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LIPOSOMES AS A MEANS TO INTRODUCE FRAGMENT A OF DIPHTHERIA TOXIN INTO CELLS

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The incorporation of fragment A of diphtheria toxin into liposomes is described. The intracellular delivery of the entrapped toxin, as evidenced by the inhibition of protein synthesis by a human lymphoblastoid cell line could be demonstrated with liposomes that contained phosphatidylethanolamine or phosphatidylserine in addition to phosphatidylcholine and cholesterol. Free fragment A, either alone or added to empty liposomes of any composition, did not affect protein synthesis, even when present in considerably higher concentrations than the liposome-entrapped form.

In studies on the potential of liposomes as carriers of anti-cancer drugs, two among the outstanding problems are: (a) the difficulty to distinguish unequivocally between cellular internalization and mere surface binding of liposomes and (b) the elimination of the possibility that the observable cytotoxic effects may result from the leakage of the drug from liposomes during contact with cells and subsequent uptake of free drug. To deal with these problems, several approaches have been used, including, fluorescent markers that are quenched while at high concentrations in liposomes [1], transport resistant cell lines [2], and inhibitors of transport [3]. It has also been shown that the encapsulation of viral DNA into liposomes can serve as a sensitive biological indicator of cell-liposome interaction [4]. We have chosen to use fragment A of diphtheria toxin as an intraliposomal marker. Diphtheria toxin is a polypeptide composed of two functionally distinct regions, A and B. B region mediates the entry of the molecule into a cell, where A region inhibits protein synthesis by catalyzing the transfer of ADP-ribose from NAD to the elongation factor 2 (EF-2). If region A is split from region B, the isolated fragment A does not enter cells (for reviews, see Refs. 5 and 6). In this report we describe the effect of liposome-entrapped fragment A on a human lymphoblastoid cell line, WiL2.

Fragment A of diphtheria toxin (Connaught Laboratories, Willowdale, Ontario, Canada) was prepared as described [7]. For the generation of liposomes by the detergent removal method [8], the following phospholipids (Avanti Biochemicals, Birmingham, AL) were used: dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), phosphatidylserine (PS), and cholesterol (Sigma). The desired lipids were suspended in a buffer of the following composition: 8.5 g NaCl and 2.38 g 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes), titrated to pH 8.0 with 1 N NaOH and the final volume

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Abbreviations: EF-2, elongation factor; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

adjusted to 1000 ml with distilled water. The concentration of the lipids was 5 μmol/250 μl of the Hepes-NaCl buffer, containing also 2.5 μmol sodium cholate and and 100 μg fragment A, if indicated. The suspension was kept for 2 h at 4°C, then sonicated for 15–30 s in a bath type sonicator (Model 350, Heat Systems-Ultrasonics) at setting 5. After sonication, the mixture was applied to a Sephadex G-50 (Pharmacia, Piscataway, NJ) column (0.5 × 25 cm) equilibrated in the Hepes-NaCl buffer and also eluted with the same. Fractions of 250 μl were collected; those with maximum opalescence (two or three fractions) were pooled, rechromatographed on a Sepharose CL-4B (Pharmacia, Piscataway, NJ) column (0.5 × 25 cm) and the elution and collection steps were repeated. The pooled fractions (two or three fractions) were used for incubation with cells, analysis of lipid phosphorus [9] and fragment A content. The amount of fragment A entrapped in liposomes was determined on the basis of a standard curve for the ADP-ribose transferase activity of known concentrations of free fragment A before and after treatment of liposomes with 0.2% Nonidet P-40 [10]. EF-2 isolated from wheat germ (Sigma) was used as the acceptor [11].

