*Biochimica et Biophysica Acta*, 690 (1982) 224–230
Elsevier Biomedical Press

BBA 71331

# LIPOSOME-MEDIATED DELIVERY OF RIBOSOME INACTIVATING PROTEINS TO CELLS IN VITRO

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(Received March 11th, 1982)

*Key words: Liposome; Gelonin; Diphtheria toxin; Protein delivery; (Tumor cell)*

**This study describes the liposome-mediated delivery of toxins to a variety of cells in vitro. Gelonin, a potent inhibitor of protein synthesis from *Gelonium multiflorum*, was delivered to the cytoplasm of TLX5 lymphoma cells most effectively by phosphatidylserine vesicles. These liposomes were also capable of inhibiting protein synthesis in XC (transformed rat fibroblasts) and phytohaemagglutinin-stimulated CBA mouse lymphocytes. Phosphatidylcholine liposomes had no capacity to deliver their contents to the cytoplasm, but the addition of cholesterol to the vesicle membrane resulted in an increased capacity. Delivery events were enhanced further by the addition of mixed bovine brain gangliosides to the membrane in the ratio 5:5:1 phosphatidylcholine/cholesterol/gangliosides. The addition of cholesterol to phosphatidylserine vesicles failed to increase the inhibitory effects of the gelonin liposomes. The A chain of diphtheria toxin encapsulated in phosphatidylserine liposomes had no inhibitory effect on the level of protein synthesis in TLX5 or Daudi cells.**

## Introduction

Liposomes have been extensively studied as possible vectors for intracytoplasmic delivery of drugs and macromolecules [1]. Biologically active molecules which produce effects when only a few enter the cytoplasm have been most effectively used with liposomes. In addition, molecules which are unable to penetrate cells are preferable because liposomes leak a large amount of their contents on contact with cells [2] and plasma components [3]. If capable, leaked material will enter cells independently, and make determination of liposome-mediated delivery impossible.

Wilson et al. [4] reported the enhanced delivery of encapsulated poliovirus RNA to HeLa cells. Whereas free polio RNA was non-infectious, encapsulated RNA had an optimal infectivity of $(1-2) \cdot 10^4$ pfu/ng. Optimal infectivity was achieved with 1–2 molecules per liposome, and, at this RNA/liposome ratio, every cell could be infected. Since a single molecule of polio RNA will produce a lytic infection of a cell, the authors concluded that the liposomes delivered their contents intracytoplasmically following fusion of the liposome with the plasma membrane. Fraley et al. [5] demonstrated that the infectivity of liposome-encapsulated SV40 DNA was 10-times higher when the liposomes were prepared from phosphatidylserine than when prepared from phosphatidylcholine. Treatment of the cells with inhibitors of oxidative phosphorylation and glycolysis to block energy-dependent uptake failed to reduce infectivity, and they concluded that phosphatidylserine liposomes delivered their contents by fusion with the plasma membrane.

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0005-2736/82/0000–0000/$02.75 

Toxins such as diphtheria toxin, abrin and ricin contain A chains which inhibit protein synthesis enzymically when only one molecule is delivered to the cytoplasm of a sensitive cell [6]. The A chain of diphtheria toxin is 1000–10000 times less toxic than the intact toxin [7] since its entry to the cytoplasm is normally mediated by the B chain. Gelonin [8], a protein isolated from the seeds of *Gelonium multiflorum*, is a potent inhibitor of protein synthesis in a cell-free lysate, and may be regarded as analagous to the A chain of a toxin. Encapsulated A chains should be toxic, if prior estimated [4,5] of the frequency with which phosphatidylserine liposomes fuse with cells are correct. The present study describes our work on the toxicity of encapsulated gelonin and diphtheria toxin A chain, and presents our conclusions of the mechanism of delivery.

## Materials and Methods

*Reagents*

Phosphatidylserine and phosphatidylcholine were obtained from Lipid Products (Redhill) and mixed bovine brain gangliosides from Sigma (London). Cholesterol was purchased from Fluka (Glossop), recrystallized three times from methanol and stored in chloroform under argon. Sodium [$^{125}$I]iodide, [$^{3}$H]leucine and sodium [$^{32}$P]phosphate were obtained from Amersham International. Iodogen was purchased from Pierce (Cheshire) and Dextran T-40 from Pharmacia (G.B.), Ltd. Gelonin and the A chain of diphtheria toxin were gifts from Dr. P.E. Thorpe. RPMI 1640, foetal and newborn calf serum were obtained from Gibco Europe Ltd. All other chemicals used were reagent grade or better.

