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Lipopolysaccharide induces raft domain expansion in membrane composed of a phospholipid–cholesterol–sphingomyelin ternary system

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The molecular behavior and interaction of Re-type lipopolysaccharide (ReLPS) and phospholipids were investigated in two different types of model membrane systems, a pure phospholipid membrane consisting of 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) and a raft-forming membrane composed of equimolar DEPE, sphingomyelin (SM), and cholesterol (Chol) by solid-state NMR spectroscopy. A remarkable influence of ReLPS on the property of lipid bilayer was found by analyzing the ^{13}C-NMR spectra. Namely, while both liquid-ordered (L_o) and liquid-disordered (L_d) phases co-exist in DEPE/SM/Chol, only the L_o phase is present in DEPE/SM/Chol/ReLPS. This clearly indicates that ReLPS induces expansion of the raft area in the raft-forming membrane. The ^{1}H spin-lattice relaxation times in the rotating frame $T^H_{1\rho}$ in the two different membranes, DEPE/ReLPS and DEPE/SM/Chol/ReLPS, indicate that the motion of DEPE is affected by the presence of ReLPS, Chol, and SM, and much faster than that of ReLPS in both membranes. The ReLPS in the raft-forming membrane, in particular, accelerated the movement of DEPE. Thus, this study shows the possibility that LPS induces the expansion of raft region and the rapid motion of the raft-forming membranes to favor molecular interactions in the animal cell membrane during innate immune recognition.

Keywords: cell membrane, molecular motion, raft domain, ReLPS, solid-state NMR

Abbreviations: Chol, cholesterol; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; FITC, fluoresceinisothiocyanate; H_{II}, inverted hexagonal phase; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LBP, LPS binding protein; LPS, lipopolysaccharide; MLV, multi-lamellar vesicle; OG, 1-octyl β-D-glucoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; SM, sphingomyelin; ReLPS, deep rough mutant LPS; TEN, triethylamine; THF, tetrahydrofuran; TLC, thin-layer chromatography; TLR, Toll-like receptor; Tris, tris(hydroxymethyl)aminomethane

Introduction

Lipopolysaccharide (LPS) is a potent inducer of the innate immune response.[1,2] When higher animals are infected by Gram-negative bacteria, macrophages activated by LPS and secrete immunoregulators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 and IL-6, that promote defense responses by other cells. However, in severe cases of bacterial infection, LPS excessively stimulates macrophages or dendritic cells, leading to extensive tissue damage and septic shock. The mechanisms of the innate immune response induced by LPS must be understood in more detail to develop therapeutic strategies against such excessive responses.

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The signal transduction cascade after LPS is transported to the MD2/Toll-like receptor-4 (TLR4) complex has been precisely elucidated.[3–5] However, the relationship between LPS and the target cell membrane and the role of the membrane in the early stage of innate immune responses are not yet well understood. Recent studies have shown the importance of the sphingolipid and cholesterol-based membrane microdomain formation for cellular activation by LPS.[6] This microdomain, called liquid-ordered phase (L_o), is floating in a sea of liquid-disordered phase (L_d)[7] like a raft, and is thus referred to as a raft domain.[8–10] Raft domains are emerging as the sites of cellular signaling and protein and lipid sorting, and they are involved in the onset of diseases such as Alzheimer's and prion disease.[11] Certain receptor proteins involved in the innate immune response, such as CD14 and heat shock proteins (hsp) 70 and 90, are constitutively found in raft domains.[6] In contrast, the LPS-cellular activation proteins, chemokine receptor 4 (CXCR4) and TLR4, are not constitutively present in lipid rafts; rather, they are recruited to the lipid rafts after LPS stimulation.[6] Thus, LPS stimulation probably induces the integration of receptor proteins into lipid rafts upon the onset of the innate immune response. Accumulation of these receptor proteins in rafts could facilitate interactions between them and the subsequent signal transduction leading to LPS-mediated cellular activation. Therefore, lipid rafts may play a significant role in the LPS-activated innate immune response.

In the present study, we investigated the molecular behavior of LPS and phospholipids and the interaction between them in two different model membranes, pure phospholipid membrane and raft-forming membrane. Our recent solid-state investigation showed that Re-type LPS (ReLPS, see below)[2] generates partial micellization of phosphatidylcholine (PC)-rich membranes, whereas this tendency is suppressed in phosphatidylethanolamine (PE)-rich membranes.[12] Therefore, in the present study, we used the 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) phospholipid bilayer as a pure phospholipid membrane. We chose the DEPE/sphingomyelin/cholesterol (DEPE/SM/Chol) ternary system for the raft-forming membrane. Because tight acyl chain packing is a key feature of the L_o phase, sphingolipids, which contain highly saturated tightly packed acyl chains, were chosen for this type of membrane. In addition, because of its ability to facilitate packing interactions with saturated lipids, Chol was selected as another component for the L_o phase in the raft-forming membrane. The L_d phase exists as a loosely packed disordered state and has a high content of unsaturated phospholipids. The two unsaturated acyl chains in DEPE make this phospholipid particularly important in producing the L_d phase in the raft-forming membrane.

We required ^{13}C-labeled, homogeneous LPS molecules for solid-state NMR measurement. For this purpose, we used the Re-type LPS (ReLPS) biosynthesized by a deep rough *Escherichia coli* K-12 mutant.[13] The Re-type LPS is the smallest natural LPS composed of lipid A and a disaccharide of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) linked to the 6′-position of the former, which is responsible for most of the endotoxic effects of LPS (Fig. 1). Re-type LPS is the sole candidate for homogeneous LPS. Non-labeled ReLPS is commercially available, but the ^{13}C-labeled specimen was prepared and purified to homogeneity as described below.

We first examined the interaction between DEPE and ReLPS by the ^{31}P-NMR spectra with varying molar ratios of DEPE/ReLPS. To characterize the membrane phase behavior, we observed the C=O line shapes of the ^{13}C-NMR spectra of the DEPE and DEPE/SM/Chol membranes in the presence and absence of ReLPS. The phase behavior was further confirmed by estimating the state of the acyl chains of ReLPS in both the DEPE and DEPE/SM/Chol membranes by observing the chemical shift values of the acyl chains in the ^{13}C-NMR spectra. Finally, we evaluated the molecular motion of the ReLPS and DEPE phospholipids in these membranes by measuring the ^{1}H relaxation time in the rotating frame ($T^H_{1\rho}$) and the temperature dependence of spectral line widths. These NMR analyses for the phase behavior and molecular motion of the DEPE and raft-forming membranes in the presence and absence of ReLPS allow us to unveil important effects of ReLPS on the property of various membranes.

