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International Journal of Pharmaceutics 123 (1995) 33–39

international journal of pharmaceutics

Preparation and characterization of interferon-γ-containing liposomes

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Received 5 October 1994; accepted 6 January 1995

Abstract

An immunoenzymatic (ELISA) assay was used to quantify the amount of interferon-γ (IFN-γ) entrapped in liposomes extruded through polycarbonate membranes of 0.2 μm pore size. Tween 20 (1% final concentration) was able to disrupt the vesicles and release the entrapped material without affecting IFN-γ activity, whereas a significant loss of activity was observed with Triton X-100 or Nonidet P-40. A substantial portion of the protein was adsorbed to glass and plastic surfaces during the preparation of IFN-γ-containing liposomes, resulting in relatively low trapping efficiencies. Saturation of the material with a blocking buffer and/or adjunction of bulk proteins (murine albumin and gelatin) to the liposomal preparation increased only slightly the amount of encapsulated IFN-γ. However, incorporation of IFN-γ within preformed blank liposomes using a freeze-thawing method yielded much better results, since up to 30% of the starting material was entrapped in the vesicles when the procedure was carried out in polystyrene tubes treated with polyether-polydimethyl-siloxane (PDMS).

Keywords: Liposome; Interferon-γ; ELISA; Detergent; Protein adsorption; Freeze-thawing; Trapping efficiency

1. Introduction

Interferon-γ (IFN-γ) exhibits a marked immunomodulatory activity (Goldstein and Laszlo, 1986) as well as antiviral and antiproliferative effects (Revel et al., 1980; Rubin and Gupta, 1980). However, the successful use of IFN-γ for in vivo immunotherapy is limited by the rapid clearance of the lymphokine from circulation (Gutterman et al., 1984; Kurzrock et al., 1985) and the potential toxicity from high dosage regimens (Bennett et al., 1986). Liposomes incorporating immunomodulators offer great potential for systemic activation of macrophages and monocytes to a tumoricidal state (Fidler, 1992). Lipid vesicles are avidly taken up by cells of the reticuloendothelial system (RES) following parenteral administration, resulting in increased delivery of encapsulated drugs to these cells (Alving, 1983; Schroit et al., 1983). In addition, in-

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SSDI 0378-5173(95)00027-5

corporation of IFN-γ into liposomes has been shown to augment the ability of this agent to induce in vitro macrophage-mediated cytotoxicity against neoplastic cells (Fidler et al., 1985; Koff et al., 1985; Hume and Nayar, 1989; Rutenfranz et al., 1990).

From a technological point of view, IFN-γ is an unstable protein that can be inactivated by physicochemical treatment during the preparation of liposomes (Ishihara et al., 1990). Furthermore, the amphipathic nature of proteins results in their adsorption at liquid-solid interfaces and this phenomenon can lead to a marked loss of compound at low protein concentration (Suelter and DeLuca, 1983; Burke et al., 1992). The aim of this study was to develop a method for the preparation of IFN-γ-containing liposomes exhibiting an acceptable degree of drug encapsulation. An immunoenzymatic assay was used for the quantification of encapsulated material following liposome solubilization by detergents. The loss of IFN-γ activity by detergents and adsorption of the protein at solid surfaces was investigated in greater detail.

2. Materials and methods

2.1. Materials

The lipids used were soybean phosphatidylcholine (SPC) (Epikuron 200 S, Lukas Meyer Inc., Hamburg, Germany). Recombinant murine interferon gamma with a specific activity of 1.7×10^7 U/mg and ELISA test kits for IFN-γ were purchased from Holland Biotechnology (Leiden, The Netherlands). Murine albumin fraction V (MuAlb), gelatin from bovine skin (type B, 2% solution), Triton X-100, Nonidet P-40 and Tween 20 were obtained from Sigma (St Louis, MO). Polyether-polydimethyl-siloxane copolymer (PDMS) was supplied by Hansa Chemie (Hamburg, Germany). All other chemicals were of analytical grade.

