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COMMUNICATION

Comparison of the Effect of Lipid A Analog E5531 and the Lipid A from *Escherichia coli* on Phospholipid Membrane Properties

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ABSTRACT

The effect of the lipid A analog E5531 on the phospholipid membrane was compared with that of the lipid A from Escherichia coli *(EC). E5531 decreased the phase transition temperature of dipalmitoylphosphatidylcholine (DPPC) membrane and increased the fluidity and micropolarity. On the other hand, the effect of EC on the membrane was contradictory. These results suggested that the reason for the difference of biological effects of these two lipid A would be caused by the differences from the effect on the cell membranes.*
Key Words: *Fluidity; Lipid A; Phase transition temperature; Phospholipid membrane; Size.*

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). Recently, researchers have focused on the effect of lipid A on the structural and dynamic properties of membranes and have revealed that most biological effects induced by lipid A are initiated by binding to a specific receptor (3,4) or by nonspecific intercalation into the lipid matrix of the cell membrane (5). The interaction and subsequent intercalation into the membrane is dependent on the fluidity of the hydrophobic region and/or the supramolecular structure of LPS and lipid A (6). Liu et al. (7) have also reported that lipid A from *Salmonella minnesota*

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decreased the membrane fluidity and raised the phase transition temperature of phospholipid membranes (7). Benedetto et al. (8) 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 important determinants of the biological activity of lipid A.

Recently, researchers have 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 tumor necrosis factor [TNF] production) possessed by lipid A (9). This compound has been found to be a specific LPS antagonist in an LPS-binding assay, and it inhibits induced TNF production in monocytes/macrophages induced by lipid A and LPS (10). Its anticipated use is as a drug for the treatment of septic shock (11,12).

The above studies for verification of the useful effects of E5531 were mainly conducted by comparing them with the toxic effects of the lipid A and LPS from *Escherichia coli* (EC). In this study, we compared the effect of E5531 and the lipid A from *Escherichia coli* (Fig. 1b) on the dipalmitoylphosphatidylcholine (DPPC) as a model membrane of cell surfaces; we used several physicochemical techniques, such as dynamic light scattering (DLS) and fluorescence spectroscopy. In addition, we investigated the correlation of these effects with the initiation of biological activities.

EXPERIMENTAL

Materials

The E5531 was obtained from Eisai Chemical Company, Limited (Ibaraki, Japan). Lipid A from *Escherichia coli* F583 (Rd mutant) was purchased from Sigma Chemical Company, Limited (St. Louis, MO). Pyrene and *N*-phenyl-1-naphtylamine (NPN) were purchased from Wako Purity Industrial, Limited (Osaka, Japan). Lactose hydrous, sodium phosphate monobasic, sodium phosphate dibasic, and sodium hydroxide were purchased from Mallinckrodt Company, Limited (Paris, KY).

Methods

Preparation of the Aggregates from the Lipid Mixtures

The aggregates from E5531/DPPC and the lipid A from EC/DPPC were prepared by the method of Dijikstra et al. (13). DPPC was dissolved in chloroform, and the

