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Liposomal Membranes. XIX. Interaction between Spermicidal Agents and Liposomes Reconstituted with Boar Spermatozoal Lipids

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To evaluate the spermicidal effect of several nonionic surfactants against human spermatozoa, the physicochemical lysis of liposomal membranes by the surfactants has been investigated. Surfactants employed in this work were menfegol (TS-88), nonoxynol-9 (INP-90), octoxynol-9 (NOP-90), hexadecyltrimethylammonium bromide (CTAB), and sodium dodecylsulfate (SDS). Lysis of liposomes by these spermicidal surfactants was quantitatively followed by monitoring the induced release of carboxyfluorescein (CF) encapsulated in the interior of liposomes. When the liposomes reconstituted with boar spermatozoal lipids and about 31% (by wt.) cholesterol were employed, the sequence in efficiency of the surfactant-induced CF release from the liposomes was significantly correlated with that of efficiency of the surfactants in immobilizing human sperm ($p<0.05$). On the other hand, when egg lecithin liposomes or the liposomes reconstituted with boar spermatozoal lipids and cholesterol less than 21% (by wt.) were utilized, the sequence in efficiency of CF release from these liposomes coincided with that in the inhibiting effect of the surfactants on the fertilizing ability of sea urchin sperm. These effects were closely correlated with the membrane fluidity as controlled by the cholesterol content or lipid composition. Among menfegol analogues from TS-40 through TS-200, the efficiency in induced CF release from the liposomes showed a maximum at around ten ethylene oxide units length of the hydrophilic moiety in the surfactant. This was also the case for spermicidal effect of the TS-series surfactants. The data obtained are discussed at the molecular level from the viewpoint of the structural characteristics of the surfactants.

Keywords——liposome; reconstituted liposome; sperm cell membrane model; boar spermatozoal lipid; menfegol (TS-88); nonoxynol-9 (INP-90); octoxynol-9 (NOP-90)

Release of a water-soluble fluorescent probe, carboxyfluorescein (CF), from the interior of egg phosphatidylcholine (egg PC) liposomes is induced by spermicidal nonionic surfactants.[1] The efficiency of the surfactants in CF release from liposomes is closely correlated with that in the inhibition of fertilizing ability of sea urchin spermatozoa.[1] Further, egg lecithin liposomes are a convenient model for sperm cells in evaluating the spermicidal activity of the surfactants, which are considered to change the permeability of spermatozoal cell

membranes.[1]

In this work, in order to evaluate the spermicidal activity of the above nonionic surfactants (TS-88, INP-90, and NOP-90) against human spermatozoa, the interaction between these surfactants and liposomes reconstituted with boar spermatozoal lipids has been investigated and compared with the effect on spermatozoa of sea urchin. The data are discussed in relation to the membrane fluidity (estimated by fluorescence polarization measurements), permeability (deduced from the CF release), and structural characteristics of the surfactants.

Experimental

Materials——Boar spermatozoal lipids were extracted from Hampshire sperm kindly supplied by the Isahaya Boar Breeding Farm, Isahaya City, Nagasaki, according to the procedure of Folch *et al.*[2] Four hundred ml of fresh sperm was centrifuged at 8000 rpm and 0 °C for 20 min. Sperm cells isolated from the plasma were suspended in 400 ml of 0.15 M NaCl at 0 °C. Sedimentation and washing were repeated three times in the same way. The washed spermatozoa were resuspended in 513 ml of chloroform–methanol (2 : 1 by vol.) and homogenized on an ice bath for several min. After filtration of the homogenate, to the filtrate was added 128 ml of distilled water. The chloroform layer containing lipids was separated after keeping it overnight at 0 °C in the dark. Upon evaporation of the solvent, lipids containing a small amount of cholesterol were obtained: yield, 256 mg; mp 155—168 °C. IR ν_{max}^{KBr} cm^{-1}: 3320 (O–H); 2920 and 2850 (CH_2); 1740 (C=O); 1660 (C=C); 1230 and 1160 (P=O); and 1070, 970; and 820 (P–O–C). IR δ_{max}^{KBr} cm^{-1}: 1470 (C–H) and 1380 (CH_3).

