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Stabilization of aerosolized IFN-γ by liposomes

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Abstract

Aerosolized IFN-γ is very unstable. We have improved the stability of IFN-γ in the jet nebulizer by adding small liposomes. Aerosolized IFN-γ was recovered in PBS solution by bubbling and its concentration was determined. After nebulization for 30 min, aerosolized IFN-γ was detected only $0.4 \pm 0.2\%$ of the initial amount in the PBS solution and $3.1 \pm 0.7\%$ in the reservoir. On the other hand, the addition of small liposomes (HSPC/DSPG = 10/1 (molar ratio), 45 ± 24 nm) in the nebulizer increased the stability of IFN-γ, $27.2 \pm 4.7\%$ of the initial amount in the PBS solution and $25.7 \pm 12.6\%$ in the reservoir. The present study also examined the effects of composition and concentration of liposomes on the stabilization of aerosolized IFN-γ. Liposome prepared from distearoyl phosphatidylcholine (DSPC) or hydrogenated soy phosphatidylcholine (HSPC) was very effective for stabilization of aerosolized IFN-γ (DSPC/DPPG = 10/1, HSPC/DSPG = 10/1). HSPC/DSPG liposome was efficient at the concentration higher than 12.5 μmols/ml for the stabilization of 5×10^5 JRU/ml of IFN-γ. In considering the mechanism of this stabilization, the results of gel filtration chromatography suggest that IFN-γ is inactivated by polymerization or aggregation in nebulization, while the inactivation is suppressed by liposomes due to their adsorption to IFN-γ.

Keywords: Stabilization; Recombinant interferon-gamma (IFN-γ); Liposome; Aerosol; Nebulization

1. Introduction

Much efforts have been made to develop ways to deliver proteins and peptides via pulmonary route (Adjei and Gupta, 1997), since these drugs are generally unstable via oral administration. Recombinant interferon gamma (IFN-γ) is one of a cytokine family that has many functions in viral infection, immune response and inflammation (Jaffe et al., 1991; Sokolova et al., 1993; Herrmann and Stricker, 1995; Adjei and Gupta, 1997). Some investigators have tried aerosolized IFN-γ for clinical use against viral infection or cancer. The efficacy of aerosolized IFN-γ against tuberculosis (Condos et al., 1997) and adult T cell leukemia–lymphoma (ATLL) (Tara, 1990) was reported. Unfortunately, IFN-γ was found to be unstable in nebulization (Tara, 1993). One possible way of stabilizing it is achieved by using liposomes, which have been tried as a carrier for introducing drugs to the lungs (Tayler et al., 1989; Vidgen et al., 1995). Liposomes can incorporate both lipophilic and hydrophilic drugs in their

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vesicles. Also, liposomes may not injure the lungs (Waldrep et al., 1997), since phospholipids are components of alveolar surfactants. We have reported a method for producing and characterizing small and homogeneous liposomes (30–50 nm) by microfluidization (Hirano et al., 1996; Jizomoto et al., 1996). The present study reports the stabilization of aerosolized IFN-γ by adding small empty liposomes in nebulization, and analysis of the mechanism for this stabilization.

2. Materials and methods

2.1. Materials

The IFN-γ used in this study was Immunomacs® with a specific activity of 3×10^6 JRU/0.683 mg protein/vial, and was product of Shionogi & Co., Ltd. (Osaka, Japan).

Fluorescein isothiocyanate-dextran 40 (molecular weight 40 000; FD-40) and human serum albumin (globulin free grade) were obtained from Sigma (St. Louis, USA). Hydrogenated soy phosphatidylcholine (HSPC), distearoyl-L-α-phosphatidylcholine (DSPC), dipalmitoyl-L-α-phophatidylcholine (DPPC), distearoyl-L-α-phosphatidyl glycerol (DSPG) and dipalmitoyl-L-α-phosphatidylglycerol (DPPG) were obtained from Nippon Fine Chemical Co., Ltd. (Osaka, Japan). Egg phosphatidylcholine (EggPC) was obtained from Asahi Chemical Industry Co., Ltd. (Nobeoka, Japan). Tertiary butyl alcohol and maltose were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). NaCl, NaH_2PO_4 and $Na_2HPO_4 \cdot H_2O$ were purchased from Nacalai Tesque Inc. (Kyoto, Japan). These reagents were all of analytical grade. Dulbecco's phosphate-buffered saline (PBS, Ca^{+2} and Mg^{+2} free) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The H_2O used was of injectable grade and obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2. Preparation of liposomes

