Cationic Vesicles Consisting of 1,2-Dioleoyl-3-Trimethylammonium Propane (DOTAP) and Phosphatidylcholines and Their Interaction with Erythrocyte Membrane

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We studied the formation and stability of vesicles consisting of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and phosphatidylcholines by electron spin resonance (ESR) analysis and observation of their hemolytic activities. In contrast with previous findings on dimethyldialkylammoniums, DOTAP formed vesicles at 37 °C with phosphatidylcholines containing either saturated acyl chains such as dimyristoylphosphatidylcholine (DMPC) or unsaturated acyl chains such as dilinoleoylphosphatidylcholine (DLPC). Phosphatidylcholines made the bilayer more rigid and significantly reduced the hemolytic activity of DOTAP. In the presence of equimolar concentration of DOTAP and phosphatidylcholines, formation of tightly aggregated structures of several erythrocytes was observed, as previously reported for the vesicles containing dimethyldipalmitylammonium. These findings indicate that DOTAP vesicles were stabilized by phosphatidylcholines with either saturated acyl chains or unsaturated acyl chains, and the interaction with the lipid bilayer of biological membranes as cationic vesicles became prominent with minimal membrane damage by DOTAP monomers.

Key words cationic vesicle; 1,2-dioleoyl-3-trimethylammonium propane (DOTAP); liposome; erythrocyte; ESR

Various kinds of double-chained cationic surfactants have been shown to form bilayer vesicles and have been applied to gene delivery.[1,2] Among them cationic surfactant 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), which has mono-unsaturated fatty acid as acyl chains, has been extensively used with helper phospholipids such as dioleoylphosphatidylethanolamine (DOPE).[3—6] Cationic vesicles designed for that purpose are generally intrinsically unstable.[3,4] Although application of cationic vesicles for delivery of drugs, peptides, vitamins and lipids and other biologically active compounds to biological cells and tissues has also been expected, vesicles used for that purpose should be intrinsically stable in order to retain their contents.[7] However, the stability of the vesicles consisting of DOTAP and various phospholipids are not clearly known. Since phosphatidylcholines themselves form stable bilayer vesicles, in this study we examined the formation and stability of vesicles consisting of DOTAP and various phosphatidylcholines. We observed their hemolytic activity using erythrocytes as a model biological membrane system as well as by spin label analysis using 5-doxylstearic acid (5-NS) as a spin label, because monomers of DOTAP seem to transfer easily to the erythrocyte membrane from unstable vesicles and induce hemolysis, as suggested for single-chained surfactants.[8] We compared the results with our previous findings on dimethyldialkylammoniums.[9] In the previous study we revealed that phosphatidylcholines enriched with unsaturated acyl chains stabilized the vesicles of dimethyldialkylammoniums such as dimethyldipalmitylammonium at physiological temperature. On the other hand, phosphatidylcholines consisting of saturated acyl chains such as dimyristoylphosphatidylcholine (DMPC) were present as solid aggregates at the same temperature when they were mixed with equimolar dimethyldipalmitylammonium.[9]

In the previous study we also revealed that vesicles consisting of dimethyldialkylammoniums such as dimethyldipalmitylammonium and phosphatidylcholines enriched with unsaturated acyl chains interacted with erythrocytes as cationic vesicles and induced the formation of tightly aggregated structures of several erythrocytes.[9] Through these findings we clarified the change in the interaction of the cationic surfactants with biological membranes due to the presence of the phosphatidylcholines. To see whether a similar phenomenon is induced by the DOTAP vesicles, we also studied the interaction of cationic vesicles with erythrocytes in the presence of various phosphatidylcholines as vesicle components.

Experimental

Materials DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Dimyristoryphosphatidylcholine (DMPC, L-α-phosphatidylcholine dimyristoyl), dipalmitoylphosphatidylcholine (DPPC, L-α-phosphatidylcholine dipalmitoyl), and dilinoleoylphosphatidylcholine (DLPC, L-α-phosphatidylcholine dilinoleoyl) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and 5-NS was from Aldrich (Milwaukee, WI, U.S.A.). Egg yolk phosphatidylcholine (egg yolk PC) and all other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of Sonicated Vesicles Consisting of Double-chained Cationic Surfactants and Phosphatidylcholines Vesicles consisting of DOTAP and phosphatidylcholines were prepared as described previously.[9] DOTAP in the presence or absence of various phospholipids was dissolved in chloroform, and the solvent was evaporated under a nitrogen stream. The dried films were prepared by removing the solvent under vacuum evaporation, then hydrated and suspended by vortex mixing in phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM phosphate buffer, pH 7.4). The suspension was then sonicated with a probe-type sonicator for 5 min at an output power of 80 W at 30 °C under a stream of nitrogen.