Materials and Methods

Materials

Deep rough mutant ReLPS from *E. coli* strain WBB06, DEPE, SM from chicken eggs, and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Uniformly ^{13}C-labeled Bioexpress cell growth media (U-^{13}C, 98%) was obtained from Cambridge Isotope Laboratory (Andover, MA, USA). Unlabeled ReLPS was used after conversion to the triethylammonium (TEN)-salt form, as described below. Other chemicals were used without further purification. 1-Octyl β-D-glucoside (OG) was purchased from Nacalai Tesque (Kyoto, Japan). Dowex 50W-X8 cation exchange resin (H^+ form) was purchased from Muromachi Technos Co., Ltd (Tokyo, Japan). Dowex MAC-3 weak acid cation-exchange resin (H^+ form) was purchased from Sigma-Aldrich. Sephadex LH-20 was purchased from GE (Uppsala, Sweden). Tris-HCl buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.6) was used for all experiments.

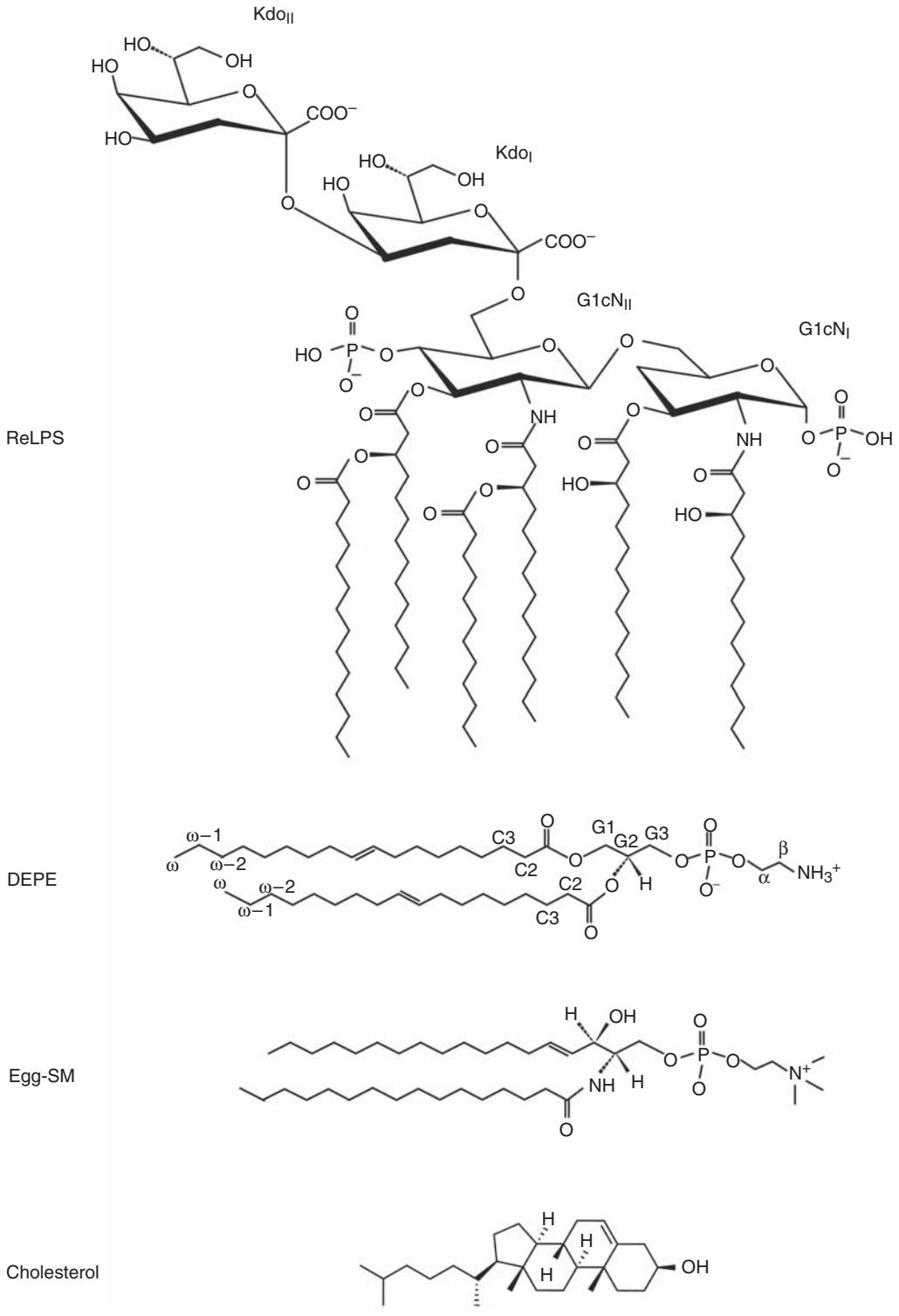

Fig. 1. Chemical structures of ReLPS, DEPE, SM, and Chol used in this study.

Culture of E. coli *WBB06 and extraction and purification of* ^{13}*C-labeled ReLPS from cells*

To prepare fully ^{13}C-labeled ReLPS, *E. coli* strain WBB06 was propagated in uniformly ^{13}C-labeled Bioexpress cell growth media supplemented with 5 mM $CaCl_2$ and 12 mg/l tetracycline.[13] Mass cultures of strain WBB06 were cultivated in a fermentor and washed with distilled water, ethanol, and acetone; then, the cells were washed twice with ether and dried.

The LPS was extracted from the dried cells by the phenol/chloroform/light petroleum (PCP) method.[14] The main component of the extract was identified with an authentic specimen of ReLPS by TLC with a mobile phase of chloroform/methanol/water (6:4:1; v/v/v). In addition to the ReLPS component, a slower-moving more hydrophilic lipid product was detected. This polar product, presumably an ReLPS derivative with an additional phosphoethanolamine (pEtN) substitution at position 7 of the outer Kdo residue,[15] was removed, and highly pure ReLPS was obtained as follows. Crude PCP extract (4 mg) was solubilized in 1 ml of 1.5% OG, the mixture was acidified (pH 2.0) on ice with HCl and quickly vortexed. The OG was removed by acetone washing (10 volumes of acetone). The insoluble precipitates were collected by centrifugation, washed with acetone again and air dried. The dried precipitates were suspended in the chloroform phase (lower phase) of a two-layer solvent system (chloroform/methanol/ethanol/H_2O, 8:2:1:2; v/v/v/v) and applied to a cation-exchange column (Dowex 50W-X8, H^+ form) equilibrated with the chloroform phase. The ReLPS was eluted with the chloroform phase. Fractions containing ReLPS (free acid form) were pooled, and the accompanying HCl was removed by washing the organic phase several times with appropriate amounts of water. ReLPS was thereby retained in the organic phase. Next, the phosphoryl residues were changed to their TEN-salt form by passing through a cation-exchange column (MAC-3 cation-exchange resin, TEN-salt form). The lipid A formed by undesired cleavage of the Kdo linkage during the purification procedure was removed by liquid–liquid partition column chromatography with a two-layered solvent system (1-buthanol/tetrahydrofuran/methanol/H_2O, 12:4.4:1:40) on Sephadex LH20 gel to yield pure ReLPS as an amorphous solid after removal of the solvent.

