Interaction of Recombinant Human Interferon- γ with Liposomes

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The interaction of recombinant human interferon- γ (IFN) with egg phosphatidylcholine liposomes was studied. IFN which binds to liposomes was dependent on the liposomal charge and pH, and a preferential binding was observed in negatively charged liposomes at pH 7.4–10. Electron-microscopic observation showed that the increased liposomal turbidity induced by IFN was due to liposomal aggregation, and the increased turbidity could be decreased by the addition of NaCl. Thus, ionic binding may participate in this interaction. But, when the incubation time was longer, the liposomal aggregation was not decreased by the addition of NaCl, and the leakage of the entrapped marker, calcein, was observed. Electron-microscopic analysis showed that this leakage resulted from the morphological change of liposomes. From these findings, ionic binding may participate in the interaction between IFN and liposomes and then develop a morphological change in negatively charged liposomes under the neutral pH condition.

Keywords interferon- γ ; liposome; interaction; binding; aggregation; leakage

Liposomes are currently being explored as a device for drug delivery in order to increase the effect of chemotherapeutic agents.¹⁾ Interferon- γ (IFN) is a peptide which has antiviral,²⁾ antiproliferative³⁾ and immunoregulatory⁴⁾ effects, and is used in the treatment of viral hepatitis^{5,6)} and certain kinds of cancer.^{7,8)}

Previously, we reported the encapsulation of IFN into asialofetuin-labeled liposomes (AF-liposomes)⁹⁾ and their receptor-mediated uptake by isolated rat hepatocytes.^{9–12)} In the preparation process of IFN-encapsulated AF-liposomes, a high apparent entrapment efficiency was obtained, which suggests that IFN interacts with the liposomal membrane. On the other hand, the interaction of peptides such as insulin,¹³⁾ neurotoxins¹⁴⁾ and immunomodulators,¹⁵⁾ with the lipid bilayer examined and induced some increase in the membrane permeability of liposomes and the liposomal fusion.

Thus, in this report, the features of the interaction between IFN and liposomes were investigated through IFN binding to liposomes, the changes in liposomal aggregation, and the leakage of calcein under the conditions of different pH and/or ionic strength. Ionic binding may participate in the interaction between IFN and liposomes, and then develop a morphological change in negatively charged liposomes.

Experimental

Materials Recombinant human IFN (Meiji Seika Kaisya Ltd., Tokyo, Japan) with antiviral activity ranging from 1×10^7 to 2×10^7 units/mg was dissolved in 50 mM phosphate buffer (pH 5.5) containing 2 mM dithiothreitol and stored at 4°C. Before the experiments, the IFN solution was dialyzed against physiological saline at 4°C for 4 h to exclude the effect of the phosphate buffer on the following experiments. Egg yolk phosphatidylcholine (PC, average molecular weight; 774) was kindly provided by Nippon Fat and Oil Co., Ltd. (Tokyo). Cholesterol (Chol) and calcein were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Egg yolk phosphatidic acid (PA, average molecular weight; 726) and stearylamine (SA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Preparation of Liposomes Saline-loaded liposomes were prepared by the vortexing method. Liposome suspension was extruded through a polycarbonate membrane (pore size 0.4 μ m, Nuclepore Inc., Pleasanton, CA, U.S.A.) and washed four times by centrifugation at $12000 \times g$ for 10 min.

Liposomes containing 90 mM calcein (pH 7.4, 97% self-quenching) were prepared by the reverse-phase evaporation technique,¹⁶⁾ and extruded through a polycarbonate membrane (pore size; 0.2 μ m). Unencapsulated

calcein was removed by gel filtration on a Sepharose 4B column (Pharmacia LKB Biotechnology, Uppsala, Sweden).

The concentration of phospholipids in the liposome suspension was measured as inorganic phosphorus by the method of Chen *et al.*¹⁷⁾ The composition of liposomes was indicated with the molar ratio of the component.

Binding Assay Liposomes composed of various membrane components (5–10 μ mol lipid) were suspended in 1 ml of buffered saline solution having different pH (3–12), and incubated with 50 μ g of IFN at 4°C for 30 min. The buffer solutions containing 0.9% NaCl were as follows: pH 3–5: 10 mM citric acid–Na₂HPO₄, pH 7.4: 10 mM Tris–HCl, pH 9–10: 10 mM glycine–NaOH, pH 11–12: 10 mM Na₂HPO₄–NaOH. The incubated mixture was centrifuged at $22000 \times g$ for 10 min, and the IFN concentration in the supernatant was determined by the method of Lowry *et al.*¹⁸⁾ The amount of IFN bound to the liposomes was expressed as percent relative to used IFN.