To prepare liposomes by microfluidization, 10 g of phosphatidylcholine and 0.9 g of phosphatiylglycerol were solubilized in 200 ml tertiary butyl alcohol at 60°C and lyophilized in a freeze drier (Type 10 l, Labconco Co., Ltd., MO, USA). They were hydrated with 200 ml sterilized 10% maltose aqueous solution using a Polytron homogenizer (Kinematica AG, Luzern, Switzerland) at room temperature. This method gave multilamellar liposomes (MLVs). The suspensions of these MLVs were reduced in size by microfluidization using a Mizuho 110H (Mizuho Industrial Co., Ltd., Osaka, Japan) in a water bath maintained above the phase transition temperature of phospholipids. The suspension was continuously sterilized with a filter (Millex GV, 0.22 μm, Japan Millipore Co., Ltd., Tokyo, Japan). The liposome size was determined by a quasi-elastic light scattering method using a submicron particle analyzer (Model N4, Coulter Co., Ltd., FL, USA). The compositions and average sizes of liposomes prepared are listed in Table 1.

2.3. Assay procedures of IFN-γ, FD-40, phospholipids, and proteins

IFN-γ was measured by immunoassay and bioassay. Immunoassay was performed using the ELISA-assay-system (Cytoscreen™, human IFN-γ) obtained from BioSource International Inc. (CA, USA). Bioassay was performed using FL-5 cells (human) and Sindvis virus. As IFN-γ is adsorbed easily onto glass or polystyrene, the containers, micropipette's tip and other components made of polypropylene were used. The concentration of FD-40 was measured fluorometrically (excitation wavelength: 495 nm, emission wavelength: 517 nm) using a Hitachi

Table 1
Liposome composition and size distribution[a]

Liposome composition	Size (nm, average ± SD)
HSPC/DSPG = 10/1	45.0 ± 24
DSPC/DPPG = 10/1	28.5 ± 19
EggPC/DSPG = 10/1	43.7 ± 23
EggPC	40.8 ± 24

[a] Liposomes were prepared by microfluidization from multilamellar vesicles (MLVs). Liposome sizes were determined by a quasi-elastic light scattering method using Coulter N4.

F-4010 instrument (Hitachi Ltd., Tokyo, Japan). The concentration of phosphatidylcholine was determined by an enzyme method using a PL assay kit obtained from Nippon Shoji Co., Ltd. (Osaka, Japan). The concentration of total protein was determined with a protein assay reagent (Bio Rad Co., Ltd., CA, USA).

2.4. Gel filtration chromatography

Bound IFN-γ to liposomes and unbound IFN-γ were separated by gel filtration chromatography, using a Sephadex G-75® column (0.9 × 25 cm) and an eluent composed of PBS containing 1% human serum albumin at a flow rate of 0.5 ml/min. The eluent was collected at every minute and the concentrations of IFN-γ and phosphatidylcholine in each fraction were measured.

The IFN-γ solutions before and after nebulization were analyzed by HPLC system of gel filtartion chromatography. The samples were injected into a HPLC system using an LKB pump connected to a TSK gel $SW3000_{XL}$ column (7.6 mm × 30 cm) (Tosoh Co., Ltd., Osaka, Japan). IFN-γ, HSA and liposome were detected at 280 nm (UV). The mobile phase of PBS containing 1% human serum albumin was kept at a flow rate of 1.0 ml/min.

2.5. Characterization of aerosols produced from a jet nebulizer

Aerosols were produced from 5 ml of IFN-γ solution by a jet nebulizer (Nippon Shoji, Osaka, Japan). The nebulizer was operated at 6 l/min. The aerosols atomized were collected in PBS solution in a tube with many small holes in the trap chamber (Fig. 1).

The size distribution of the aerosols produced by the jet nebulizer was determined by a light scattering method using Galai *cis*-1 (Gala) Production Ltd., Israel) in nitrogen gas flow.

2.6. Evaluation for the mass balance of aerosols produced from a jet nebulizer

FD-40 is stable in the nebulization (data not shown). FD-40 solution was aerosolized in a jet nebulizer. At 5, 10, 20 and 30 min, the amount of FD-40 recovered from the reservoir and the trap chamber were calculated (Fig. 2). The sum of FD-40 recovered from the reservoir and the trap chamber was almost 90% of the initial amount independent of the sampling time.

