International Journal of Pharmaceutics, 80 (1992) 93–100

IJP 02645

Research Papers

Use of liposomes as carriers for immunomodulatory polypeptides: Studies on thymostimulin encapsulation and retention

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(Received 12 July 1991)
(Accepted 2 September 1991)

Key words: Thymostimulin; Dimyristoylphosphatidylcholine; Dipalmitoylphosphatidylcholine; Liposome; Encapsulation efficiency

Summary

The incorporation of thymostimulin, an immunoactive hormone extracted from calf thymus, into liposomes was characterized by means of DSC analysis. Changes in vesicle composition, as well as in the presence of cholesterol, led to variations in thermotropic behaviour and influenced their incorporation efficiency and stability. Among the prepared formulations, liposomes prepared using equimolar amounts of DPPC and DMPC showed the highest value (80%) for drug incorporation.

Introduction

The transport of therapeutic agents by means of liposomes is a well-established technique.

In recent years, several kinds of macromolecules, such as hormones, enzymes and proteins, have been incorporated into multilamellar liposomes for use as in vivo delivery systems (Gregoriadis, 1976; Tyrrell et al., 1976; Galzigna et al., 1981; Sakaguchi, 1988).

The use of liposomes as drug carriers could be effective in increasing the concentration of the agent incorporated into the target tissue as well as in enhancing the stability and lifetime of the drug, i.e., incorporation of enzymes or proteins into multilamellar vesicles can protect such agents from proteolytic degradation. Moreover, by specific modification of the liposomal surface, they could be directed toward a specific target cell and be restricted to a particular site of action of the entrapped drug (Shek and Barber, 1986).

However, in order to achieve the maximum extent of incorporation and to control the rate of release of the enclosed molecules, a critical evaluation of the factors affecting the entrapment and release of different drugs, such as the phospholipid composition and size of the liposomes, is essential.

The present paper represents the completion of a previous work (Panico et al., 1991), in which we examined the in vitro inhibitory effect on human erythroleukemic cells (K562), of thy-

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mostimulin (Ts), a polypeptidic multifactorial hormone, extracted from calf thymus, when carried by liposomes consisting of pure dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylcholine (DMPC). Ts-loaded liposomes appeared to be more effective inhibitors of cell growth than drug alone, thus demonstrating that delivery of the drug through fusion between vesicles and the cell membrane is a process that is facilitated to a greater extent than the simple uptake, perhaps through a specific receptor, of free Ts by the cells.

The results of these orientative tests prompted us to extend the study to phospholipid formulations containing Ts, to perform DSC (differential scanning calorimetry) analysis of the kind of interaction between the Ts macromolecule and liposomal bilayers, and to establish the optimal conditions for the preparation of stable and effective vesicles capable of controlling and increasing the delivery of Ts into the bloodstream and its in vivo activity.

The encapsulation efficiency and stability of vesicles at different temperatures of preparation were then evaluated as a function of lipid composition and of the presence of cholesterol in the formulations.

Materials and Methods

L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-dimyristoylphosphatidylcholine (DMPC) and cholesterol (Chol) were obtained from Fluka (Switzerland); their purity was greater than 99% as judged by two-dimensional thin-layer chromatography on silica gel plates (Merck 60 F_{254}) loaded with solutions of the lipids in $CHCl_3$ and developed first with $CHCl_3/CH_3OH/(7\ N)\ NH_4OH$ (60:3:5, v/v) and successively with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (12:60:8:2.5, v/v).

Tetranitrobenzenesulfonic acid (TNBS) was purchased from Aldrich Chimica S.r.l. (Milan, Italy).

Thymostimulin (TP-1) (average MW 12000) was kindly donated by Serono (Italy). NaCl was recrystallized from double-distilled water and tested for the absence of calcium by atomic absorption spectroscopy. Deionized double-distilled water was used in this work.

Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared following the film method (Bangham et al., 1965).