Measurement of Hemolysis and Observation of Aggregation and Shape Change of Erythrocytes Guinea-pig erythrocyte suspension was prepared as described in the previous report.[9] The erythrocytes were suspended in PBS at a hematocrit value of about 20% and incubated with the vesicles at 37 °C. A sample of this mixture was taken out after 1 min and 60 min incubation. The extent of hemolysis was obtained by measuring the hemoglobin release in the supernatant after centrifugation at 2000 ***g*** for 2 min. Hemoglobin was determined spectrophotometrically at 541 nm according to the cyanmethemoglobin method.[10] Percentage of hemolysis was determined by comparing the hemoglobin release with that of control samples completely hemolysed with distilled water. Shape change and aggregation of the erythrocytes were observed using Olympus phase-contrast microscopy equipment (Tokyo, Japan).

Measurement of Electron Spin Resonance (ESR) Spectra Measure-

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ment of ESR spectra was carried out using 5-NS as a spin label. Vesicles were incubated with 25 μM 5-NS at 37 °C for 2 min and transferred to duplicate 20 μl capillaries. One end of the capillaries was sealed with Hematoseal (Terumo, Tokyo, Japan) and inserted into ESR tubes. The ESR spectra were measured at 37 °C with a TE-200 (X-band) spectrometer (JEOL, Tokyo, Japan) with a 100-kHz field modulation frequency and 0.1 mT modulation amplitude at an output power of 8 mW. The order parameter of 5-NS was calculated from the ESR spectra as described previously.[11)]

Statistical Analysis Mann-Whitney's U-test was used to analyze differences between sets of data. The level of significance was adjusted by Bonferroni's method. A p-value less than 0.05 was considered statistically significant.

Results and Discussion

To investigate the molecular assemblies of DOTAP in the presence of various phospholipids, we observed the ESR spectra of 5-NS with a total concentration of DOTAP and phosphatidylcholines of 2 mM. 5-NS shows an anisotropic spectrum when it is present in bilayer vesicles. On the other hand, it shows a relatively isotropic spectrum in micelles and a broad spectrum in solid aggregates,[9,12—14)] as shown in Fig. 1d for 2 mM cetyltrimethylammonium micelles and in Fig. 1e for vesicles consisting of 1 mM dimethyldipalmitylammonium and 1 mM DMPC. According to the anisotropic spectrum shown in Fig. 1a, DOTAP itself seems to form bilayer vesicles, although direct confirmation by electron micrograph is necessary. The order parameter value of the spin label in DOTAP vesicles shown in Table 1 is larger than those in alkyltrimethylammonium vesicles (less than 0.470[13)]), but still much smaller than those in the vesicles of phosphatidylcholines such as DMPC, shown in Table 1, and egg yolk PC (0.578 ± 0.004[13)]). This is probably due to the repulsive interactions among the cationic trimethylammonium groups in DOTAP vesicles.

According to their anisotropic ESR signals shown in Figs. 1b and 1c for vesicles containing DMPC and DLPC, respectively, DOTAP seemed to form bilayer vesicles either with phosphatidylcholines consisting of saturated acyl chains (DMPC, DPPC) or those enriched with unsaturated acyl chains (egg yolk PC, DLPC). The bilayer vesicles appeared to become more rigid in the presence of all phospholipids tested, according to the increase in order parameters shown in Table 1 and, shown in Fig. 2, the dose-dependent effects of DMPC. The lipid bilayer formed with DLPC, which has two *cis*-double bonds in each acyl chain, was more fluid than those formed with other phosphatidylcholines tested according to the smaller value of the order parameter.