*Preparation of vesicles*

The vesicles used were unilamellar reverse phase evaporation vesicles, 0.1 $\mu$m in diameter, prepared by the method of Szoka and Papahadjopoulos [9]. 300 $\mu$l Hepes/NaCl/EDTA buffer pH 7.4 with or without $^{125}$I-labelled-toxin fragment was added to 10 $\mu$mol phospholipid in 1 ml diethyl ether. The mixture was sonicated under $N_2$ for 5 min and the solvent was removed by rotary evaporation under reduced pressure. Liposomes were extruded through a series of polycarbonate membranes of pore size 0.4–0.1 $\mu$m (Biorad U.K., Ltd) using an ultrafiltration cell (Millipore U.K., Ltd) with $N_2$ at pressures of $(4–7) \cdot 10^{-5}$ N $\cdot$ m$^{-2}$ in order to produce vesicles of uniform size [10]. Non-entrapped solute was separated from the vesicles by flotation in discontinuous dextran gradient with centrifugation at $50000 \times g$ for 20 min [11].

The percentage entrapped protein was calculated by the addition of $^{125}$I-labelled material with subsequent gamma counting of the pure vesicle fraction. Phospholipid yield was determined from the phosphorus content [12].

*Tissue culture and toxicity testing*

TLX5, a CBA/Ca mouse T lymphoma, and Daudi, a human lymphoblastoid cell line, were routinely maintained in RPMI 1640 medium in 5% $CO_2$ with 10% fetal calf serum. XC is a Rous sarcoma virus transformed rat fibroblast grown in minimal essential medium at 10% fetal calf serum and 10% $CO_2$. Cell suspensions from axillary and mesenteric lymph nodes of CBA/Ca mice were obtained by gently squeezing the tissue through stainless steel sieves into cold phosphate-buffered saline. Clumps were allowed to settle and cells in the supernatant were washed three times in buffer.

All suspension cultures were plated out in 96 well micro-test plates (Nunclon). Cell concentrations were $2 \cdot 10^5$ per well for Daudi and TLX5 and $1 \cdot 10^6$ per well for CBA lymphocytes. Cells were exposed to vesicles for 1 h at 37°C and 5% $CO_2$ and then washed three times in buffer and incubated in RPMI 1640 containing 5% heat inactivated fetal calf serum for various times before pulsing with [$^{3}$H]leucine. After 6 h cells were harvested onto glass fibre discs in cold 5% trichloroacetic acid with a final wash of 95% ethanol. Discs were then counted for [$^{3}$H]leucine incorporation in a liquid scintillation counter.

For experiments with the XC rat fibroblasts, suspensions of 300 cells in 2 ml of Eagles medium containing 10% newborn calf serum were incubated in 30 mm plastic Petri dishes for 7 h at 37°C. The cells were washed once with 2 ml of cold balanced salt solution and 0.5 ml of liposomes, A chain or buffer was added. The dishes were shaken for 20 min at 4°C, warmed to 37°C and incubated for a further 20 min. The plates were washed once and incubated in fresh medium

until colonies appeared at 4 days. The cells were finally fixed with methanol and stained with Giemsa before counting the colonies.

*Rabbit reticulocyte assay*

Reticulocyte lysate was prepared by the method of Clemens et al. [13]. Freshly thawed lysates were assayed for their ability to incorporate [$^3$H]leucine (65 Ci/mmol) into protein. 50 $\mu$l lysate was mixed with 4 $\mu$l [$^3$H]leucine, 51 $\mu$l phosphate-buffered saline, 13 $\mu$l A chain (or buffer for controls), and 12 $\mu$l of RPMI 1640 to which was added: 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 60 $\mu g \cdot ml^{-1}$ creatinephosphate kinase, 0.01 mM cysteine, 0.05 mM alanine, 100 mM ammonium acetate and 2 mM magnesium acetate.

The lysate mixture was incubated at 30°C for 10 min after which amino acid incorporation into protein was assayed in 10-$\mu$l aliquots of the incubation mixture on Whatman 3 MM filter discs using the procedure of Mans and Novelli [14] with modifications.