Phospholipids (PL) (10 mg), either singly or in a mixture, and Chol, if needed, were dissolved in chloroform and the solvent removed at 30°C under a nitrogen stream. The resulting lipid film was kept overnight at 60°C under high vacuum.

Liposomes were obtained by adding 100 μg of Ts in 200 μl of 0.9% NaCl solution; the mixture was heated to a temperature above that of its gel-to-liquid crystal phase transition (T_m) (70°C) to allow full hydration of the sample and then vortexed for 5 min at 30°C. The liposomal suspension was centrifuged for 15 min at 100000 $\times g$ at 4°C, in a 50 Ti type rotor of a Beckman L8-60 M ultracentrifuge, in order to separate the incorporated from the free Ts. This washing step was repeated twice.

Vesicle size and morphology were determined under a photomicroscope (Zeiss III RS, Germany). The method used for the preparation of liposomes gives rise to a rather homogeneous population of multilamellar liposomes; the mean diameter of vesicles ranged between 0.1 and 0.5 μm (Fig. 1). No clusters or formation of crystals was observed for all the preparations.

Determination of Ts

The amount of non-incorporated Ts was determined by reaction of the supernatant solutions with TNBS as described by Fields (1972) and evaluation of the increase in absorbance at 420 nm on a Varian 640 Spectrophotometer. A standard working curve was prepared daily using known concentrations of Ts. The limit of detection of the assay was as low as 2 μg/ml of Ts.

The incorporation of Ts was determined based on the difference from the initial amount of drug added and was expressed as binding capacity (mg Ts ml^{-1}) (Tables 2 and 3) calculated from the mean of at least three experiments.

TABLE 1

Peak phase-transition temperature (T_m) of DMPC / DPPC liposomes at various composition

DMPC:DPPC molar ratio	T_m (°C)
Pure DMPC	25.1
10:1	28.0
7:1	24.6
5:1	25.3
2:1	30.8
1:1	34.7
0.5:1	37.7
Pure DPPC	42.0

DSC analysis

Transition temperatures (T_m) for each sample were determined on a Mettler TA 3000 calorimeter, equipped with a DSC 30 cell and a TC 10 processor.

Palmitic acid was employed to calibrate the temperature scale and ΔH. Samples were subjected to several heating and cooling cycles in the temperature range 20–80°C at a scanning rate of 2 °C/min. Enthalpy changes were calculated from the peak areas.

The T_m values measured for vesicles of different compositions are listed in Table 1.

Results and Discussion

Calorimetric analyses play a fundamental role in determining both the type and the strength of the interactions occurring between a lipid, biological or synthetic, bilayer and a 'host' molecule (Bach, 1984). The nature of the latter affects the thermotropic behaviour of the phospholipids, i.e., it gives rise to a shift in the temperature of the midpoint of transition, T_m, according to the type of interaction, viz., electrostatic or non-polar, that occurs in the case of hydrophilic and lipophilic compounds, respectively.