As can be seen from the typical data presented in Table I, liposomes of all compositions studied incorporated approximately the same amount of fragment A, from 431 to 647 ng/μmol P, resulting in 2.16 to 3.24% entrapment. Ribosylation activity of liposomal suspensions containing fragment A in the absence of the detergent did not exceed 15%, suggesting that most of the fragment A associated with the liposomes was, indeed, entrapped rather than bound to liposome surface. DOPC/cholesterol liposomes showed consistently 10% or less of the total activity in the absence of the detergent. Electron microscopy of negatively stained preparations [12] revealed that most of the vesicles were unilamellar. Their size varied from 43 to 81 nm with an average of 69 nm. Assuming that the surface area of a lipid molecule is 0.7 nm^2 and the thickness of the bilayer is 3.7 nm [8], an average vesicle contains $3.8 \cdot 10^4$ molecules and there are $1.6 \cdot 10^{13}$ such vesicles per μmol lipid. On the basis of these calculations and the data presented in Table I there are between 0.8 and 1.2 molecules of fragment A per average vesicle in the different preparations listed.

TABLE I

ENTRAPMENT OF FRAGMENT A OF DIPHTHERIA TOXIN INTO LIPOSOMES

Liposomal composition	Molar ratio	Fragment A entrapped [a]	% entrapment [b]
DOPC/cholesterol	2.1	636 [c]	3.18
		540	2.70
DOPC/cholesterol/ DOPE	4:2.5:1	431	2.16
		526	2.63
DOPC/cholesterol/ DOPE	1:1:1	496	2.48
		560	2.80
DOPC/cholesterol/ PS	4:2.5:1	522	2.61
		647	3.24

[a] ng/μmol lipid P.
[b] Percent of initial concentration of fragment A (20 μg/μmol lipid P) present in all lipid suspensions.
[c] Data from two separate preparations.

To measure the effect of liposome-entrapped fragment A on cellular protein synthesis, WiL2 cells, kindly provided by Dr. Salisbury, were used [13]. The cells were maintained in RPMI 1640 medium with 10% fetal calf serum (Flow Laboratories, McLean, VA). For experiments, cells were always harvested from the logarithmic growth phase, i.e., $(2–3) \cdot 10^5$ cells/ml. To determine [^{14}C]leucine incorporation, $1 \cdot 10^5$ cells in 24-well tissue culture plates were incubated at 37°C in 5% CO_2/95% air atmosphere for indicated lengths of time with 140 μl of leucine-free Eagle's minimum essential medium with 10% calf serum to which 100 μl of liposomal suspension or free fragment A in the Hepes-NaCl buffer were added, as indicated. After the preincubation, 1 μCi of [^{14}C]leucine (300 Ci/mol; Amersham, Arlington Heights, IL) was added and incubation was continued for an additional 90 min. Acid precipitable radioactivity was determined by collecting cells on Millipore filters, washing with RPMI 1640 medium without serum, precipitating and washing with 5% trichloroacetic acid (60 ml per sample). Filters were cleared with ethylene monomethyl ether and counted in Aquasol (New England Nuclear, Boston, MA).

Although all the liposomal suspensions contained approximately the same amount of entrapped fragment A, inhibition of protein

TABLE II

EFFECT OF FRAGMENT A OF DIPHTHERIA TOXIN ON PROTEIN SYNTHESIS BY WiL2 CELLS

Cells were preincubated as indicated for 1 h then 1 μCi of [^{14}C]leucine was added and incubation was continued for a further 90 min. [^{14}C]leucine incorporation was determined as described in the text.

Fragment A		[^{14}C]Leucine incorporation	
Concentration in incubation mixture (μg)	Form added	Cpm	% of control
0	N.A.	51327 ± 5379 [a] (6) [b]	100 ± 6.97
10.0	Free, no liposomes	55882 ± 5017 (3)	108.7 ± 8.98
$6.9 \cdot 10^{-2}$	Captured in DOPC/ cholesterol liposomes	60564 ± 2642 (3)	118.0 ± 4.36
$6.7 \cdot 10^{-2}$	Captured in DOPC/cholesterol/DOPE liposomes	25071 ± 2069 (3)	48.8 ± 8.36
3.0	Free, with DOPC/cholesterol/DOPE liposomes	59379 ± 4483 (3)	115.7 ± 7.54
$6.3 \cdot 10^{-2}$	Captured in DOPC/cholesterol/PS liposomes	27.515 ± 2913 (3)	53.6 ± 10.59
3.0	Free with DOPC/cholesterol/PS liposomes	49990 ± 2906 (3)	97.4 ± 5.81