The fatty acid composition of the extracted spermatozoal lipids was determined by gas-liquid chromatography (GLC) after saponification of the lipids followed by esterification with diazomethane.[3] To 28.6 mg of the lipid was added a mixture of 3 ml of methanol, 3 ml of water, and 0.8 ml of 85% KOH. The resulting mixture was hydrolyzed above pH 10 at 70—80 °C for 90 min under stirring on a magnetic stirrer. The mixture was acidified to below pH 1 with cold conc. HCl, then 18 ml of dry ether was added and the whole was stirred for 40 min at 0 °C. The reaction mixture was centrifuged at 1000 rpm and 0 °C for 5 min. The supernatant ether layer containing fatty acids was mixed with 4 ml of 2% aq. KCl solution, and the mixture was stirred for 15 min on a magnetic stirrer equipped with an ice-bath. The ether layer was separated and washed thrice with distilled water upon centrifugation at 1000 rpm and 0 °C for 5 min. After evaporation of the solvent, 10 ml of diazomethane–ether solution was added and the mixture was kept for 1 h at room temperature to obtain the fatty acid methyl esters. Excess diazomethane and ether were removed by passing dry gaseous nitrogen through the mixture, then 15 μl of ether was added to provide a sample for GLC (Shimadzu GC-7A FID gas chromatograph with 20% EGSS-X on Chromosorb WAW 80/100 (i.d. 3 mm × 3 m); column temperature, 180 °C; injection temperature, 210 °C; mobile phase, N_2 at a flow rate of 40 ml/min). The fatty acid residues of parent spermatozoal lipids as assigned using the calibration curve obtained beforehand,[4,5] the retention times (min), and the contents (%) in total fatty acids were respectively as follows: $C_{6:1}$, 1.44 min, 0.25%; $C_{8:0}$, 1.95, 2.33; $C_{8:1}$, 2.39, 0.06; $C_{8:2}$, 2.14, 0.04; $C_{10:0}$, 2.95; 0.03; $C_{10:1}$, 3.80, 0.02; $C_{10:2}$, 5.74, 0.07; $C_{12:0}$, 5.40, 0.10; $C_{14:0}$, 9.84, 8.11; unknown, 13.8, 2.14; $C_{14:2}$, 17.44, 17.53; $C_{16:2}$, 30.57, 4.78; $C_{18:1}$, 35.90, 5.05; unknown, 45.6, 3.72; $C_{20:2}$, 96.30, 0.55; $C_{22:1}$, 110.44, 2.78; $C_{24:1}$, 200.04, 0.87; $C_{26:0}$, 232.57, 13.60; $C_{26:1}$, 320.04, 37.53; and $C_{26:2}$, 424.57, 0.21. The extracted lipids were subjected also to thin-layer chromatography (TLC) on a silica gel plate (Merck) developed with $CHCl_3$: MeOH : H_2O (65 : 25 : 4 by vol.).[6] Most of the lipids were phosphatidylcholines (Rf=0.16 and 0.25) and phosphatidylglycerol (Rf=0.49). Neither phosphatidylethanolamine nor lysophosphatidylcholine was detected, but small amounts of phosphatidylserine (Rf=0.19) and sphingomyelin (Rf=0.12) were observed. The present extraction procedures[2] could not completely exclude free cholesterol from the extracts. Hence, the cholesterol content was also determined using a TLC/hydrogen flame ionization detector (FID) analyzer (Iatron TH-10 with a Chromarod-SII, developed with hexane : ethyl ether : formic acid (90 : 10 : 0.2 by vol.) and detected by FID with H_2 (160 ml/min) and air (240 ml/min)). Total phospholipids and free cholesterol were 87.0 and 10.9 wt%, respectively, together with 1.8 wt% of unknown compounds. However, no cholesterol esters, triglycerides, or free fatty acids were detected at all. The present result on the lipid composition is not exactly the same as that recently reported by Nissen and Kreysel using a high performance liquid chromatography (HPLC) technique.[7] The difference between the results must be ascribed to the difference in the extraction procedures.