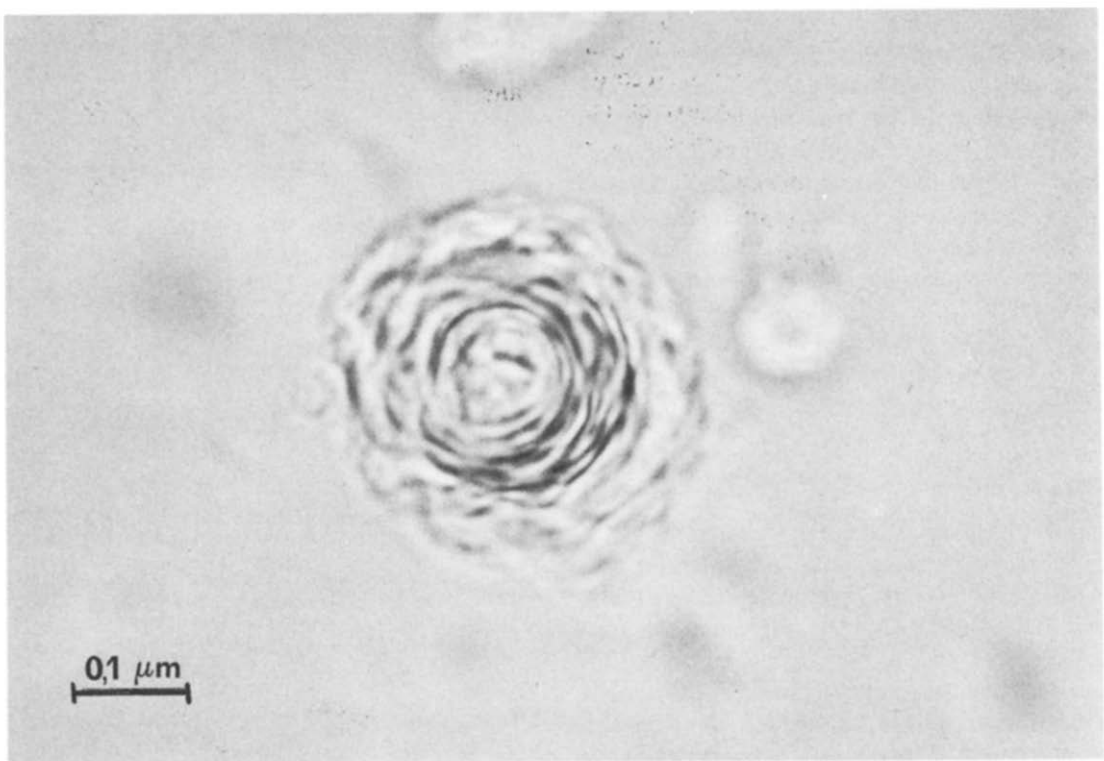

Fig. 1. Microphotography of a Ts-loaded DPPC liposome.

Moreover, modifications in the enthalpy of transition, ΔH, demonstrate the ability of extraneous molecules to penetrate into the hydrocarbon chains of phospholipid bilayers.

Therefore, we submitted liposome samples of different compositions to DSC scanning, either free or loaded with Ts.

As expected (Table 1), T_m decreased with increasing concentration of DMPC in the mixture, reaching a minimum at a DMPC/DPPC molar ratio of 7.0, and then slowly increased again.

These data are consistent with the observation that phospholipids containing shorter chain fatty acids have lower transition temperatures with respect to long-chain fatty acids containing phospholipids.

DSC analyses of vesicles containing Ts, as reported in Tables 2 and 3, demonstrated that the drug does not undergo any substantial interaction with phospholipid vesicles: the phase transition temperatures and ΔH showed no significant change with respect to pure phospholipid. Ts might in fact be too weakly lipophilic to fit into their hydrocarbon matrix, as well as having a molecular size too great to permit hydrogen bonding with the lipid polar head groups. According to the findings of Papahadjopoulos et al. (1975), this could indicate that only surface electrostatic interactions occur between lipid and polypeptide molecules, without penetration of the latter into phospholipid bilayers taking place.

Furthermore, as regards the presence of Chol in the bilayers, changes in neither T_c nor ΔH were observed for the tested mixtures, indicating that the steroid has no effect on the kind of interaction occurring between the drug and the lipids.

Drug incorporation

As for any other water-soluble compound, due to its low affinity toward lipid chains in liposomal bilayers, Ts resides within the interlamellar aqueous spaces.

The rate of encapsulation from the vesicles is then correlated to the volume of the aqueous phase entrapped during their formation. A measure of the latter can be obtained from a representative parameter, the encapsulation capacity (EC), which corresponds to the fraction of aqueous space encapsulated in the vesicles per mmol of lipid (Benita et al., 1984). EC values for the prepared formulations are reported in Tables 2 and 3.