Preparation of phospholipid vesicles

To prepare multilamellar vesicles (MLVs), specified amounts of DEPE, SM, and Chol, with or without ReLPS, were co-solubilized in chloroform/methanol (2:1; v/v), and the mixtures were dried under a nitrogen gas stream. Since a uniform lipid film did not form after a single procedure, chloroform was added to the residual slurry, and the mixture was dried again. This drying procedure was repeated several times, until a uniform lipid film was formed. Then, the film was further dried under high vacuum for about 20 h. For ^{31}P measurement, the lipid film was hydrated with Tris-HCl buffer and extensively vortexed. The suspension was freeze-thawed for 10 cycles and centrifuged. A calculated amount of the supernatant was removed to adjust the water content in the remaining mixture to about 80% (w/w). The suspension was transferred to NMR glass tubes and capped to prevent dehydration. For ^{13}C measurement, the lipid film was hydrated with Tris-HCl buffer to 35% water by weight, further hydrated with 1 ml of de-ionized water, and extensively vortexed. The suspension was freeze-thawed for 10 cycles and lyophilized. The resulting dry powder was rehydrated to 35% water by weight and transferred to a glass tube insert and sealed with Araldite® standard epoxy resin (Huntsman Advanced Materials, UT, USA) to prevent dehydration. The glass tube was inserted into an NMR rotor for magic angle spinning (MAS).

Solid-state NMR spectroscopy

All solid-state NMR spectra were acquired on a CMX Infinity 300 spectrometer (Chemagnetics, Varian, Palo Alto, CA, USA) operating at a proton resonance frequency of 300 MHz. ^{31}P-Spectra were acquired by using a 5 μs single excitation pulse with a 30 kHz continuous wave (CW) ^1H decoupling during acquisition. The dwell time was 50 μs, and 1024–60,000 transients were accumulated for each free induction decay (FID) with a 5 s delay. All ^{31}P-spectra were processed using 30 Hz line broadening. For ^{13}C-NMR measurements, 1000–12,000 transients were accumulated for each FID with a 60 μs dwell time and a 5 s relaxation delay. Spectra were acquired by using 4 μs excitation pulses with 75 kHz CW ^1H decoupling. ^{13}C-Spectra were processed using a 10-Hz line broadening for Figures 3 and 5, and 3 Hz for Figure 4. The ^1H $T^H_{1\rho}$ relaxation times were measured by using a spin-lock field strength of 60 kHz. The actual temperature in the sample under 5 kHz MAS and 75 kHz ^1H CW decoupling was calibrated by using chemical shift differences between water and methyl peak in the DEPE 35% hydrated sample.[16] Except for the temperature dependence measurement, every experiment was performed at the temperatures (40°C or 37°C) where all phospholipids and ReLPS formed lamellar phase. The ^{31}P and ^{13}C chemical shifts were externally referenced to 85% H_3PO_4 (0 ppm) and the methine carbon of adamantane (29.5 ppm), respectively.

RESULTS

Interaction between ReLPS hand DEPE in the membrane

We previously examined the effects of incorporated ReLPS on the morphology of various lipid membranes by observing the ^{31}P-NMR spectra at a molar ratio of phospholipid/ReLPS (10:1; mol/mol; hereafter, relative

amounts of lipid components are expressed as molar ratios).[12] We found that ReLPS generated partial micellization of PC-rich membranes, whereas this tendency was suppressed in PE-rich membranes. The DEPE membrane, in particular, showed strong tendency to suppress micellization in the presence of ReLPS. Therefore, we used DEPE-based membranes in the present study. Figure 2 shows the ^{31}P-NMR spectra of lipid bilayers composed of various ratios of DEPE and ReLPS at 40°C. When membranes were made only of DEPE, the spectrum showed the axially symmetric powder pattern, suggesting that the membranes formed a bilayer (Fig. 2a). When membranes were prepared from a mixture of DEPE/ReLPS (20:1), the spectrum also showed the axially symmetric powder pattern (Fig. 2b). This spectrum clearly indicated that all ReLPS was incorporated into the DEPE membranes. By contrast, when bilayers were prepared from a mixture of DEPE/ReLPS (10:1), a small isotropic signal was observed, which suggests the partial micellization of the membrane superimposed on the bilayer component (Fig. 2c).[12] The critical ratio at which all ReLPS is uniformly incorporated into DEPE membranes is DEPE/ReLPS (20:1). We used this ratio in all subsequent experiments. In membrane composed only of ReLPS, the spectrum showed a complicated pattern (Fig. 2d), which indicates that ReLPS forms a randomly associated mixture of isotropic phase, for example micelles, small unilamellar vesicles, or cubic phase, and lamellar phases.[17]

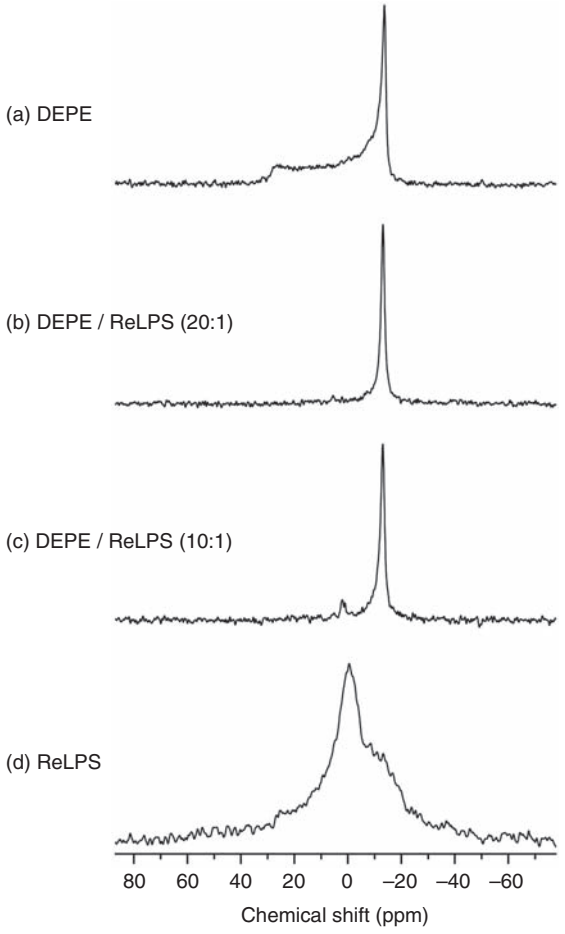

Fig. 2. ReLPS concentration dependence of ^{31}P-NMR spectra of the DEPE lipid bilayers at 40°C. The DEPE/ReLPS molar ratios were (a) 100:0, (b) 20:1, (c) 10:1, and (d) 0:100. The gel-lamellar phase transition temperature T_m of DEPE is ~35°C; the lamellar-inverted hexagonal (H_{II}) phase transition temperature T_h of DEPE is ~60°C;[48] and the gel-lamellar phase transition temperature T_m of ReLPS (ethanolamine-salt form) reported by Seydel *et al*.[17] is ~33°C.