Egg phosphatidylcholine (egg PC) was isolated and purified from fresh egg yolk according to the method described previously.[8,9] Dipalmitoyl-DL-α-phosphatidylcholine, dipalmitoyl-DL-α-phosphatidylethanolamine and L-α-phosphatidyl-L-serine were purchased from Sigma Chemical Co., St. Louis, Mo. Carboxyfluorescein (CF) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Eastman Kodak, Rochester, N.Y. and Aldrich Chemical Co., Millwaukee, Wi., respectively. Cholesterol (Nakarai Chemical Co., Ltd., Kyoto) was purified by recrystallization prior to use.

p-(Menthanyl)phenylpolyoxyethylenyl(8.8)ether (TS-88), *p*-(menthanyl)phenyl polyoxyethylenyl (4.0 through

20.0) ethers, such as TS-40, -60, -71, -80, -90, -112, -131, -150, and -200, *p*-(1,3,5-trimethylhexyl)phenyl polyoxyethylenyl (9) ether (INP-90), *p*-(1,1,3,3-tetramethylbutyl)phenyl polyoxyethylenyl (9) ether (NOP-90), sodium dodecyl sulfate (SDS), and hexadecyltrimethylammonium bromide (CTAB) were all the same as those used in the previous work.[1,10,11] All the other reagents were commercially available as analytical grade and used without further purification.

Preparation of Liposomes——Small unilamellar vesicles of egg PC and boar spermatozoal lipids were prepared and isolated by essentially the same method as described previously.[1,8] All the vesicle formations were directly visualized on a JEOL JEM-100 electron microscope by negative staining method with 2% sodium phosphotungstate.[9]

Surface Charge of Liposomes——Surface charge of the reconstituted liposomal membranes was measured spectrophotometrically by using methylene blue as a dye probe according to the method established by Nakagaki and his coworkers.[12] As standard samples, egg PC liposomes containing dicetylphosphate at various concentrations from 5 through 20 mol% were employed. The surface charge of the reconstituted liposomes was anionic and comparable to that of egg PC liposomes containing 20 mol% dicethylphosphate.

Miscellaneous Measurements——Procedures to prepare CF-encapsulated liposomes and to monitor CF release from liposomes upon perturbation with surfactants were the same as those employed previously.[1]

The membrane fluidity of the reconstituted liposomes containing cholesterol at various concentrations was examined by fluorescence polarization measurements using a hydrophobic fluorescent probe, DPH, as described elsewhere.[13,14]

Effective concentrations of surfactants for immobilization of human sperm were measured at various concentrations of the surfactants in 5% (w/v) glucose solution (glucose injection, JPX) by the same method as described previously.[10] The minimum spermicidal concentration able to irreversibly immobilize all the sperm at zero time was estimated.

Results and Discussion

Interaction of the Spermicidal Surfactants with Liposomal Membranes

Previously, it was found that CF release from egg PC liposomes is significantly induced by several spermicidal surfactants and that the efficiency in induction of CF release is closely correlated with that in the inhibition of fertilizing ability of sea urchin sperm.[1] In this work, we employed liposomes reconstituted with boar spermatozoal lipids and investigated the difference and/or correlation in the interaction with the same spermicidal surfactants between the liposomes and human sperm.

In phospholipids extracted from egg yolk, only about 50% of the fatty acids are saturated and the longest carbon skeleton is twenty carbons. On the other hand, in phospholipids from boar spermatozoa more than about 70% of the fatty acids are unsaturated and the longest one is twenty-six carbons. For boar spermatozoal lipids, even in the presence of ethylene

TABLE I. Fluidity of Various Liposomal Bilayer Membranes[a]

Membrane	Fluorescence polarization (p)	Order parameter
Egg PC	0.10	0.27[g]
Egg PC-10[b]	0.15	0.42[g]
Egg PC-20[c]	0.20	0.48[h]
Boar SL-11[d]	0.14	0.34[g]
Boar SL-21[e]	0.23	0.60[g]
Boar SL-31[f]	0.27	0.66[h]

a) [Phospholipid] = 1.0×10^{-4} M; [DPH] = 1.0×10^{-7} M; pH 8.6, at 37.0 °C.
b) Egg PC with 10% (w/w) cholesterol.
c) Egg PC with 20% (w/w) cholesterol.
d) Boar spermatozoal lipids with 11% (w/w) cholesterol.
e) Boar spermatozoal lipids with 21% (w/w) cholesterol.
f) Boar spermatozoal lipids with 31% (w/w) cholesterol.
g) Calculated by the method described in ref. 13.
h) Calculated by the method described in ref. 14.