*Calculation of capture*

The number of protein molecules captured per vesicle is calculated from the expression:

$$\frac{(\mu g \text{ protein}^*/\mu mol \text{ lipid}) \cdot (\text{mol protein}/\mu g)}{(\text{liposomes}/\mu mol \text{ lipid})}$$

In order to calculate the number of liposomes/$\mu$mol lipids certain assumptions are made. These are, that the surface area of a lipid molecule is 0.7/nm$^2$ and that the thickness of a lipid bilayer is 4.5 nm. For a 0.1 $\mu$m reverse phase evaporation vesicle there are $7.341 \cdot 10^{12}$ vesicles per $\mu$mol lipid, and the captured volume per liposome is $3.95 \cdot 10^{-13}$ $\mu$l. This gives a figure of 2.896 $\mu$l of captured volume per $\mu$mol of lipid.

## Results

*Co-encapsulation with [$^{32}$P] phosphate*

$^{125}$I-labelled gelonin and [$^{32}$P]phosphate, an aqueous phase marker, were simultaneously encapsulated in phosphatidylserine liposomes to determine if gelonin association with vesicles was due to aqueous capture. For [$^{32}$P]phosphate, the capture was 5.34 litre $\cdot$ mol$^{-1}$, while for $^{125}$I-labelled gelonin it was 7.05 litre $\cdot$ mol$^{-1}$. The ratio of entrapment of $^{32}$P/$^{125}$I was 1:1.3 indicating that both molecules were trapped to a similar extent. It therefore seems that the extent of gelonin binding to the liposome membrane is small.

*Activity of gelonin and diphtheria toxin A chain after encapsulation in liposomes and after iodination*

The activity of gelonin and diphtheria toxin A chain after iodination was assayed in the rabbit reticulocyte cell free system (data not shown). Both were found to be fully active. The encapsulated material was released from the vesicles by incubation with 0.05% Triton-X 100 for 30 min at room temperature. The degree of inhibition of protein synthesis by the released material was compared to that of untreated gelonin or diphtheria toxin A chain on a dose response curve. Released gelonin was found to retain 90% of the control activity and the A chain of diphtheria toxin, 100%. Lysed liposomes containing buffer had no inhibitory effect on the levels of protein synthesis. These results indicate that the process of encapsulation seems not to impair the activity of the inhibitor and that the iodinated material included in the entrapment mixture as a tracer molecule is fully active.

*Toxicity of gelonin in phosphatidylserine vesicles. Time-course*

TLX5 cells were exposed to 200 nmol lipid, 200 nmol lipid, containing $2.75 \cdot 10^{-11}$ mol gelonin, 200 nmol lipid mixed with $2.75 \cdot 10^{-11}$ mol gelonin or $2.75 \cdot 10^{-11}$ mol gelonin. The lipid + free gelonin control was included in case of a synergistic effect induced by the negative charge of the phosphatidylserine vesicles and the positive charge of gelonin. Assuming $7.341 \cdot 10^{12}$ vesicles per $\mu$mol and a molecular weight for gelonin of 30500 this corresponds to a capture of 11 molecules per vesicle. Triplicates were pulsed with [$^3$H]leucine at various times after exposure to liposomes and harvested at various times after pulsing. No inhibition of leucine incorporation was observed for any of the treatments when pulsed less than 21 h after incubation. Consequently, a 21 h incubation before pulse was used in all subsequent experiments.

* Experimentally determined.