2.2. Liposome preparation

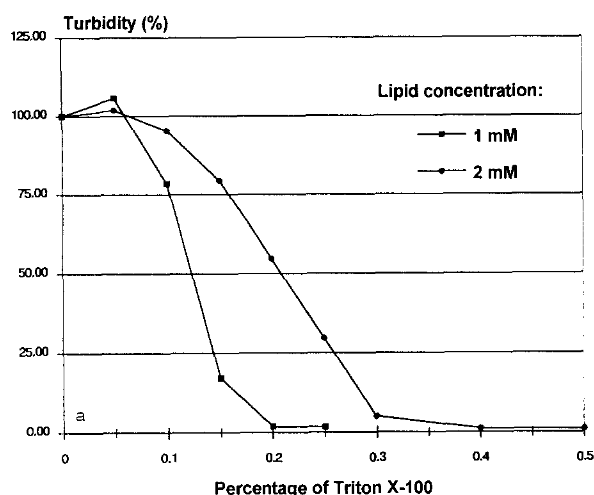
Multilamellar vesicles composed of SPC (200 mM) were prepared by an extrusion technique similar to that described by Olson et al. (1979). Briefly, the lipids were dissolved in methanol and the organic solvent was slowly removed under reduced pressure on a rotary evaporator at 30° C. The thin film of dried lipids was hydrated at room temperature for 30 min with PBS (pH 7.4) containing IFN-γ (22000 U/ml). The resulting liposomes were calibrated by extrusion through polycarbonate membrane filters of 0.2 μm pore size (Nuclepore Corp., Pleasanton, CA) using an Extruder® (Lipex Biomembranes Inc., Vancouver, Canada). Unincorporated IFN-γ was removed by three ultracentrifugation cycles at $200000 \times g$ for 1 h with buffer washes. Blank liposomes were prepared in PBS alone and extruded through 0.2 μm polycarbonate membranes. When IFN-γ was encapsulated using the freeze-thawing procedure, blank liposomes were mixed with IFN-γ solution to achieve a final concentration of 22000 U/ml. The mixture was frozen in liquid nitrogen (−196° C) and thawed at room temperature with subsequent extrusion through 0.2 μm polycarbonate membranes. The amount of lipids in the initial and final liposome preparations was quantified according to the method of Stewart (1980). The vesicle size distribution was determined by quasi-elastic light scattering using a laser particle sizer (Model N4 SD, Coulter Electronics Ltd, Luton, UK). For all the preparations tested, the vesicle mean diameter ranged from 180 to 200 nm when liposomes were calibrated through 0.2 μm polycarbonate membranes.

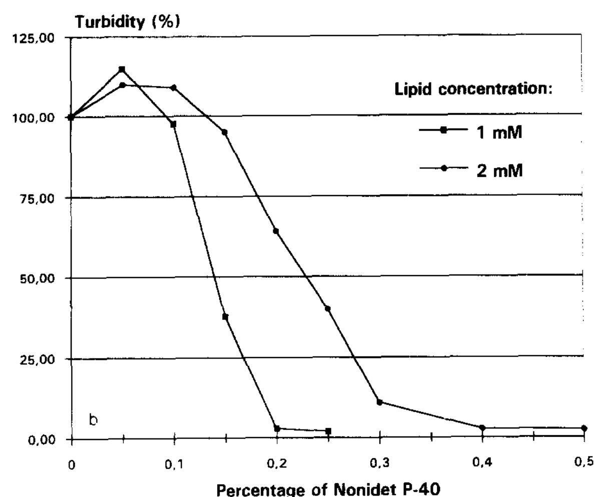
2.3. Liposome solubilization by detergents

Blank liposomes diluted in PBS (1 or 2 mM) were incubated for 30 min at room temperature with increasing amounts of Triton X-100, Nonidet P-40 or Tween 20. Vesicle solubilization was assessed as the decrease in turbidity of liposomal dispersions by measuring the absorbance at 400 nm using a Shimadzu UV-2101 PC spectrophotometer (Shimadzu Corp., Kyoto, Japan).