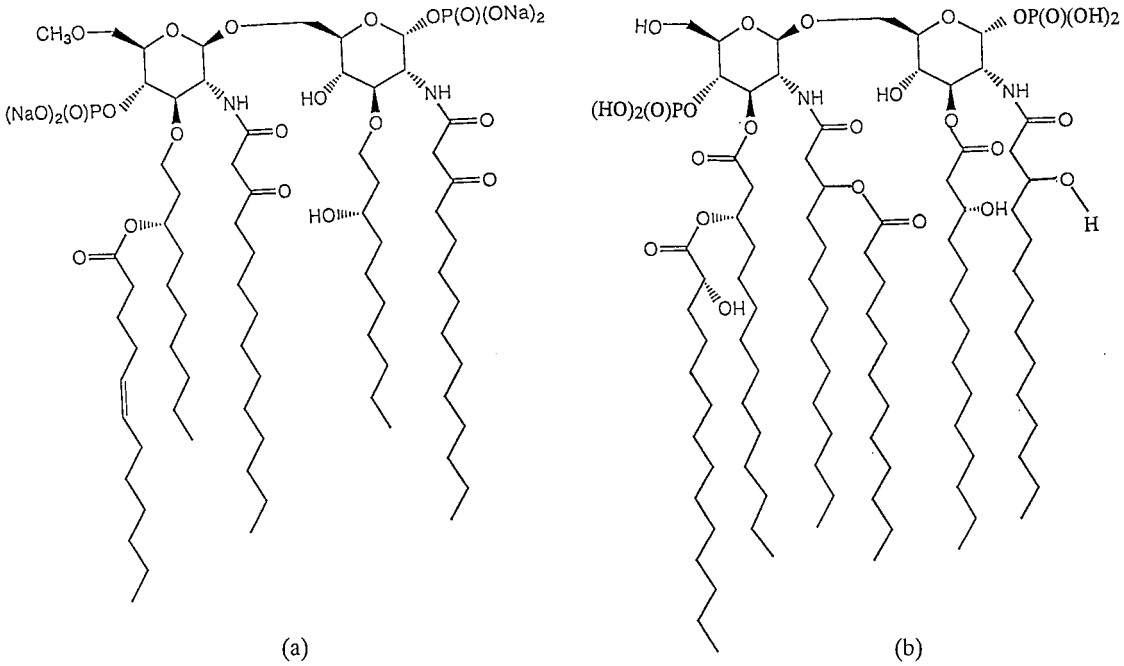

Figure 1. (a) Chemical structure of the synthetic lipid A analog E5331. (b) Chemical structure of the lipid A from *Escherichia coli*.

E5531 or EC were dissolved in methanol. These stock solutions were then mixed at a suitable ratio. The solvents were evaporated under a steam 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 Co., Ltd., Tokyo, Japan) at 50°C for 10 min.

Determination of the Size of the Lipid Aggregates

The size of the aggregates in the lipid mixtures was determined at 25°C by the DLS techniques using a laser particle analyzer (model DLS-7000DL, Ohtsuka Electronics Co., Ltd., Osaka, Japan). The data were analyzed by the histogram method (14), and the sizes of the weight-averaged aggregates were evaluated.

Determination of the Phase Transition Temperature of the Lipid Mixtures

The phase transition temperatures of the E5531/DPPC and EC/DPPC aggregates were determined using a fluorescence polarization technique (probe: NPN). The fluorescence measurements were performed on a Hitachi F-4500 spectrophotofluorometer with a temperature-controlled cuvette holder. Phase transition temperature was taken as the midpoint of the respective transition regions, determined from the changes in the slopes of the fluorescence intensity at the beginning and the end of the transition (15).

Effect of E5531 and EC on Pyrene Diffusion in DPPC Membrane

To evaluate the effect of E5531 and EC on the micropolarity around pyrene in DPPC membrane, the fluorescence spectrum of pyrene was measured. Steady-state fluorescence spectra were obtained at 37°C. The excitation wavelength used was 337 nm. The intensity ratio of pyrene fluorescence peaks I_{475}/I_{393} reflects the polarity of the microenvironment of pyrene, and I_{385}/I_{375} reflects the membrane fluidity of the aggregates (16).

RESULTS

Effects of E5531 and *Escherichia coli* on the Size of Dipalmitoylphosphatidylcholine Liposomes

Table 1 shows the weight-averaged size of the aggregates of the E5531/DPPC and EC/DPPC mixtures evaluated by DLS measurements at different E5531 mole fraction range X_{E5531} and EC mole fraction range X_{EC}. The mean diameters for E5531/DPPC and EC/DPPC mixtures were almost 20 and 25 nm, respectively, and independent of X_{E5531} and X_{EC}.

Effects of E5531 and *Escherichia coli* on the Phase Transition Temperature of Dipalmitoylphosphatidylcholine

The effects of E5531 and EC on the phase transition temperature of DPPC are shown in Fig. 2. The phase transition temperature of DPPC was 41°C. The phase transition of the lipid mixtures was dependent on X_{E5531}. At $X_{E5531} = 0.1$, 0.3, and 0.5, the phase transition temperatures were 36°C, 34°C, and 32°C, respectively. On the other hand, at $X_{EC} = 0.1$, 0.3, and 0.5, the phase transition temperatures were 42°C, 43°C, and 44°C, respectively. The interaction of EC on the phase transition temperature is confirmed to be different from that of E5531.