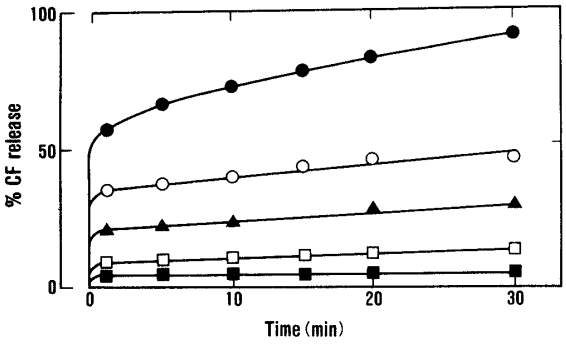

Fig. 1. Carboxyfluorescein Release Induced by Several Surfactants from the Liposomes Reconstituted from Boar Spermatozoal Lipids and 31% Cholesterol at 37.0 °C and pH 8.6

—●—, INP-90; —○—, TS-88; —▲—, NOP-90; —□—, CTAB; and —■—, SDS.

TABLE II. Percent Release of Carboxyfluorescein (R_{CF}) Induced by Surfactants from Various Liposomes[a)]

Surfactant	R_{CF} (%)				
	Egg PC-10	Egg PC-20	Boar SL-11	Boar SL-21	Boar SL-31
TS-88	96.0	89.0	36.0	63.0	40.0
INP-90	92.0	81.0	32.5	50.0	72.5
NOP-90	33.0	43.0	20.3	23.0	25.0
CTAB	57.5	39.5	10.0	15.0	10.0
SDS	2.0	3.0	2.5	3.0	5.0

a) At 5 min after injection of an aqueous surfactant solution at 37.0 °C and pH 8.6. Values are averages of triplicate determinations. All the abbreviations are the same as in the footnote to Table I. [Phospholipid] = 1.3×10^{-4} M, [Surfactant] = 1.0×10^{-4} M.

glycol,[15)] no discernible phasetransition could be observed over the range from −40 °C through +40 °C, though about 11% cholesterol was contained. Nevertheless, the fluorescence polarization studies showed that the membrane of the reconstituted liposomes is barely more fluid than that of egg PC liposomes, as seen in Table I. In any event, small unilamellar liposomes of boar spermatozoal lipid could be well prepared and could encapsulate CF in the same way as egg PC liposomes.

When an aqueous solution of the surfactants was injected into the liposome suspension after an appropriate preincubation (usually for 5 min) at 37.0 °C, an instantaneous and rapid CF release was observed followed by a slower release then a levelling off. The extent of the CF release was affected by the concentration and structure of surfactants and the cholesterol content. A typical example of the surfactant-induced CF release from the liposomes reconstituted with boar spermatozoal lipids is shown in Fig. 1. These phenomena were the same as those observed in the previous study for egg PC liposomes.[1)] The results are summarized in Table II. Clearly, the efficiencies of the CF release induced by the surfactants employed were rather different, especially in the effect of cholesterol content, between liposomes prepared from egg PC and from boar spermatozoal lipids.

Since it seemed that the CF release induced by the surfactants is closely correlated with membrane fluidity, we investigated this point first. As expected, the order parameter calculated from the fluorescence polarization, p-values, showed that adding cholesterol decreases the membrane fluidity (Table I) and the CF release is effectively depressed by adding cholesterol in the case of egg PC liposomes (Table II); namely, the more fluid the membrane is, the more CF release is accelerated. Thus, the egg PC liposomes were more perturbed by the surfactants than the spermatozoal lipid liposomes.

