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Effect of dicetylphosphate or stearic acid on spontaneous transfer of protein from influenza virus-infected cells to dimyristoylphosphatidylcholine liposomes

Kentaro Kogure [a], Chiharu Nakamura [a], Otomo Okuda [a], Kyoko Hayashi [b], Masaharu Ueno [a,*]

[a] *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan*
[b] *Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan*

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Abstract

Membrane proteins, such as viral spike, were transferred spontaneously from influenza virus-infected cells to various liposomes. The protein transfer was enhanced by the presence of negative charged component dicetylphosphate (DCP) or stearic acid (SA) in dimyristoylphosphatidylcholine (DMPC) liposomes. The lowering of membrane fluidity did not relate to the effect of DCP or SA on protein transfer in this study. We considered that the alteration of membrane properties, such as construction of the surface or stability of transferred protein in liposomes, due to the specific structure of DCP or SA is responsible for the enhancement of spontaneous protein transfer by the presence of the amphiphilic components. © 1997 Elsevier Science B.V.

Keywords: Protein transfer; Dicetylphosphate; Stearic acid; Liposome; Virosome

1. Introduction

In order to reconstitute a transmembrane protein of cells or organelles, various detergents or organic solvents are generally used. However, treatment with these reagents has the possibilities to cause denaturation and inactivation of the target protein.

In recent years, it has been reported that the various membrane proteins are transferred spontaneously to liposomal membranes without a solubilization and reconstitution step using detergents or organic solvents [1–15]. Transfer of cytochrome b_5, which binds to the membrane surface due to its hydrophobic tail, was studied by many investigators [1–7]. That protein was considered to pass to liposomes by the

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DCP, dicetylphosphate; SA, stearic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; DDPC, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide; HA, hemagglutinin; PBS, phosphate buffered saline; Pi, phosphorus; FITC, fluorescein isothiocyanate; *P*, fluorescence polarization; Control-lipo, DMPC liposomes; DCP–lipo, DCP-containing DMPC liposomes; SA–lipo, SA-containing DMPC liposomes; Chol, cholesterol; Chol–lipo, Chol-containing DMPC liposomes

* Corresponding author. Fax: +81-764-345050.

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release into aqueous phase from lipid membrane as monomer, and transferred favorably to small unilamellar vesicles (SUV) rather than to large unilamellar vesicles (LUV) [4–7]. On the other hand, Huestis and his coworkers' study focused on the transfer of acetylcholinesterase and band 3 protein from erythrocytes to liposomes [8–10,15]. They found that transferred band 3 protein in liposomes held original activity and orientation in lipid bilayer as in erythrocytes [10]. Therefore, application of spontaneous protein transfer is appropriate for the reconstitution of membrane protein without detergent. In addition, they reported that acetylcholinesterase transfers more to liposomes when the recipient membrane is more fluid than the donor [8]. Further, Sunamoto and his coworkers reported that the addition of artificial lipid 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) into phosphatidylcholine (PC) liposomes enhanced protein transfer from a biological membrane such as erythrocytes and platelets [11,13,14]. They considered that the reason for the effect of DDPC on protein transfer was the enhancement of the holding ability of transferred protein into recipient membrane by intermolecular hydrogen bonding of those lipids [16,17]. Thus, many investigations have been done. Until now, however, no clear explanation has yet been made for the mechanism and regulatory factors of spontaneous protein transfer from biological membranes to liposomes.

Recently, for the purpose of preparing artificial membrane vaccine, virosomes; we examined spontaneous transfer of viral protein from influenza virus-infected cells to dimyristoylphosphatidylcholine (DMPC) liposomes, and succeeded in it [18]. Further, we found that the amount of transferred protein of liposomes incubated with influenza virus-infected cells was higher than that with uninfected cells, and that the ratio of the amount of transferred protein of the former to the latter increased in proportion to the diameter of liposomes except for SUV [18]. It was considered that the feature is due to the physical and structural properties of the phospholipid membrane based on the diameter of vesicles [18]. Thus, in this study, to obtain more information of spontaneous protein transfer for the efficient preparation of virosomes, we examined the effect of negative charged amphiphilic molecules dicetylphosphate (DCP) or stearic acid (SA) as the component of DMPC liposomes on spontaneous protein transfer from influenza virus-infected cells.