The Effect of E5531 and *Escherichia coli* on Pyrene Diffusion in Dipalmitoylphosphatidylcholine Membrane

The influence of E5531 and EC on pyrene diffusion in DPPC membrane was evaluated by monitoring the fluorescence of pyrene. The micropolarity in the hydrophobic regions of the aggregates, as reflected by the intensity of pyrene at 385 nm (I_{385}) relative to that at 375 nm (I_{375}) (14) is shown in Fig. 3a. The ratio I_{385}/I_{375} increased with an increase of X_{E5531}, indicating that, with the addition of the E5531, micropolarity around pyrene became more hydrophilic. On the other hand, the ratio I_{385}/I_{375} decreased with an increase of X_{EC}, indicating that, with the addition of EC, micropolarity around pyrene became more hydrophobic. The membrane fluidity of the aggregates, as reflected by the ratio of the intensity ratio of pyrene at 475 nm I_{475} relative to that at 393 nm I_{393} (14), is also shown in Fig. 3b. The ratio I_{475}/I_{393} increased with an increase in X_{E5531}, indicating that, by the addition of E5531, the membrane fluidity increased. On the other hand, the ratio I_{475}/I_{393} decreased with increase in X_{EC}, indicating that, by the addition of EC, the membrane fluidity decreased.

DISCUSSION

In this study, we concluded from our results that E5531 decreased the phase transition temperature of DPPC membrane and increased the fluidity and the mi-

Table 1

Size of Aggregates and Phase Transition Temperature for the Mixtures of DPPC and E5531 or the Lipid A from Escherichia coli

	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
Phase transition temperature determined by fluorescence intensity (probe: NPN) (°C)				
E5531	41	36	34	32
Escherichia coli	41	42	43	44

cropolarity. On the other hand, the addition of EC increased the phase transition temperature and decreased the membrane fluidity and the micropolarity. These differences may be correlated with the initiation of biological effects induced by these lipids.

Endotoxic reactions like pyrogenicity and lethal toxicity (i.e., cytokine production) proceed via binding of lipid A (or LPS) to the surface of monocytes/macrophages. With respect to the interaction mechanism, some investigators have shown that lipid A may intercalate into the lipid matrix of target cells (5,17).

For the mechanism of initiation of various biological responses during endotoxin shock induced by lipid A, it has been proposed that lipid A triggers its noxious effects by acting specifically on a receptor site (18) or nonspecifically through membrane lipids (8,19), while others have observed specific binding proteins in the serum or at the cell surfaces (3,4,20,21). For example, the 60-kDa

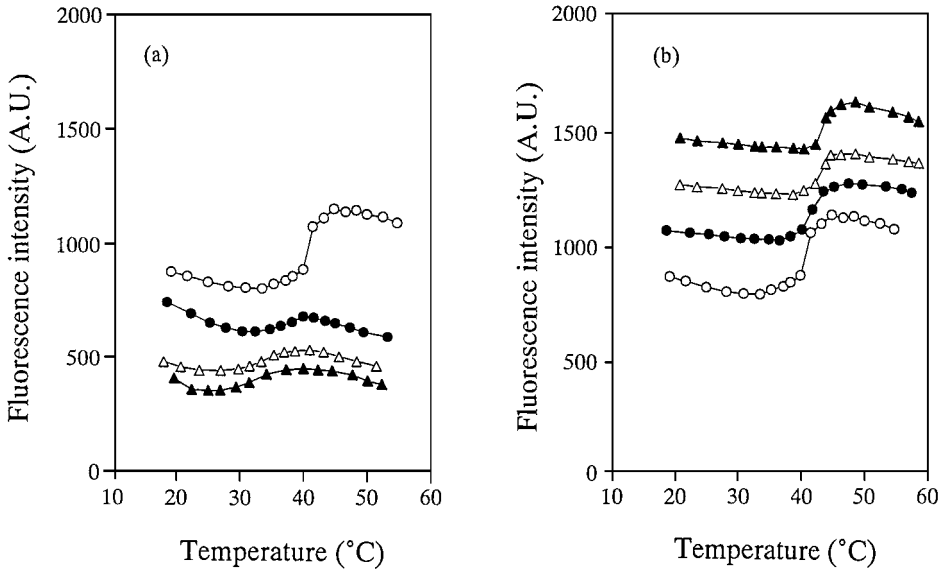

Figure 2. Relationship between incubation temperature and fluorescence intensity (arbitrary unit; probe: NPN) as a function of the lipid mole fraction in the lipid mixture: (a) E5331/DPPC mixtures, $X_{E5331} = 0$ (○), 0.1 ●, 0.3 (△), 0.5 (▲). (b) EC/DPPC mixtures, $X_{EC} = 0$ (○), 0.1 (●), 0.3 (△), 0.5 (▲).