Fluorescence Leakage Assay Liposomes (200 nmol lipid) encapsulating self-quenched calcein were incubated in 2 ml of buffered-saline solution (pH 7.4–11) with IFN (25–100 μ g). The fluorescence was measured at 37°C (Ex. 495 nm, Em. 520 nm) with a fluorescence spectrophotometer Model 650-40 (Hitachi Co., Ltd., Tokyo, Japan), and the fluorescence intensity was expressed as percent of the fluorescence obtained by the addition of Triton X-100 (final concentration; 0.1%). The leakage rate was defined as the maximum differential value in the change of the relative fluorescence.

Turbidity Development Liposomes were incubated in 2 ml of buffered-saline solution (pH 7.4–11) at 37°C with a varying amount (50–200 μ g) of IFN. After rapid mixing, the turbidity was measured as absorbance at 400 nm with a spectrophotometer model U-3200 (Hitachi Co., Ltd.) for 5 or 25 min. The initial absorbance of liposome suspension was adjusted to 0.3 (2.4 μ mol lipid/2 ml) and the changes in turbidity were indicated as Δ absorbance.

Electron Microscopic Observation Liposomes (molar ratio; PC:PA:Chol=4:3:6, 500 nmol of lipid), which were incubated with or without IFN (50 μ g) in 10 mM Tris–HCl buffered saline (pH 7.4, 1 ml) at 37°C for 1, 5, or 30 min, were placed on a 300-mesh copper grid. After 1 min, excess liposome suspension was removed with the edge of a piece of filter paper. The liposomes on the grid were negatively stained with 3% phosphotungstic acid, pH 7.4, for 1 min. These samples were observed with an electron microscope model H-600 (Hitachi Co., Ltd.).

Results

Binding of IFN to Liposomes Figure 1 shows the binding of IFN to liposomes of various lipid compositions at pH 7.4. IFN hardly bound to neutral (molar ratio; PC:Chol=7:6) and positively charged (PC:SA:Chol=6:1:6) liposomes, but bound to negatively charged (PC:PA:Chol) liposomes. The more PA that was contained in the negatively charged liposomes, the more binding was observed. Figure

Fig. 1. Effect of Lipid Composition on the Binding of IFN to Liposomes

IFN was added to each liposomal suspension (10 g IFN/mol lipid) in 10 mM Tris-HCl buffered saline (1 ml, pH 7.4). The experiment was carried out at 4°C. Each bar indicates the average of two experiments. A, PC:Chol=7:6; B, PC:SA:Chol=6:1:6; C, PC:PA:Chol=6:1:6; D, PC:PA:Chol=5:2:6; E, PC:PA:Chol=4:3:6.

Fig. 2. pH Dependency of the Binding of IFN to Negatively Charged Liposomes

Each point represents the mean $\pm$ S.D. ($n=3$). Liposomes (PC:PA:Chol=6:1:6) were incubated with IFN (5 g IFN/mol lipid) in various pH of buffered saline solution (1 ml). The experiment was carried out at 4°C.

2 shows the effect of pH on the IFN binding to negatively charged (PC:PA:Chol=6:1:6) liposomes. IFN showed a higher rate of binding at the pH region of 7.4–10, but hardly bound to the liposomes at and above pH 11.

Leakage of Calcein from Liposomes Figure 3 is a typical result of the calcein leakage from negatively charged (PC:PA:Chol=4:3:6) and neutral (PC:Chol=7:6) liposomes induced by the addition of IFN in 10 mM Tris-HCl buffered saline (pH 7.4). In the case of the negatively charged liposomes, the intensity of fluorescence began to increase after a lag time of several minutes, and it reached about 60% relative to the total fluorescence intensity after an adequate incubation time. On the other hand, calcein was not leaked from the neutral liposomes by the addition of IFN.

At pH 7.4, the leakage rate of calcein from negatively charged liposomes increased with increasing PA content and IFN concentration (Fig. 4). Table I indicates that the leakage rate of calcein from negatively charged liposomes (PC:PA:Chol=4:3:6) was affected by pH.

