ASSOCIATION OF MOUSE INTERFERON WITH LIPOSOMES

C. LA BONNARDIÈRE

Station de Recherches de Virologie et d'Immunologie (INRA), 78850 Thiverval-Grignon, France

Received 21 February 1977

1. Introduction

Since their definition by Bangham in 1965 [1], liposomes have been extensively used in model membrane systems, and more recently as carriers of drugs [2,4], enzymes [5,9], antibodies [10] and interferon inducers [11], either in model systems, or in vivo. Gregoriadis and Ryman described the fate of liposome-entrapped ^{131}I-labelled albumin injected intravenously into rats [12], showing first that there was no significant leakage of the entrapped protein during the blood transit, and secondly that liposomes were rapidly concentrated in certain organs, mainly the liver. It has been recently demonstrated that the inoculation route as well as the size of liposomes can influence their fate in vivo [4]. It is also possible to considerably enhance the cellular uptake of liposomes by coating these with isologous heat-aggreagated IgG [9] or IgM [8] and even to direct liposomes more specifically towards certain cells with cell-specific IgG [3].

We thought that this double function of liposomes, namely protection and transport, could be positively applied to increase the in vivo interferon activity. The efficiency of exogenous interferon as a prophylactic or therapeutic anti-viral and anti-tumor agent has been demonstrated, particularly in the mouse [13,14]. However, because of a rapid dilution and/or degradation of interferon in the organism [15,16] successful treatments have necessitated rather large amounts of interferon and/or repeated inoculations. Entrapment of interferon in liposomes might obviate these disadvantages and render it more efficient.

It seems now well established that mouse interferon is a glycoprotein with two molecular forms: form A, with mol. wt 38 000 and an isoelectric point of 7.35; form B, with mol. wt 22 000 and an isoelectric point of 7.15 [17,18]. Since much larger proteins have been entrapped in liposomes [7,8,10], the size of the interferon molecule was not 'a prior' a limiting factor.

In the present report we have characterized the association between liposomes and interferon through the anti-viral state induced in Mouse L cells by the complex.

2. Materials and methods

2.1. *Cells and viruses*

Mouse L_{929} cells, kindly provided by Dr E. de Maeyer (Institut du Radium, Orsay), were grown in monolayers in Eagle's Minimum Essential Medium (MEM) supplemented with 10% calf serum.

$C_{243\text{-}3}$ Cells [19], a gift from Dr M. Tovey (Institut de Recherches sur le Cancer, Villejuif) were grown in monolayers in MEM plus non-essential amino acids, which noticeably reduced their doubling time.

Newcastle Disease Virus (NDV), strain Hertfordshire, was grown in the allantoic cavity of 10 day-old embryonated chicken eggs.

Vesicular Stomatitis Virus (VSV, serotype Indiana) was kindly provided by Dr A. Flamand (Faculté des Sciences, Orsay).

2.2. *Interferon*

Interferon (IF) was produced in $C_{243\text{-}3}$ cells according to the procedure described by Gresser et al. [20], except that cells were kept in monolayers throughout induction. Crude mouse interferon, kindly supplied by Dr Desrumeaux (Laboratoire Roger Bellon), was used in some experiments.

Crude IF was semi-purified as follows: IF was

acidified to pH 2.0, held at 4°C for 4 days, clarified by centrifugation, then exposed to a step-wise ammonium sulfate precipitation at pH 2.0 (30–80% saturation). Finally, interferon was dialysed against 0.05 M phosphate buffer, pH 7.4, and stored at − 70°C.

Interferon (free or liposome-bound) was titrated in L_{929} cells as follows: serial 2-fold dilutions in MEM + 5% calf serum were incubated in duplicate with 24 h-old L cell monolayers for 17 h at 37°C. Monolayers were then washed with MEM, infected with 100 plaque forming units of VSV, overlaid with nutrient agarose, then incubated at 37°C for 24 h in a humidified incubator with 5% CO_2. IF titers were expressed as the reverse of the dilution which reduced the number of VSV plaques by 50%.

2.3. *Protein*

Protein was quantified by the method of Lowry [21]; our preparations of interferon had spec. act. $5 \times 10^5 - 10^6$ units/mg protein.