2. Materials and methods

2.1. Materials

Dimyristoylphosphatidylcholine (DMPC) was obtained from Nichiyu Liposome, (Tokyo). Dicetylphosphate (DCP) was purchased from Sigma (St. Louis). Stearic acid (SA) was obtained from Tokyo Kasei Kogyo (Tokyo). Other materials and reagents were of the highest grade commercially available.

2.2. Culture of CV-1 cells and infection with influenza virus

The experimental procedures of cell culture and of infection with influenza virus were the same as described previously [18]. CV-1 cells from monkey kidney were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, and were cultured in a CO_2 incubator at 37°C with CO_2 in humidified air. The cells, which grew in a monolayer, were infected with influenza virus NWS strain (H1N1) at 10 plaque-forming units per cell, and incubated at 37°C for 20–24 h. Expression of a viral glycoprotein, hemagglutinin (HA), on the cell surface was confirmed by using chicken erythrocytes, which bind to HA.

2.3. Preparation of liposomes

Liposomes were prepared by reverse phase evaporation method [19] in phosphate-buffered saline (PBS). Then, the liposome suspension was extruded several times through polycarbonate membrane filters for regulation of diameter about 200 nm. Diameter of liposome was determined by the quasi-elastic light scattering method using a laser particle analyzer (LPA 3000/3100, Otsuka Electronics, Osaka). The amount of lipid in liposome suspension was estimated as phosphorus (Pi) by the method of Ames [20]. The liposomal suspension was diluted to 0.1 mMPi with PBS before use.

In order to entrap the calcein into liposomes, 100

mM calcein/PBS solution was added instead of PBS at the process of reverse phase evaporation. Then, the size regulation of these liposomes, referred to as calcein–liposomes, was done in the same manner as described above. The unentrapped calcein was removed by ultra centrifugation at 284 400 × g for 30 min at 4°C just before use. For complete removal of unentrapped calcein, this centrifugal procedure was repeated 5 times.

2.4. Incubation of liposomes with CV-1 cells

CV-1 cell monolayer in 35 mm dish was washed twice with PBS. 1 ml of 0.1 mMPi liposome suspension was added into the dish and incubated at 37°C without shaking for 1 h. The length of an incubation was decided by our previous experience, i.e., the amount of transferred protein after 30 min incubation was almost the same after 1 h (unpublished data), and a number of cells removed from dish by the incubation for over 1 h. After the incubation, the liposome suspension was recovered from the dish and centrifuged at 2000 × g for 1 min. The supernatant was subjected to the assay.

2.5. Estimation of protein in recovered liposome suspension

The protein in recovered liposome suspension was estimated by the method of Lowry [21] using bovine serum albumin as a standard.

2.6. Determination of the amount of transferable protein on cell surface, and that of transferred protein to liposomes

The amount of transferable protein on surface of CV-1 cells was determined by using a fluorescent labeling reagent, fluorescein isothiocyanate (FITC). After cell monolayer in dish was washed twice with PBS, 1.5 ml of 0.1 mM FITC was added to the dish, and the cells were incubated for 2 h at 4°C for labeling the protein of cell surface with the fluorescent reagent. After the removal of the labeling reagent and the washing with PBS, 1.5 ml of glycine buffer (0.3 M glycine, 91.3 mM NaCl, 1.79 mM KCl, 8.94 mM Na_2HPO_4, 0.98 mM KH_2PO_4; pH 7.4) was added to the cell dish, and incubated for 5 min to terminate the labeling reaction. To determine the amount of labeled protein on cell surface, the cells, which were recovered from the dish by the use of a cell-scraper, were subjected to sonication to destroy membrane structure, and were solubilized with 10% SDS solution. The fluorescence intensity of the solubilized cell suspension was measured at 520 nm with excitation at 490 nm.

