The effect of the lipid A analog E5531 on phospholipid membrane properties

Yasuyuki Asai[a,*], Kiyoshi Iwamoto[a], Sumio Watanabe[b]

[a] *Formulation Research Laboratory, Kawashima, Eisai Co., Ltd., Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501-61, Japan*
[b] *Tsukuba Research Laboratories, Eisai Co., Ltd., Tokodai, Tsukuba, Ibaraki 300-26, Japan*

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Abstract In order to determine the effect of the lipid A analog, E5531, on phospholipid membranes, we used dipalmitoylphosphatidylcholine (DPPC) and investigated the physicochemical interaction between E5531 and DPPC membranes. E5531 and DPPC are miscible in the bulk phase at 25°C. Within the E5531 mole fraction range (X_{E5531}) of 0–0.5, E5531 decreased the zeta potentials of DPPC membranes but did not change the size of the DPPC liposomes. E5531/DPPC mixtures formed liposome-like structures. E5531 increased the fluidity of the DPPC membrane and decreased pyrene diffusion in the membrane. E5531 decreased the phase transition temperature and the cooperative interactions between DPPC molecules. These effects of E5531 on phospholipid membranes were different from those of lipid A from *Escherichia coli* and *Salmonella minnesota*.

Key words: DPPC; E5531; Phospholipid membrane

1. Introduction

Lipid A is a lipid anchor in lipopolysaccharide (LPS) that exists on the outer membrane of Gram-negative bacteria. Lipid A induces undesirable toxic effects such as fever and the Schwartzmann bleeding reaction [1,2]. Recent research has focused on the effect of lipid A on the structural and dynamic properties of membranes and revealed that most lipid A-induced biological effects are initiated by binding to a specific receptor [3,4] or by non-specific intercalation into the lipid matrix of the cell membrane [5]. The interaction and subsequent intercalation into the membrane are dependent on the fluidity of the hydrophobic region and/or the supramolecular structure of LPS and lipid A [6]. Rottem [7] has reported that lipid A from *Proteus mirabilis* decreased the membrane fluidity and permeability of phospholipid bilayers. Lei et al. [8] have also reported that lipid A from *Salmonella minnesota* decreased the membrane fluidity and raised the phase transition temperature of phospholipid membranes. In addition, Benedetto et al. [9] have suggested that some of the effects produced by lipid A are mediated by a specific molecular reaction at the cell surface membrane and that the physicochemical properties of the membrane may be an important determinant of the bioactivity of lipid A.

Recent research has also focused on the synthesis of lipid A analogs with low toxicity. The synthetic disaccharide lipid A analog E5531 (Fig. 1a) has low toxicity and retains various useful biological activities (e.g. reduction of TNF production) possessed by lipid A [10]. This compound has been found to be a specific LPS antagonist in an LPS-binding assay, and it inhibits LPS-induced TNF production in monocytes/macrophages. Its anticipated use is as a drug for the treatment of septic shock.

In order to investigate the effect of E5531 on the cell surface membrane, we used dipalmitoylphosphatidylcholine (DPPC) as a model membrane and we investigated the physicochemical properties using several techniques, monolayer to evaluate the miscibility, zeta potentials, trapped volume of fluorescence dye and fluorescence spectrometry to study the fluidity and phase transition temperature of the membranes in the E5531/DPPC mixtures. In addition, the effect of E5531 on DPPC membranes was compared with that for lipid A from *Escherichia coli* and *Salmonella minnesota* (Fig. 1b, Table 1).

2. Materials and methods

2.1. Materials

E5531 was obtained from Eisai (Ibaraki, Japan). The lipids A from *Escherichia coli* F583 (Rd mutant) and *Salmonella minnesota* Re 595 (R mutants) were purchased from Sigma (St. Louis, MO, USA). Calcein (3,3′-bis[*N,N*-bis(carboxymethyl)aminomethyl]-fluorescein) was supplied by Dojin (Kumamoto, Japan). L-α-Dipalmitoylphosphatidylcholine (DPPC), pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Wako (Osaka, Japan). Lactose hydrous, sodium phosphate monobasic, sodium phosphate dibasic and sodium hydroxide were purchased from Mallinckrodt (Paris, KY, USA).