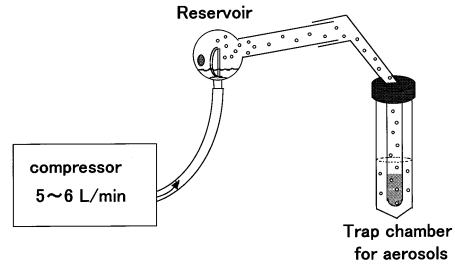

Fig. 1. Aerosols produced by jet nebulizer. These aerosols were trapped in a container (trap chamber) filled with 7 ml PBS, after being led there by a tube with a lot of small holes at its tip.

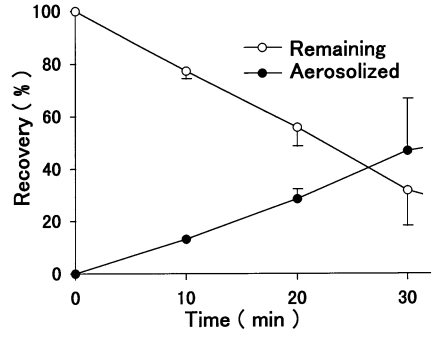

Fig. 2. Time profiles of the recovery of FD-40 solution from jet nebulizer were tested. The FD-40 solution (5 ml) was nebulized in the jet nebulizer. The recovery percentages of the initial amount in the reservoir and the trap chamber were calculated at 0, 10, 20 and 30 min. N = 3. ○, Recovery of the reservoir; ●, recovery of the trap chamber.

3. Results

3.1. Stabilization of IFN-γ in nebulization

For 30 min nebulization, the aerosolized IFN-γ was being collected in the PBS solution in trap chamber. IFN-γ solution without liposomes was recovered only 0.4 ± 0.2% of the initial amount in the trap chamber (aerosolized) and 3.1 ± 0.7% in

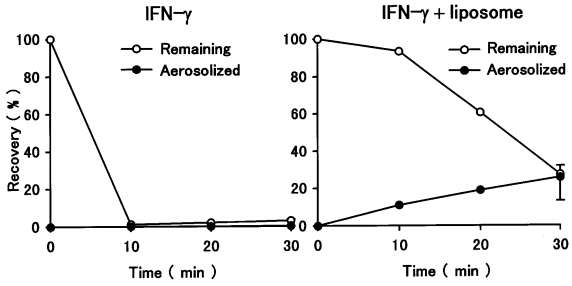

Fig. 3. (a) Stability of IFN-γ during nebulization by jet nebulizer. Each point is represented the mean value. IFN-γ, 5×10^6 JRU/ml; 5 ml. ○, Recovery of IFN-γ remaining in reservoir; ●, recovery of aerosolized IFN-γ. (b) Stability of IFN-γ with liposome. Each point represents the mean value. IFN-γ, 5×10^5 JRU/ml; Liposome, HSPC/DSPG = 10/1, 25 μmols/ml. ○, Recovery of IFN-γ remaining in the reservoir; ●, recovery of aerosolized IFN-γ.

the reservoir (remaining) (Fig. 3a). On the other hand, HSPC–DSPG liposome added to the reservoir increased the recovery of IFN-γ to 25.7 ± 12.6% in the trap chamber and 27.2 ± 4.7% in the reservoir (Fig. 3b). The effect of the liposome composition on the stabilization of IFN-γ was also examined. Table 2 shows the recovery percentage of aerosolized or remaining IFN-γ after 30 min nebulization. The DSPC–DPPG and HSPC–DSPG liposomes were very efficient for the stabilization of nebulized IFN-γ, while EggPC liposome was inefficient. The biological activity of IFN-γ was kept in the presence of liposome (data not shown).

Table 2
Stability of IFN-γ in the jet nebulizer[a]

Liposome	% of Recovery	
	Remaining	Aerosolized
None	3.1 ± 0.7	0.4 ± 0.2
HSPC/DSPG	27.2 ± 4.7	25.7 ± 12.6
DSPC/DPPG	29.8 ± 2.6	43.1 ± 16.6
EggPC/DSPG	16.2 ± 13.0	15.8 ± 2.6
EggPC	3.7 ± 1.0	1.2 ± 0.4

[a] The % of IFN-γ remaining in the reservoir and the % of aerosolized IFN-γ are listed with or without liposome. IFN-γ was 5.0×10^5 JRU/ml and liposome was 25 μmols/ml in the jet nebulizer. The values are represented mean ± SD. ($N = 3$).