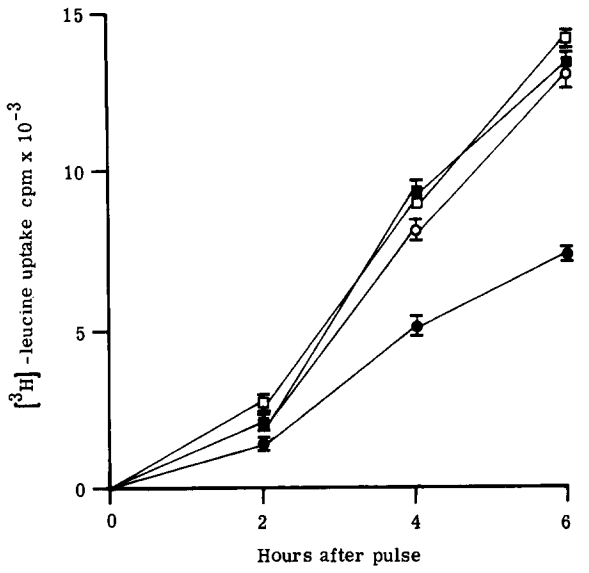


Fig. 1. The [$^3$H]leucine incorporation of triplicate cultures of TLX5 cells given four different treatments. All groups were pulsed 21 h after treatment and harvested 2, 4 and 6 h after the pulse. ●———●, 200 $\mu$mol phosphatidylserine liposomes containing $2.75 \cdot 10^{-11}$ mol gelonin; ○———○, 200 $\mu$mol phosphatidylserine liposomes containing buffer; ■———■, 200 $\mu$mol phosphatidylserine liposomes + $2.75 \cdot 10^{-11}$ mol gelonin; □———□, $2.75 \cdot 10^{-11}$ mol gelonin.

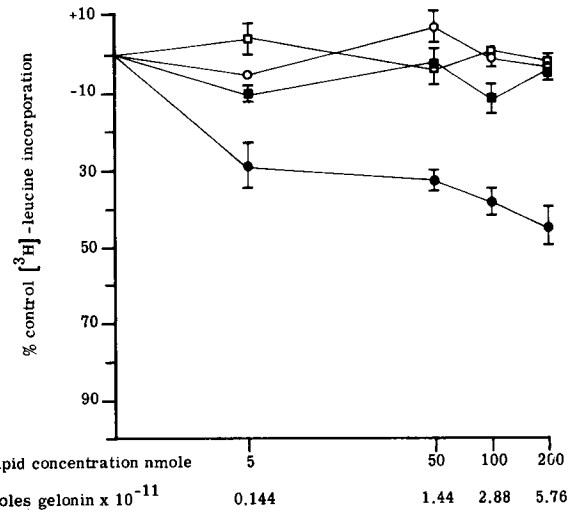


Fig. 2. Effect of phosphatidylserine liposomes each containing 11 molecules of gelonin, on TLX5 lymphoma cells. Triplicate cultures of $2 \cdot 10^5$ cells in microtitre plates were treated with: ●———●, liposomes containing gelonin; ○———○, liposomes containing buffer; ■———■, liposomes + free gelonin; □———□, free gelonin.

Fig. 1 shows the timecourse of leucine incorporation for samples pulsed at 21 h and harvested at various times after pulse. The incorporation of leucine proceeds linearly for up to 6 h and in all later experiments at 6 h pulse was used. Inhibition of leucine incorporation was observed for cells treated with encapsulated gelonin, and the inhibition amounted to 45% of control levels for samples harvested at 6 h. Treatment with lipid alone, gelonin alone or lipid mixed with gelonin had no effect on cell growth, indicating that the protein must be encapsulated to have a toxic effect. To correlate protein synthesis inhibition with cytotoxicity some cell samples were stained with Trypan blue and assessed for viability, the inability to exclude the dye being indicative of cell death. In all cases toxicity and protein synthesis inhibition were closely related.

Fig. 2 shows the toxicity of gelonin encapsulated in phosphatidyl serine vesicles when between 5 and 200 nmol of lipid, and $1.44 \cdot 10^{-12}$ mol gelonin and $5.76 \cdot 10^{-11}$ mol gelonin is added. Encapsulated gelonin exhibits rapidly increasing toxicity between 0 and 10 nmol lipid and thereafter levels off at 45% inhibition of protein synthesis. As before, gelonin and lipid alone or mixed show no toxicity over the range of addition.

The above experiments were all performed with vesicles calculated to contain 11 molecules of gelonin per vesicle. If this ratio was increased to 54 molecules per vesicle the results shown in Fig. 3 were obtained. Toxicity is again only observed for encapsulated gelonin and is maximally 84% inhibition of protein synthesis. Preparations containing 4 molecules per vesicle were found to be non-toxic.

