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Membrane-damaging action of staphylococcal alpha-toxin on phospholipid-cholesterol liposomes

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The mechanism of membrane damage by staphylococcal alpha-toxin was studied using carboxyfluorescein (internal marker)-loaded multilamellar liposomes prepared from various phospholipids and cholesterol. Liposomes composed of phosphatidylcholine or sphingomyelin and cholesterol bound alpha-toxin and released carboxyfluorescein in a dose dependent manner, when they were exposed to alpha-toxin of concentrations higher than 1 or 8 μg/ml, respectively. In contrast, the other liposomes composed of phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol or phosphatidylinositol plus cholesterol were not susceptible to the toxin even at high concentrations up to 870 μg/ml. The insensitive liposomes containing either phosphatidylserine or phosphatidylglycerol were made sensitive to alpha-toxin by inserting phosphatidylcholine into the liposomal membranes. In addition, phosphorylcholine inhibited the toxin-induced marker release from liposomes. These results indicated that the choline-containing phospholipids are required for the interaction between alpha-toxin and liposomal membranes. Susceptibility of liposomes containing phosphatidylcholine or sphingomyelin increased with the increase in cholesterol contents of the liposomes. Based on these results, we propose that the choline-containing phospholipids are possible membrane components or structures responsible for the toxin–membrane interaction, which leads to damage of membranes. Furthermore, cholesterol may facilitate the interaction between alpha-toxin and membrane as a structural component of the membrane.

Introduction

Staphylococcal alpha-toxin is a 34 kDa, extracellular polypeptide produced by most strains of *Staphylococcus aureus* isolated from human infections. It is considered to be an important pathogenic factor in staphylococcal infections [1–3]. This toxin exhibits membrane-damaging effects on various types of cells including erythrocytes [4–6], although no enzymatic activity has been found so far in alpha-toxin preparations. Extensive studies on the mechanism of membrane damage by alpha-toxin were performed with erythrocytes [7–11], suggesting that erythrocyte lysis occurs as the result of an osmotic process: monomeric toxin molecules bind to and polymerize on/in the membrane to form transmembrane ring-shaped structures with diameter of approx. 90 Å, which may allow small ions and molecules to pass through

Abbreviations: PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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the membrane and induce osmotic lysis. Similar ring-shaped structures were observed on/in the membranes of other types of cells as well as erythrocytes, when they were exposed to the toxin [5,6].

Weissmann et al. [12] and Freer et al. [13] showed that alpha-toxin acted on liposomes composed of egg phosphatidylcholine, cholesterol and dicetyl phosphate, and induced the release of internal marker such as CrO_4^{2-} or glucose from the liposomes. Thus, the lipid bilayer was suggested to be the primary target of alpha-toxin [12–14]. Ring-shaped structures were also observed on the surface of liposomes exposed to the toxin. Moreover, liposomes bearing the ring-shaped structures were permeable to the silicotungstate negative stain, whereas liposomes without the structures were not stained with silicotungstate [9,13,15]. Therefore, the marker release from liposomes is thought to occur through the transmembrane ring-shaped structures. Although the formation of ring-shaped structure may play a key role in the early stages of membrane damage by the toxin, little is known about the membrane components which are involved in the formation of transmembrane channels. To analyze the initial interaction between alpha-toxin and the membrane components, we employed liposomes as model membranes and examined the membrane-damaging action of alpha-toxin on various liposomes of defined compositions. Our results indicated that choline-containing phospholipids were required for the interaction between alpha-toxin and liposomal membrane, and that the susceptibility of liposomes to the toxin increased as the cholesterol content in the liposomal membrane increased.