Table 3
Effects of liposome concentration on IFN-γ stability[a]

Liposome concentration (μmol/ml)	% of Recovery	
	Remaining	Aerosolized
0.0	3.1 ± 0.7	0.4 ± 0.2
2.5	5.2	14.7
5.0	4.1	9.1
12.5	15.6	22.1
25.0	27.2 ± 4.7	25.7 ± 12.6

[a] The effects of the liposome concentration were tested on the stabilization of IFN-γ. The % recovery of aerosolized and remaining IFN-γ after 30 min nebulization in the jet nebulizer are listed. Liposome, HSPC/DSPG = 10/1; IFN-γ, 5×10^5 JRU/ml.

3.2. Effects of liposome concentration on IFN-γ stability

The recovery of aerosolized IFN-γ (5.0×10^6 JRU/ml) was examined at the various concentrations of the liposome (HSPC–DSPG) from 2.5 to 25 μmols/ml. With the increase in the concentration, aerosolized IFN-γ was stabilized. The concentration of liposome higher than 12.5 μmols/ml was effective on IFN-γ stability (Table 3).

3.3. Analysis of aerosols produced by nebulizer

The concentration of each component of the aerosols produced by the jet nebulizer was deter-

Table 4
Components of aerosols produced by jet nebulizer[a]

Liposome	% of Recovery (mean ± SD)		
	IFN-γ	Total protein	Phospholipids
None	0.4 ± 0.2	23.1 ± 5.6	–
HSPC/DSPG	25.7 ± 12.6	33.8 ± 11.2	44.1 ± 5.3
DSPC/DPPG	43.1 ± 16.6	34.1 ± 3.0	32.2 ± 4.0
EggPC/DSPG	15.8 ± 2.6	35.7 ± 4.7	41.8 ± 5.4
EggPC	1.2 ± 0.4	32.6 ± 3.2	38.4 ± 7.1

[a] The components of aerosols produced by the jet nebulizer were analyzed, which had been collected in the trap chamber for 30 min. The concentrations of IFN-γ, total protein and phospholipids of aerosols were measured. $N = 3$.

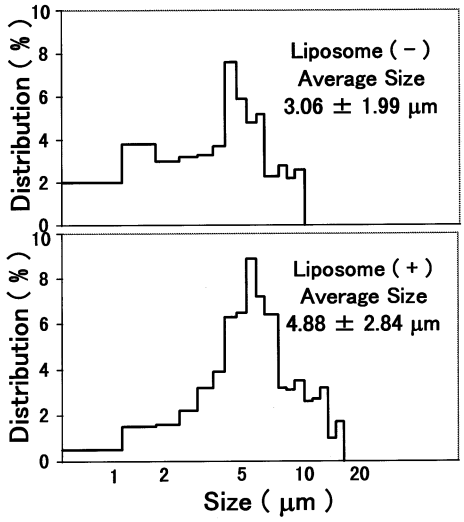

Fig. 4. Size distributions of aerosols produced by jet nebulizer with or without liposome determined by a light scattering method using Galai-*cis*-1. The upper column shows that without liposome and the lower that with liposome. Liposome, HSPC/DSPG = 10/1.

mined. Table 4 shows the recoveries of IFN-γ, total protein and phospholipids in aerosols after 30 min of nebulization. The recovery of IFN-γ was dependent on the kind of the liposomes. However, the total protein and phospholipids were recovered equally independent of them. These results support that the nebulizer produce the aerosols normally in our any investigation with the different liposome composition.

3.4. Pharmaceutical characterization of the aerosols

Fig. 4 shows the typical size distributions of aerosol droplets produced by the jet nebulizer without or with liposome. The mean values of them without and with the liposome were 3.06 ± 1.99 μm and 4.88 ± 2.84 μm, respectively. The latter shifted slightly larger than the former, and the distribution percentage of aerosol droplets over 10 μm increased a little. Table 5 shows that similar results were obtained from other liposome compositions.

Table 6 shows the average sizes of liposomes did not change before and after the nebulization.

Table 5
Sizes of aerosols with or without liposome[a]

Liposome	Size (μm, average ± SD)
None	3.06 ± 1.99
HSPC/DSPG	4.88 ± 2.84
EggPC/DSPG	3.79 ± 2.29
EggPC	4.99 ± 3.06

[a] The sizes of the aerosol droplets produced by the jet nebulizer were measured by a light scattering method using Galai-*cis*-1.