2.2. Evaluation of miscibility of E5531 and DPPC by measurement of spreading pressure

In order to evaluate the miscibility of E5531, lipid A from *Escherichia coli* (EC), lipid A from *Salmonella minnesota* (SM) and DPPC in bulk phase, spreading pressures of the lipid mixtures were measured. E5531, EC and SM were dissolved in methanol and DPPC were dissolved in chloroform, respectively, and mixed in a suitable ratio. After evaporation of the solvent, the dried lipid mixtures were added to distilled water in a surface tensiometer (Model CBVP-A3, Kyowa Kaimenkagaku, Tokyo, Japan). The spreading pressures of the lipid mixtures at an air/water interface (surface pressure of the bulk lipid mixture) were obtained from the steady value of surface pressure at 1–2 h after addition of the lipid or the lipid mixture to the water. Spreading pressures were determined at 25°C. The data are given as mean values of triplicate measurements. The details of the monolayer techniques have been described elsewhere [11,12].

2.3. Preparation of the aggregates from E5531/DPPC mixtures

The aggregates from E5531/DPPC, EC/DPPC and SM/DPPC mixtures were prepared by the method of Dijkstra et al. [13]. DPPC was dissolved in chloroform and E5531, EC and SM were dissolved in methanol. These stock solutions were then mixed at a suitable ratio. The solvents were evaporated under a stream of nitrogen gas at 70°C. The lipid film was hydrated to give a total concentration of the total lipids of 1 mM with 4.25 mM phosphate-NaOH buffer containing 10% lactose (pH 7.3). The lipid dispersion was then sonicated with a probe-type sonicator (Tomy Seiko, Tokyo, Japan) at 50°C for 10 min.

2.4. Determination of the size of the E5531/DPPC aggregates

The size of the aggregates in the lipid mixtures was determined at

*Corresponding author. Fax: (81) (586) 89 3910.

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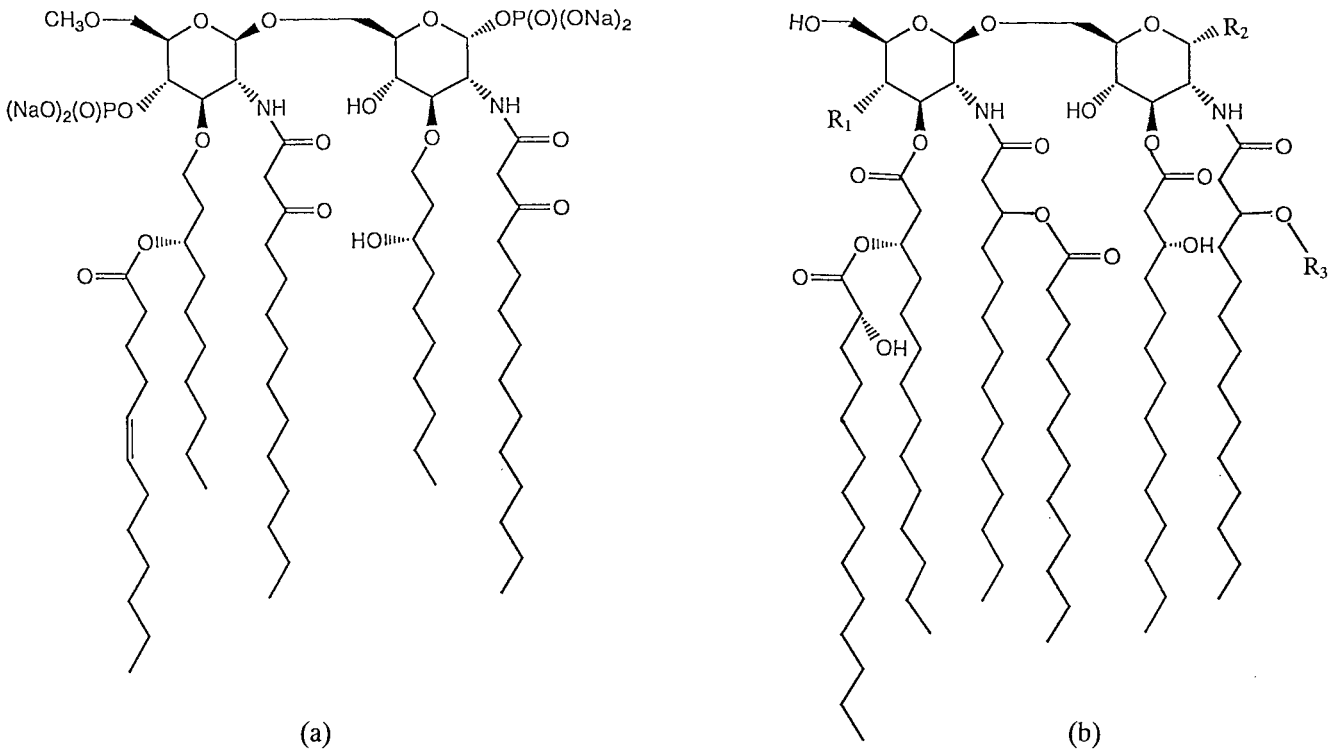