2.4. Loss of IFN-γ activity in the presence of detergents

IFN-γ solutions diluted to 65 U/ml in dilution buffer (ELISA test kit) were incubated for 30 min





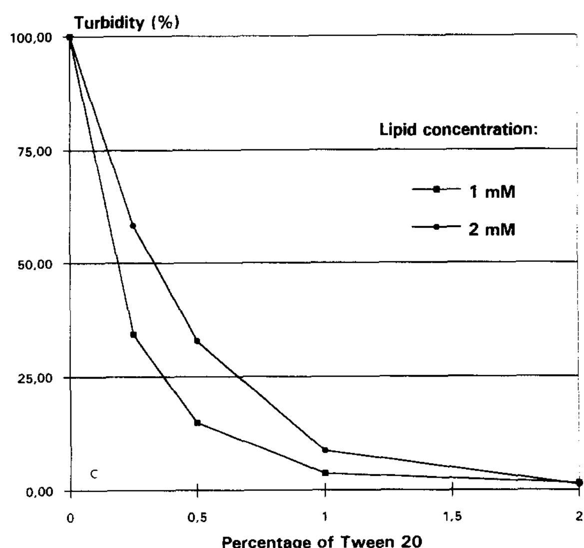


Fig. 1. Disruption of SPC liposomes extruded through 0.2 μm polycarbonate membranes as a function of Triton X-100 (a), Nonidet P-40 (b) or Tween 20 (c) concentrations. Turbidity (A_{400}) of liposomal dispersions (1 or 2 mM) was measured following incubation with detergents. Results are expressed as the mean of two experiments.

with Triton X-100 (0.5% w/v), Nonidet P-40 (0.5% w/v) or Tween 20 (1–3% w/v). The activity of IFN-γ was determined using a standardized immunoenzymatic assay having specificity for biologically active natural or recombinant mouse IFN-γ. The concentration of diluted IFN-γ solution (65 U/ml) was in the detection range of the ELISA assay.

2.5. Adsorption of IFN-γ to glass and plastic materials

A 0.2 ml volume of IFN-γ solution diluted to 22 000 U/ml in PBS was incubated for 1 h at 4° C in glass or polystyrene tubes (13 × 75 mm). The amount of IFN-γ recovered was quantified by sampling two aliquots and performing ELISA assay. Standards were diluted in dilution buffer (ELISA test kit) containing additives to prevent IFN-γ adsorption. In some cases, bulk proteins (0.1 mg/ml MuAlb and gelatin) were added to IFN-γ solution. Tubes were treated or not by incubation for 24 h (4° C) with a blocking buffer (5% dried skimmed milk and 0.05% thimerosal in PBS) or PDMS (1% in ethanol). After the incubation period, tubes were rinsed three times with PBS and distilled water and dried at 50° C.

2.6. Preparation of IFN-γ-containing liposomes

Liposomes incorporating IFN-γ were prepared in material (round-bottom flask, polycarbonate membranes, polystyrene and centrifuge tubes) which was treated or not by incubation for 24 h (4° C) with blocking buffer. The material was then rinsed with PBS and distilled water and dried at 50° C. Liposomes were prepared in the absence or presence of bulk proteins (0.1 mg/ml MuAlb and gelatin). When IFN-γ was encapsulated using the freeze-thawing procedure, polystyrene tubes were treated with PDMS as described above. Phospholipids and IFN-γ were

quantified at each step of the preparation following liposome solubilization by 1% Tween 20. Trapping efficiencies were determined after removal of unencapsulated material by ultracentrifugation.

3. Results and discussion

Several methods have been previously described to quantify the amount of IFN-γ encapsulated in liposomes, including antiviral assay (Rutenfranz et al., 1990), immunoassay (Ishihara et al., 1990) and radiolabelling of the protein (Hume and Nayar, 1989; Ishihara et al., 1990). We used an immunoenzymatic (ELISA) assay for the quantification of liposomal IFN-γ. In order to release the entrapped material, this method requires vesicle solubilization by detergents prior performing the assay.

