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ENCAPSULATION OF HUMAN FIBROBLAST INTERFERON ACTIVITY IN LIPOSOMES

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SUMMARY: The preparation is described of three types of liposomes containing biologically active human fibroblast interferon. Depending on the preparative method, up to 50% of the initial interferon activity could be recovered associated with the liposomes, 15-30% being entrapped into the aqueous space of the vesicles. Encapsulation into negatively charged liposomes is dependent on the acidic phospholipid content; liposomes bearing a net positive charge could capture more interferon than those with a negative charge but were toxic to the target cells. Expression of biological activity of liposomes encapsulated interferon was demonstrated by their antiviral activity and their ability to prime interferon induction.

INTRODUCTION

Interferon is presently under consideration for treatment of a wide range of diseases, both viral and oncogenic in nature[1]. To achieve adequate therapeutic benefit from the administration of interferon, it appears that high doses must be injected at frequent intervals. In a search for more effective utilization of interferon, liposomes have been used in the past *in vivo* in animal model systems as carriers for the interferon inducer (poly I:poly C)[2] and, *in vitro*, for interferon itself[3,4]. Recently, clear evidence has been presented[5,6] that human leukocyte interferon can be entrapped into the aqueous space of cationic liposomes of various types and compositions. In addition, human leukocyte interferon was found to bind to preformed multilamellar liposomes containing fully saturated long chain (C_{16}), fatty acids. In other systems[3], the majority of liposome-associated mouse interferon seems to be attached to the outer vesicle membrane by electrostatic interaction. While human leukocyte interferon apparently did not lose its biological activity during liposome preparations, Anderson *et al.*[5], were not able to entrap human fibroblast interferon (β-IFN) into liposomes,

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due to its pronounced lability to mechanical agitation. In addition, no data were presented as to the expression of the biological competence of the encapsulated interferon species[5,6]. In this communication, we describe the preparation of liposomes containing biologically active human fibroblast interferon, as demonstrated by their antiviral activity and their ability to prime interferon.

MATERIALS AND METHODS

Interferon preparation: Interferon was prepared from human fibroblast cells FS-11 essentially according to the procedure of Havell and Vilcek[7]. Purification on columns of Blue-Sepharose (Pharmacia) resulted in interferon with a specific activity of not less than 10^7 units/mg. Human serum albumin was added as stabilizer (final concentration 0.1%) and dialysis was carried out against phosphate buffered saline, pH 5.5. For liposome-preparations the interferon concentration was about 200,000 units/ml.

Interferon assay: The activity of free and liposome-entrapped fibroblast interferon was measured by estimation of antiviral activity in the cytopathic effect assay[8], using human foreskin fibroblast FS-11 cells challenged with vesicular stomatitis virus. Interferon activity is expressed in International Reference Units (fibroblast interferon standard GO23-901-527 kindly supplied by the Infectious Diseases Branch, NIAID, Bethesda, Md).

Liposome preparations: Liposome preparations were carried out essentially by methods described by Yatvin and Lelkes[9], with care being taken to minimize mechanical and temperature stress. Liposome-associated interferon was separated from free interferon either by column chromatography on Sephacryl S-200 (Pharmacia 1x20 cm, with PBS + 0.1% HSA, pH 5.5, flowrate = 12 ml/hr, as eluant) or by repeated centrifugation (3x20,000 x g for 15 min.). Lipid recovery in liposome preparations was monitored at all stages by phosphate analysis[10], or by estimations of ^{14}C-dipalmitoyl phosphatidyl choline (Amersham Radiochemicals, U.K.) incorporated as radiotracer into the lipid membrane. Recovery by both methods was greater than 95% in all experiments.

Interferon induction and priming: Interferon induction experiments were carried out in FS-11 cells essentially using the superinduction technique of Havell and Vilcek[7]. Priming[11] prior to induction was with free or liposome-entrapped interferon with concentrations and times as shown. The priming effect in control experiments (cells primed with free interferon as compared to unprimed cells) was 5-10 fold.

RESULTS AND DISCUSSION

In preliminary experiments, we established that human β-interferon retained essentially fully activity (70-90%) on treatment by one of the following techniques subsequently used to prepare liposomes[9]: (1) freezing and thawing of presonicated vesicles (FTV), (2) fusion of small sonicated vesicles by addition of 10 mM Ca^{++} and subsequent chelation of the cations with 20 mM EDTA (37°C, 30 mins) to yield large unilamellar vesicles (LUV),

(3) gentle (30 strokes/min) shaking of an aqueous lipid dispersion to produce large multilamellar vesicles (MLV). Other techniques such as vigorous vortex-mixing, sonication, high pressure extrusion or reverse-phase evaporation, resulted in up to 95% loss of β-IFN activity. Subsequently, we entrapped β-IFN into liposomes composed of various amounts of egg-phosphatidylcholine (PC) phosphatidylserine obtained from bovine brain (PS), and cholesterol (CL), by each of the three techniques described above.