Fig. 1. a: Chemical structure of the synthetic lipid A analog, E5531. b: Chemical structure of the lipid A from *Escherichia coli* and *Salmonella minnesota* (see also Table 1).

25°C by dynamic light scattering (DLS) techniques using a laser particle analyzer (model DLS-7000DL, Ohtsuka Electronics, Osaka, Japan). The data were analyzed by the histogram method [14] and the weight-averaged aggregate sizes were evaluated.

2.5. Determination of zeta potential

The zeta potentials of the lipid mixtures were measured at 25°C using a model ELS-800 analyzer (Ohtsuka Electronics, Osaka, Japan). The data are given as mean values of duplicate measurements.

2.6. Determination of the rapped volume in the aggregates of the E5531/DPPC mixtures

In order to obtain information on the structure of the aggregates in E5531/DPPC, EC/DPPC and SM/DPPC mixtures, the trapped volume inside the aggregates was determined using fluorescence techniques [15]. The mixtures was dispersed in 2.5 ml of 70 mM calcein solution (pH 7.3) with sonication at 50°C for 60 min and then cooled to 25°C. The total lipid concentration was 2 mM.

The untrapped calcein was removed by gel filtration (Sephadex G-50) at 25°C. The volume of the calcein solution trapped in the dispersed aggregates was determined fluorometrically [16] after solubilization of the lipid aggregates by the addition of 10% Triton X-100, and the aqueous volume trapped per mol of DPPC was evaluated. DPPC in the dispersion was assayed by HPLC (detection wavelength 210 nm).

2.7. Determination of the membrane fluidity of the E5531/DPPC mixtures

The membrane fluidity of the E5531/DPPC, EC/DPPC and SM/DPPC aggregates was determined using a fluorescence polarization technique (Probe: DPH) as reported by Iwamoto et al. [17]. DPH was added at 1 mol% of total lipids. All fluorescence measurements were carried out using a Model F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with a thermoregulated cell compartment, Atago Coolnics Model REX-C10 (Atago, Tokyo, Japan). The degree of polarization (P) was calculated using the following equation:

$$P = (I_{VV} - C_f \cdot I_{VH})/(I_{VV} + C_f \cdot I_{VH})$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($= I_{HV}/I_{HH}$) is the grating correction factor.

2.8. Determination of the phase transition temperature by pyrene diffusion

In order to determine the phase transition temperature of the E5531/DPPC, EC/DPPC and SM/DPPC membranes with a method other than fluorescence polarization (probe: DPH), the fluorescence spectra of pyrene embedded in E5531/DPPC, EC/DPPC and SM/DPPC membranes were measured. Pyrene was added at 1 mol% to total lipid. Measurements were made at increasing temperatures with an excitation wavelength of 330 nm and emission wavelength of 480 nm. The intensity ratio of the pyrene fluorescence peak (I_{480}/I_{376}) is the ratio of excimer fluorescence intensity (480 nm, I') to the monomer fluorescence intensity (376 nm, I) and is reported to correlate with the diffusion of pyrene in the lipid membrane [18].