We next examined the hemolytic effects of vesicles consisting of DOTAP and phosphatidylcholines. DOTAP vesicles without phosphatidylcholines rapidly induced hemolysis as shown in Table 2, just like the dimethyldialkylammonium

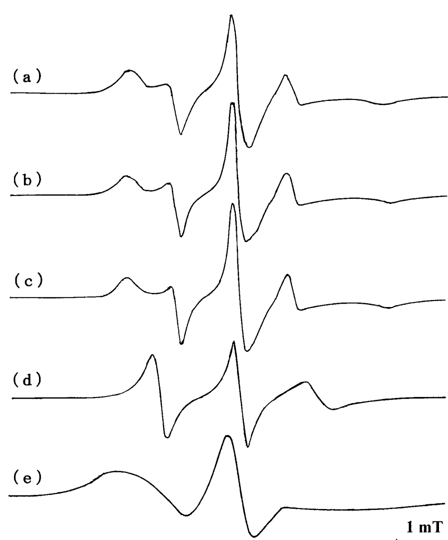

Fig. 1. ESR Spectra of 5-NS in Vesicles Consisting of 1 mM DOTAP in the Presence of 1 mM Phosphatidylcholines at 37 °C

(a) DOTAP only; (b) with DMPC; (c) with DLPC. ESR spectrum of 5-NS in 2 mM cetyltrimethylammonium micelles[13)] (d), which were prepared without sonication, and that in vesicles consisting of 1 mM dimethyldipalmitylammonium and 1 mM DMPC[9)] (e), which were prepared by a similar sonication method, are shown for comparison.

Table 1. Order Parameter of 5-NS in Vesicles Consisting of 1 mM DOTAP and 1 mM Phosphatidylcholines at 37 °C

DOTAP	Phosphatidylcholine	Order parameter
None	DMPC	0.580 ± 0.003***
DOTAP	None	0.532 ± 0.002
	DMPC	0.568 ± 0.002***
	DPPC	0.570 ± 0.004***
	Egg Yolk PC	0.559 ± 0.002***
	DLPC	0.545 ± 0.002***

DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DLPC, dilinoleoylphosphatidylcholine. Data are means±S.D. for four experiments. ***$p<0.001$ compared with the value in DOTAP vesicles without phosphatidylcholines.

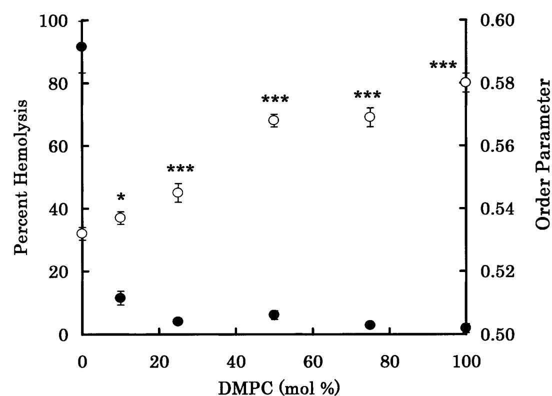

Fig. 2. Dose-Dependent Effects of DMPC in Vesicles Consisting of DOTAP and DMPC on Hemolysis after 60 min Incubation (●) and Order Parameter of 5-NS (○) at 37 °C

Total concentration of DMPC and DOTAP was 2 mM. Data of hemolysis are means±S.D. for three experiments and all the values in the presence of DMPC are significantly different from the value in DOTAP vesicles without DMPC (***$p<0.001$). Data of order parameter are means±S.D. for four experiments. *$p<0.05$, ***$p<0.001$ compared with the value in DOTAP vesicles without DMPC.

Table 2. Hemolysis Induced by Vesicles Consisting of 1 mM DOTAP and 1 mM Phosphatidylcholine after 1 min or 60 min Incubation at 37 °C

DOTAP	Phosphatidylcholine	Hemolysis (%)	
		After 1 min	After 60 min
None	None	0.4 ± 0.2	0.7 ± 0.1
DOTAP	None	2.2 ± 0.2***	83.0 ± 8.5***
	DMPC	0.5 ± 0.2	2.9 ± 0.2***
	DPPC	0.5 ± 0.3	3.0 ± 0.3***
	Egg Yolk PC	0.3 ± 0.2	3.0 ± 1.5
	DLPC	0.6 ± 0.2	14.4 ± 3.0**

Data are means±S.D. for three experiments. **$p<0.01$, ***$p<0.001$ compared with the value without vesicles.

vesicles previously reported.[9] The addition of phosphatidylcholines as vesicle constituents decreased the hemolytic activity of cationic vesicles. As shown in Fig. 2, the presence of 10 mol% of DMPC increased the order parameter of 5-NS and significantly reduced hemolysis. In the presence of 25 mol% or more, the order parameter increased more significantly and hemolysis was only slightly observed. Likewise, in the presence of egg yolk PC, which is enriched with oleic acid and linoleic acid in its acyl chains,[15] as well as DPPC, vesicles induced only slight hemolysis.