Materials and Methods

Chemicals

Carboxyfluorescein was purchased from Eastman Kodack (Rochester, NY). Soyabean phosphatidylcholine (PC) and dioleoylphosphatidylcholine (DOPC) were the gifts from Nippon Shoji Kaisha Ltd (Osaka). Egg yolk PC was purchased from Nippon Fine Chemicals (Osaka). Bovine brain sphingomyelin, bovine erythrocyte sphingomyelin, egg yolk sphingomyelin, egg yolk phosphatidylethanolamine (PE) and egg yolk phosphatidylglycerol (PG) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine, prepared from egg yolk PC, were obtained from Avanti Biochemical (Birmingham, AL). Crude preparation of soyabean phosphatidylinositol (PI) was obtained from Hohnen Oil Co. (Tokyo), and purified by silica gel column chromatography (Iatrobeads 6RS-8060; Iatron Chemical Products, Tokyo) using chloroform/methanol (4 : 1, v/v) as elution solvent. Bovine brain phosphatidylserine (PS) was purified as follows: Total lipids, extracted from bovine brain with chloroform/methanol (2 : 1, 1 : 1 and 1 : 2, v/v) solutions, were dissolved in a chloroform/methanol (2 : 1, v/v) solution. After removal of insoluble materials by filtration, one quarter volume of distilled water was added into the mixture. The lower phase was collected, evaporated to dryness and treated with acetone. The acetone precipitate was dissolved in chloroform, and applied to an Iatrobeads 6RS-8060 column. The column was eluted by a linear gradient of chloroform/methanol (9 : 1 to 4 : 6, v/v). Combinned PS-rich fractions were then applied to DEAE-Toyopearl 690C column (acetate form) (Toyo Soda, Tokyo), and PS was eluted with a linear gradient of 0 to 0.4 M ammonium chloride/chloroform/methanol (30 : 60 : 8, v/v). The lipid preparations used in this study were checked by thin-layer chromatography on silica gel plates (Merck, Darmstadt), and all of them exhibited single spots on the plates.

Staphylococcal alpha-toxin

Alpha-toxin was purified from the culture supernatant of *Staphylococcus aureus* Wood 46 as previously described [16]. Purified toxin exhibited a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3), and contained 19000 hemolytic units per mg protein. Treatment of the toxin preparation with heat at 60 °C for 1 h abolished the hemolytic activity (Ref. 17; Fig. 7), indicating that the toxin preparation was not contaminated with heat stable hemolysisn such as delta toxin.

Staphylococcal delta-toxin

Delta toxin was purified from the culture su-

pernatant of *Staphylococcus aureus* RN25 as described previously [18], i.e., according to the method of Smith and Shaw [19], a modified method of Heatley [20].

Preparation of liposomes

Multilamellar liposomes composed of phospholipid and cholesterol were prepared as previously described [21]. Briefly, a mixture of phospholipid (0.5 μmoles) and cholesterol (0.5 μmoles) in chloroform was evaporated under reduced pressure to form a lipid film on the wall of a conical-bottomed flask. After drying under reduced pressure for 1 h, the lipid film was hydrated by vortexing at 50 °C in 0.1 ml of either 0.1 M carboxyfluorescein (for marker-loaded liposomes) or phosphate-buffered saline (for marker-unloaded liposomes), respectively. The liposome suspensions were centrifuged at 23 000 × *g* for 20 min to remove the nonencapsulated marker, and washed three times by centrifugation in veronal-buffered saline (pH 7.3) containing 0.4% gelatin.

The size of carboxyfluorescein-loaded liposomes was measured with Microtrac SPA (Leeds and Northrup, St. Petersburg, FL). Mean diameter of the liposomes of different compositions varied within the range between 7.64 and 5.70 μm.

Assay of marker release from liposomes

This was done as described previously [21,22]. 5 μl of liposome suspension were added to 50 μl of serially diluted toxin solution (0.5 to 3200 μg/ml) per well in 96-well U-bottomed plate (Nunc, Denmark), and incubated at 25 °C for 30 min. Fluorescence intensity was measured with a MTP-12 F fluorescence microplate photometer (Corona Electric, Katsuta) at an excitation of 490 nm and an emission of 520 nm. 100% release was defined as the fluorescence intensity which was gained upon exposure of the liposomes to 5% Triton X-100. The concentration of liposomes was adjusted on the basis of the fluorescence intensities obtained upon 100% release, since the amounts of entrapped carboxyfluorescein per lipids in the used liposomes were within the relevant range. The assay was performed in duplicate, and repeated at least three times.

Indirect measurement of toxin binding to liposomes

This was done by using marker-unloaded liposomes as test liposomes and carboxyfluorescein-loaded, DOPC-cholesterol (1 : 1, molar ratio) liposomes as indicator liposomes. 5 μl of serially diluted marker-unloaded liposomes were added to 50 μl of toxin solution (2.5 μg/ml) in 96-well microplate. After incubation at 25 °C for 30 min, 5 μl of indicator liposomes were added to the reaction mixture to assay the residual marker release activity of the toxin solutions.