2.9. Thermotropic behavior of E5531/DPPC mixtures

In order to investigate the phase transition of the lipid mixtures, differential scanning calorimetry (DSC) was performed using a Model DSC-100 (Seiko-Denshi, Tokyo, Japan). E5531/DPPC, EC/DPPC and SM/DPPC mixtures (total 1.5×10^{-6} mol) in 40 μl of 4.25 mM phosphate-NaOH, 10% lactose buffer (pH 7.3) were placed in a DSC pan and sealed. An equal volume of the buffer solution (pH 7.3) was placed in the reference pan. Temperature scans were made from

Table 1
Chemical structure of lipid A from *Escherichia coli* and *Salmonella minnesota*

Preparation	Chemical nature of		
	R_1	R_2	R_3
E. coli lipid A	*P*	*P*	H
S. minnesota lipid A	*P*- - -AraN	*P*- - -*P*-EtN	16:0

Dotted lines indicate incomplete subscription. AraN = β-linked 4-amino-4-deoxy-L-*arabino*pyranose; EtN = ethanolamine; 16:0 = hexadecanoyl.

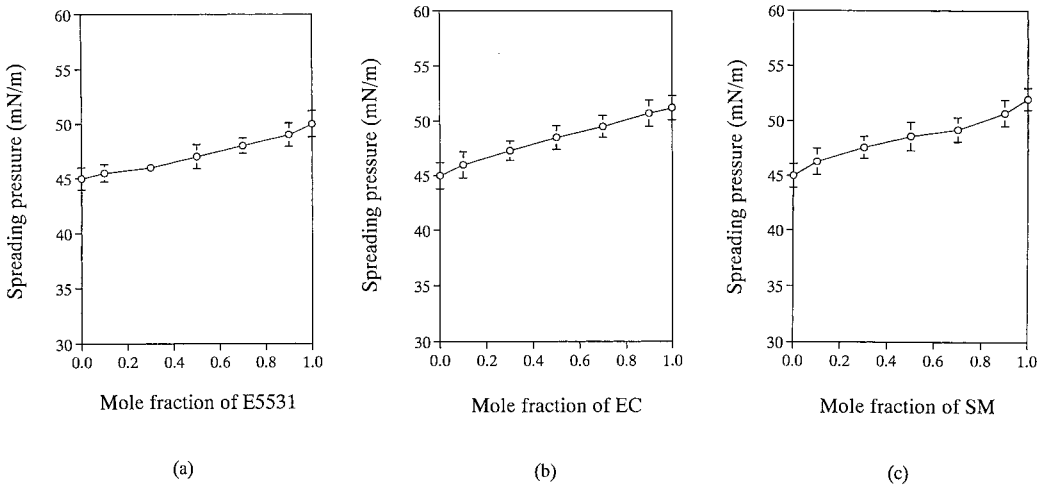

Fig. 2. Spreading pressures of the lipid mixtures at an air/water interface at 25°C. Each point represents the mean ± S.E.M. of triplicate measurements. a: E5531/DPPC mixtures; b: *E. coli*/DPPC mixtures; c: *S. minnesota*/DPPC mixtures.

10°C to 70°C with heating rates of 2°C/min. All calorimetric data were obtained from samples during the heating phase.

3. Results

3.1. Evaluation of miscibility of E5531 and DPPC

Fig. 2 shows the spreading pressures of E5531/DPPC, EC/DPPC and SM/DPPC mixtures. The spreading pressure of hydrated DPPC (lamellar bilayers of DPPC) was 45.0 mN/m. Those for E5531, EC and SM were 50.0, 51.2 and 52.0 mN/m, respectively. The spreading pressure of a lipid mixture depends on the miscibility of the lipids in the bulk phases [19,20]. The spreading pressure of E5531/DPPC, EC/DPPC and SM/DPPC mixtures varied with the mole fraction of E5531 in the lipid mixture (X_{E5531}), EC in the mixture (X_{EC}) and SM in the mixture (X_{SM}). On the basis of the surface phase rule [12,21] it was concluded that E5531, EC, SM and DPPC were miscible in the bulk phases, respectively. Based upon these results, we decided to evaluate the effect of E5531, EC and SM on DPPC membranes in the range 0–0.5.

3.2. Determination of the size of the aggregates and the zeta potentials of the E5531/DPPC mixtures

Table 2 shows the weight averaged size of the aggregates of the E5531/DPPC, EC/DPPC and SM/DPPC mixtures evaluated by DLS measurements and their zeta potentials at different X_{E5531}, X_{EC} and X_{SM}. The mean diameters for E5531/DPPC, EC/DPPC and SM/DPPC mixtures were almost 18, 24 and 25 nm, respectively, and independent of X_{E5531}, X_{EC} and X_{SM}. The zeta potentials were negative and decreased as X_{E5531}, X_{EC} and X_{SM} increased. Since E5531, EC and SM are negatively charged in neutral aqueous solution, the phosphate group at the head sugar moiety of E5531, EC and SM was considered to be at the surface of the lipid membrane and this group conferred the negative charge on the aggregates.