^{13}C-NMR spectra of fully ^{13}C-labeled ReLPS in membranes

Figure 3a,b shows the direct polarization spectra of a membrane prepared from a mixture of DEPE/fully ^{13}C-labeled ReLPS (20:1) and that from DEPE alone, respectively. We can distinguish the sharp DEPE signals in Figure 3a from those of ReLPS and readily assigned[18] the former signals by comparing with Figure 3b. Owing to the structural similarity between ReLPS and DEPE, many peaks in Figure 3a, especially those in the aliphatic region, are overlapping. But the following four resonances of ReLPS were assigned by referring to its solution NMR chemical shifts:[19,20] $GlcN_{I,II}$ C_2 (54 ppm), Kdo_{II} $C_{4,5}$ (69 ppm), $GlcN_I$ C_1 (94 ppm), and $Kdo_{I,II}$ C_2 (100 ppm). Figure 3d shows the direct polarization spectrum of DEPE/SM/Chol membrane. Figure 3c is the corresponding spectrum of a membrane containing fully ^{13}C-labeled ReLPS as an additional component (DEPE/SM/Chol/ReLPS 20:20:20:1). The ReLPS is homogeneously incorporated into the lipid bilayer as judged from ^{31}P spectra of the latter membrane where only superposition of axially symmetric powder patterns were observed without any sign of isotropic signals that would indicate partial micellization (data not shown). Although additional SM and Chol peaks overlap around the small $GlcN_{I,II}$ C_2 (54 ppm) peak, the other three peaks (Kdo_{II} $C_{4,5}$, $GlcN_I$ C_1, and $Kdo_{I,II}$ C_2) can barely be distinguished from the DEPE, SM and Chol peaks. The chemical shift value of the $(CH_2)_n$ peak of ^{13}C-labeled ReLPS was 31.0 ppm in DEPE/ReLPS (20:1) membranes (Fig. 3a) and 32.3 ppm in DEPE/SM/Chol/ReLPS (20:20:20:1) membranes (Fig. 3c), indicating that the acyl chain adopts a *trans-gauche* conformation in DEPE/ReLPS and an *all-trans* conformation in DEPE/SM/Chol/ReLPS.[21]

Influence of ReLPS on the phase state of DEPE and DEPE/SM/Chol lipid bilayers

The effects of ReLPS present in lipid bilayers were analyzed for both DEPE and DEPE/SM/Chol membranes.

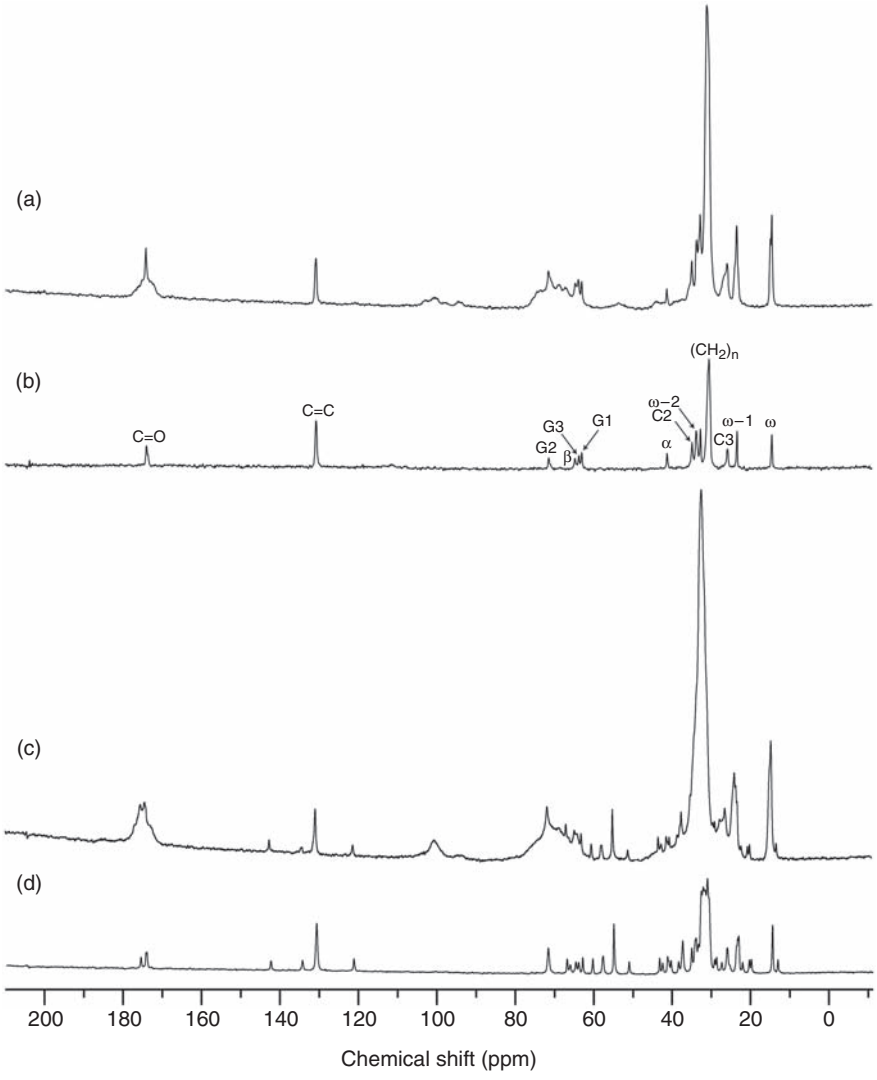

Fig. 3. Direct polarization ^{13}C-NMR spectra of a membrane composed of (a) DEPE/^{13}C-labeled ReLPS (20:1), (b) DEPE alone, (c) DEPE/SM/Chol/^{13}C-labeled ReLPS (20:20:20:1), and (d) DEPE/SM/Chol (1:1:1) under MAS with a spinning speed of 5 kHz measured at 37°C. The DEPE^{13}C assignment is indicated.

Non-labeled ReLPS was used here in order not to disturb unequivocal observation of the C=O signals of the phospholipid components in ^{13}C-NMR spectra. Figure 4a shows the carbonyl region of ^{13}C MAS NMR spectra of both the DEPE and DEPE/Chol/SM membranes in the presence and absence of ReLPS. The presence of ReLPS in the DEPE bilayer changes the chemical shift of C=O in DEPE from 173.99 ppm to 174.08 ppm. Hydrogen bonding is known to alter the chemical shifts of C=O to lower fields.[22] The shift is thus supposed to indicate hydroxy or amide groups of ReLPS form a hydrogen bond with the C=O of DEPE, though possible influence of conformational changes of DEPE molecules in the presence of ReLPS can not be excluded.[23] A similar chemical shift change of C=O in the presence of ReLPS also occurred in the DEPE/SM/Chol membrane; the chemical shift of C=O of SM changed from 175.45 ppm to 175.49 ppm. This change is thought to be likewise due to intermolecular hydrogen bonding between the hydroxy or amide groups of ReLPS and the C=O of SM or due to conformational change of SM molecules. Whatever the reason, these results suggest that ReLPS penetrates the DEPE and DEPE/SM/Chol membranes.