TABLE 2

Incorporation of thymostimulin by DMPC / DPPC liposomes

Lipid composition (mol/mol)	Ts incorporated [a] ($\mu g\ ml^{-1}$)	EC (ml $mmol^{-1}$)	% Drug content
DMPC	413 ± 17	11.50	0.82
DMPC/DPPC (10:1)	120 ± 16	3.35	0.24
DMPC/DPPC (7:1)	213 ± 47	5.97	0.42
DMPC/DPPC (5:1)	205 ± 11	5.78	0.41
DMPC/DPPC (2:1)	230 ± 51	6.57	0.46
DMPC/DPPC (1:1)	420 ± 54	12.16	0.83
DMPC/DPPC (0.5:1)	280 ± 40	8.21	0.56
DPPC	280 ± 34	8.42	0.55

[a] Mean ± S.D. of three determinations.

Furthermore, the amount of Ts entrapped by liposomes may be expressed as the drug content (DC), according to:

$$DC = \frac{W_{Ts}}{W_{Ts} + W_l} \times 100$$

TABLE 3

Incorporation of Ts by cholesterol containing DMPC or DPPC liposomes

Lipid composition (mol/mol)	Ts incorporated [a] ($\mu g\ ml^{-1}$)	EC (ml $mmol^{-1}$)	% Drug content
DMPC	413 ± 17	11.50	0.82
DMPC/Chol 2:1	451 ± 40	10.70	0.89
DMPC/Chol 1:1	287 ± 18	6.20	0.57
DPPC	280 ± 34	8.42	0.55
DPPC/Chol 2:1	300 ± 8	7.60	0.60
DPPC/Chol 1:1	214 ± 14	4.87	0.43

[a] Mean ± S.D. of three determinations.

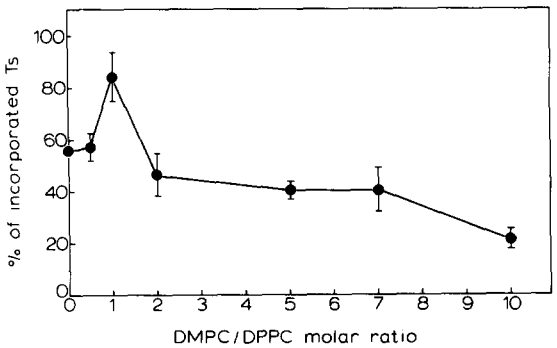

Fig. 2. Percent incorporation of thymostimulin by liposomes at different DMPC/DPPC molar ratios. Mean ± S.D. of three experiments.

where W_{Ts} represents the amount of drug found in the vesicles and W_l is the quantity of lipids which form the membrane bilayers.

As demonstrated in Fig. 2 and Table 2, DMPC liposomes permit more extensive incorporation of the immunomodulatory extract than pure DPPC vesicles.

On addition to DMPC of a small amount of DPPC (10:1 molar ratio), EC fell sharply; in contrast, reduction of the molar ratio of the above phospholipids led to an increase in the extent of drug incorporation, reaching a maximum (EC = 12.16 ml $mmol^{-1}$) in liposomes composed of equimolar amounts of DMPC and DPPC, which were found to retain more than 80% of the initial dose of Ts (Fig. 2). Finally, the presence of DPPC in excess over DMPC (2:1) further reduced drug encapsulation efficiency toward that of pure DPPC.

The role of cholesterol in the capacity of DMPC and DPPC vesicles to incorporate drug is evident from the data presented in Table 3. Chol has previously been demonstrated to modify the fluidity of lipid membranes, thereby influencing both the extent of incorporation and the degree of retention of drugs by vesicles (Taylor et al., 1990). Chol may act as a 'spacer' in phospholipid bilayers: it lowers the cohesive forces between the hydrocarbon chains, prevents their crystallization and increases the fluidity of the bilayer in the gel state (Bach, 1984). Moreover, the closer 'packing' of phospholipid molecules also results in the greater resistance of liposomes to mechanical strain.