These findings by ESR analysis and the experiments on hemolytic activity suggest that the molecular packing of DOTAP vesicles became tighter and the stability of the vesicles was improved by the presence of phosphatidylcholines with saturated acyl chains such as DMPC and DPPC, as well as those with unsaturated acyl chains. This is consistent with the results by Campbell *et al.* on the effects of DPPC, from the studies using differential scanning calorimetry and Laurdan as a fluorescence probe,[7] but is quite different from our previous findings on dimethyldialkylammoniums[9] DOTAP seems to be favorable for stable bilayer vesicle formation even with phosphatidylcholines containing saturated acyl chains at 37 °C, because it has a low phase transition temperature (−11.9 °C) due to its unsaturated acyl chains.[16]

In the presence of DLPC hemolysis was gradually induced (Table 2). The hemolytic activity of the vesicles was possibly affected not only by the molecular packing of the vesicle bilayers but also by other factors such as the lamella structure of the vesicles. In the case of DLPC-containing vesicles, oxidative damage of the erythrocyte membrane seemed to be induced, accompanied by the oxidation of linoleic acid in its acyl chains, although hemolysis by a DOTAP monomer may have also been involved due to the loose packing of the bilayer containing DLPC.

In the DOTAP vesicles without phosphatidylcholines, a mass formation of aggregated erythrocytes was observed before hemolysis was fully induced (data not shown), as reported in the previous paper for dimethyldialkylammoniums.[9] On the other hand, vesicles consisting of equimolar ratios of DOTAP and DMPC formed characteristic tightly aggregated structures of several erythrocytes, as shown in Fig. 3. Similar structures were observed in the presence of DOTAP vesicles containing equimolar DPPC, egg yolk PC or DLPC (data not shown). Without DOTAP, these aggregated structures were not observed (data not shown). Like dimethyldipalmitylammonium vesicles,[9] these DOTAP vesicles seem to interact with erythrocyte membranes as vesicles, change their surface charge and fluidity, and induce interaction among erythrocytes.[9] It has been reported that rabbit erythrocyte fusion was induced by cationic vesicles consisting of DOTAP and phosphatidylethanolamine.[17] Some erythrocyte fusion may have been induced in this study, although it has been reported that fusion is induced much less by vesicles containing phosphatidylcholines than by those containing phosphatidylethanolamines.[18]

Cationic vesicles designed for cellular nucleic acid delivery are generally intrinsically unstable.[3,4] On the other hand, those designed for delivery of drugs, peptides, vitamins, lipids and other small molecules to biological cells and tissues require stability as vesicles in addition to reactivity to the cells and tissues. As revealed in this study, cationic vesicles consisting of DOTAP are stabilized with a small amount of phosphatidylcholines. The cationic DOTAP vesicles containing phosphatidylcholines seem to interact with biological membranes as vesicles with minimal toxicity of DOTAP. Therefore, these vesicles have a wide range of future applications for delivery of various compounds especially to tissues like skin, because the lipid lamella of the stratum corneum of skin contains a high ratio of negatively charged lipids, which are expected to interact with cationic liposomes.

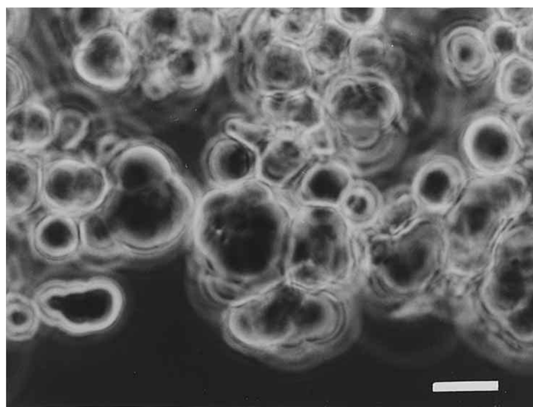

Fig. 3. Phase-Contrast Microscopic Photo of Tightly Aggregated Structures of Guinea-Pig Erythrocytes Induced by 60-min Incubation with Vesicles Consisting of 1 mM DOTAP and 1 mM DMPC at 37 °C

The bar is 10 μm.

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