In the DEPE/SM/Chol membrane, the C=O peak of DEPE also shifted to lower field in the presence of ReLPS; but direct estimation of the shift value was difficult because the C=O peak splits into a triplet in the absence of ReLPS and into a doublet in its presence (Fig. 4a). This complex line structure probably results from the interaction between ReLPS, DEPE, and Chol. To examine the effect of Chol concentration on the DEPE C=O signal structure and positions, we measured

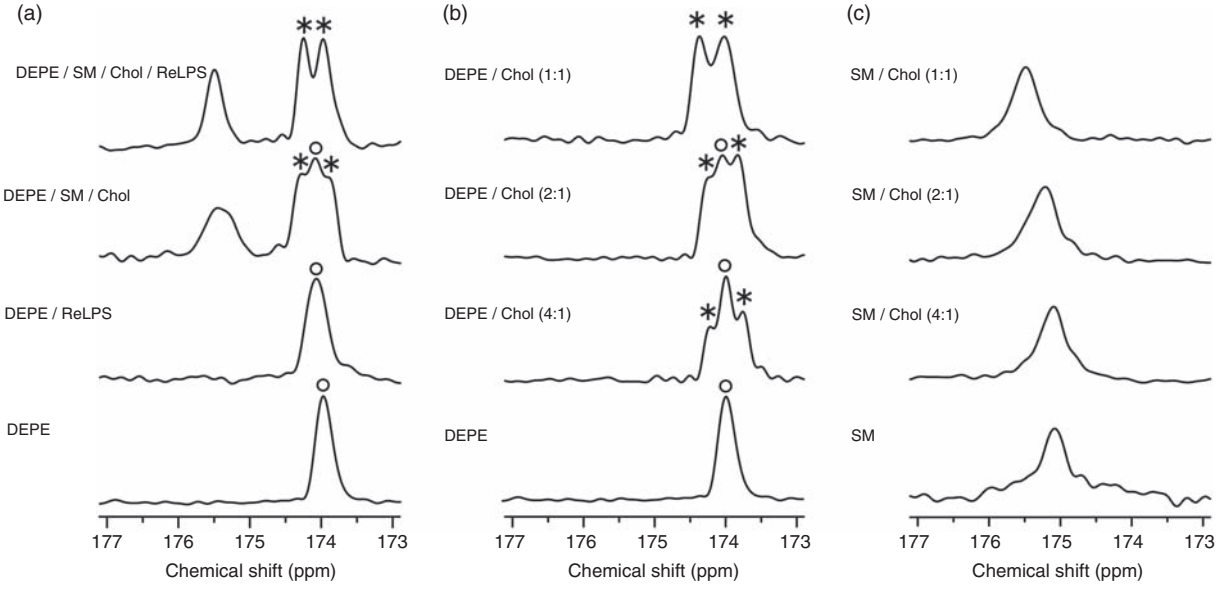

Fig. 4. (a) Carbonyl carbon region of direct polarization ^{13}C-NMR spectra of various membranes composed of DEPE/SM/Chol/ReLPS (20:20:20:1), DEPE/SM/Chol (1:1:1), DEPE/ReLPS (20:1), and DEPE alone, where non-labeled ReLPS was used. (b,c) Carbonyl carbon signal of the (b) DEPE/Chol and (c) SM/Chol membrane with varying Chol contents, as indicated. The central and both side peaks are marked with circles and asterisks, respectively. All spectra were obtained under MAS with a spinning speed of 5 kHz at 37°C and processed with 3 Hz line broadening.

the ^{13}C MAS NMR spectra of the DEPE/Chol lipid bilayer with varying Chol contents (Fig. 4b). At 20 mol% Chol, the C=O peak split into a triplet. As the molar ratio of Chol to DEPE increased, the intensity of the central peak (marked with circle) gradually decreased, and at 50 mol% Chol, the central peak disappeared and the two side peaks (marked with asterisk) remained. This observation suggests that the central peak represents the disordered liquid (L_d) phase and the left and right peaks represent the ordered liquid (L_o) phase. In the L_o phase, non-identical *sn*-1 and *sn*-2 C=O peaks in phospholipids are resolved,[24] whereas these two carbonyl carbons become indistinguishable in the L_d phase. Therefore, in the pure DEPE lipid bilayer, the phospholipid is in the L_d phase, but then the ratio of the L_o phase in the L_o/L_d mixed phase increased with increasing Chol content. At 50 mol% Chol, the L_d phase finally disappeared and a pure L_o phase was attained. Figure 4c shows the C=O region of ^{13}C MAS NMR spectra of the SM/Chol lipid bilayer with varying Chol content. Since SM has only one carbonyl carbon, the spectra contain only one C=O signal. As the Chol content increased, the SM signal shifted downfield as observed in the DEPE/Chol membrane. Comparing the Chol content dependence of the C=O signals of DEPE/Chol (Fig. 4b) and SM/Chol (Fig. 4c) with the spectra of DEPE/SM/Chol and DEPE/SM/Chol/ReLPS (Fig. 4a), we can clearly see that both the L_o and L_d phases co-exist in the DEPE/SM/Chol membrane, whereas the DEPE/SM/Chol/ReLPS membrane is only in the L_o phase.

Motion of ReLPS and DEPE phospholipid in different membranes

Figure 5a shows the spectra for DEPE/fully ^{13}C-labeled ReLPS (20:1) at four different temperatures. As the temperature decreased, both the ReLPS and DEPE lines became broad. Figure 5b shows the relationship between temperature and the line width of separated peaks, *i.e.* $(CH_2)_n$ (31 ppm), Kdo_{II} $C_{4,5}$ (100 ppm), and $GlcN_{I,II}$ C_2 (54 ppm) of ReLPS and CH=CH (130 ppm) of DEPE peaks. The peaks of $(CH_2)_n$, Kdo_{II} $C_{4,5}$ of ReLPS and CH=CH of DEPE showed increasing line width with decreasing temperature. Thus, these sites are in the region where the molecular motion becomes faster as relaxation times are longer, and their motional rates are all faster than the time scale of the inverse of the proton decoupling field strength (75 kHz, 13 μs) in the temperature range examined.[25] By contrast, the peak of $GlcN_{I,II}$ C_2 (54 ppm) in ReLPS showed two maxima. This observation suggests the presence of a distribution of motional correlation times.[26]

To compare the motion of ReLPS and phospholipid in DEPE and DEPE/SM/Chol membranes, we evaluated the ^{1}H spin-lattice relaxation time in the rotating frame $T^H_{1\rho}$. The $T^H_{1\rho}$ values are sensitive to molecular motion that has the time scale of the inverse of the spin-lock field strength (60 kHz, 16 μs). The $T^H_{1\rho}$ values for some peaks of ReLPS in DEPE membranes are shown in Table 1. These peaks have $T^H_{1\rho}$ values of approximately 1–4 ms. We also measured the $T^H_{1\rho}$ for DEPE peaks in the presence and absence of ReLPS, and these values are