And to determine the amount of transferred protein from FITC-labeled cells to DMPC liposomes, liposome suspension was added into the labeled cell dish, followed by incubation for 1 h at 37°C. Then, liposome suspension was recovered, and the fluorescence intensity of it was measured at the same wavelength described above.

2.7. Estimation of the amount of remained calcein into liposomes after incubation with cells

After the incubation of 1 ml of 0.1 mMPi calcein-liposomes with cells at 37°C for 1 h, the concentration of calcein, which leaked out from liposomes, was measured by the fluorescence intensity at 520 nm with excitation at 490 nm (F_{leak}), but that of the remained calcein into vesicles was not detected due to self-quenching. Then, 10% Triton X-100 was added into the suspension for solubilization of liposomes to release the rest of calcein, and the fluorescence intensity was also measured as the amount of total calcein (F_{total}). Percentage of the amount of remained calcein into DMPC liposomes (F_{remain}) was calculated by Eq. (1).

$$F_{remain}(\%) = \{(F_{total} - F_{leak})/F_{total}\} \times 100 \quad (1)$$

The values of remained calcein percentage varied from ca. 10 to 40 depending upon the experimental conditions, but the reproducibility of the data was confirmed.

2.8. Measurement of the fluidity of liposomal membrane

The fluidity of liposomal membrane was measured as fluorescence polarization P of 1,6-diphenyl-1,3,5-hexatriene (DPH) [22] and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) [23] as a probe at hydrophobic and hydrophilic regions of lipid bilayer, respectively. 2 μl of 0.1 mM

DPH (or 0.25 mM TMA-DPH) was added into 2 ml of 0.1 mMPi liposome suspension (final concentration of DPH was 0.1 μM) and incubated for 1 h at 37°C in the dark. Then, P of DPH (or of TMA-DPH) was calculated from polarized intensities in the horizontal ($I_{\parallel}$) and vertical ($I_{\perp}$) directions, respectively, at 425 nm (or at 430 nm) excited at 360 nm by Eq. (2) [24]. G is the correction factor for instrument polarization.

$$P = (I_{\parallel} - I_{\perp} G)/(I_{\parallel} + I_{\perp} G) \quad (2)$$

2.9. Estimation of ζ-potential of liposomes

ζ-potential of liposomes was estimated by a LASER ZEE, model 501 apparatus (PEN KEM, New York). The liposome suspension was applied into the device, and ζ-potential was determined electrophoretically at 20°C [25].

3. Results

3.1. The amount of transferred protein from influenza virus-infected or uninfected cells to DMPC liposomes

For transfer of protein from cells to liposomes, DMPC liposome suspension was added into the cell dish, and incubated at 37°C for 1 h. After incubation of liposomes with influenza virus-infected cells or uninfected cells, we recovered liposome suspension from the dish, and determined the amount of protein. The protein content in recovered liposome, which was incubated with virus-infected cells, was slightly higher than that with uninfected cells (Table 1), but the difference between both was small. We reported previously that the amounts of transferred protein from influenza virus-infected and uninfected cells to the liposomes, the diameter of which was about 200 nm, were almost the same [18]. The result of Table 1 was consistent with our previous report.

Table 1
The amount of protein of recovered DMPC liposomes, which were incubated with uninfected cells or influenza virus-infected cells

The cells incubated with DMPC liposomes	Protein (μg ml^{-1})
Uninfected cells	6.72 ± 1.4
Influenza virus-infected cells	7.62 ± 0.94

These data are means of five different samples at the same time with standard deviation.
The reproducibility of these data was confirmed in experiments at different times. Experimental conditions are described in Section 2.