The surface charge of egg PC liposomes is neutral, while that of the liposomes of boar

TABLE III. Effective Concentrations of Surfactants to Inhibit Fertilizability of Sea Urchin Sperm and to Immobilize Human Sperm

Surfactant	C_{f_0} [a] (μM)	MSC_0 [b] (mM)
TS-80	2.88	1.31
INP-90	5.50	0.86
NOP-90	5.37	1.71
CTAB	10.47	5.12[c]
SDS	31.62	5.79

a) Minimum effective molar concentration for 100% inhibition of fertilizability of sea urchin sperm.[1,10]
b) Minimum spermicidal concentration for irreversibly immobilizing human sperm at 0 min.[11]
c) This work.

spermatozoal lipids is relatively anionic (see Experimental). It is, hence, expected that a cationic surfactant will interact more strongly with and perturb the latter liposomes more than the former. Contrary to what would be expected, however, CTAB was not effective enough to perturb even the boar spermatozoal lipid liposomes. Thus, the membrane fluidity must be a more significant factor in the interaction with these surfactants.

An additional interesting finding is that there exists a significant correlation between the CF release induced by and the spermicidal activity of these three spermicidal surfactants. First, we determined the minimum concentration for a spermicidal agent to irreversibly immobilize human spermatozoa at zero time (MSC_0).[10] Data are listed in the second column of Table III. The sequence of MSC_0 for the surfactants employed coincided exactly with that of induced CF release from the reconstituted liposomes of boar spermatozoal lipids containing 31 wt% cholesterol. For other liposome systems, on the other hand, the sequence of efficiency in induced CF release was roughly consistent with that of the inhibition in the fertilizing ability of sea urchin sperm.[1] Hence, a regression analysis of the obtained data (Tables II and III) was considered. As in the previous case,[1] a reasonable linear correlation was obtained between R_{CF} (the percent release of CF from the Boar SL-31 liposomes (Table II)) and $\log MSC_0$: $\log MSC_0 = -0.0125R_{CF} - 2.27$ with $r = -0.924$, $n = 5$, and $p < 0.05$ for $R_{CF} > 0$. On the other hand, also in the case of the inhibition of fertilizing ability of sea urchin sperm, there exists a significant correlation between $\log C_{f_0}$ and the percent release of CF from egg PC-20 liposomes, R_{CF}: $\log C_{f_0} = -0.0104R_{CF} - 4.58$ with $r = -0.917$, $n = 5$, and $p < 0.05$, for $R_{CF} > 0$.

Very recently, Davis has proposed[16] that the initiation of the sperm acrosome reaction among mammals should be the first fusion process found to be physiologically modulated through the membrane bilayer cholesterol level and that during the capacitation of sperm the concentration level of cholesterol decreases. In addition, the capacitation interval (T) of spermatozoa is closely correlated with the cholesterol/phospholipid ratio (R-value); for example, for boar sperm T is 2 h and the R-value is 0.35, while for human sperm, T and the R-value are 7 h and 0.99, respectively. These findings suggest that if the cholesterol content in the boar spermatozoal lipid liposomes were increased, the membrane fluidity of the reconstituted liposomes would more closely resemble that of human spermatozoa. Our present findings are consistent with this. These data reveal that the membrane fluidity as controlled by the concentration level of cholesterol is very important in both the capacitation and inhibition of the acrosome reaction and/or the fertilization of sperm. We would like to propose, in addition, that the reconstituted liposomes of animal spermatozoal lipids are a convenient model system for human sperm.

Effect of Chemical Structure of the Surfactants on the Perturbation of Liposomal Membranes

The effect of the structure of the hydrophobic moiety in nonionic surfactants on the CF release from liposomes is also another important factor. Not only the hydrophobicity but also the bulkiness must be considered.[1] In fact, Ishii and his coworkers[17] have revealed that the structure of the hydrophobic part in nonionic surfactants derived from octylphenyl greatly affects their solution behavior.