Assay of toxin binding to multilamellar liposomes by using SDS-PAGE

Alpha-toxin (40 μg in 20 μl) and liposomes (0.3 μmoles phosphate in 30 μl) were mixed and incubated at 25 °C for 30 min. To remove unbound toxin, the mixture was centrifuged at 21 000 × *g* at 4 °C for 20 min. The precipitated liposomes were washed three times by centrifugation with 0.5 ml of phosphate-buffered saline. The washed liposomes were subsequently boiled for 5 min in 50 μl of the sample buffer, containing 62.5 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.005% Bromophenol blue [23]. SDS-PAGE was carried out on the slab gels containing 10% acrylamide, according to the method of Laemmli [23]. The following proteins were used as the molecular weight strandards: β-galactosidase from *Escherichia coli,* 116 000; bovine serum albumin, 66 000; ovalbumin, 45 000; and carbonic anhydrase from bovine erythrocytes, 29 000. The gels were stained with Coomassie brilliant blue R250 as described by Fairbanks et al. [24].

Determination of protein and phosphate

Protein determination was carried out according to the method of Lowry et al. [25] using bovine serum albumin as a standard. Phosphate was assayed by the method of Gerlach and Deuticke [26].

Results

Requirement of choline-containing phospholipids for the interaction between alpha-toxin and liposomal membrane

To study whether or not the polar region of

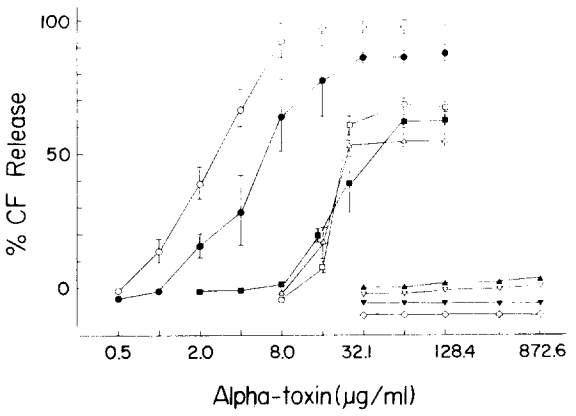

Fig. 1. Alpha-toxin-induced marker release from various phospholipid-cholesterol liposomes. Marker release from various liposomes upon exposure to alpha-toxin was assayed as described in Materials and Methods. The liposomes used in the experiments were composed of soyabean PC (○), egg yolk PC (●), bovine brain sphingomyelin (■), bovine erythrocyte sphingomyelin (△), egg yolk sphingomyelin (□), egg yolk PE (◇), bovine PS (▼), soyabean PI (▽) or egg yolk PG (▲) plus cholesterol at 1 : 1 molar ratio. Results are expressed as means ± S.E. ($n = 5$), CF, carboxyfluorescein.

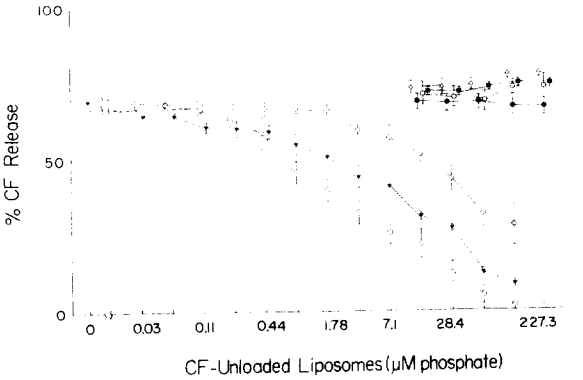

Fig. 2. Toxin binding ability of various liposomes composed of phospholipid and cholesterol. Binding of alpha-toxin to liposomes was indirectly assayed as described in Materials and Methods. The liposomes used were composed of egg yolk PC (▼), DOPC (○), egg yolk sphingomyelin (◇), egg yolk PE (△), bovine brain PS (■), egg yolk PG (●), or soyabean PI (□) plus cholesterol at 1 : 1 molar ratio. Results are represented as means ± S.E. ($n = 4$). CF, carboxyfluorescein.