[a] Standard deviation.
[b] Number of determinations.

synthesis could only be demonstrated if the liposomes contained other lipids in addition to DOPC and cholesterol (Table II). In the presence of 69 ng of fragment A entrapped into DOPC/ cholesterol/DOPE liposomes (100 nmol) the inhibition of protein synthesis was about 49%. This inhibition was achieved when the molar ratio of DOPE to DOPC was 1 : 4. Increasing the DOPE/DOPC to 1 : 1 did not increase the inhibition. Similar, i.e., about 54% inhibition was also demonstrable with DOPC/cholesterol/PS liposomes containing 63 ng fragment A/100 nmol phospholipid. Cells were also incubated with empty liposomes at comparable lipid concentrations to which increasing amounts (up to 3 μg) of free fragment A were added. Even with these concentrations of fragment A, no inhibitory effect on protein synthesis could be observed. Free fragment A alone (10 μg) also failed to affect protein synthesis.

The data obtained when cells were preincubated with DOPC/cholesterol/PS liposomes containing fragment A (56 ng/100 nmol) for varying periods of time are represented in Fig. 1. A slight decrease in [^{14}C]leucine incorporation could be observed already after 0.1 h preincubation with liposomes; there was about 55% inhibition after 1 h and a slightly higher level of inhibition after preincubation for 2 h. There was no further increase in the inhibition of [^{14}C]leucine incorporation when the preincubation period was prolonged to 7 h or even overnight (data not shown). Cells incubated with 100 nmol of empty DOPC/ cholesterol/PS liposomes to which 1 μg of free fragment A was added did not show any inhibition.

The 50%–60% inhibition of [^{14}C]leucine incorporation achieved when 10^5 cells were preincubated for 1 h with 100 nmol of lipid vesicles containing 62.3 ng fragment A could not be increased with higher concentrations of the same liposomes (up to 200 nmol). However, when cells were incubated with the same vesicles but at lesser lipid concentrations (50 nmol, 25 nmol and 10

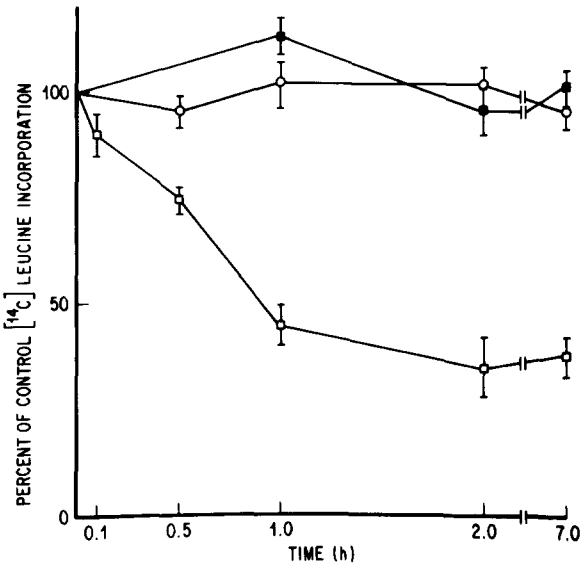

Fig. 1. Effect of preincubation time with liposomes on [^{14}C]leucine incorporation by WiL2 cells. All incubation mixtures ($1 \cdot 10^5$ cells/well) were treated with 100 nmol lipid. □, DOPC/cholesterol/PS liposomes, 56 ng entrapped fragment A; ○, DOPC/cholesterol liposomes, 64 ng entrapped fragment A; ■, empty DOPC/cholesterol/PS liposomes, 1 μg free fragment A added. Vertical bars denote S.D. of triplicate determinations.

nmol, respectively) a decrease in the inhibition was observed. With a different DOPC/cholesterol/PS liposome preparation containing 39.4 fragment A per 100 nmol phospholipid, a lesser extent of inhibition was observed. DOPC/cholesterol/PS liposomes containing 10.5 ng fragment A per 100 nmol lipid did not inhibit [^{14}C]leucine incorporation at any lipid concentration studied (Fig. 2).