2.4. *Liposomes*

2.4.1. Preparation

Egg lecithin-(L-α-phosphatidyl-choline, Sigma), stearylamine or dicetyl phosphate, and cholesterol were used in the molar ratio 7:2:1; 50 mg lipids in $CHCl_3$ were added to a round-bottom flask, and using a rotary evaporator, a uniformly thin film was formed. Subsequently 5 ml IF in 0.05 M phosphate buffer was added. From this stage, two different procedures were alternatively used:

Procedure 1 (Sonicated liposomes)

Flasks were kept at 4°C for 2 h and then sonicated for 4 min using a 12 mm probe (Siduse sonicator); rather good clarification of the suspension was obtained. Crude suspension (0.5 ml) was layered onto a Sephadex G-150 column (1 × 20 cm) and eluted with 0.05 M phosphate buffer, NaCl 0.4 M, pH 7.4. Relative lipid concentration was determined in each 2 ml fraction of the eluate by measuring the apparent absorbance at 410 nm. Liposomes were isolated in the exclusion peak. Free IF eluted much later than lipid-containing fractions.

Procedure 2 (Unsonicated liposomes)

The flasks were mechanically shaken for 1.5 h at 20°C (Griffin Shaker). Under these conditions the lipid film was completely suspended into a milky suspension of large liposomes; 5 ml of this crude suspension was centrifuged at $10^5 \times g$ for 2.5 h at 4°C. The clear supernatant was carefully removed, and the pellet was resuspended into 5 ml phosphate buffer.

2.4.2. Determination of liposome-bound IF activity

IF associated with liposomes was titrated in L cells by the same method as free IF (see 2.2.), the anti-viral titer obtained is referred to as liposome-bound IF.

2.4.3. Solubilisation by Trition X-100

Crude liposome suspensions were incubated for 10 min at 20°C in the presence of 1% (v/v) Triton X-100 (Rohm and Haas). A good clarification of the suspension was observed. At the end of the reaction, samples were diluted 100-fold in phosphate buffered saline plus 1‰ bovine serumalbumin (BSA) before titration.

2.4.4. Treatment with trypsin

Trypsin, 50 μg/ml (B grade, Calbiochem) was added to soluble or liposome-bound IF at 37°C, pH 7.6, in the presence of 1‰ BSA. Samples were withdrawn at intervals, supplemented with 50 μg/ml soybean trypsin inhibitor (Calbiochem), then diluted 100-fold in MEM + 5% calf serum before titration.

3. Results

3.1. *Preliminary data*

Our anti-viral substance fulfilled all the physico-chemical and biological properties of interferon: non dialysable, not precipitable at $10^5 \times g$ for 2.5 h; sensitive to trypsin and resistant to pH 2.0. It was mouse-specific since it inhibited the growth of VSV and EMC virus in mouse cells, but had no effect on VSV growth in bovine cells or hamster cells.

We designed a control experiment to ascertain whether the presence of liposomes influenced the titer of soluble interferon. Two sets of L cell monolayers were incubated in parallel with interferon alone or with interferon plus preformed liposomes. Interferon titers were identical in both cases.

Table 1
Interferon activity (% initial activity) associated with sonicated liposomes (Procedure 1)

Expt. No.	Control IF	IF + sonication	IF + lipids + sonication		
			Net charge liposomes	Crude suspension[a]	Liposome-associated IF[b]
1	100	10	–	17	9.6
2	100	17	+	30	20

[a] Interferon titer of the suspension before chromatography
[b] Total interferon activity (% initial activity) in liposome-containing fractions of the eluate

Expt 1: Liposomes were made of lecithin, dicetyl-phosphate, cholesterol (–)
Expt 2: Liposomes were made of lecithin, stearylamine, cholesterol (+)

3.2. *Determination of liposome-bound interferon activity*

Initially the lipids were suspended in interferon solutions by sonication. The resulting small liposomes could easily be separated from free interferon by column chromatography (Procedure 1). Table 1 shows the results of 2 experiments involving respectively anionic and cationic liposomes; in spite of the drastic inactivation of control interferon by sonication (about 90%), it was possible to recover respectively 9.6% and 20% of initial activity in anionic and cationic liposomes. This result was probably relevant to the greater uptake of cationic liposomes by the cells [7,11].

In a second set of experiments, sonication was avoided, and replaced by mechanical shaking; the resulting large liposome could not be passed through a column, and consequently were separated from free interferon by ultracentrifugation (Procedure 2). In this set of experiments (including only anionic liposomes) we tested the protective effect of BSA. We observe (table 1) that, in the absence of BSA, there was a marked inactivation of interferon, and here again liposomes exhibited an apparently protective effect (most residual activity in this case was recovered in the pellet after centrifugation). On the other hand, if BSA was effective in preventing inactivation of control interferon, its presence did not significantly increase the final yield of liposome-bound interferon. A possible competition between interferon and BSA for binding to liposomes might explain this result. As far as comparison between interferon titers in control solutions and in liposome suspensions is possible, we