phospholipids is involved in the interaction between alpha-toxin and liposomes, we examined the membrane-damaging effects of alpha-toxin on carboxyfluorescein-loaded multilamellar liposomes each composed of various phospholipids and cholesterol at 1 : 1 molar ratio. As shown in Fig. 1, the liposomes composed of phosphatidylcholine (PC) or sphingomyelin (SM) and cholesterol released the internal marker in a dose-dependent manner, when exposed to alpha-toxin of concentrations higher than 1 or 8 μg/ml, respectively. In contrast, the liposomes composed of phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) or phosphatidylglycerol (PG) plus cholesterol were insensitive to the toxin even at high concentrations up to 870 μg/ml (Fig. 1). These results suggested that the polar region of phospholipids in liposomal membrane was directly involved in the interaction between alpha-toxin and liposomes, and that the choline-containing phospholipids may be required for the toxin–membrane interaction which leads to the release of the internal marker.

To investigate further the toxin–membrane interaction, we also measured the binding of alpha-toxin to the liposomes of various compositions. Toxin-binding ability of the liposomes was assayed by using marker-unloaded liposomes (as test liposomes) and carboxyfluorescein-loaded, dioleoylphosphatidylcholine (DOPC)-cholesterol liposomes (as indicator liposomes), i.e., after the incubation of a constant amount of alpha-toxin with the test liposomes, residual marker release activity of the toxin solution was measured with the indicator liposomes. The marker release activity of alpha-toxin was markedly reduced by the incubation with the test liposomes containing PC or sphingomyelin (Fig. 2), indicating that these liposomes bound the toxin. Such reduction in marker release activity of the toxin solution was not observed when the toxin solution was preincubated with the other liposomes containing PE, PS, PI or PG (Fig. 2). These results suggested that the liposomes containing PC or sphingomyelin did bind alpha-toxin, but the other liposomes did not bind the toxin or bound very small amount of the toxin.

Binding of alpha-toxin to the liposomes was further assayed directly by using SDS-PAGE. After liposomes and alpha-toxin were mixed and incubated, the mixture was centrifuged to remove unbound toxin. The precipitated liposomes were washed, solubilized and subjected to SDS-PAGE. As shown in Fig. 3, large amount of alpha-toxin

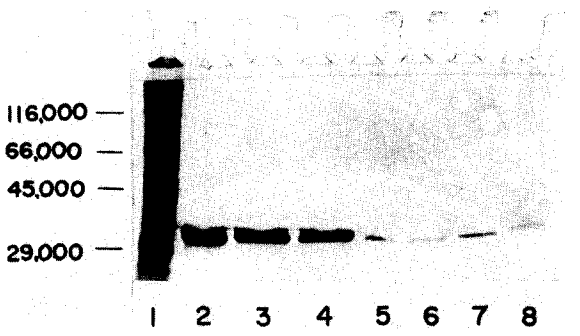

Fig. 3. Assay of toxin binding to liposomes of various compositions by means of SDS-PAGE. SDS-PAGE was carried out as described in Materials and Methods. Lane 1: molecular weight standards; lane 2: alpha-toxin (20 μg); lane 3 to lane 8: PC-, sphingomyelin-, PS-, PG-, PI- or PE-cholesterol liposomes, respectively, which had been incubated with alpha-toxin. CF, carboxyfluorescein.

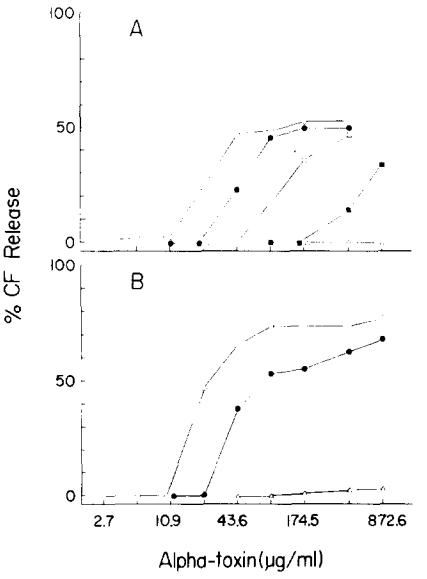

Fig. 4. Effect of PC insertion into PS- or PG-cholesterol liposomal membrane on the susceptibility of liposomes to alpha-toxin. Various amounts of DOPC were added into liposomal membranes composed of bovine PS (A) or egg yolk PG (B) and cholesterol of a molar ratio 1:1. The molar ratio of DOPC to PS (or PG) was 0 (△), 0.125 (■), 0.25 (□), 0.5 (●) or 1.0 (○). Marker release assay was performed as described in Material and Methods. The experiment was repeated three times, and the figures show the representative data. CF, carboxyfluorescein.