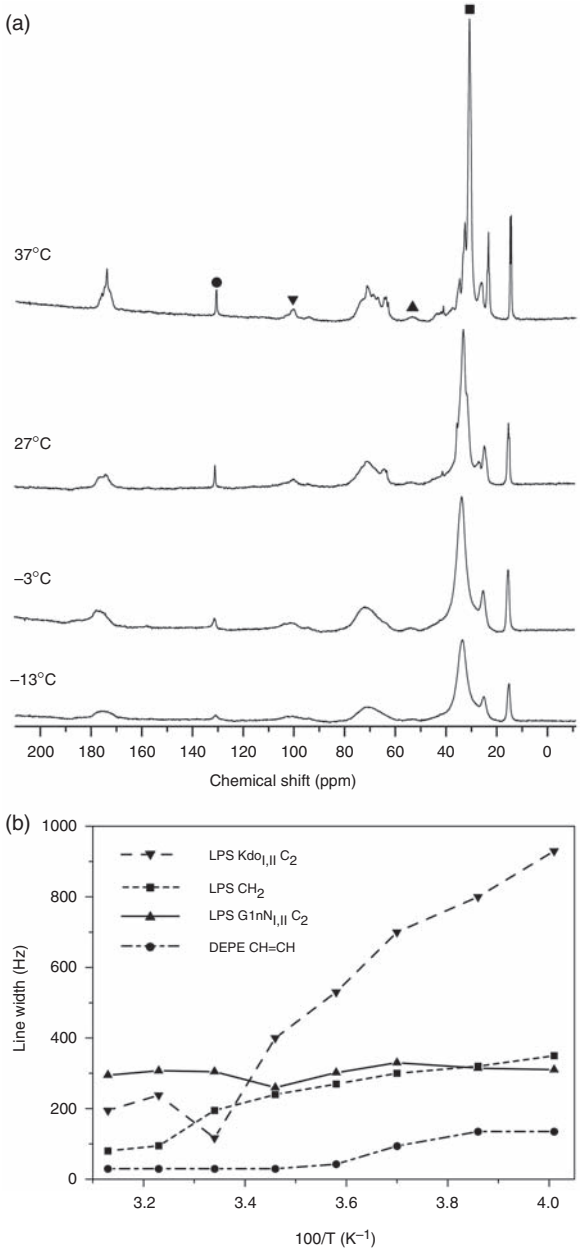

Fig. 5. (a) ^{13}C NMR spectra of a DEPE/^{13}C-labeled ReLPS (20:1) sample at 37, 27, –3, –13°C under MAS with a spinning speed of 5 kHz. (b) Temperature dependence of the line width of the $(CH_2)_n$ and Kdo_{II} $C_{4,5}$, $GlcN_{I,II}$ C_2 in ReLPS and DEPE CH=CH peaks.

Table 1. ^{13}C $T^H_{1\rho}$ relaxation times (ms) of ReLPS in the DEPE or DEPE/SM/Chol membrane

	DEPE/ReLPS	DEPE/SM/Chol/ReLPS
$GlcN_{I,II}$ C_2	1.4 ± 0.1	–
Kdo_{II} $C_{4,5}$	3.4 ± 0.1	3.1 ± 0.5
$GlcN_I$ C_1	1.7 ± 0.2	1.2 ± 0.1
$Kdo_{I,II}$ C_2	1.0 ± 0.1	–

$T^H_{1\rho}$ values are for 35% hydrated samples under MAS ($\omega_r = 5$ kHz).

summarized in Table 2 (left two columns). In the absence of ReLPS, $T^H_{1\rho}$ values for DEPE peaks were approximately 60–500 ms, whereas in the presence of ReLPS, the $T^H_{1\rho}$ values were smaller. These smaller $T^H_{1\rho}$ values of DEPE carbons in the presence of ReLPS are caused by the slow movement of ReLPS that disturbs the DEPE lateral diffusion.[12]

The $T^H_{1\rho}$ values for some peaks of ReLPS in DEPE/SM/Chol membranes (DEPE/SM/Chol/ReLPS (20:20:20:1)) are approximately 1–4 ms, which is almost the same as those in DEPE membranes (Table 1). This result indicates that the motion of ReLPS is not affected by the membrane environment. The $T^H_{1\rho}$ values for DEPE carbon in the DEPE/SM/Chol system in the presence and absence of ReLPS are summarized in Table 2 (right two columns). In the absence of ReLPS, $T^H_{1\rho}$ values for the acyl chain region became smaller than those in DEPE membranes, whereas the values for the head-group became larger. Smaller $T^H_{1\rho}$ values for the acyl chain region are explained by partitioning of Chol between the DEPE molecules in the bilayer, which restricts the motion of the DEPE acyl chains. The DEPE head-group can move relatively freely around the molecular axis, because the cholesterol molecule does not interact with the DEPE head-group moiety which is extended outside of the lipid bilayer. In the presence of ReLPS, the $T^H_{1\rho}$ values of both the acyl chain and the head-group of DEPE became larger than those in its absence. The effect of ReLPS on the motion of the DEPE/SM/Chol membranes is the opposite of the effect of ReLPS on the motion of the DEPE membranes.

DISCUSSION

We investigated the molecular behavior and mutual interactions of LPS and phospholipids in two different types of membranes, a pure DEPE membrane and a raft-forming DEPE/SM/Chol membrane, by using the solid-state NMR method. Our goal was to understand the possible role of cell membranes during the early stage of LPS-activated innate immune responses.

Morphological changes in DEPE/ReLPS membranes at various molar ratios were examined by solid-state ^{31}P-NMR spectra (Fig. 2). When the membrane is composed of only ReLPS, a randomly associated mixture of isotropic and lamellar phases was formed. The content of the lamellar phase became larger as the DEPE concentration increased. The DEPE/ReLPS membrane became pure lamellar phase at molecular ratios of DEPE greater than DEPE/ReLPS (20:1). The LPS is a major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria, and PE is a major constituent of the inner leaflet. The LPS is assumed to assemble

Table 2. ^{13}C $T_{1\rho}^{H}$ relaxation times (ms) of DEPE in the DEPE or DEPE/SM/Chol membrane in the presence and absence of ReLPS

Carbon position	DEPE	DEPE/ReLPS	DEPE/SM/Chol	DEPE/SM/Chol/ReLPS
α	67.0 ± 4.2	104.9 ± 6.9	101 ± 11	135.1 ± 1.0
β	84.6 ± 3.2	24.1 ± 2.0	–	–
G3	91.9 ± 4.9	34.0 ± 2.4	–	–
G2	91.7 ± 2.2	32.8 ± 4.9	108 ± 11	109.5 ± 7.8
G1	197.3 ± 5.0	64.8 ± 4.1	–	–
C1	99.0 ± 2.9	67.2 ± 9.1	–	–
C2	118.0 ± 5.4	39.6 ± 1.4	74 ± 19	246.2 ± 18.4
C3	157.1 ± 22.4	47.3 ± 2.9	125 ± 2	169.0 ± 13.2
C=C	462.4 ± 62.8	160.3 ± 34.0	692 ± 137	No relaxation
CH_2	146.0 ± 13.3	125.5 ± 21.2	127 ± 4	731.9 ± 111.9
ω-2	354.7 ± 30.9	133.7 ± 24.6	163.7 ± 14	257.8 ± 25.7