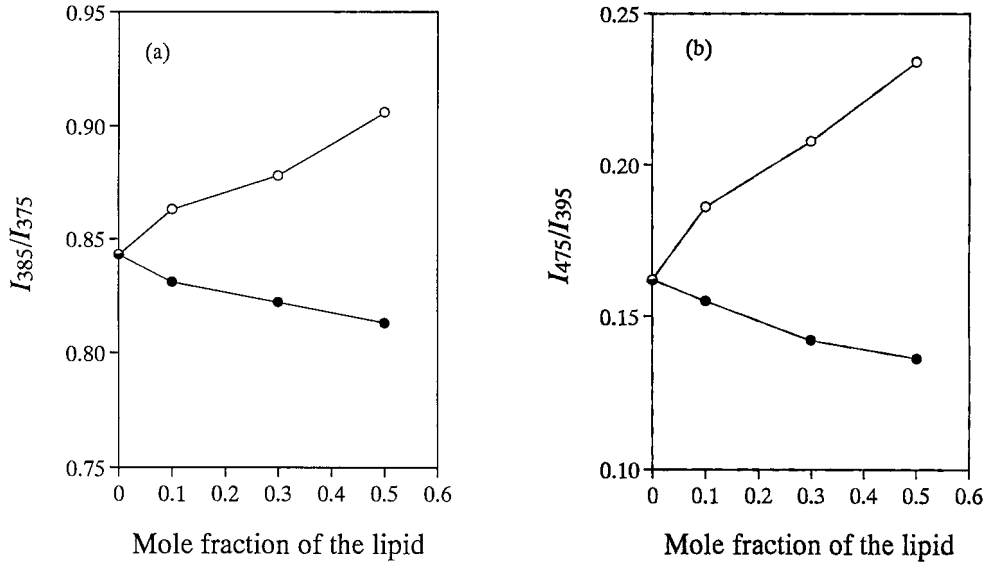

Figure 3. (a) Relationship between mole fraction of the lipid in the lipids mixture and the ratio of fluorescence intensity of pyrene I_{385}/I_{373} at 37°C. ○, E5331/DPPC mixtures; ●, EC/DPPC mixtures.

glycoprotein LBP (lipopolysaccharide-binding protein) was found to complex with LPS by binding to the lipid A portion of LPS independent of the saccharide moiety (20). The resulting LPS-LBP complex is then recognized by a membrane-bound cell surface ''receptor'' protein called CD14, which finally triggers the biological response (4). In any case, it seems important to note that, after binding of LPS to the membrane, whether specific or unspecified, in a second step, the decisive signal that causes the biological response must be given, probably by a membrane protein. For the signal translation, the formation of a nonlamellar inverted structure of lipid A, leading to disturbances at or around the ''binding places'' at the cell surface due to the concave curvature of the lipid A bilayer regions, might be a prerequisite.

Based on our results, it will be assumed that the intercalation of the E5531 increased the fluidity of that region of the cell membranes, and that the biological action of E5531 and molecular mechanism of E5531 interaction with cell membranes will be different from those of EC. Christ et al. (9,12) have reported that E5531 is a lipid A antagonist, that it will bind the LPS receptor, and that the affinity of E5531 to bind the receptor is larger than that for lipid A. Kawata et al. (10) have also reported that E5531 blocked the induction of TNF-α by lipid A and LPS from EC and reduced LPS-induced lethality in mice (10). In addition, Kobayashi et al. (11) suggested that E5531 suppressed the hepatic injury in mice induced by LPS from EC in an apparently competitive manner. Based on the results from our study and these reports, it will be assumed that these useful effects of E5531 will be obtained from not only the difference from the affinity to the LPS receptor, but also from the difference from the interaction with the cell membrane. In other words, for initiation of the useful biological effects, E5531 will bind specifically on a receptor site; in addition, it will intercalate nonspecifically through membrane lipids and increase the membrane fluidity.

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