Zaslavsky and his coworkers have shown that the effect of nonionic surfactants derived from fatty acids on the hemolysis of human erythrocytes cannot be simply interpreted in terms of the hydrophile-lipophile balance (HLB).[18] Figure 2 shows the effect of the number of ethylene oxide units in the TS-88 analogues on the induced CF release from egg PC liposomes. The efficiency increased suddenly from the compound carrying around eight units of ethylene oxide (TS-80) up to TS-110. Further increase in the length of the ethylene oxide moiety, which corresponds to an increase in the hydrophilicity, did not cause any further significant increase in the efficiency. This is in good agreement with the data recently reported by one of the present authors (K.F.), who found that the effect of TS-88 analogues on the induction of the acrosome reaction and the inhibition of fertilizing ability of sea urchin sperm was also enhanced over the range of TS-80 to TS-150.[11] Similarly to the case of egg PC liposomes, in the reconstituted liposomes of boar spermatozoal lipids containing 31% cholesterol, the profile of the efficiency in induced CF release with TS-series surfactants showed a bell shape with a maximum around TS-90 and TS-110 (Fig. 3). A bell-shaped profile has also been observed in the efficiency of hemolysis of human erythrocytes with nonionic surfactants, Triton-X series.[18] This relationship is again consistent with the recent finding that the spermicidal activity of such a surfactant series on human sperm was highest with TS-90 and TS-110.[10] At present, the reason why a certain length of the hydrophilic moiety is required for bilayer membranes to be effectively perturbed and the detailed mechanism are not clear.

Hansch and his coworkers have proposed that the mobility of drug through biological materials is an important factor in considering the drug activity and is significantly correlated with HLB.[19] In fact, one of the present authors (K.F.) also showed that a good correlation exists between the biological activities of the present surfactants to animal sperm and the partition coefficients in the apolar organic solvent-water system.[10,11] However, if one recognizes that the first interacting point of lipophilic drugs in biological systems after administration must be at the cell membrane level, liposomes appear to be a better tool to

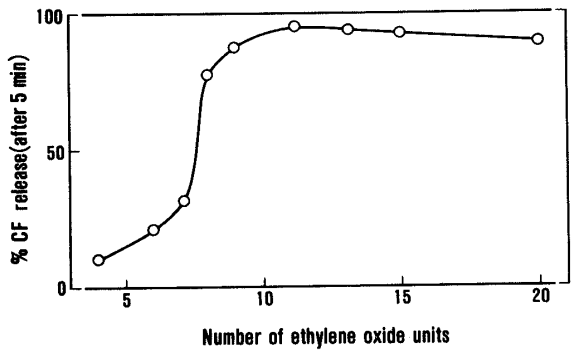

Fig. 2. Carboxyfluorescein Release Induced by TS-(40 through 200) Surfactants from Egg PC Liposomes without Cholesterol at 37.0 °C and pH 8.6

The extent of release (%) was obtained at 5 min after injecting surfactants into the pre-incubated liposome suspension.

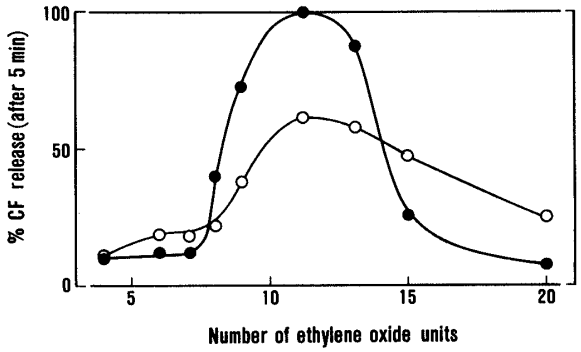

Fig. 3. Carboxyfluorescein Release Induced by TS-Series Surfactants from Liposomes Reconstituted from Boar Spermatozoal Lipids Containing 11% (by wt.) Cholesterol (Boar SL-11, —○—) and 31% (by wt.) Cholesterol (Boar SL-31, —●—) at 37.0 °C and pH 8.6

The extent of release was determined at 5 min after injecting the surfactants into liposome suspension.

rationalize the drug activity with the structural characteristics. Furthermore, we consider that the reconstituted membrane of boar spermatozoal lipids is more sensitive to the chemical structure of the spermicidal surfactants than the conventional liposomes of egg PC.

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