3.2. Confirmation of holding of liposomal structure during the incubation of DMPC liposomes with cells

To know whether the liposome membrane was destroyed in the process of protein transfer from cells in these experimental conditions, we measured fluorescent intensity of retained calcein, which was entrapped into liposomes, after incubation in the cases of without cells, with uninfected cells and with virus-infected cells (Table 2). In Table 2, the percentages of retained calcein were almost the same, indicating that calcein did not leak from liposomes by the incubation with cells except for passive transport in these conditions. Therefore, it was considered that liposome membrane was not disrupted in the process of spontaneous protein transfer.

3.3. The comparison of the amount of transferred protein between influenza virus-infected and uninfected cells

Further, we attempted to know the amounts of transferable protein of uninfected and virus-infected cells. As the transferable protein was assumed to exist on the surface of cells, we measured the fluorescent intensity, which may correspond to the amount of FITC-labeled protein on plasma membrane surface

Table 2
Effect of incubation at 37°C for 1 h with or without cells on the amount of entrapped calcein within DMPC liposomes

Conditions of incubation	Percentages of remained calcein after incubation (%)
Without cell	13.8 ± 4.3
With uninfected cells	11.5 ± 1.9
With influenza virus-infected cells	12.2 ± 0.90

We measured fluorescence intensity after the incubation, and estimated the percentage of the remaining calcein. The data are the average of five samples at the same time with standard deviation in each case.

Table 3
The fluorescence intensity of FITC-treated cells, which were infected with or without influenza virus, before incubation with DMPC liposomes (total), and of recovered liposomes after incubation with both cells (transferred)

FITC-labeled cells	Fluorescence intensity	
	Total	Transferred
Uninfected cells	16.1 ± 3.4	14.7 ± 3.1
Influenza virus-infected cells	24.5 ± 1.4	13.2 ± 1.8

The data are means of five samples at the same time with standard deviation. The reproducibility was confirmed in experiments at different times.

of both cells, before incubation with DMPC liposomes, and of recovered liposomes after treatment with cells as the content of transferred protein (Table 3). Although the total amount of FITC-labeled protein of virus-infected cells was significantly higher than that of uninfected cells, the contents of transferred FITC-labeled protein from both cells to liposomes were almost the same. This was consistent with the result of Table 1. As shown in Table 3, it appeared that little amount of labeled protein on the surface of uninfected cells remained after treatment with liposome suspension, whereas much labeled proteins still existed on the surface of virus-infected cells after incubation with liposomes. The remained fluorescent protein had transferability, because all of them transferred to liposomes by the reincubation of the post-treated cells with new liposome suspension (data not shown). Thus, we focused on the spontaneous protein transfer from influenza virus-infected cells.

3.4. The effect of DCP or SA on the amount of transferred protein from virus-infected cells

Next, we examined the effect of negative charged amphiphilic molecules dicetylphosphate (DCP) or stearic acid (SA) as the component of DMPC liposome on the amount of transferred protein from virus-infected cells (Fig. 1). As shown in Fig. 1, we found that the protein transfer from influenza virus-infected cells to DMPC liposomes was enhanced by the presence of such negative charged components. Thus, the amount of transferred protein of DCP-containing DMPC liposomes (DCP–lipo) increased with an increase in DCP content (Fig. 1), in particular, that of 30 mol% DCP–lipo was double to DMPC liposomes (Control-lipo). On the other hand, in the case of SA-containing DMPC liposomes (SA–lipo), the content of transferred protein was also enhanced by the presence of SA in DMPC liposomes, but that did not depend on the molar percentage of SA to DMPC (Fig. 1).

3.5. The electrostatic conditions and the amount of transferred protein of various liposomes

We measured ζ-potential, which reflects the electrostatic condition of membrane surface, of various liposome suspensions (Fig. 2). The ζ-potential of Control-lipo was almost zero, and that value became minus with the presence of negative charged components in DMPC liposomes. The value of negative ζ-potential of DCP–lipo increased with an increase in the content of DCP, that is, the value of ζ-potential indicated the content of negative charged component in liposomal membrane. But those of SA–lipos were almost the same independently of SA content at the preparation (Fig. 2A). Then, the amounts of transferred protein of various liposomes (data from Fig. 1) were plotted against ζ-potentials of them. As shown in Fig. 2B, a correlation seemed to exist between the content of transferred protein and ζ-potential of liposomes.