Turbidity Development and Its Decrease By the addition of IFN to the liposomal suspension at pH 7.4 (Fig. 5), an increase in turbidity was observed in negatively charged

Fig. 3. Typical Leakage of Calcein from Liposomes Induced by IFN

Negatively charged (PC:PA:Chol=4:3:6, —) and neutral (PC:Chol=7:6, ---) liposomes encapsulating 90 mM calcein were incubated with IFN (100 μ g IFN; 500 g/mol lipid) at 37°C under neutral conditions (pH 7.4). The arrow indicates the addition of Triton X-100 to disrupt liposomes.

Fig. 4. Effect of Lipid Composition on the Leakage Rate of Calcein from Liposomes by IFN

Liposomes encapsulating 90 mM calcein were incubated with IFN (125–500 g IFN/mol lipid) at 37°C under the neutral conditions (pH 7.4). Each point indicates the average of two experiments. ●, PC:PA:Chol=4:3:6; ▲, PC:PA:Chol=6:1:6; ■, PC:Chol=7:6.

TABLE I. Effect of pH on the IFN-Induced Leakage of Calcein from Liposomes

pH	Leakage rate (%/min)
7.4	11.1
9.0	7.0
11.0	4.0

Lipid composition; PC:PA:Chol=4:3:6. One hundred micrograms of IFN was added to liposome suspension (670 g IFN/mol lipid).

liposomes (PC:PA:Chol=4:3:6), but not in neutral liposomes (data not shown). The intensity of turbidity in the suspension of the negatively charged liposomes was dependent on IFN concentration.

Table II shows the effects of pH and NaCl concentration on the turbidity development of negatively charged liposomes (PC:PA:Chol=4:3:6). At pH 7.4, the turbidity development until 5 min after the IFN addition was inversely proportional to the NaCl concentration, and when the NaCl

Fig. 5. Effect of IFN Concentration on Turbidity Development in Liposome Suspension

Liposomes and IFN (20.8–83.3 g IFN/mol lipid) were incubated at 37°C under neutral conditions (pH 7.4). Lipid composition; PC:PA:Chol=4:3:6. ●, 100 μg IFN/ml; ▲, 50 μg IFN/ml; ■, 25 μg IFN/ml; ○, without IFN.

TABLE II. Effect of pH and NaCl Concentration of IFN-Induced Turbidity Development of Liposome Suspension

pH	NaCl conc. (%)	Δ Absorbance
7.4	0.45	1.18
7.4	0.90	0.39
7.4	1.80	0.09
9.0	0.90	0.14
11.0	0.90	0.02

Lipid composition; PC:PA:Chol=4:3:6. One hundred micrograms of IFN was added to liposome suspension (41.7 g IFN/mol lipid).

Fig. 6. Effect of NaCl Addition on the Turbidity Development Induced by IFN

Each arrow indicates the timing of NaCl addition after IFN addition (83.3 g IFN/mol lipid, pH 7.4). Lipid composition; PC:PA:Chol=4:3:6. —; IFN (100 μg) was added. ---; NaCl (final 2.7%) was added. ----; NaCl (final 2.7%) was added after the addition of IFN (100 μg).

concentration remained constant (0.9%), a lower turbidity development was observed in a higher pH region.

The results in Fig. 6 show the change in the IFN-induced turbidity by the addition of NaCl. When NaCl (final concentration; 2.7%) was added to negatively charged liposomes (PC:PA:Chol=4:3:6), a slight increase in turbidity was observed (control experiment). When NaCl was added to the liposomal suspension at 1 or 2 min after

Fig. 7. Negative-Stain Electron Micrographs of Liposomes

Liposomes (PC:PA:Chol=4:3:6, 500 nmol lipids) were incubated with IFN (50 μg/ml) under the neutral conditions (pH 7.4) and were stained with 3% phosphotungstic acid at indicated time. A, before the addition of IFN; B, 1 min after the addition of IFN; C, 5 min after the addition of IFN; D, 30 min after the addition of IFN; magnification, ×10000.

the IFN addition, the increased turbidity induced by IFN decreased to the control level. However, the addition of NaCl at 5–17 min after the IFN addition could not decrease the turbidity to the control level.