Depending on the preparative method and the separation technique used, up to 50% of the initial ($9x10^4$ - $2.5x10^5$ units) interferon activity could be recovered in association with liposomes. This value was not increased by using higher initial concentrations ($1-2x10^6$ units/ml) of interferon; similar results were reported by Eppstein and Stewart[6]. The interferon titres of the various liposome preparations before trypsinization are not given since they reflect the efficiency of the procedure utilized to separate liposomes from free excess interferon. Trypsin digestion (200 μg/ml, 37°C, 40 mins.) reduced this figure to 1.5-5.0% (≈ 70-180 units/μmol lipid) which appeared to be lipid associated and trypsin resistant (Table I). This trypsin resistant activity may indicate a partial stabilization of the membrane-associated interferon, which in its free form is completely inactivated by trypsin. A similar stabilization phenomenon was observed with α-IFN associated with liposomes[6]. Alternatively, these results are in line with findings on the adjuvant properties of antigen-containing liposomes which are mainly due to the exposure of the antigens on the outer surface of the liposomes[12]. Following trypsin digestion, trypsin inhibitor (400 μg/ml) was added and the vesicles were disrupted with sodium cholate (0.8% v/v). Depending on the lipid composition and liposome type used, between 1,000 and 2,200 units of interferon per μmole lipid could be recovered, which were considered truly incorporated (Table I). These values are comparable to those obtained by Eppstein and Stewart, and two- to three-fold higher than those of Anderson et al.,[5] both using leukocyte interferon in the absence of albumin stabilizer. From these data, we conclude that approximately 50%

TABLE 1. ASSOCIATION OF INTERFERON WITH VARIOUS LIPOSOME PREPARATIONS

Lipid Composition (molar ratios)	Type of Liposome	Interferon titre, units/μmol lipid (a)	(b)
PC PS:CL 10:2:10	FTV	183	1333
	LUV	183	1267
	MLV	not determined	985
PS:SA:CL 10:2:10	MLV	not determined	2133
PC:PS 7:3	FTV	74	1720
	LUV	80	1640
	MLV	72	1280
PC:PS 5:5	MLV	92	1730
	FTV	120	2214

Liposomes were prepared using cited lipids at 10-30 μmoles, essentially according to Yatvin and Lelkes[9]. (phosphatidylcholine and phosphatidyl serine, chromatographically pure, were from Lipid Products Inc., Nutfield, U.K; cholesterol was obtained from Merck Darmstadt FGR and recrystallized twice from ethanol). Care was taken to minimize mechanical agitation and exposure to heat. Association of interferon with liposomes is given in terms of units activity per μmole lipid. (a) liposomes treated with trypsin (200 μg/ml, 37°C, 40 mins.) followed by trypsin inhibitor (400 μg/ml, 22°C, 20 mins.). (b) liposomes treated as in (a) followed by sodium cholate 0.8% v/v.

of the total interferon activity is associated with the liposomes, 15-30% of which are entrapped into the aqueous space of the vesicles.

IFN encapsulation into negatively charged liposomes is dependent on the acidic phospholipid content, as shown in Fig. 1 for FTV and MLV. Similar data have been presented for α-IFN[5]. We noted that liposomes containing 90% PC and 10% stearylamine (thus bearing a net positive charge) could capture up to two times more interferon than those with a negative charge probably due to electrostatic interaction between the proteins and the vesicle surface. However, these liposomes proved to be toxic to the target cells (human fibroblasts) at relatively low concentrations (6 μM lipid/10^6 cells) as has been shown previously by others[13]. Negatively-charged liposomes showed a toxic effect on these cells only at concentrations

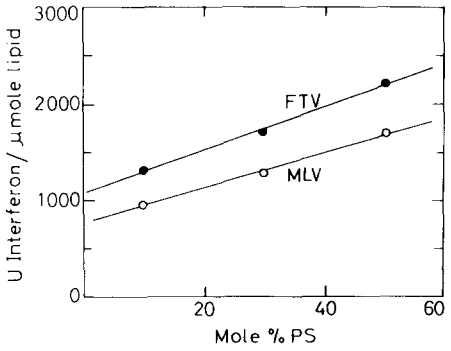

Fig. 1. Association of human fibroblast interferon with cationic liposomes as a function of phosphatidyl serine content. Liposome preparations and assays for interferon activity were performed as described. Each experimental point is based on data from at least two independent experiments, carried out in duplicate. Experimental error in all cases was less than 10%.

of above 150 $\mu M/10^6$ cells in agreement with other findings[14]. A further attempt to improve interferon entrappment by reducing the ionic strength[9] (using ten-fold diluted phosphate-buffered saline) during the entrappment procedure, resulted in osmotically unstable liposomes when these were incubated with cells in biological media.