*The toxicity of encapsulated diphtheria toxin A chain*

The A chain of diphtheria toxin was encapsulated in phosphatidylserine liposomes at 57 molecules per vesicle and tested against TLX5 lymphoma cells. No toxicity was observed for the trapped A chain (Fig. 4). This result is in contrast to similar experiments performed with liposomes containing gelonin (Fig. 3). In a cell free assay, the a chain of diphtheria toxin is a better inhibitor of protein synthesis in the presence of added $NAD^+$ which is a co-factor required for the ADP ribosylation of elongation factor 2 (EF2). It seemed possi-

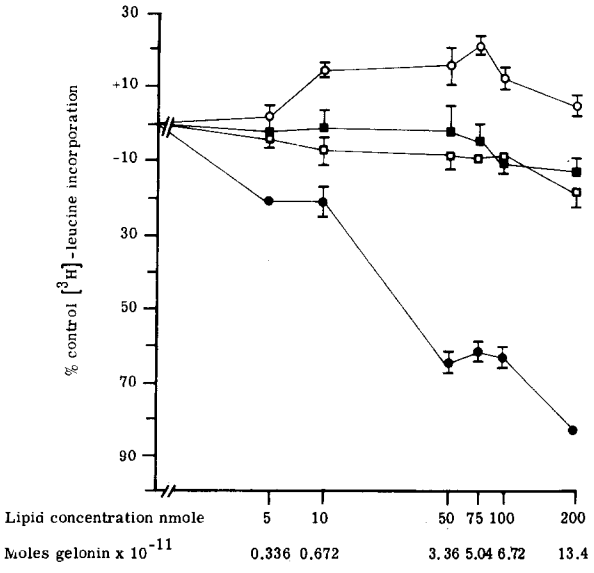


Fig. 3. Effect of phosphatidylserine liposomes each containing 54 molecules of gelonin on TLX5 lymphoma cells (methods as in Fig. 2). ●——●, Liposomes containing gelonin; ○——○, liposomes containing buffer; ■——■, liposomes + free gelonin; □——□, free gelonin.

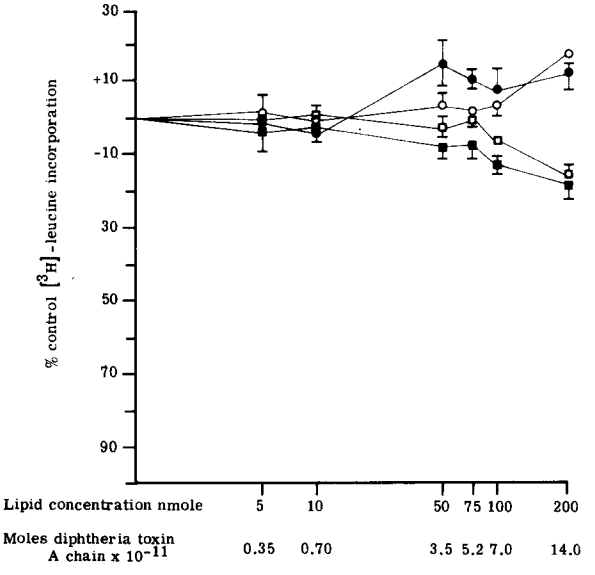


Fig. 4. Effect of diphtheria toxin A chain entrapped in phosphatidylserine liposomes on TLX5 cells. Experimental conditions as in Fig. 2. ●——●, Liposomes containing diphtheria toxin A chain; ○——○, liposomes containing buffer; ■——■, liposomes containing buffer + free diphtheria toxin A chain; □——□, free diphtheria toxin A chain.

ble that additional intracellular $NAD^+$ might be required to potentiate the activity of diphtheria toxin A chain. When co-encapsulated with $NAD^+$ 100 molecules of diphtheria toxin A chain per liposome did not inhibit protein synthesis in TLX5 lymphoma cells, but caused a small increase in [$^3$H]leucine incorporation. Daudi, a human lymphoblastoid cell line known to be sensitive to whole diphtheria toxin was also found to be insensitive to the A chain of diphtheria toxin entrapped in phosphatidylserine liposomes.