$T_{1\rho}^{H}$ values are for 35% hydrated samples under MAS ($\omega_r = 5$ kHz).
For carbon position numbers see Figure 1.

uniformly on the outer leaflet of the bacterial cells. However, uniformly assembled pure lamellar structures were not reproduced in the artificial membrane made only of ReLPS (Fig. 2d). To form a pure lamellar structure, ReLPS is assumed to require other unknown factors. The ReLPS is the simplest LPS molecule present on living bacterial cells. Natural LPS specimens in wild-type bacteria have heterogeneous structures because they contain various sizes of sugar chains (30–300 residues).[27] The lack of long sugar chains linked to the Kdo disaccharide might be the main reason that ReLPS does not form a pure lamellar phase under experimental conditions. Despite this fact, we used ReLPS in this study because it is the sole homogeneous LPS readily obtained from natural sources.

The chemical shift values of the $(CH_2)_n$ peak in the DEPE/ReLPS (20:1) and DEPE/SM/Chol/ReLPS (20:20:20:1) membranes suggest that the acyl chain adopts a *trans-gauche* conformation in DEPE/ReLPS and an *all-trans* conformation in DEPE/SM/Chol/ReLPS (Fig. 3). These results suggested that almost all ReLPS exists in the L_d phase in DEPE/ReLPS and in the L_o phase in DEPE/SM/Chol/ReLPS. It was further confirmed by the results shown in Figure 4. Figure 4b shows the C=O region of the ^{13}C-NMR spectra of DEPE in various molar ratios of DEPE/Chol. The C=O line shape and chemical shift values changed depending on the Chol content. By analyzing relationship between the C=O line shape and the molar ratios of DEPE/Chol in the DEPE/Chol membrane, we estimated that the central peak represents the L_d phase and two side peaks show the L_o phase. From comparison of the dependency of the C=O line shapes in Figure 4a on the Chol content (in Fig. 4b), we concluded the membrane phase behavior as follows: (i) only the L_d phase exists in the DEPE and DEPE/ReLPS membrane; (ii) the L_o and L_d phases co-exist in the DEPE/SM/Chol membrane and (iii) only the L_o phase exists in the DEPE/SM/Chol/ReLPS membrane. These results imply that ReLPS induces the expansion of the raft area in the raft-forming membrane. Figure 6 schematically illustrated the phase states of the DEPE/SM/Chol membrane in the absence and presence of ReLPS.

Suzuki *et al.*[28,29] reported that the binding of CD59, a glycosylphosphatidylinositol (GPI)-anchored receptor, with its ligand, C8, induces the clustering of about six CD59 molecules. Then, Chol and glycolipid accumulate around the CD59 cluster and form a raft. Intracellular signaling molecules, Gαi2 and Lyn, are recruited to the raft and form a complex leading to Lyn activation. Subsequently, the inositol-(1,4,5) triphosphate (IP_3)–Ca^{2+} signal is transduced. Their study is the first report that protein clustering induces the raft formation that triggers signal transduction. Our present study shows that ReLPS incorporated into the raft-forming membrane induces an expansion of the raft area. This raft-expanding ability of LPS is regarded to be similar to that of the above mentioned CD59 clustering induced raft.

The GPI anchor is a glycolipid composed of a phosphatidylinositol group connected through a carbohydrate-containing linker (glucosamine and mannose glycosidically bound to the inositol residue) to the C-terminal amino acid of a protein to form a GPI-anchored protein. The acyl chains in their phosphatidylinositol moiety are all saturated, which is considered to be important for binding with the raft.[30,31] The molecular structure of GPI is similar to that of ReLPS in that both structures consist of saturated acyl groups linked to carbohydrate residues. Accordingly, the raft-forming mechanisms generated by the CD59 cluster and ReLPS are supposed to be similar. The six saturated

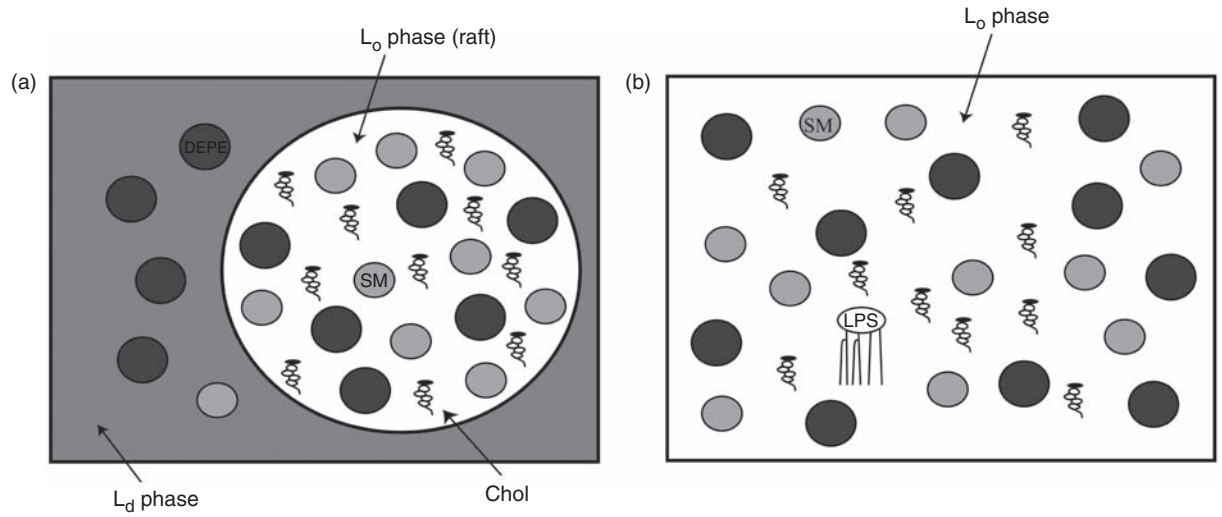

Fig. 6. Schematic models of phase states of the (a) DEPE/SM/Chol and (b) DEPE/SM/Chol/ReLPS membrane. The L_d and L_o phase domains are shown by gray and white, respectively.

acyl chains in ReLPS give the molecule a stronger tendency to associate, and this tendency may favor raft formation. The hydrogen-bonding ability and sterically rigid architecture of the sugar moieties are also likely to favor raft formation.[32] The amide and hydroxy groups around the acyl chains of ReLPS, common with SM, may also be important.