was detected on the liposomes containing PC or SM (lane 3 and 4). However, only trace amount of the toxin was associated with the liposomes containing PS, PG, PI or PE (lane 5–8). These results indicated that the choline-containing phospholipids were required for the binding of alpha-toxin to the liposomal membranes.

Subsequently we examined the effect of PC contents on the toxin–membrane interaction by using liposomes prepared from PS (or PG), cholesterol and PC at the molar ratios 1:1:0–1. As shown in Fig. 4, the liposomes containing PS or PG were sensitive to alpha-toxin only when DOPC was included in their membranes, indicating that the choline-containing phospholipids were required for the toxin–membrane interaction. Susceptibility of the PS-cholesterol-PC liposomes to alpha-toxin increased as the DOPC content increased (Fig. 4A), but the level of susceptibility of the liposomes containing both PS and DOPC did not reach that of the liposomes containing DOPC alone. Similar results were obtained with the PG-cholesterol-DOPC liposomes (Fig. 4B). Binding of alpha-toxin to PS- or PG-containing liposomes was also observed when DOPC was added to the liposomes (data not shown). These results indicated that the epitope density of the choline residues may be important for the toxin–membrane interaction, and that the polar head group of PS or PG apparently interfered with the interaction between alpha-toxin and the polar region of PC.

To determine whether or not the choline-residues in phospholipids are indispensable for the toxin–membrane interaction, we compared alpha-toxin susceptibilities of liposomes which contained phospholipids of structures intermediate between PC and PE, i.e. phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine. As shown in Fig. 5, the liposomes composed of phosphatidyl-*N*-monomethylethanolamine and cholesterol released the internal marker upon exposure to alpha-toxin of concentrations higher than 321 μg/ml, whereas the liposomes containing phosphatidyl-*N*,*N*-dimethylethanolamine were sensitive to the toxin of concentrations higher than 64 μg/ml. These results suggested that phosphoryl-*N*,*N*-dimethylethanola-

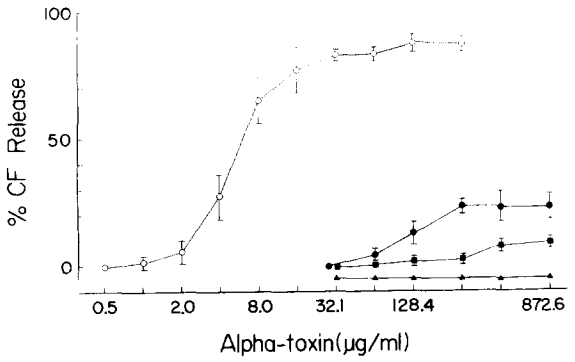

Fig. 5. Comparison of alpha-toxin susceptibilities between liposomes composed of PC, phosphatidyl-*N*,*N*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine or PE plus cholesterol. Liposomes were prepared from PC (○), phosphatidyl-*N*,*N*-dimethylethanolamine (●), phosphatidyl-*N*-monomethylethanolamine (■) or PE (▲) and cholesterol at a 1:1 molar ratio. Assay of marker release from the liposomes was done as described in Materials and Methods. Results are expressed as means ± S.E. ($n = 3$). CF, carboxyfluorescein.