*Lipid composition*

Studies by Fraley et al. [5] on the infectivity of encapsulated SV40 DNA have shown negatively charged liposomes to be the most effective in delivering their contents and have demonstrated that the inclusion of cholesterol in the vesicle membrane can promote delivery by reducing serum and cell-mediated leakage of vesicle contents. Fig. 5 shows the toxicity of gelonin encapsulated at 32

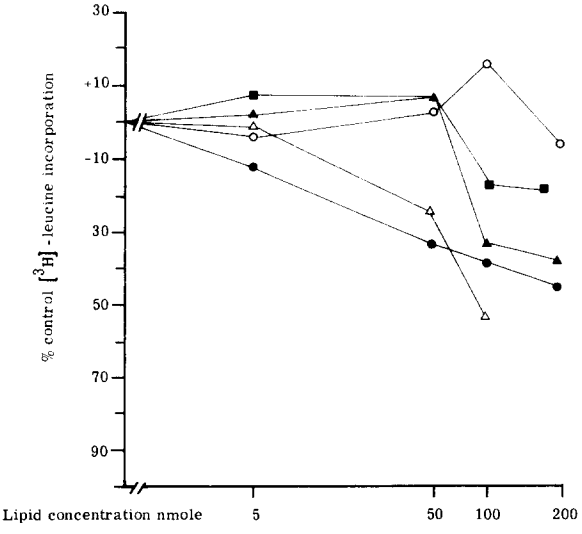


Fig. 5. Effect of different lipid compositions on gelonin delivery to TLX5 cells. Experimental conditions as in Fig. 2. ●——●, Phosphatidylserine liposomes each containing 11 molecules of gelonin; ▲——▲, phosphatidylserine/cholesterol 1 : 1 liposomes each containing 32 molecules gelonin; ■——■, phosphatidylcholine/cholesterol 1 : 1 liposomes each containing 7 molecules gelonin; ○——○, phosphatidylcholine liposomes each containing 11 molecules of gelonin; △——△, phosphatidylcholine/cholesterol/ganglioside 5 : 5 : 1 each containing 1 molecule of gelonin, All standard deviations are below 10%.

molecules per vesicle in phosphatidylserine/cholesterol 1:1 vesicles. No protein synthesis inhibition occurred at additions less than 75 nmol lipid and reached only 37% at 200 nmol added. The addition of cholesterol has therefore decreased the ability of gelonin encapsulated in liposomes to inhibit cellular protein synthesis.

Fig. 5 also shows the toxicity of gelonin encapsulated in phosphatidylcholine or phosphatidylcholine/cholesterol lipomes. No toxicity was observed for phosphatidylcholine encapsulated gelonin at 11 molecules per vesicle when up to 200 nmol lipid were added per well. Gelonin encapsulated in phosphatidylcholine/cholesterol 1:1 at 7 molecules per vesicle showed some toxicity, maximally 16% inhibition when 50–200 nmol were added per well. Hence the inclusion of cholesterol renders phosphatidylcholine encapsulated gelonin marginally toxic. Gelonin encapsulated in vesicles prepared from 5:5:1 phosphatidylcholine/cholesterol/gangliosides also exhibits marked toxicity which is maximally 52% inhibition at 100 nmol lipid added.

*Toxicity of encapsulated gelonin for other cell types*

Gelonin encapsulated in phosphatidylserine liposomes was also tested for toxicity against phytohaemagglutinin stimulated CBA mouse lymph node cells. The result is similar to that for TLX5 and the inhibition of protein synthesis was 50% when 200 nmol lipid containing 11 molecules of gelonin per vesicle was incubated with the cells.

The effect of phosphatidylserine-encapsulated gelonin on the plating efficiency of rat fibroblasts has also been examined. Treatment of the cells with 100 nmol lipid (11 molecules of gelonin per vesicle) reduced colony formation by 43%. Treatment with gelonin, buffer containing vesicles or gelonin mixed with vesicles had no effect on colony formation.

## Discussion

The results demonstrated the enhancement of cytotoxicity of gelonin when it is encapsulated in liposomes. In the range of concentrations used, gelonin is toxic neither free nor mixed with buffer loaded liposomes. The toxicity is therefore due to the association of the gelonin with the vesicles. This association appears to be due to encapsulation since gelonin does not bind to preformed vesicles and is associated upon encapsulation to the same extent as [$^{32}$P]phosphate, a marker for the internal aqueous space of the liposomes. Gelonin is fully active as a protein synthesis inhibitor when released from liposomes with detergent.