Electron Microscopy The effects of IFN on the change in the size and morphology of liposomes were examined with electron microscopic observation. One minute after the addition of IFN liposomal aggregation appeared, and with a lapse of time the aggregation advanced and each liposome became smaller in size (Fig. 7).

Discussion

The binding of IFN to liposomes was dependent on the liposomal charge and pH, and a preferential binding was observed in negatively charged liposomes at pH 7.4–10 (Figs. 1 and 2). The IFN-induced turbidity development in the suspension of negatively charged liposomes at pH 7.4 was dependent on the IFN concentration (Fig. 5), and was inversely proportional to the NaCl concentration (Table II). Because the pI value of IFN has been reported to be 8.6–10.4,^{9,19)} this binding may be caused by the ionic interaction between positively ionized IFN and negatively charged PA, one of the components of liposomal phospholipids. This consideration may be supported by the fact that human IFN is quite basic in character, having 27 basic amino acids, lysine and arginine, and especially in that some of them are localized on the amino acid sequences of 89–92 (Lys–Lys–Lys–Arg) and 131–134 (Lys–Arg–Lys–Arg).²⁰⁾ From the results of Fig. 6, the presence of two kinds of interactions between negatively charged liposomes and IFN are suggested: one is an ionic interaction which aggregates negatively charged liposomes and decreases by the addition of NaCl, and the other is a non-ionic interaction which may accompany a leakage of entrapped calcein and does not decrease by the addition of NaCl.

The lag time observed in the leakage of calcein (pH 7.4)

from negatively charged liposomes (Fig. 3) was in accordance with the time at which the complete reversion of IFN-induced turbidity development could no longer be observed by the addition of NaCl (Fig. 6). Thus, the leakage of calcein may be a subordinate phase following the transition from ionic to non-ionic interaction. If the calcein leakage results from the latter step, a change in membrane fluidity or liposomal fusion should occur. But no proof for either of these phenomena could be obtained using the fluorescence polarization or the resonance energy transfer technique (data not shown). Electron microscopic analysis showed that the liposomal aggregation grew tight and the liposomal size gradually became smaller with incubation time after adding IFN. Thus, the calcein leakage may have resulted from this morphological change of liposomes induced by IFN.

Under alkaline pH conditions, the calcein leakage (Table I) and turbidity development (Table II) in negatively charged liposomes were lower than those under neutral pH conditions. However, at pH 9.0, IFN showed a high binding to negatively charged liposomes (Fig. 2). Since the ionization of IFN hardly occurs at pH 9.0, IFN may bind to negatively charged liposomes through a non-ionic rather than an ionic interaction. Walter *et al.* reported that the polylysine-induced fusion of liposomes containing an acidic phospholipid, phosphatidylserine, occurred at an acidic pH, in which condition the negative charge of phosphatidylserine is considered to be neutralized.²¹⁾ Oku *et al.* reported that the tumor necrosis factor increased the permeability of negatively charged liposomes by hydrophobic interaction under the acidic condition, but the tumor necrosis factor did not affect the permeability of neutral liposomes.²²⁾ Polylysine is a basic peptide and the tumor necrosis factor is an acidic peptide. From these reports, the neutralization of a negative charge on the liposomal membrane may be important to the perturbation of the liposomal membrane. However, in the case of IFN, the ionic interaction with a negatively charged phospholipid under the neutral pH condition is the trigger for aggregation and calcein leakage. Thus, the cationic portions of an IFN molecule may bind to the negatively charged phospholipid and then induce a change in the integrity of liposomes. At pH 9.0, IFN does not ionize, and the observed binding (Fig. 2) may be caused by non-ionic interaction through a hydrophobic portion developed in an IFN molecule. To aggregate liposomes, an IFN molecule needs more than two binding sites for cross-linking of liposomes. The decrease in liposomal aggregation at pH 9–11 (Table II), indicates a reduction of multivalent binding between IFN and liposomal membrane. This consideration is based on the finding that the net charge and the conformation of peptides, such as

polyglutamate, in aqueous solution are affected by the pH and ionic strength of the solution.²³⁾ The magnitude of the cationic and hydrophobic portions in IFN molecules, which interact with the liposomal surface, may change according to the characteristics of the solution in which IFN is dissolved. IFN may show an affinity to liposomes through different types of interactions at different pH and ionic strength.

The above findings suggest that IFN bound to the outer surface of the liposome bilayer should be removed to keep negatively charged liposomes stable.

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