3.5. Mechanism of IFN-γ stabilization by liposome

A mixture of IFN-γ and liposome was eluted by gel filtration chromatography (Sephadex G-75®). The concentrations of IFN-γ and phospholipid were measured in each fraction. Fig. 5 shows the elution profiles of IFN-γ resulting from the trials of two mixtures of IFN-γ and liposome, which was composed of HSPC–DSPG or EggPC. In the former, IFN-γ was mainly detected in the liposome fractions (nos. 10–16). While in the latter, it was detected not in those fractions, but in the IFN-γ fractions (nos. 16–). These suggest that IFN-γ is adsorbed more strongly to the HSPC–DSPG liposome than EggPC liposome.

The IFN-γ solution remaining in the reservoir was analyzed by HPLC system of gel filtration chromatography (Fig. 6a), after 30 min nebulization. An unknown peak (P) was detected in the front of IFN-γ in the chromatogram, which had

Table 6
Sizes of liposome after nebulization[a]

Liposome	Liposome size (nm, average ± SD)		
	Reservoir[b]		Aerosol[c]
	Initial	30 min	30 min
HSPC/DSPG	45.0 ± 24	42.5 ± 23	45.1 ± 20
EggPC	40.8 ± 16	60.8 ± 120	47.3 ± 62

[a] The liposome sizes were measured by a quasi-elastic light scattering method using Coulter N4 before and after nebulization by the jet nebulizer.
[b] The sample remaining in the reservoir of the nebulizer.
[c] The sample collected in the trap chamber.

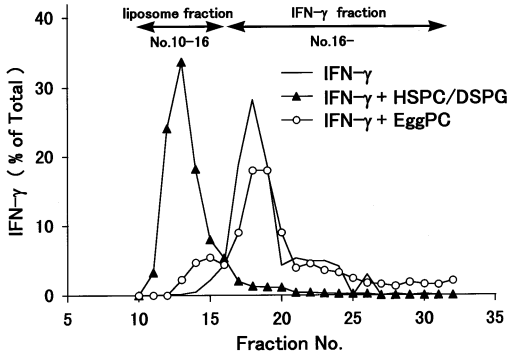

Fig. 5. Gel filtration chromatography of IFN-γ and some of its mixtures with liposome. The percentage of IFN-γ in each fraction to the total IFN-γ recovery was plotted. —, IFN-γ; ▲, the mixture of IFN-γ and HSPC/DSPC liposome; ○, the mixture of IFN-γ and EggPC liposome.

not been observed in that of the initial sample (data not shown). It was clear that the height of peak P decreased when the liposome was added to the solution (Fig. 6b). The percentage of P production in relation to the initial IFN-γ (on the assumption that the UV absorption intensity of P is same as that of IFN-γ) with or without liposome (HSPC–DSPG) was 27 and 97%, respectively. These results clearly demonstrate that the addition of the liposome strongly protect the formation of P in nebulization.

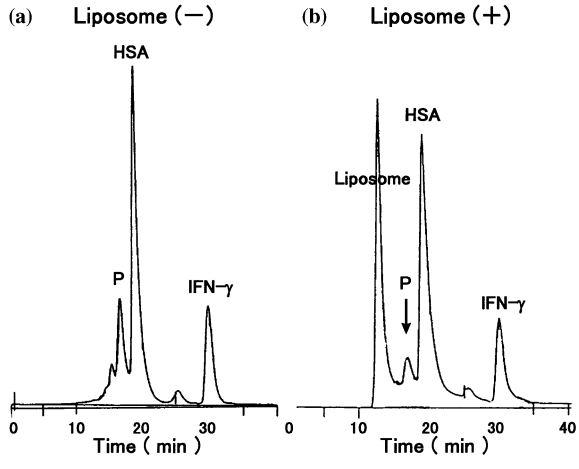

Fig. 6. (a) The typical chromatogram of gel filtration chromatography of nebulized IFN-γ solution remaining in the jet nebulizer reservoir. A new product (P) was detected in the chromatogram, it had not been present before nebulization. IFN-γ, 5×10^5 JRU/ml. (b) The typical chromatogram of gel filtration chromatography of nebulized IFN-γ solution with liposome remaining in the reservoir. Liposome, HSPC/DSPG = 10/1, 25 μmols/ml.