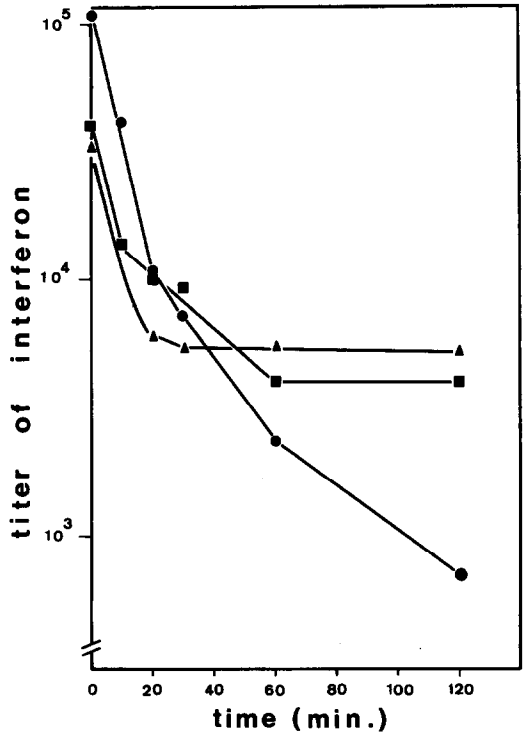

Fig.1. Action of trypsin on liposome-associated interferon. IF was dialysed against 0.05 M phosphate buffer pH 7.6, and liposomes (anionic and cationic) were made as described, and sonicated for 4 min. Trypsinization was performed as described in Materials and methods (○——○) Control IF; (▲——▲) IF + anionic liposomes; (■——■) IF + cationic liposomes.

Table 2
Interferon activity (% initial activity) associated with non-sonicated liposomes (Procedure 2)

		IF + lipids + shaking		
Control IF	IF + shaking	Crude suspension	Supernatant	Pellet
(a) 100	6.0	30	1.4	18
(b) 100	72	44	16.7	17

(a) Average of 2 experiments – No BSA added
(b) Average of 4 experiments – 1‰ BSA added
In these experiments, liposomes contained dicetyl phosphate (anionic liposomes)

recovered here again an appreciable amount of the initial activity in liposomal fractions (18% and 17%).

3.3. *Fraction of interferon activity resistant to trypsin*

Figure 1 shows the kinetics of hydrolysis by trypsin of crude liposome–interferon suspensions and of control interferon. We trypsinized crude suspensions instead of isolated liposomes to avoid potential problems arising from centrifugation, namely a loss of interferon by handling, and a loss of the smaller liposomes in the supernatant.

The results clearly show that in either type of liposomes (anionic or cationic) there was a fraction of interferon which was simultaneously resistant to trypsin and still biologically active in cell culture. This fraction was 4% of the total interferon activity in anionic liposomes and 5% of the total in cationic ones. From the preceding results (tables 1 and 2), the trypsin-resistant fraction could therefore be estimated to be 20 – 5 = 15% of initial activity. These two fractions will be referred to as Fraction 1 (trypsin sensitive) and Fraction 2 (trypsin resistant).

All the results presented so far concern titratable interferon, i.e., anti-viral activity, which could be expressed in cell culture without disruption of the liposome structure.

3.4. *Action of Triton X-100 on liposome associated interferon*

Triton X-100 has been widely used to demonstrate the latency of various molecules in liposomes [5,7–9]. In our system we examined the detergent-induced release of interferon from liposomes. The results had to be analysed more qualitatively than quantitatively, since liposome solubilisation by Triton is never complete and this detergent exerts a degrading effect upon control interferon solutions under the conditions used. Table 3 shows two different experiments which demonstrate, one with anionic, the other with cationic liposomes, the effect of Triton X-100. If we consider the loss of activity in control solutions (about 60%) it is clear that in both experiments solubilisation of liposomes led to the release of a masked fraction of interferon (Fraction 3).

Table 3
Action of Triton X-100 on crude suspensions of liposome-bound interferon

Expt No.	Triton concentration (% v/v)	Control IF (%)	IF + liposomes (units/ml)
1	0	100	21 000
	1	40	30 000
2	0	100	36 000
	1	45	40 000

Expt 1: Sonicated, anionic liposomes
Expt 2: Non-sonicated, cationic liposomes

4. Discussion

We have shown an association between mouse interferon and liposomes made of egg-lecithin, cholesterol and a charged lipid. Our results suggest the existence of three different fractions of liposome-associated interferon: the first fraction being titratable in cell culture and trypsin sensitive, amounted to 14–16% of the initial activity (Fraction 1). Another fraction was also biologically active, but trypsin resistant, representing 4–5% of the initial activity (Fraction 2). A third fraction, of unknown amount, was latent, i.e., unable to trigger the anti-viral state in the cell unless it was liberated by Triton X-100 (Fraction 3).