3.3. Structure of the aggregates in the E5531/DPPC mixtures

Neutral lipids such as α-tocopherol and phosphatidylcholine are miscible in the bulk phase and form a bilayer and hexagonal (H_{II}) phases [22]. E5531, EC or SM and DPPC are also miscible in the bulk phase and we investigated the phase structure of E5531/DPPC, EC/DPPC and SM/DPPC mixtures. Table 2 represents the volumes of trapped inner space in the aggregates per mol of the E5531/DPPC, EC/DPPC and SM/DPPC mixtures. The trapped volumes of small unilamellar vesicles (diameter 20–50 nm), large unilamellar vesicles (200–1000 nm) and multilamellar vesicles (400–3500 nm) of phosphatidylcholine have been estimated to be 0.2–0.5, 3–4

Table 2
Size of aggregates, zeta potentials and trapped volumes for E5531, *Escherichia coli*, *Salmonella minnesota* and DPPC mixtures

	Mole fraction of the lipids in the mixtures			
	0	0.1	0.3	0.5
Size of aggregates (nm)				
E5531	18.9 ± 7.9	20.0 ± 7.3	17.4 ± 6.5	19.4 ± 7.9
Escherichia coli	18.9 ± 7.9	24.8 ± 5.2	25.2 ± 3.8	24.3 ± 4.0
Salmonella minnesota	18.9 ± 7.9	25.6 ± 6.1	27.1 ± 4.6	26.5 ± 5.2
Zeta potential (mV)				
E5531	−1.3	−26.9	−46.4	−58.1
Escherichia coli	−1.3	−24.8	−43.5	−55.3
Salmonella minnesota	−1.3	−25.1	−44.8	−56.9
Trapped volume (liter/mol)				
E5531	0.36	0.36	0.37	0.35
Escherichia coli	0.36	0.45	0.44	0.45
Salmonella minnesota	0.36	0.43	0.47	0.45

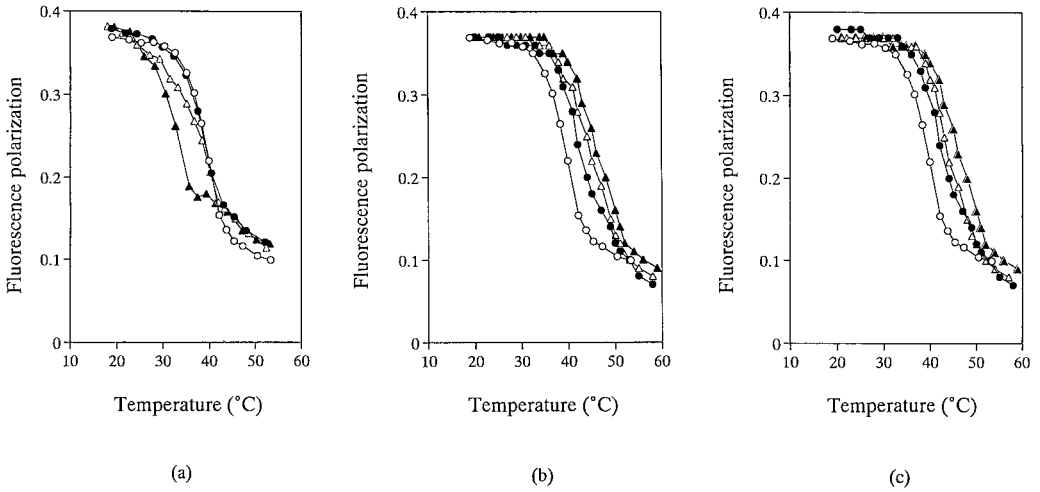

Fig. 3. Relationship between incubation temperature and fluorescence polarization using DPH as a function of the lipid mole fraction in the lipid mixture. a: E5531/DPPC mixtures; $X_{E5531} = 0$ (○- - -○), 0.1 (●- - -●), 0.3 (△- - -△), 0.5 (▲- - -▲). b: *E. coli*/DPPC mixtures; $X_{EC} = 0$ (○- - -○), 0.1 (●- - -●), 0.3 (△- - -△), 0.5 (▲- - -▲). c: *S. minnesota*/DPPC mixtures; $X_{SM} = 0$ (○- - -○), 0.1 (●- - -●), 0.3 (△- - -△), 0.5 (▲- - -▲).