4. Discussion

The efficacy of liposome for the stabilization of aerosolized IFN-γ was clearly dependent on its composition (Table 2). HSPC–DSPG and DSPC–DPPG liposomes were much more effective to stabilize IFN-γ than EggPC liposome in our investigations. The results of gel filtration chromatography shows that the stronger the interaction between IFN-γ and liposome is, the greater the effect of stabilization of IFN-γ is (Fig. 5). It is considered that a part of the IFN-γ molecule was adsorbed to liposomes and protected against polymerization or aggregation of it (Fig. 6a and 6b).

IFN-γ, with a molecular weight of about 17 000 (146 amino acids), is known to be inactivated by mechanical stress (Cartwright, 1977). As the formulation of IFN-γ on the market (Immunomacs®) contains cysteine hydrochloride as a stabilizer, its solution is stable. However, it is not effective for stabilization of aerosolized IFN-γ, because the mechanical stress is very strong in nebulization. It is likely that the distilfide bond is formed intra- or intermediate molecule of IFN-γ in nebulization, because IFN-γ has two cysteines. It must be very easy to aggregate IFN-γ by such a strongly mechanical shearing.

Ishihara et al. (1991) reported an interaction between IFN-γ and liposome. In their study, IFN-γ was shown to interact with the negatively charged part of the liposome. Our results suggest two possibilities about its interaction, one is the negative charge of phosphatidylglycerol and the other is the hydrophobic interaction with the acyl chain of phospholipid. Three negative charged liposomes, HSPC–DSPG, DSPC–DPPG and EggPC–DSPG liposome, were compared on the stabilization effect of IFN-γ in nebulization. HSPC–DSPG and DSPC–DPPG liposome, which strongly adsorb IFN-γ rather than EggPC–DSPG one, could stabilize IFN-γ effectively com-

pared to EggPC–DSPG liposome. The hydrophobic interaction between IFN-γ and liposome seems to be necessary on the stabilization in nebulization. EggPC–DSPG liposome was more effective than EggPC liposome, because DSPG seems to give not only a negative charge but also a hydrophobicity to EggPC liposome.

HSPC–DSPG liposome could stabilize 5×10^5 JRU/ml of IFN-γ at higher than 12.5 μmols/ml of lipid concentration (Table 3). In this condition, the number of liposome associated with a IFN-γ molecule can be calculated. This liposome was considered as a small unilammelar vesicle (SUV, size 45 nm). The number of liposome (N_{liposome}) is given by a following equation.

$$N_{\text{liposome}} = \frac{C_{\text{liposome}}}{(4\pi r^2 d \times D)}$$

where C_{liposome}, r, d and D are concentration of liposome, radius of liposome, lipid bilayer thickness and density of liposome suspension, respectively. When d is 5 nm (Goldeliy et al., 1996), D is 1.0 g/ml and the concentration of IFN-γ is 0.114 mg/ml ($= 5.0 \times 10^5$ JRU/ml, molecular weight: 17 000), the number of liposome associated with a IFN-γ molecule ($N_{\text{liposome}}/N_{\text{IFN-}\gamma}$) is calculated to about 100.

The size distributions of aerosol droplets produced by nebulizers showed almost no change with or without liposomes (Fig. 4 and Table 5). This shows that the addition of liposomes will not interfere with the delivery of IFN-γ to the pulmonary alveoli. Some researchers reported that the size of liposomes changed during nebulization depending on the time or composition of liposomes (Waldrep et al., 1994; Leung et al., 1996). In this study, the average sizes of liposomes in both the reservoir and the trap chamber did not change after nebulization (Table 6). Because, our liposomes, especially HSPC–DSPG and DSPC–DPPG, were too small and too rigid to change by fusion, aggregation and disruption.

IFN-γ administered via the pulmonary route is expected to be efficacious against lung cancer and some infections. Goldbach et al. have already reported the utility of IFN-γ incorporated into liposomes (Friede et al., 1994; Goldbach et al., 1995). When liposomes are used as a carrier to the lungs, they enable the sustained release of a drug (Bennett, et al., 1994; Arppe et al., 1998) and special delivery to alveolar macrophages (Schreirer et al., 1993; Tayor and Farr, 1993). In conclusion, our most useful finding in this study is that the simple addition of empty small liposomes to the IFN-γ solution can stabilize the aerosolized IFN-γ. This method means that we need neither incorporating IFN-γ into liposome vesicles nor removing free IFN-γ from them. The effect of liposomes was also observed using with an ultrasonic nebulizer (data not shown). Therefore, the simple addition of empty liposome should be useful for the IFN-γ nebulization clinically.

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