Amselem, S. and Barenholz, Y., Liposome-associated doxorubicin: preclinical pharmacology and exploratory clinical phase. In Lopez-Berestein, G. and Fidler, I. (Eds), *UCLA Symposia on Molecular and Cellular Biology New Series. Liposomes in the Therapy of Infectious Diseases and Cancer,* A.R. Liss, New York, 1989, pp. 391–402.

Gregoriadis, G. and Neerunjun, D., Tumor regression with liposome-entrapped L-Asparaginase: some immunological advantages. *Biochem. Soc. Trans.,* 4 (1976) 133–134.

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

Gregoriadis, G., Davies, A. and Davies, D., Liposomes as immunological adjuvants: antigen incorporation studies. *Vaccine,* 5 (1987) 145–151.

Gregoriadis, G., 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.

Gruner, S.M., Material properties of liposomal bilayer in liposomes. In Ostro, M.J. (Ed), *From Biophysics to Therapeutics,* Dekker, New York, 1987 pp. 1–38.

Jayaram, H.N., Cooney, D.A. and Jayaram, S., A simple and rapid method for the estimation of L-asparaginase in chromatographic and electrophoretic effluents: comparison with other methods. *Anal. Biochem.,* 59 (1974) 327–346.

King, E.J., The colorimetric determination of phosphorus. *Biochem. J.,* 26, (1932) 292–297.

Lopez-Berestein, G., Fainstein, V., Hopfer, R., Metha, K., Sullivan, M.P., Keating, M., Rosenblum, M.G., Metha, R., Luna, M., Hersh, E.M., Reuben, J., Juliano, R.L. and Bodey, G.P., Liposomal amphotericin-B for the treatment of systemic fungal infections in patients with cancer: a preliminary study. *J. Infect. Dis.,* 151, 4 (1985) 704–710.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.,* 193 (1951) 265–275.

Martins, M.B.F., Jorge, J.C.S. and Cruz, M.E.M., Acylation of L-asparaginase with total retention of enzymatic activity. *Anal. Biochem.,* 72 (1990) 671–675.

Michailin, V.S., Kondrashin, A.A. and Berezov, T.T., Peculiarities of immobilization and catalytic properties of native commercial L-Asparaginase in liposomes formed from soybean phospholipids. *Vopr. Med. Khim,* 32 (1986) 68–72.

Ohsawa, T., Miura, H. and Harada, K., Evaluation of a new liposome preparation technique, the freeze - thawing method, using L-ASNase as a model drug. *Chem. Pharm. Bull,* 33 (1985) 2916–2923.

Perez-Soler, R., Khokhar, A.R., Hacker, M.P. and Lopez-Berestein, G., Toxicity and anti tumor activity of *cis*-bis-cyclopentenecarboxylato-1,2-diaminocyclohexane platinum(II) encapsulated in multilamellar vesicles. *Cancer Res.,* 46 (1986) 6269–6273.

Reed, L.J. and Muench, H., A simple method of estimating fifty percent endpoints. *Am. J. Hyg.,* 27 (1938) 825–876.

Senior, J.H., Fate and behavior of liposomes in vivo: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.,* 3 (1987) 123–193.

Stamp, D. and Juliano, R.L., Factors affecting the encapsulation of drugs within liposomes. *Can. J. Physiol. Pharmacol.,* 57 (1979) 535–539.

Ullman, E.F., Tarnowski, T., Felgner, P. and Gibbons, I., Use of liposome encapsulation in a combined single liquid reagent for homogeneous enzyme immunoassay. *Clin. Chem.,* 33 (1987) 1579–1584.

Vadiei K., Lopez-Berestein G., Perez-Soler R. and Luke, D.R., In vitro evaluation of liposomal cyclosporine. *Int. J. Pharm.,* 57 (1989) 133–138.