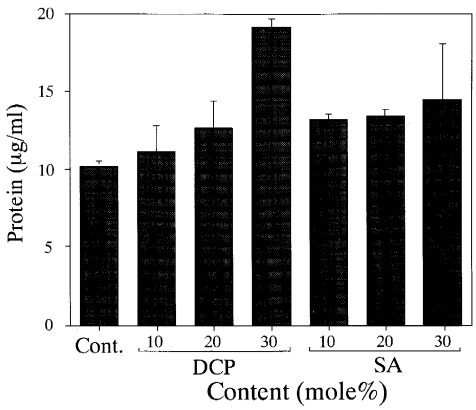

Fig. 1. The amount of transferred protein of various liposomes, which were recovered after incubation with influenza virus-infected cells at 37°C for 1 h. DMPC liposomes (Control-lipo) are shown as Cont. The numbers, which are displayed under each column, indicate the mol% of DCP or SA in DMPC liposomes. The data are means of five different samples with standard deviation in each case.

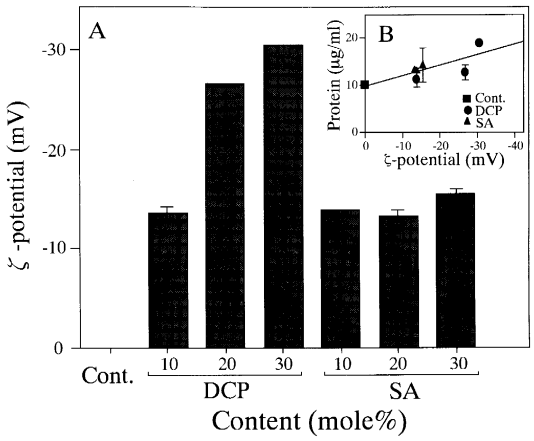

Fig. 2. (A) ζ-potential of various liposomes and (B) plots of the amount of transferred protein of various liposomes (data from Fig. 1) against ζ-potential of each vesicle. DMPC liposomes (Control-lipo) are shown as Cont. The numbers, which are displayed under each column, indicate the molar percentages of DCP or SA in DMPC liposomes. The data of ζ-potential are means of five different measurements at the same time with standard deviation.

3.6. The relation between membrane fluidity of various liposomes and protein transfer

As it was presumed that the change of membrane fluidity may be responsible for the enhancing effect of DCP or SA on spontaneous protein transfer in these conditions, we measured fluidity of liposome membrane by using fluorescence probes. The values of fluorescence polarization P, which are indicators of membrane fluidity at hydrophobic and hydrophilic regions of lipid membrane, were measured with DPH and TMA-DPH, respectively. As shown in Table 4, membrane fluidity of various liposomes and the amount of transferred protein were compared. In hydrophobic regions (Table 4), the fluidity of 30 mol% DCP–lipo was significantly lower than that of Control-lipo, while the content of transferred protein of this liposome was the highest. On the other hand, 50 mol% cholesterol-containing DMPC liposome (Chol–lipo) had the lowest fluidity, but the amount of transferred protein was the same as that of SA–lipo (Table 4). In addition, the fluidity at the hydrophilic region of all liposomes were almost the same, while the amounts of transferred protein of those varied (Table 4).