Before further discussion, we should point out the distinction to be made between interferon activity (expressed in IF units/ml) and real interferon concentration (unknown). It is probable that binding of interferon to a liposome will modify its intrinsic anti-viral activity, basically because the cell recognizes the liposomes and treats them correspondingly and any bound molecules will be dependant on cell–liposomes interactions. An example is given by the work of Magee et al. [7] who found that liposome-entrapped horseradish peroxidase was taken up by HeLa cells at least 300-times as efficiently as free enzyme.

Considering that 15–20% of our initial activity was found associated with liposomes, even in conditions where most control interferon was inactivated, and that the major part of this activity was trypsin-sensitive (Fraction 1), we suspect that externally-bound molecules acquire a higher intrinsic activity. This would explain the apparent 'protective' effect of liposomes against sonication or shaking (see tables 1 and 2). This potentiating effect was more pronounced with cationic liposomes.

Fraction 3 might include molecules of internal location. By some mechanism, perhaps endocytosis of liposomes and fusion with cellular liposomes, the most internal molecules are prevented from reaching their site of action. The work of Chany et al. [22] with Sepharose-bound interferon suggested that interferon can trigger the anti-viral state from outside the cell.

It will be difficult to assess precisely the spread of interferon within the different liposomal compartments as long as the biological activity is the only available marker for this protein. Even so, our results suggest that liposomes might be promising tools for improving therapeutic action of interferon in vivo. In particular, we demonstrated that a fraction of interferon activity (Fraction 2) was simultaneously active in cell culture and trypsin-resistant; even if this fraction is, a priori, the most likely to utilize the previously mentioned functions of liposomes, we should not minimize the possible roles of Fraction 1 and Fraction 3.

Acknowledgements

We are grateful to Dr Desrumeaux for his advice and to Christiane de Vaureix for her excellent technical assistance.

References

[1] Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965) J. Mol. Biol. 8, 660–668.
[2] Gregoriadis, G. (1973) FEBS Lett. 36, 292–296.
[3] Gregoriadis, G. and Neerunjun, E. D. (1975) Biochem. Biophys. Res. Commun. 65, 537–544.
[4] Dapergolas, G., Neerunjun, D. and Gregoriadis, G. (1976) FEBS Lett. 63, 235–239.
[5] Sessa, G. and Weissmann, G. (1970) J. Biol. Chem. 25, 3295–3301.
[6] Gregoriadis, G., Leathwood, P. D. and Ryman, B. E. (1971) FEBS Lett. 14, 95–100.
[7] Magee, W. E., Goff, C. W., Schoknecht, J., Smith, M. D. and Cherian, K. (1974) J. Cell. Biol. 63, 492–504.
[8] Weissmann, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gotlier, A. and Nagle, D. (1975) Proc. Natl. Acad. Sci. USA 72, 88–92.
[9] Cohen, C. M., Weissmann, G., Hoffstein, S. and Anasthi, Y. C. (1976) Biochemistry 15, 452–460.
[10] Magee, W. E. and Miller, O. V. (1972) Nature 235, 339–340.
[11] Straub, S. X., Garry, R. F. and Magee, W. E. (1974) Infect. Immun. 783–792.
[12] Gregoriadis, G. and Ryman, B. E. (1972) Eur. J. Biochem. 24, 485–491.
[13] Gresser , I., Fontaine, D., Coppey, J., Falcoff, R. and Falcoff, E. (1968) Proc. Soc. Exp. Biol. Med. 127, 491–496.
[14] Gresser, I., Fontaine-Brouty-Boye, D., Bourali, C. and Thomas, M. T. (1969) Proc. Soc. Exp. Biol. Med. 130, 236–242.
[15] Gresser, I., Fontaine, D., Coppey, J., Falcoff, R. and Falcoff, E. (1964) Proc. Soc. Exp. Biol. Med. 124, 91–94.

[16] Subrahmanyan, T. P. and Mims, C. A. (1966) Brit. J. Exp. Path. 47, 168–176.
[17] Carter, W. A. (1970) Proc. Natl. Acad. Sci. USA 67, 620–628.
[18] Stewart, W. E. (1974) Virology 61, 80–86.
[19] Oie, H. K., Gazdar, A. F., Buckler, C. E. and Baron, S. (1972) J. Gen. Virol. 17, 107–109.
[20] Tovey, M. G., Begon-Lours, J. and Gresser, I. (1974) Proc. Soc. Exp. Biol. Med. 146, 809–815.
[21] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[22] Ankel, H., Chany, C., Galliot, B., Chevalier, M. J. and Robert, M. (1973) Proc. Natl. Acad. Sci. USA 70, 2360–2363.