3.1. Detergent disruption of liposomes

The first set of experiments was designed to determine the effective concentrations of Triton X-100, Nonidet P-40 and Tween 20 for the disruption of SPC liposomes extruded through 0.2 μm polycarbonate membranes. Fig. 1 shows the variation in turbidity of liposomal dispersions (1 or 2 mM) as a function of the amount of detergent added. It can be seen that Triton X-100 and Nonidet P-40 were effective at 0.2 and 0.4% (w/v) for the disruption of liposomes diluted to 1 and 2 mM lipids, respectively. The initial increase in turbidity observed upon addition of sublytic detergent levels is a general phenomenon indicating swelling, aggregation and fusion of the vesicles (Lasch and Schubert, 1993). Tween 20 was also effective at disrupting lipid vesicles but at higher concentration, since 2% (w/v) was required to solubilize liposomes diluted to 2 mM. On a molar basis, however, the effectiveness of the three detergents was equivalent as approx. 3 mol of detergent were required to solubilize 1 mol of liposomal phospholipids.

3.2. Loss of IFN-γ activity by detergents

The incubation of IFN-γ with detergents such as sodium deoxycholate can lead to a significant loss of radioimmunoassay activity of the protein (Ishihara et al., 1990). Thus, it was important to check whether Triton X-100, Nonidet P-40 or Tween 20 did not denature IFN-γ and/or interfere with the ELISA assay at detergent concentrations which are effective at disrupting liposomes. When IFN-γ was diluted to 65 U/ml and incubated for 30 min with Triton X-100 (0.5%) or Nonidet P-40 (0.5%), a significant loss of activity was detected, whereas Tween 20 used at 1–3% affected only slightly IFN-γ activity (Table 1). Consequently, Tween 20 was chosen to disrupt liposomes prior to performing the ELISA assay for quantification of encapsulated material.

3.3. Adsorption of IFN-γ at solid surfaces

Adsorption of peptides and proteins at liquid-solid interfaces is a well-known phenomenon largely described in the literature (Suelter and DeLuca, 1983; Burke et al., 1992). Undesired adsorption represent a significant problem for the preparation of IFN-γ-containing liposomes as it can lead to physical loss of protein and conse-

Table 1
Loss of IFN-γ activity upon incubation with detergents

Detergents	IFN-γ recovered (U/ml)	Loss of IFN-γ activity (%)
Tween 20 (1%)	61.19	5.86
Tween 20 (2%)	59.98	7.72
Tween 20 (3%)	58.17	10.51
Nonidet P-40 (0.5%)	31.54	51.48
Triton X-100 (0.5%)	21.68	66.65

IFN-γ solution diluted to 65 U/ml in dilution buffer (ELISA test kit) was incubated with Tween 20 (1–3%), Nonidet P-40 (0.5%) or Triton X-100 (0.5%). Results are expressed as the mean of two experiments.

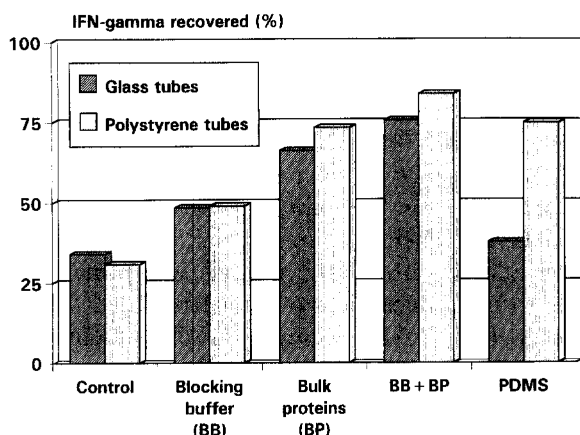


Fig. 2. Percentage recovery of IFN-γ diluted to 22000 U/ml in PBS when incubated in glass or polystyrene tubes. Effect of the treatment with blocking buffer, adjunction of bulk proteins (MuAlb and gelatin) and saturation of the tubes with PDMS. Results are expressed as the mean of two experiments.