4. Discussion

Previously, Huestis and Newton [10] reported that transmembrane protein band 3 transferred into liposomal membrane with native orientation, and they presumed the mechanism of protein transfer among lipid membranes. Their hypothesis was: The hydrophobic region of protein flips into liposomal membrane when liposomes contact with cell membranes accompanying transient merging of outer monolayer of each lipid bilayer [10]. In Table 2, as the entrapped calcein did not leak from liposomes by incubation with cells except for passive transport, it was confirmed that the structure of the vesicle is not disrupted in the process of spontaneous transfer of membrane protein. This result is consistent with the hypothesis of Huestis and Newton [10], because, based on their presumption, only the outer monolayer of liposomes may interact

Table 4
Comparison between the values of fluorescent polarization (P) and the amounts of transferred protein of various liposomes

Liposomes	P ($\times 10^{-2}$)		Transferred protein (μg ml^{-1})
	Hydrophobic region	Hydrophilic region	
Control	7.87 ± 0.82	11.0 ± 0.37	9.57 ± 0.69
DCP (30)	12.6 ± 0.082	12.4 ± 0.33	15.4 ± 1.9
SA (30)	8.83 ± 0.42	11.0 ± 0.39	12.0 ± 1.1
Chol (50)	15.1 ± 0.21	12.5 ± 0.69	11.8 ± 0.32

DMPC liposomes are described as Control. DCP, SA and Chol represent the liposomes which contain DCP, SA and cholesterol with DMPC, respectively. The mol% of DCP, SA and cholesterol in DMPC liposomes are displayed in each parentheses. The P values at hydrophobic and hydrophilic regions were measured using DPH and TMA-DPH as fluorescent probes, respectively. The amounts of transferred protein of recovered liposomes were estimated after incubation with influenza virus-infected cells at 37°C for 1 h. The data are means of five samples with standard deviation.

with the outer one of the cell membrane, and it is predicted that inner membranes of both are not affected significantly during protein transfer. Therefore, in our experimental conditions, the membrane proteins on the surfaces of virus-infected and uninfected cells were considered to transfer also to liposome membrane in the same manner as presumed by Huestis and Newton [10].

Recently, we found that the amount of transferred protein from virus-infected cells increased with an increase in diameter (above 100 nm) of liposomes, but that from uninfected cells did not change in all sizes of the vesicles [18]. At that time, we considered that the excess amount of transferred protein from virus-infected cells over that of uninfected cells may be virus protein on the surface of virus-infected cells, and the size dependent protein transfer was caused due to the easily transferable structure of viral protein [18]. But in this study, we found the reason for this phenomenon in our previous report. Table 3 showed that more excess amount of FITC-labeled protein retained on the surface of virus-infected cells than on uninfected cells after incubation with liposomes. This advantage was considered to be responsible for that the content of transferred protein from virus-infected cells increased with an increase in the size of recipient [18].

In this study, to obtain more information about spontaneous protein transfer, we examined the effect of negative charged component DCP or SA on the amount of transferred protein from influenza virus-infected cells. As shown in Fig. 1, the amount of transferred protein increased by the presence of negative charged components in liposomes. Further, we estimated the value of the electrostatic conditions on membrane surface, ζ-potential, of various liposomes (Fig. 2A), and plotted the amount of transferred protein against ζ-potential (Fig. 2B). Thus, in Fig. 2A, there were no differences in the values of ζ-potential of SA–lipos, indicating SA content did not correspond to that at the preparation of liposomes and would be almost the same in all SA–lipos. Thus, from the plot in Fig. 2B, the effect of DCP or SA on protein transfer was confirmed to depend on the content of them in liposomal membrane, and to be caused by the presence of these components. From these results, we found that each DCP and SA, especially the former, is the useful reagent for enhancement of protein transfer like the artificial phospholipid 1,2-dimyristolylamido-1,2-deoxyphosphatidylcholine (DDPC) [11–14].

Recently, however, Waters et al. reported that the inclusion of anionic lipid dipalmitoylphosphatidylglycerol (DPPG) in the recipient vesicle membrane did not alter the yield of transferred protein, and concluded that the electrostatic factors do not limit intermembrane transfer of protein [15]. Therefore, our results are in contrast to their report, and might be specific in the case of DCP or SA.