and 7–10 l/mol, respectively [23]. The size of the aggregates of DPPC liposomes was 18 nm and the trapped volume was 0.36 l/mol. The size of the aggregates and trapped volume of the E5531/DPPC, EC/DPPC and SM/DPPC mixtures remained constant over the X_{E5531}, X_{EC} and X_{SM} range of 0–0.5. These data indicate that within this range E5531/DPPC, EC/DPPC and SM/DPPC molecules form liposome-like structures (small unilamellar vesicles) and have a membrane.

3.4. Membrane fluidity of the E5531/DPPC mixtures

The membrane fluidity of the E5531/DPPC, EC/DPPC and SM/DPPC mixtures were evaluated using fluorescence polarization techniques (Fig. 3). The fluorescence polarization of DPH in DPPC liposomes decreased markedly around 40°C, indicating that the phase transition of the DPPC bilayer from gel to liquid-crystal state occurs at this temperature. This result is in good agreement with the reported value [24]. The phase transition of the lipid mixtures was dependent on X_{E5531}. At $X_{E5531} = 0.1$, 0.3, 0.5, the phase transition temperatures were 38, 37 and 33°C, respectively. As X_{E5531} increased the fluorescence polarization decreased. These results indicate that with the increase in X_{E5531} a more fluid membrane was formed and the cooperative interaction between the DPPC molecules decreased. On the other hand, at $X_{EC} = 0.1$, 0.3, 0.5, the phase transition temperatures were 44, 46 and 47°C, respectively. At $X_{SM} = 0.1$, 0.3, 0.5, the phase transition temperatures were 42, 43 and 45°C, respectively. These results indicate that with the increase in X_{EC} and X_{EC} more rigid membranes were formed and the cooperative interaction between the DPPC molecules increased.

3.5. Diffusion of pyrene in E5531/DPPC membrane

The pyrene fluorescence intensity ratio I'/I was plotted as a function of the temperature (Fig. 4). The lipid phase transition

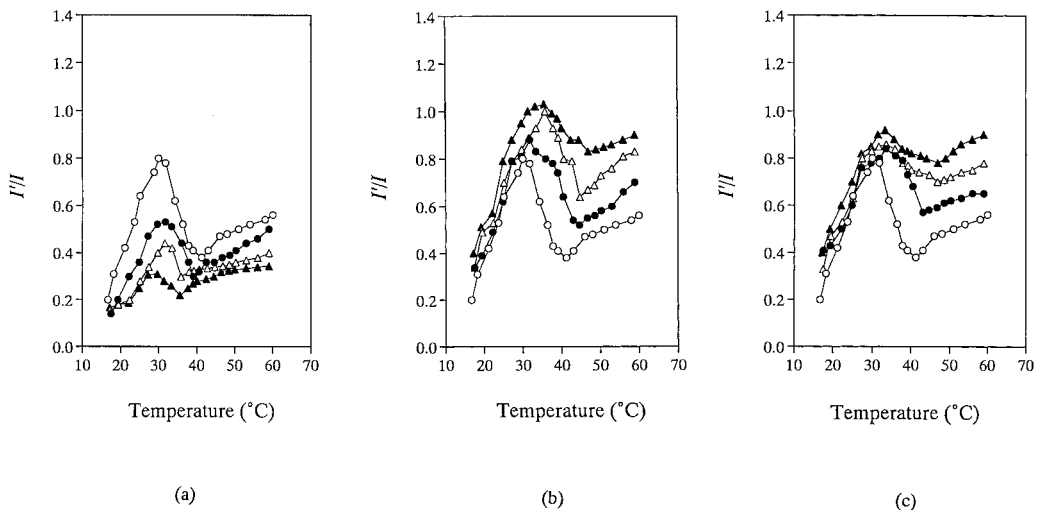

Fig. 4. Relationship between incubation temperature and I'/I for pyrene diffusion in the lipid mixtures. a: E5531/DPPC mixtures; $X_{E5531} = 0$ (○- - -○), 0.1 (●- - -●), 0.3 (△- - -△), 0.5 (▲- - -▲). b: *E. coli*/DPPC mixtures; $X_{EC} = 0$ (○- - -○), 0.1 (●- - -●), 0.3 (△- - -△), 0.5 (▲- - -▲). c: *S. minnesota*/DPPC mixtures; $X_{SM} = 0$ (○- - -○), 0.1 (●- - -●), 0.3 (△- - -△), 0.5 (▲- - -▲).