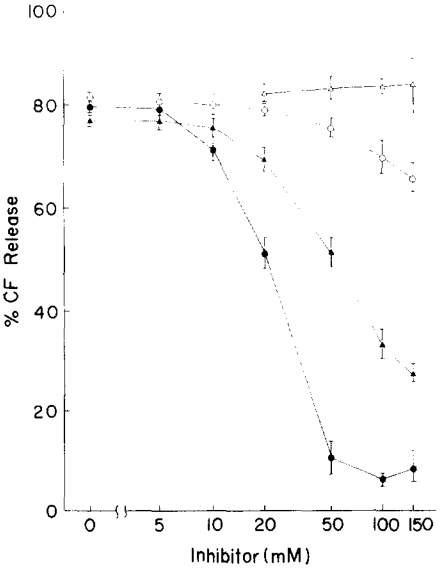

Fig. 6. Inhibition of toxin-induced marker release from liposomes by phosphorylcholine chloride and choline chloride. Marker release from DOPC-cholesterol liposomes was assayed as described in Materials and Methods except that the amounts of phosphorylcholine chloride (●), phosphorylethanolamine chloride (▲), choline chloride (○) or glycerol 3-phosphate (△) were shown on the abscissa, and 3 μg/ml alpha-toxin was used. The results are represented as means ± S.E. ($n = 3$). CF, carboxyfluorescein.

mine residue interacted with alpha-toxin more efficiently than phosphoryl-*N*-monomethylethanolamine residue. Thus, choline-containing structure was dispensable for the interaction between alpha-toxin and liposomal membrane. However, the choline-containing structure was much more favorable for the toxin–membrane interaction than the other structures (Fig. 5).

Inhibitory effect of free phosphorylcholine, phosphorylethanolamine, choline, glycerol 3-phosphate and 4-amino-*n*-butyric acid on the membrane-damaging action of alpha-toxin was investigated. As shown in Fig. 6, toxin-induced marker release from DOPC-cholesterol liposomes was significantly inhibited by phosphorylcholine at concentrations higher than 20 mM. Phosphorylethanolamine and choline exhibited less inhibitory effect in comparison with phosphorylcholine, and no inhibitory effect was observed with glycerol 3-phosphate (Fig. 6) and 4-amino-*n*-butyric acid (data not shown). These results suggested that alpha-toxin interacted directly with phosphorylcholine residues of the phospholipids on/in the liposomal membrane.

Insertion of 10 mol% dipalmitoylphosphatidic acid or stearylamine into DOPC-cholesterol membranes did not significantly alter the susceptibility of the liposomes to alpha-toxin, suggesting that surface charge on the liposomal membranes has little effect on the interaction between alpha-toxin and liposomes.

It was suggested that the alpha-toxin preparation used in the early studies of Weissmann et al. [12] was contaminated with delta-toxin [27]. To avoid such possibility, we checked the heat stability of the alpha-toxin preparation used in this study. Hemolytic and carboxyfluorescein-release activities of the alpha-toxin preparation were inactivated by the treatment with heat at 60 °C for 1 h (Fig. 7). Under the conditions, however, delta-toxin did not lose the hemolytic activity (Fig. 7). We also examined the carboxyfluorescein-release activity of delta-toxin with the liposomes of various compositions. Delta-toxin induced carboxyfluorescein-release from all the liposomes composed of PC, SM, PS, PI or PE plus cholesterol (Fig. 8). These results clearly indicated that the alpha-toxin preparation used was not con-

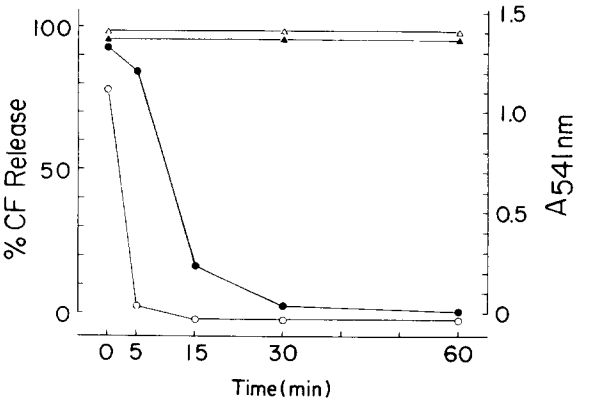

Fig. 7. Heat stability of hemolytic and carboxyfluorescein (CF)-release activity of alpha- and delta-toxin. After the treatment of alpha-toxin (32.1 μg/ml) or delta-toxin (20 μg/ml) with heat at 60°C for the indicated periods, residual hemolytic and carboxyfluorescein-release activities of the toxins were measured. Hemolytic activity was assayed with rabbit erythrocytes, as described previously [16]. Assay of carboxyfluorescein release was performed with DOPC-cholesterol liposomes, as described in Materials and Methods. Residual hemolytic and carboxyfluorescein-release activities of alpha-toxin (●, ○) and delta-toxin (▲, △) are expressed as means of three experiments.

taminated with appreciable amount of delta-toxin, and that the requirement of choline-containing phospholipids for the toxin–membrane interaction was specific for alpha-toxin.