Since Cook et al. reported that relative fluidity between donor and recipient membrane may relate to protein transfer [8], we measured the fluorescent polarization P of various liposomes. As shown in Table 4. However, no-relation was observed between membrane fluidity and protein transfer. For example, the fluidity in hydrophobic region of DCP–lipo and Chol–lipo were lower significantly than that of Control-lipo, but the amount of transferred protein of DCP–lipo was distinct from that of Chol–lipo. The reason for lowering the membrane fluidity by the existence of DCP in DMPC liposomes may be due to the length of saturated hydrocarbon chains of that negative charged component, because the branch of DCP was two carbon atoms longer than that of DMPC. However, since the phase transition temperature of DCP in water-system exists around 66°C [26], the possibility of phase separation should be considered. Thus, we have an intention of clarifying it and the relationship to protein transfer. On the other hand, in the hydrophilic region of membrane, the content of transferred protein of liposomes varied, but the values of P were almost the same. Although examined TMA-DPH probe at hydrophilic region of membrane was possible to interact with the head group of DCP or SA, the P values of all samples showed little difference. If the motion of positive charged probe were to be restricted by interaction with the negative charged components, the P value of them would have differed significantly from that of the Control-lipo. In addition, Shimooka et al. [24] also reported that the P values of bovine heart cardiolipin-containing egg PC liposomes was the same as that of egg PC liposomes. Therefore, membrane surface charge was considered to be ineffective on membrane fluidity of liposomes in this study. Consequently, in these experimental conditions, it was suggested that the effect of

DCP or SA on the spontaneous protein transfer from influenza virus-infected cells to DMPC liposomes did not depend on the membrane fluidity of recipient vesicles.

However, the amount of transferred protein of Chol–lipo, which showed the highest P value in this study, was almost the same as that of SA–lipo (Table 4). From the result of Table 4 and the report of Cook et al. [8], probably alteration of membrane construction, except for lowering fluidity, by the presence of cholesterol would be responsible for it in these conditions. Yeagle et al. reported that the presence of cholesterol in egg PC bilayers increases the separation between phospholipids until intermolecular interactions between head groups of lipids can no longer occur [27]. In addition, they described that this insertion frees head groups, increasing the freedom of motion of the phosphorylcholine moiety, and increasing the hydration of the bilayer surface [27]. Presumably, such modifications of membrane surface construction by the presence of cholesterol would be convenient for protein transfer.

Similarly, the cause of the enhancing effect of DCP or SA would not be the change of membrane fluidity, but be other factors caused by the presence of these components. In addition, although a good relation between ζ-potential and the amount of transferred protein seemed to exist (Fig. 2B), as Waters et al. suggested that the electrostatic factors do not limit intermembrane transfer of protein [15], negative charge on membrane surface might not be responsible for the stimulation of protein transfer. Thus, we considered two other possibilities based on the specific structure of DCP or SA. One possibility is the direct effect of structure of the amphiphilic components such as longer acylchain of them than that of DMPC, i.e., the improvement of stability of transferred protein in liposomal membrane described by Waters et al. [15]. The other possibility is the indirect effect of the specific structure of DCP or SA on the membrane construction. As the head group of DCP or SA are much smaller than that of DMPC, membrane surface construction, such as the distance among each head group of lipid or the hydration, of liposomes containing these amphiphilic components with DMPC are considered to differ from that of DMPC liposomes like the effect of cholesterol [27]. Therefore, the constructive change of membrane surface might facilitate protein transfer into DCP–lipo or SA–lipo. Thus, although both DPPG and DCP (or SA) are negative charged amphiphilic components, the structural difference of them was considered to make the contradiction between the results of Waters et al. [15] and ours.

Consequently, we considered that the alteration of membrane conditions by specific structure of DCP or SA is responsible for the enhancement of spontaneous protein transfer from influenza virus-infected cells to DMPC liposomes by the presence of them. However, it is still unclear what the specific constructive modification of DMPC liposomal membrane by the presence of DCP or SA would be. Now, we are making efforts to confirm the mechanism of the effect of DCP or SA on protein transfer.

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