In addition to their antiviral activity, interferon-containing liposomes were shown to exhibit a priming effect[11] in induction of FS-11 cells for interferon production (Fig. 2). In control experiments using free fibroblast interferon for priming, it was found that with low concentrations of interferon (below 20 U/ml) a priming effect was observed only with long time periods of incubation (usually 18 hrs.). To obtain a priming effect with only short times of incubation of interferon with cells (2 or 4 hrs) higher concentrations are required. When priming was carried out using liposome-entrapped fibroblast interferon, overnight (18 hr.) incubation resulted in a priming effect only equal to that of an equivalent concentration of free interferon, whereas, a far greater potentiation of the cells to produce interferon on induction was obtained in short priming periods. The priming effect after a 2-hour incubation with typsinized liposomes was most significant (Fig. 2) and suggests a "potentiation" of interferon activity when entrapped within the liposomes, with respect to this activity. We attempted to increase the

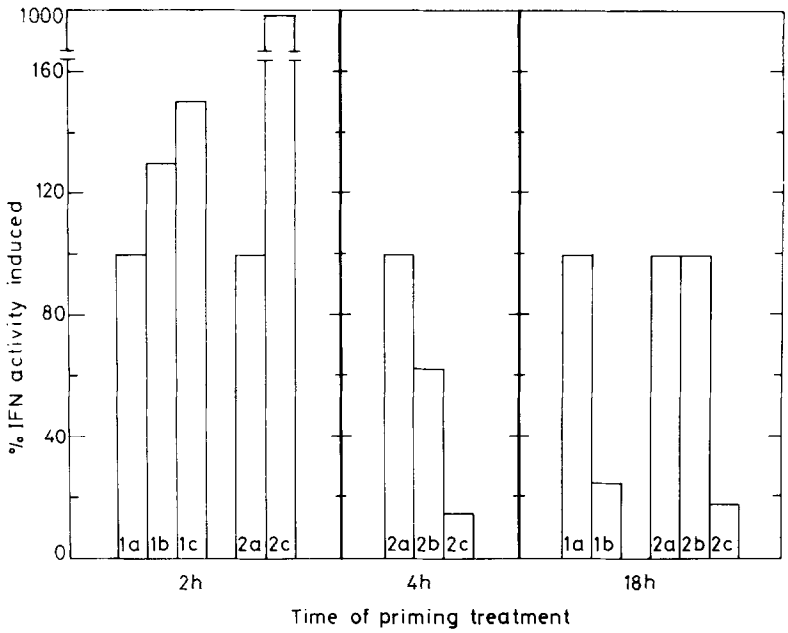

Fig. 2. Effect of liposome encapsulated fibroblast interferon in priming cells for interferon production. Human foreskin fibroblast cells FS-11 (10^6 cells/60 mm plate) were treated for the times indicated with free interferon or liposome preparations as shown. Cells were then superinduced for interferon production and assayed as usual[7,13]. Interferon titres are expressed as percent of titres obtained on priming with free interferon, which were 5-10 fold higher than titres obtained with unprimed fibroblasts.
a: free interferon control; b: untreated liposomes; c: trypsinized liposomes.

Preparation 1: FTV liposomes containing PC:PS:CL (10:2:10).
Preparation 2: FTV liposomes containing PC:PS (7:3).

priming effect of liposome encapsulated interferon on fibroblast cells by use of polyethylene glycol to initiate fusion[15]. Polyethylene glycol was found to have a toxic effect on the cells and impaired their ability to produce interferon on induction.

At present, it is not known how the effects of liposomal-encapsulated interferon are manifested in the target cells, i.e. human fibroblasts entering the antiviral state or undergoing priming for interferon induction. The possibility should be considered that interferon-associated with liposomes could leak out due to cell-mediated lysis, and may thus be subsequently expressed by a receptor-mediated process. However, such mechanisms are not likely to be significant for large multilamellar vesicles during the short time periods during which liposome-mediate effects were

observed. According to current views[16], the liposomes may either fuse with their target cells or be endocytosed by them. In both types of internalization, the liposomal contents are released within the cell, either directly into the cytosol or via the lyosomal apparatus. This, therefore, represents a useful system for future study of the mechanism of interferons action, without involvement of the cell-surface receptor. Other liposomal-encapsulated lymphokines, like macrophage activating factor or muramyl dipeptide, have been shown to be potent augmentors of the host-defence mechanism upon internalization of their carrier vesicles, by-passing the receptor-recognition mechanism[17]. Because of the species-specificity of interferon[18], it will be of interest to use liposome-encapsulated interferon of, for example, mouse origin, to enable the study of interferon's action in vivo.

With regards to the therapeutic uses of interferon, there is a possibility that the liposomes become attached to the cell surface, acting as a depot providing high local concentrations by sustained release. In addition,there is some indication that the encapsulation of interferon in liposomes does achieve, at least partially, the desired result of stabilization, as inferred from the residual activity after trypsinization (Table I, col (a)). There is also an apparent enhancement of liposome-encapsulated interferon activity as compared to free interferon, with respect to priming of fibroblast cells for interferon production, during short incubation times.

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