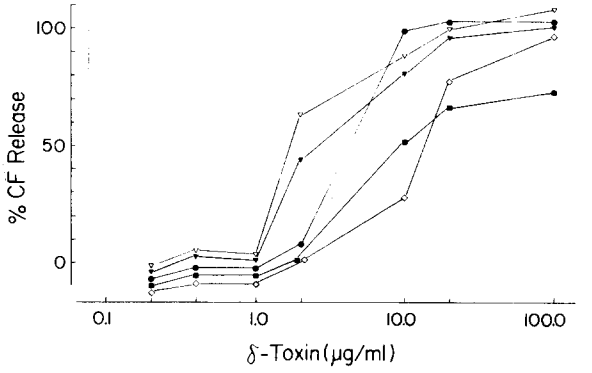

Fig. 8. Membrane-damaging effect of delta-toxin on liposomal membranes of various compositions. Carboxyfluorescein (CF) release upon exposure to delta-toxin was assayed as described in Materials and Methods. The liposomes were prepared from egg yolk PC (●), egg yolk sphingomyelin (■), bovine PS (▼), soyabean PI (▽) or egg yolk PE (◇) plus cholesterol at 1:1 molar ratio. The results are represented as means of three experiments.

Relationship between cholesterol contents in liposomes and their susceptibility to alpha-toxin

Effect of cholesterol contents on the susceptibility of PC- or sphingomyelin-containing liposomes to alpha-toxin was examined. As shown in Fig. 9A, the marker release from DOPC-containing liposomes increased with the increase in cholesterol contents between 0 and 50 mol%, although the liposomes without cholesterol were also susceptible to the toxin. Toxin binding activity of the liposomes also increased, as the cholesterol content in the liposomes increased (data not shown). Similar tendency was observed with liposomes containing egg yolk sphingomyelin (Fig. 9B). Since the liposomes composed of sphingomyelin alone spontaneously released CF at the incubation temperature (25°C), we could not determine whether alpha-toxin induced marker release from these liposomes or not. We, therefore, assayed the toxin-binding activity of these liposomes, and found that the sphingomyelin liposomes had no significant toxin-binding activity (data not shown). These results indicated that cholesterol-containing membranes are more susceptible to alpha-toxin than those devoid of cholesterol.

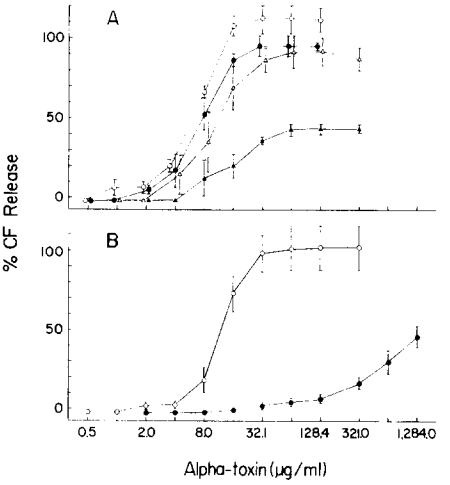

Fig. 9. Effect of cholesterol contents on the susceptibility of liposomes to alpha-toxin. Liposomes were composed of DOPC (A) or egg yolk sphingomyelin (B) plus cholesterol. Molar ratio of cholesterol to PC was 0 (▲), 0.2 (△), 0.5 (●) or 1.0 (○). Marker release assay was performed as described in Materials and Methods. Results are expressed as means ± S.E. ($n = 3$). CF, carboxyfluorescein.

Discussion

In this paper we showed that the choline-containing phospholipids in the membrane are required for the interaction of alpha-toxin with liposomes, and that the susceptibility of liposomal membranes to alpha-toxin was augmented as cholesterol contents in the membranes increased. Based on the results obtained, we propose that the choline-containing phospholipids are possible membrane constituents or structures responsible for the toxin–membrane interaction which leads to the damage of membrane, perhaps through the formation of transmembrane channels in the cell membranes. Furthermore, cholesterol may facilitate the interaction between alpha-toxin and phosphorylcholine residues of the phospholipids as a structural component of the membrane.