Our results show that the intracellular delivery of fragment A into WiL2 cells depends on lipid composition. Fraley et al. [4] found that among the different liposome populations they studied, the most efficient delivery of encapsulated viral DNA into CV-1P cells was achieved with PS/cholesterol liposomes. While this work was in progress a report by McIntosh and Heath [14] was published. They found that fragment A entrapped into reverse phase evaporation vesicles [15] did not inhibit protein synthesis in their system. There are several differences between our work and theirs, including the cell lines studied, assay conditions, method of preparation of liposomes and lipid composition. Uchida and coworkers could not demonstrate any toxic effect on mouse L cells by fragment A entrapped into lecithin cholesterol liposomes [16]. Norrie et al. [17] in a recently published study reported that small fluid anionic and small solid cationic liposomes containing fragment A were more efficient than large cationic vesicles in killing HeLa cells.

The maximum effect we could demonstrate never exceeded 70% although more consistently 50–60% inhibition of [^{14}C]leucine incorporation was found. It is difficult to explain this lack of total inhibition with the knowlege available at the present time. If one molecule of diphtheria toxin can kill a cell [18] the available number of fragment A-containing vesicles ($1.6 \cdot 10^{12}$ vesicles per 10^5 cells) should have been sufficient for total inhibition. Norrie et al. [17], who reported that small cationic liposomes were more efficient than large ones of the same composition in delivering fragment A into HeLa cells, attribute this difference to the mechanism of cell-liposome interaction. They suggest that the enhancement is due to the delivery of fragment A into the cytoplasm during the fusion of liposomal and plasma membrane, although endocytotic mechanisms could not be excluded because of technical difficulties. It is obvious that further work directed to dissect the steps of uptake and/or intracellular fate of liposome-delivered fragment A is necessary.

The emphasis and the main finding of this study is that low concentrations of incorporated fragment A affect WiL2 cells. This effect depends on lipid composition. Furthermore, free fragment A even at much higher concentrations does not shown such an effect. Therefore, the possible leakage of the toxin from liposomes during liposome-cell contact does not influence the results. Furthermore, it has been shown previously by us [12] and others [3,19] that a major fraction of liposomes of varying compositions remains adsorbed to cell surfaces. Under such conditions it has been difficult to follow the effect of the minor fraction that may enter cells and to investigate the possible modification of these effects by different experimental parameters, including ligand mediated mechanism of entry. The fact that free fragment A does not enter cells combined with its high intracellular toxicity [14] makes such studies possible.

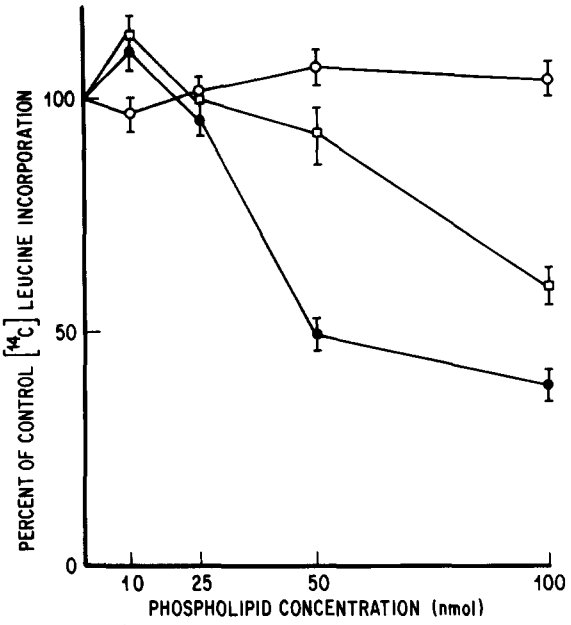

Fig. 2. Effect of lipid and fragment A concentration on [^{14}C]leucine incorporation by WiL2 cells. Cells ($1 \cdot 10^5$/well) were preincubated for 1 h with DOPC/cholesterol/PS liposomes, as indicated. Fragment A concentration/100 nmol lipid: ●, 62.3 ng; □, 39.4 ng; ○, 10.5 ng. Vertical bars denote S.D. of triplicate determinations.

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