Owing to these structural characteristics, some ReLPS are likely to form domains like rafts by itself in the both DEPE/ReLPS and DEPE/SM/Chol/ReLPS membranes. In fact, both Forestier *et al*.[33] and Lapaque *et al*.[34] have shown by immunofluorescence assays that *Brucella abortus* LPS forms microdomains at the macrophage surface and in this domain some proteins related to the innate immune system segregate. Henning *et al*.[35] demonstrated, by observing the images of FITC-LPS fluorescence, that smooth LPS aggregates and concentrates in solid-ordered (S_o) microdomains when both S_o and L_d phases co-exist in the DOPC/DPPC/FITC-LPS (1:1:1) GUVs at 25°C. By contrast, LPS homogeneously distributes when only the L_d phase is present in the GUV at 60°C. The latter result is consistent with our previous results in the egg-PC/ReLPS (10:1) GUVs by dark-field and fluorescence microscopic observations.[12] In the egg-PC/POPG/ReLPS (9:1:1) GUVs, however, ReLPS aggregates on the bilayer surface. This aggregation is probably due to the electrostatic repulsion between POPG and ReLPS.[12] In our observation, the chemical shift value of $(CH_2)_n$ peaks of ^{13}C-labeled ReLPS in DEPE/ReLPS (20:1) (31.0 ppm) is a little higher than the typical chemical shift values of CH_2 peaks with a complete *trans-gauche* conformation (30.0 ppm).[21] This result suggests the probability of the LPS forming aggregates in the DEPE/ReLPS membrane. Considering these results, LPS is likely to aggregate in raft domains in the raft-forming membrane. On the other hand, the aggregate formation of LPS in the L_d phase depends on the miscibility between ReLPS and individual phospholipid components.

In the LPS cellular activation mechanism, LPS initially binds to the LPS-binding protein (LBP),[36] and then LPS/LBP associates with the GPI-anchored protein CD14.[37] Next, LPS is transferred to the MD2/TLR4 complex,[3,5] which transduces the signal into the cell. Triantafilou *et al*.[6] reported that CD14 exists in the raft domain prior to LPS stimulation. After LPS stimulation, TLR4 is recruited to the raft domain. Therefore, we anticipate that during the LPS-activated signal transduction process, clustering of CD14 induces the raft formation in a similar manner as observed with CD58 described above, and then LPS, when incorporated into the cell membrane, expands the raft area. In fact, Gutsmann *et al*.[38] previously observed the smooth incorporation of LPS into the cell membrane in the presence of LBP. When such consecutive events would occur, synergistic effects result in the increased chance of LPS binding to its ultimate signaling receptor MD2/TLR4 complex in the expanded raft area.

The $T^H_{1\rho}$ relaxation time measurements of ReLPS and the phospholipid in the DEPE and DEPE/SM/Chol membranes clearly demonstrate that the motion of DEPE in both membranes is much faster than that of ReLPS and is affected by the presence of ReLPS, Chol, and SM. By contrast, the motion of ReLPS in the same two membranes is almost unchanged (Tables 1 and 2). From the similar molecular architecture of ReLPS and phospholipids, we can closely compare the motion of these molecules in relation to their relaxation times. The differences in the rate of motion between ReLPS and DEPE in both DEPE and DEPE/SM/Chol membranes

can primarily be attributed to the difference in their molecular sizes. The rate of diffusion in lipid membranes depends on the sizes of the molecules.[39,40] Since the molecular mass of ReLPS is about three times as large as that of DEPE, it is reasonable that ReLPS moves slower than DEPE. The shorter $T_{1\rho}^{H}$ relaxation time of DEPE in the presence of ReLPS suggests that the slow movement of ReLPS retards the lateral diffusion of DEPE. This observation is consistent with our previous results regarding the lipid lateral diffusion coefficient measurement at various molar ratios of egg-PC/ReLPS by microscopic observations.[12]

Furthermore, in the DEPE/SM/Chol membrane even in the absence of ReLPS, the $T_{1\rho}^{H}$ values of the acyl chain region of DEPE became smaller than those in the uniform DEPE membranes, whereas the $T_{1\rho}^{H}$ values of the head-group became larger in the former ternary membrane. Thus, the DEPE acyl chain region moves slower and the head-group moves faster in the DEPE/SM/Chol membrane than in the membrane composed of DEPE alone. This result is consistent with previously reported ^{31}P- and ^{2}H-NMR measurements.[41] Such motional change probably occurs due to a partitioning of Chol between the acyl chain moiety of the DEPE molecules in the bilayer.[42] In the presence of ReLPS, by contrast, the $T_{1\rho}^{H}$ values of both the acyl chain and the head-group of DEPE became larger compared to those in its absence. This result suggests that the presence of ReLPS reduces the packing effect of Chol to the DEPE and releases of the DEPE acyl chain from Chol. It must be emphasized that the effect of ReLPS on the entire motion of the DEPE/SM/Chol membrane is opposite to the effect of ReLPS in the DEPE membrane. The rapid motion of raft-forming membranes is expected to increase further the chance of LPS binding to its receptor, the MD2/TLR4 complex, in the raft region.

This study demonstrates the possibility that the LPS-activated innate immune response is accelerated by LPS itself. When LPS is inserted into the raft-forming cell membrane of host animals, it expands the raft area and accelerates the movement of phospholipids in the membranes. The innate immune response of higher animals is induced not only by LPS but also by other pathogen-associated molecules (PAMPs), such as ssRNA, dsRNA, fragelin, and lipoprotein. These PAMPs are specifically recognized by their respective receptors in the Toll-like family[3,43] to induce the host responses, although the PAMPs have broad structural and physicochemical characteristics. Among the PAMPs, the bacterial lipoprotein and its active entity, lipopeptide, which corresponds to the N-terminal partial structures of the former, have structural similarities with LPS; both lipoprotein (lipopeptide) and LPS contain saturated acyl chains linked to hydrophilic head groups. Thus, only lipoproteins (lipopeptides) may have the ability to be inserted into the membrane and expand the raft area, as LPS described in this paper. In fact, Schromm *et al*.[44] observed intercalation into the liposome of synthetic lipopeptides, Pam_2CSK_4, Pam_3CSK_4, and lipolan, by FRET measurements.

Crystal structures have been reported for the dimmer of the TLR4/MD2 complex bound to LPS,[45] the TLR1–TLR2 heterodimer bound to a tri-acylated lipopeptide,[46] and the TLR3–TLR3 homodimer bound to dsRNA.[47] These structures show that PAMPs bind to the extracellular domains of their receptors, which are not close to the transmembrane domains. Therefore, the direct transport of these ligands to the binding site of the TLR receptors would be difficult when the ligands are once incorporated into cell membranes. If such transport from membrane to receptors occurs, a yet unknown process or player may participate there. In our next study, we plan to elucidate by solid-state NMR the interaction between other protein factors, such as CD14, and ReLPS on the membrane.

Conclusions

In the raft-forming membrane, ReLPS induced the expansion of the raft area and accelerated the movement of phospholipids. These results imply that, when higher animals infected by Gram-negative bacteria, LPS possibly acts autocatalytically in macrophages or dendritic cells and facilitates the innate immune recognition.

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