The toxicity of encapsulated gelonin varies with the lipid composition of the vesicle. The greatest inhibition of protein synthesis is observed with phosphatidylserine or phosphatidylserine/cholesterol vesicles. Of these two compositions phosphatidylserine is the most effective at low lipid concentrations while comparable preparations are of equal potency at higher lipid concentrations. Gelonin is non-toxic when encapsulated in phosphatidylcholine liposomes but marginally toxic in phosphatidylcholine/cholesterol liposomes. These results agree with those of Fraley et al. [5] for the delivery of SV40 DNA in all but one respect. Fraley et al. [5] found phosphatidylserine liposomes to be 10-times more effective in delivering SV40 DNA than phosphatidylcholine liposomes, but found that cholesterol improved the ability of all liposomes to deliver their DNA content. The cholesterol effect was primarily on the leakage of liposome contents, bovine brain phosphatidylserine vesicles being least affected since they were relatively resistant to leakage.

The results also demonstrate the complete non-toxicity of diphtheria toxin A chain when encapsulated in phosphatidylserine liposomes. The A chain of diphtheria toxin has been shown to be active after encapsulation and has been shown to be inactive on two cell lines which were sensitive to phosphatidylserine encapsulated gelonin. This result agrees with the findings of Uchida et al. [15] who, in several studies, have never observed toxicity of diphtheria toxin A chain for normal cells when encapsulated in pure lipid liposomes.

The difference between encapsulated gelonin and diphtheria toxin A chain is very striking and leads to consideration of the mechanism of delivery and how it might affect the toxicity. If the delivery of liposome contents were a result of fusion with the plasma membrane, both gelonin and the A chain of diphtheria toxin would be

injected into the cytoplasm and would exert their effects equally. Moreover, it would be difficult to explain why preparations containing 54 molecules per liposome are more toxic than those with only 11 molecules per liposome. Yamaizumi et al. [6] have demonstrated that only one diphtheria toxin A chain molecule may be needed completely to inhibit protein synthesis in a cell. Gelonin has been shown in cell free systems to be as effective as the A chain of diphtheria toxin in inhibiting protein synthesis. Consequently one would expect fusion with a liposome containing 56 molecules of diphtheria toxin A chain to have no lesser effect than fusion with a liposome containing 11 molecules of gelonin on protein synthesis.

If the delivery of liposome contents involved the endocytosis of liposomes by the cell, then the entry of the fragment into the cytoplasm might involve processing in the lysosomal or pinosomal compartment. The latent period of 21 h seen in the time course experiment supports this notion. Such processing might result in the successful delivery of gelonin while not permitting delivery of the A chain of diphtheria toxin. Processing of gelonin could involve a lectin-like receptor for the glycosylated portion of the molecule and might rely on partial proteolytic cleavage or the low pH of the lysosomal compartment. It is clear that by no means all of the gelonin is successfully delivered since in most circumstances only 50% inhibition of protein synthesis is achieved. Moreover gelonin/vesicle ratios far in excess of one are required for detectable effects. An endocytic mechanism for delivery would also explain the effects of cholesterol on the toxicity of gelonin in phosphatidylserine liposomes. Increased stability of vesicles might reduce the rate of lysosomal-cytoplasmic transfer of gelonin which in a short-term assay may reduce the effects observed.

Whilst care should be exercised in comparing these results to those of others it is interesting to speculate on the possible role of endocytosis in the delivery of liposome contents to cells. It is surprising that a cell such as a T lymphoma which would be expected to be poorly endocytic, should take up sufficient material to exhibit toxicity. While the mechanism of delivery is clearly not fusion, qualitative differences relating to lipid type are very similar to those in other studies. This leads to the suggestion that endocytosis may indeed be a major reason for the effectiveness of phosphatidylserine liposomes in delivering their contents to cells. This suggestion is confirmed by recent observations on the toxicity of γ-aspartyl methotrexate which is more toxic to L929 fibroblasts when encapsulated in phosphatidylserine/cholesterol liposomes than when encapsulated in phosphatidylcholine/cholesterol liposomes (unpublished data).

## Acknowledgments

We thank Dr. D.C. Edwards, Dr. D. Papahadjopoulos and Professor A.J.S. Davies for useful discussion, and Miss M. Butt for secretarial assistance. This work was supported by a joint grant to the Institute of Cancer Research from the Cancer Research Campaign and the Medical Research Council.

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