In our hands, the introduction of Chol in increasing molar ratios yielded a pattern of Ts incorporation similar to that of both DMPC and DPPC formulations (Table 3).

In fact, at a phospholipid/Chol molar ratio of 2:1, a slight increase (about 10%) in drug entrapment was observed with respect to pure phospholipid vesicles. Simultaneously, an equivalent reduction in EC was determined. Such behaviour could indicate that Chol did not result in greater aqueous volumes in the liposomes, and thus that the small increase in drug content could be ascribed to the greater physical stability of vesicles during steps involving mechanical stress, e.g., centrifugation.

This protective effect, however, disappears at higher concentrations of Chol (1:1 molar ratio vs phospholipid), since a drastic decrease in the entrapped aqueous space (above 40%) is associated with a reduction in the degree of drug incorporation that is almost equally significant (Table 3). It appears that, at this dose, Chol exerts such a strongly fluidizing effect on liposomal membranes that only a small proportion of the added drug is retained.

Liposome stability studies

In our experiments, the final concentration of entrapped Ts has been taken to be the difference between the initial drug dosage and the amount released during the washing step.

The stability of Ts-loaded liposomes would then be of crucial importance, since the use of liposomes as pharmaceutical devices would necessitate, in addition to a high trapping efficiency, that they are also both physically and chemically resistant for extended periods of time.

Furthermore, studies on the ability of liposomes to retain the dose of drug initially incorporated can help in establishing whether the observed effects of Chol are due to the membranes having too great a resistance to Ts inflow or if they result from an increase in permeability to leakage of the entrapped drug.

If, as suggested, the fluidity of the membrane phase controls the uptake and the relative rate of

TABLE 4

Effect of temperature of preparation on Ts incorporation into liposomes

Composition (molar ratio)	% of incorporated Ts	
	4°C	25°C
DMPC	82.5	69.6
DPPC	56.0	54.8
DMPC/DPPC (1:1)	83.7	67.5
DMPC/Chol (2:1)	90.2	75.5
DMPC/Chol (1:1)	57.3	53.2
DPPC/Chol (2:1)	60.0	56.0
DPPC/Chol (1:1)	42.8	37.9

Mean of three determinations.

release of the drug, it could be possible to increase the stability of loaded liposomes by storage at lower temperatures.

In order to verify this assumption, we measured the amount of Ts incorporated during washing of liposomes at 4°C, as in the normal procedure, or at 25°C. The results obtained in such experiments are listed in Table 4. Liposomes prepared from pure DPPC remain unaffected by the temperature of the washing step. In contrast, incorporation into DMPC liposomes or mixtures with DPPC or Chol is strongly dependent upon washing temperature, such that, when carried out at 25°C, almost 70% of the originally entrapped material was lost.

Moreover, the time-dependent release course of Ts from liposomes was also assessed on the basis of drug content at various times (15, 30 and 60 min) after the preparation of liposomes. The results are reported in Figs 3 and 4 for DMPC and DPPC formulations, in the absence and presence, respectively, of Chol in the fractions. DPPC liposomes were found to be capable of retaining the initial amount of material entrapped throughout the course of the experiment. DMPC alone or in an equimolar mixture with DPPC, instead, slowly released Ts with a half-time of 45 and 50 min, respectively (Fig. 3).

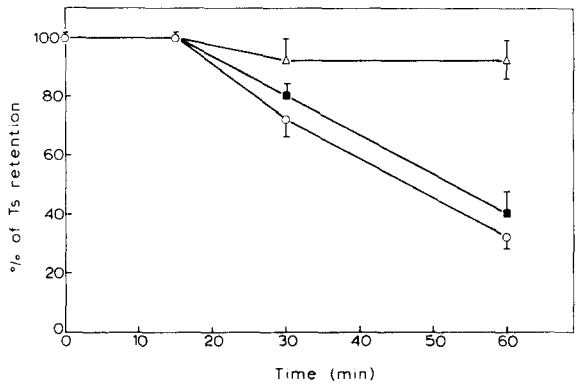

Fig. 3. Time-release course of thymostimulin from DMPC (○) DPPC (△) or DMPC/DPPC (1:1, mol/mol) (■) liposomes. Mean ± S.D. of three determinations.