quently low trapping efficiencies. Glass and polystyrene tubes were used to investigate IFN-γ adsorption at solid surfaces and to determine the effectiveness of different treatments in preventing the loss of protein. When IFN-γ was diluted to 22000 U/ml in PBS and incubated for 1 h in the tubes, a marked loss of activity was detected for both glass and polystyrene containers (Fig. 2). This phenomenon was partially inhibited by saturation of the tubes with a blocking buffer containing milk proteins or by adjunction of bulk proteins (MuAlb and gelatin) in IFN-γ solution. The best results were obtained by combining the two procedures, since 75–85% of the active compound was recovered after incubation of IFN-γ solution containing bulk proteins in tubes saturated with blocking buffer. Interestingly, PDMS treatment of polystyrene tubes prevented IFN-γ adsorption to a great extent, but this treatment was ineffective with glass tubes. We believe this difference to be the result of a stronger interaction between PDMS and polystyrene than glass surfaces.

3.4. Preparation of IFN-γ-containing liposomes

When IFN-γ-containing liposomes were prepared in untreated material, a substantial loss of protein was observed after each preparation step (Fig. 3). Only 62% of the initial amount of IFN-γ was recovered after hydration of the dry lipid film and 25% after extrusion of the vesicles. Saturation of the material (round bottom flask, polycarbonate membranes and polystyrene tubes) with blocking buffer increased only slightly the amount of IFN-γ recovered before extrusion and practically not after extrusion. One explanation is that adsorption of the protein occurred at the surface of untreated material such as micropipette tips and the extrusion device. Similar results were obtained when bulk proteins (MuAlb and gelatin) were added to the liposomal preparation. It may be possible that a substantial proportion of bulk proteins was lost during hydration of the lipid film and consequently that prevention of IFN-γ adsorption was no longer effective during extrusion. On the other hand, the combination of the two procedures (i.e., saturation of the material and adjunction of bulk proteins) yielded a much better result as 72% of the starting IFN-γ was recovered in the liposomal dispersion after extrusion. In this case, adsorption of both IFN-γ and bulk proteins was probably reduced during the hydration step and subsequent loss of IFN-γ was

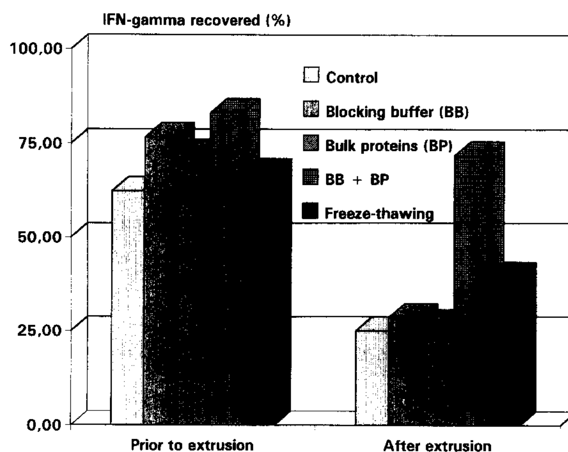


Fig. 3. Recovery of IFN-γ in liposomal preparations before and after extrusion through 0.2 μm polycarbonate membranes. Effect of the treatment with blocking buffer and/or adjunction of bulk proteins. The freeze-thawing procedure was carried out in PDMS-treated polystyrene tubes. Results are expressed as the mean of two experiments.

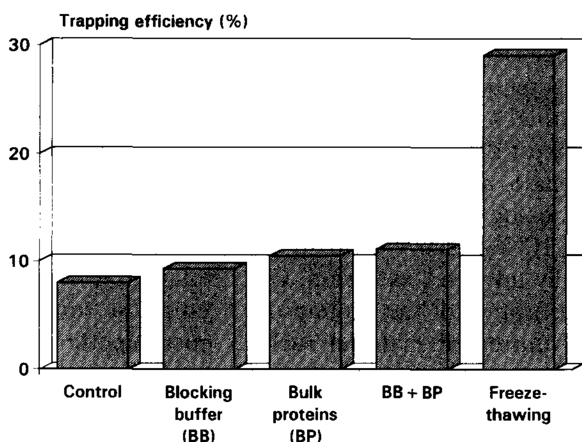


Fig. 4. Trapping efficiencies of SPC vesicles incorporating IFN-γ and extruded through 0.2 μm polycarbonate membranes. Liposomes were prepared in either untreated or treated material (blocking buffer) with or without the presence of bulk proteins. IFN-γ was also encapsulated by freeze-thawing procedure in PDMS-treated polystyrene tubes. Results are expressed as the mean of two experiments.

prevented during extrusion by the presence of bulk proteins in sufficient quantities.