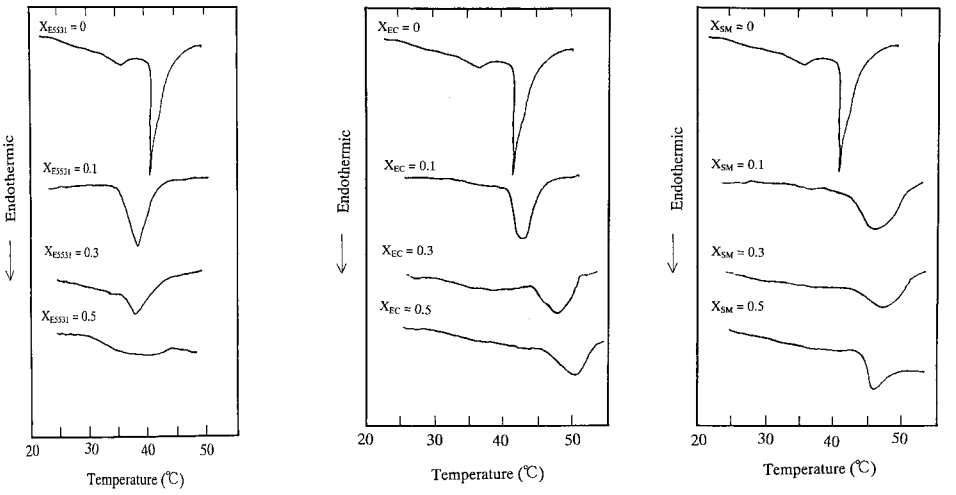

Fig. 5. DSC thermogram of the lipid mixtures. Scan speed was 2°C/min and temperature ranged from 10°C to 60°C. a: E5531/DPPC mixtures. b: *E. coli*/DPPC mixtures. c: *S. minnesota*/DPPC mixtures.

is characterized by a sharp decrease in the intensity ratio I'/I [18]. The I'/I ratio of E5531/DPPC mixtures at $X_{E5531} = 0$, 0.1, 0.3 and 0.5 sharply decreased around 41, 39, 36 and 35°C, respectively, and these temperatures were suggested to be phase transition temperatures. On the other hand, the I'/I ratio of EC/DPPC mixtures at $X_{EC} = 0.1$, 0.3 and 0.5 sharply decreased around 44, 45 and 47°C, respectively. The I'/I ratio of SM/DPPC mixtures at $X_{SM} = 0.1$, 0.3 and 0.5 sharply decreased around 43, 45 and 47°C, respectively. These results are similar to the fluorescence polarization (probe: DPH) results (Fig. 3). The change in I'/I at the phase transition decreases with decreasing pyrene concentration and correlating with diffusion coefficients (D_{diff}) for lateral diffusion of pyrene in lipid membranes [18]. The I'/I at $X_{E5531} = 0$, 0.1, 0.3 and 0.5 at 37°C were 0.43, 0.36, 0.32 and 0.25, indicating that as X_{E5531} increases, D_{diff} will decrease and there will be a reduction for the lateral diffusion. The I'/I at $X_{EC} = 0.1$, 0.3 and 0.5 at 37°C were 0.78, 0.93 and 0.99. The I'/I at $X_{SM} = 0.1$, 0.3 and 0.5 at 37°C were 0.79, 0.84 and 0.88. These data indicate that as X_{EC} and X_{SM} increase, D_{diff} will increase and there will be an increase in lateral diffusion.