Inclusion of Chol in phospholipids further enhances membrane permeability: a 2:1 DMPC/Chol mixture displays a reduction of 35 and 60% in Ts encapsulation after 15 and 30 min, respectively. A higher proportion of Chol (DMPC/Chol 1:1) has an initially protective effect against drug leakage, which however becomes significant in its influence after 30 min (Fig. 4). This result is not surprising, in that it confirms that a lower phase transition temperature of vesicles is often associated with greater bilayer fluidity and, hence, per-

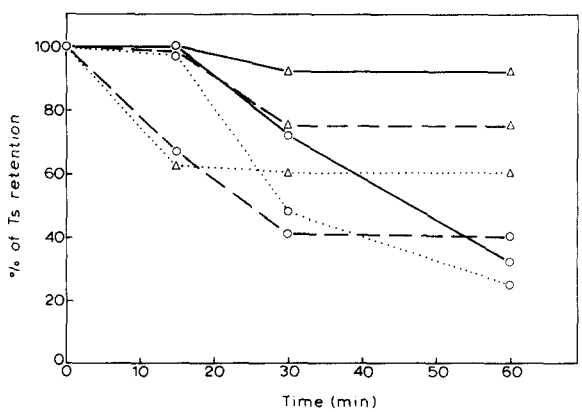

Fig. 4. Time-release course of thymostimulin from liposomes in the presence of cholesterol. (○) DMPC liposomes; (△) DPPC liposomes. (———) Pure phospholipid; (· · · · · ·) phospholipid/Chol molar ratio 2:1; (— — —) phospholipid/Chol molar ratio 1:1. Mean of three determinations; S.D. bars have been omitted for clarity.

meability at temperatures above T_c (Knight and Shaw, 1979; Ryman and Tyrrell, 1979).

With respect to the higher stability shown by DPPC liposomes, at both doses, the presence of Chol induces the loss of drug, which is significant during the first 15 min, being about 40% for the 1:1 DPPC/Chol mixture; however, incorporation of Ts remains constant during the following period of observation.

Conclusions

The present findings show that liposomes can reliably be used as potential therapeutic devices if properly prepared. We have in fact observed that the fluidity of multilamellar vesicles affects the incorporation as well as the rate of retention of the high molecular weight polypeptide, thymostimulin, which is known to modulate macrophage stimulation and cell proliferation (Wigzell and Aiuti, 1980).

In our experiments, the highest degree of incorporation was determined for a mixture of DMPC and DPPC (1:1, mol/mol); pure DMPC liposomes were found to be able to entrap more Ts than DPPC, confirming our observations that the fluidity of the membrane phase plays a key role in the process of uptake. Further, at 25°C the release of drug from DMPC vesicles is very rapid and almost 60% of the entrapped Ts leaks out within 1 h. In DPPC liposomes, in contrast, no significant loss of drug was observed under the same conditions: this might indicate that less fluid liposomes could be more reliable, as drug carriers, in those cases where such preparations are stored for prolonged time periods before use.

On considering the validity of immunoactive drugs in oncological therapy (Wigzell and Aiuti, 1980; Nagel et al., 1988), the results of this study, supported by our preliminary evaluation of the in vitro efficacy of Ts-loaded liposomes against tumoral cell proliferation (Panico et al., 1991), should be very interesting in the application of these formulations to in vivo experimental models of tumoral diseases. This work is in fact part of a wider national research program, in which the advantages of a therapeutic strategy, involving the combination of immunomodulating and antineoplastic agents for the cure of solid tumors, are currently being investigated.

Acknowledgements

This work was supported by Italian MURST 60 and 40% grants.

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