When IFN-γ was encapsulated in preformed blank liposomes by the freeze-thawing procedure, about 33% of the protein was lost during performance of the freeze-thawing cycle in a PDMS-treated polystyrene tube. This loss may be attributed both to partial IFN-γ adsorption to the tube and denaturation of the protein upon freeze-thawing. In an independent experiment, it was found that about 25% of IFN-γ activity was lost upon freezing IFN-γ solution (22 000 U/ml in dilution buffer) in liquid nitrogen. Since adsorption was not prevented during extrusion, only 40% of the initial amount of IFN-γ was recovered after calibration of the vesicles.

3.5. Trapping efficiency of IFN-γ in liposomes

Fig. 4 demonstrates the percentage of IFN-γ encapsulated in vesicles prepared in untreated or saturated material with or without adjunction of bulk proteins. It can be seen that surface treatment and/or the presence of bulk proteins improved only slightly the amount of entrapped IFN-γ. In the best case, only 11% of IFN-γ was encapsulated in the liposomes when the material was saturated with blocking buffer and bulk proteins were added. This value is relatively low in comparison with the trapping efficiency of 17% obtained with carboxyfluorescein, a solute marker of the passively entrapped aqueous volume of liposomes. Thus, the improved recovery of liposomal IFN-γ when prepared in saturated material with adjunction of bulk proteins was principally due to the reduced loss of unencapsulated IFN-γ rather than to an increase in the amount of entrapped active compound. The presence of MuAlb and gelatin certainly reduced IFN-γ adsorption to the round-bottom flask during hydration of the lipid film but the bulk proteins also prevented interactions with phospholipid bilayers and consequently limited the amount of liposome-associated IFN-γ.

On the other hand, the freeze-thawing method was much more effective for the encapsulation of IFN-γ in liposomes, as 29% of the starting material was associated to the vesicles following removal of unencapsulated active compound. In this case, adsorption on the tube was prevented by PDMS treatment and the protein interacted preferentially with preformed phospholipid vesicles. The encapsulation of IFN-γ probably resulted from the fusion of small extruded liposomes (200 nm mean diameter) during the freeze-thawing cycle, producing larger vesicles which encapsulated a significant proportion of the active material. Furthermore, it has been reported that lipid vesicles become transiently permeable to solutes during freeze-thawing in the absence of cryoprotectants (Strauss and Hauser, 1986). The freeze-thawing procedure has been successfully employed by others to encapsulate proteinaceous drugs in liposomes (Ohsawa et al., 1985; Ishihara et al., 1990), but adsorption at liquid-solid interfaces was not prevented by surface treatment such as in our work.

4. Conclusion

We have shown here that IFN-γ encapsulated in liposomes can be quantified by immunoenzymatic assay following vesicle disruption by Tween

20. This detergent used at 1% final concentration was able to solubilize liposomal phospholipids without denaturing the protein and/or interfering with the ELISA assay.

The adsorption of IFN-γ at solid surfaces was partially prevented by saturation of the material with blocking buffer and/or adjunction of bulk proteins (MuAlb and gelatin). However, the reduced loss of IFN-γ during the preparation of liposomes did not result in a marked improvement of trapping efficiencies. The freeze-thawing procedure performed in PDMS treated tubes was much more appropriate for the encapsulation of IFN-γ in liposomes as trapping efficiencies up to 30% were achieved.

Acknowledgements

This work was supported by a grant from the French Ministry of Research and Technology.

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