Table 3
Phase transition temperature and enthalpy for E5531, *Escherichia coli*, *Salmonella minnesota* and DPPC mixtures

	Mole fraction of the lipids in the mixtures			
	0	0.1	0.3	0.5
Phase transition temperature determined by DSC (°C)				
E5531	33.1 40.6	36.0	37.4	32.4
Escherichia coli	33.1 40.6	42.3	45.1	47.8
Salmonella minnesota	33.1 40.6	41.2	44.6	45.8
Phase transition enthalpy determined by DSC (kJ/mol)				
E5531	2.7 35.8	36.6	13.2	6.5
Escherichia coli	2.7 35.8	30.1	25.3	17.8
Salmonella minnesota	2.7 35.8	29.5	26.8	23.4
Phase transition temperature determined by fluorescence polarization (probe: DPH) (°C)				
E5531	40	38	37	33
Escherichia coli	40	44	46	47
Salmonella minnesota	40	42	43	45
Phase transition temperature determined by pyrene diffusion (°C)				
E5531	41	39	36	35
Escherichia coli	41	44	45	47
Salmonella minnesota	41	43	45	47

3.6. Thermotropic behavior of E5531/DPPC mixtures

The thermotropic behavior of the lipid mixtures was determined by differential scanning calorimetry (DSC). The thermograms of E5531/DPPC, EC/DPPC and SM/E5531 mixtures are shown in Fig. 5. The pretransition of DPPC was not completely abolished at $X_{E5531} = 0.1$, rather it was shifted to a lower temperature as a shoulder in the endothermic peak. The shape of the main transition of DPPC was not changed at $X_{E5531} = 0.1$. At $X_{E5531} = 0.3$, E5531 broadened the half-height width of the excess heat curve of DPPC, while the curve shape became asymmetric as the phase transition temperature decreased with a shoulder at the higher temperature. Both the onset and the end of the transition were shifted to lower temperatures as the concentration of E5531 was increased. The suppression of the endothermic transition is presented in Table 3. E5531 decreased the phase transition enthalpy of the DPPC membrane. On the other hand, the addition of EC and SM increased the phase transition temperature. These findings obtained from EC were similar to the lipid A from *Escherichia coli* K-12 cells [25] and this lipid A increased the phase transition temperature of DPPC membrane.

4. Discussion

Lipid A is a complex macromolecule which produces a number of pathophysiological events when introduced into the bloodstream of higher animals. For the mechanism responsible for the initiation of various biological responses during endotoxin shock, it has been proposed that lipid A

triggers its noxious effects by acting specifically on a receptor site [26], or non-specifically through membrane lipids [9,27]. Regarding the latter concept, Jacobs [28] has shown that lipid A associates with cells in a manner that is suggestive of a two-step process. The first step is ionic in nature. Based on the results from zeta potentials measurements, E5531, EC and SM gave a negative charge to DPPC membranes. However, it is not known whether such charge-mediated interaction can provide an activation signal to cells from the cell surface. The charge-mediated association of macromolecules with cell surfaces inactivates, rather than activates, cells in one system [29]. The second of these steps, proposed to be the intercalation of the lipid A into the membrane lipid bilayer, is not unexpected since lipid A interacts with phospholipid monolayers [30]. The intrusion of such a large segment of lipid would be expected to alter the order of fluidity of the membrane lipid bilayer. Indeed, lipid bilayers that are reconstituted with lipopolysaccharide or lipid A are less fluid than reconstituted bilayers consisting of the lipid alone [25]. Therefore, we designed a test system to examine whether or not E5531 could interact with a membrane bilayer to alter its fluidity.

In this study, we concluded from our results that E5531 interacted with the lipid bilayer and increased the fluidity of that bilayer. On the other hand, the addition of EC and SM decreased the membrane fluidity of the bilayer. In the case of lipopolysaccharide, cell activation is associated with changes in cell membrane fluidity [31,32]. It will be assumed that the intercalation of E5531 increased the fluidity of that region of the cell membranes and that the biological action of E5531 and the molecular mechanism of E5531 interaction with cell membranes will be different from those of EC and SM. Christ et al. [10] have reported that E5531 is a lipid A antagonist and will bind the LPS receptor, and the affinity of E5531 to bind the receptor is larger than that for lipid A. Kawata et al. [33] have also reported that E5531 blocked the induction of TNF-α by LPS and reduced LPS-induced lethality in mice. Based upon the results from our study and these reports, it is assumed that these useful effects of E5531 are obtained not only from the difference in the affinity to the LPS receptor but also from the difference in the interaction to the cell membrane. In other words, E5531 will bind specifically on a receptor site, and in addition it will intercalate non-specifically through membrane lipids and give the useful biological effects.

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