Free phosphorylcholine inhibited the membrane-damaging action of alpha-toxin (Fig. 6), suggesting that phosphorylcholine residues are directly involved in the interaction between alpha-toxin and membrane. However, 1000-fold more free phosphorylcholine molecules were required for the inhibition, compared with the PC-cholesterol liposomes on the basis of phosphate concentration (Figs. 2 and 6). It is, therefore, feasible that phosphorylcholine residues have more chance to react with alpha-toxin when they are membrane-associated, i.e., when they are properly oriented on/in the membrane. Although free phosphorylethanolamine exhibited inhibitory effect (Fig. 6), PE-cholesterol liposomes did not inhibit the interaction bteween alpha-toxin and DOPC-cholesterol liposomes (Fig. 2). A possible explanation of this apparent discrepancy would be as follows: alpha-toxin can interact with phosphorylethanolamine residue of membrane-associated PE (perhaps less efficiently than with phosphorylcholine residue; Fig. 3 and Fig. 6), but it can not be bound to or integrated into the PE-cholesterol membrane.

In terms of net charge on the liposomal surface at neutral pH, the liposomes used are divided into two groups, i.e., the liposomes containing PC, sphingomyelin, phosphatidyl-*N*,*N*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine or PE which have no net charge, vs. the others containing PS, PG or PI which have negative net charge. Since the latter liposomes are insensitive to alpha-toxin, the negative charge may have inhibitory effect on the interaction between liposomes and alpha-toxin. However, difference in the surface net charge is not enough to explain the different susceptibilities of various liposomes to alpha-toxin, since PE-containing liposomes as well as those containing PC, sphingomyelin, phosphatidyl-*N*,*N*-dimethylethanolamine or phosphatidyl-*N*-monomethylethanolamine have no net charge, but their susceptibilities to alpha-toxin are different. Furthermore, the addition of 10 mol% dipalmitoylphosphatidic acid (negative charge) or stearylamine (positive charge) into DOPC-cholesterol liposomes did not alter the susceptibility of the liposomes.

Bernheimer et al. [15] reported that the ring-shaped structures were formed when alpha-toxin was incubated with the liposomes prepared from PC, PS, PI or cardiolipin, but not with those composed of PE. However, they observed the ring-shaped structures, not on the liposomes composed of PI or cardiolipin, but in the background. Moreover their results varied with different commercial lots of PC, i.e., the ring-shaped structures were not observed on the liposomes but mainly in the background, when some lots of PC were used. Other studies on the formation of ring-shaped structures were performed with the PC-containing liposomes [9,13,14]. Taken together, it is not clear whether or not the ring-shaped structures were formed on the liposomes composed of the phospholipids other than PC.

Incubation of alpha-toxin with cholesterol did not inactivate the toxin (unpublished data), and the liposomes composed of DOPC (Fig. 9), egg yolk PC or soyabean PC alone were also susceptible to alpha-toxin (unpublished data). Therefore, cholesterol may not directly interact with alpha-toxin, but rather may facilitate the interaction as a structural component of the membrane. The enhancing effect of cholesterol would be explained by the increased mobility of choline head group and the increased water accessibility in the hydrophilic loci of the membrane [28,29]. As stated in Results, the liposomes composed of sphingomyelin alone exhibited no significant toxin-binding activity, suggesting that cholesterol is indispensable for the interaction between alpha-toxin and

sphingomyelin. It is not clear why PC liposomes were sensitive to alpha-toxin, whereas liposomes composed of sphingomyelin alone did not bind the toxin. A possible explanation would be that because of the structural difference between glycerophospholipid and sphingophospholipid, phosphorylcholine residues of sphingomyelin are more properly oriented on membrane to react with alpha-toxin only when cholesterol is present.

Our preliminary experiments indicated that DOPC-cholesterol liposomes were more susceptible to alpha-toxin that distearoylphosphatidylcholine-cholesterol liposomes at 25 °C, and liposomes containing PC with shorter acyl chains were more sensitive to the toxin, compared with those containing PC with longer acyl chains (unpublished data). These results suggested that the fluidity of hydrophobic region of PC also affects the susceptibility of membrane to alpha-toxin.

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