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ESR study on synthetic glyceroglycolipid liposomal membranes

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We previously reported that glyceroglycolipid liposomes without cholesterol activated mouse peritoneal macrophages in vivo and in vitro, whereas glyceroglycolipid liposomes containing equimolar cholesterol did not. In order to characterize the properties of the glyceroglycolipid membranes, ESR spectroscopic studies were carried out with an acyl spin-labeled galactosyl ceramide (SL-GC) or a headgroup spin-labeled phospholipid (SL-6-DPPA) in 1,2-dipalmityl[β-cellobiosyl-(1′ → 3)]glycerol (Cel-DAG) liposomal membranes. The ESR spectrum of the SL-GC in the Cel-DAG liposomes at 37°C was a single broad line, indicating that the SL-GC molecules were excluded almost completely from Cel-DAG domains and formed clusters in the membranes. The spectrum of SL-6-DPPA in the Cel-DAG liposomes at 37°C showed broad resonance lines with the central peak being the highest, while that at 60°C gave narrow lines with the low-field peak being the highest. This observation and rotational correlation time analysis showed that the molecular motions of spin-label moiety of the SL-6-DPPA were extremely restricted at 37°C but not above T_c. These results suggest that below T_c the Cel-DAG molecules are packed tightly and restricted in motion in the membrane. Incorporation of cholesterol into the Cel-DAG liposomal membranes gave (1) the spectra of the SL-GC triplet, and (2) the spectra of the SL-6-DPPA narrow resonance with the low-field peak being the highest. These results suggest that cholesterol disturbs the rigid-packed structure of the Cel-DAG membrane and increases the molecular motions of the Cel-DAG. The DSC analysis of Cel-DAG with and without cholesterol agreed well to the results of the ESR technique. Thus we assume that peritoneal macrophages recognize the rigid-packed carbohydrate residues which are restricted in motion on the Cel-DAG membranes.

Introduction

Glyceroglycolipids comprise one of the most abundant lipid classes in plant tissues, bacteria and mycoplasma membranes [1,2]. They are also present in animals in small amounts [3]. We previously reported that the synthetic glyceroglycolipids having disaccharides alone form liposomes and function as a barrier against water-soluble materials, as do phospholipids [4]. We also reported that the synthetic glyceroglycolipid liposomes activate mouse peritoneal macrophages in vitro and in vivo, and show prophylactic activities against ascites tumor cells [5]. On the other hand, glyceroglycolipid liposomes containing cholesterol do not exhibit such activities [5]. These differences could be due to the alteration of properties of the glyceroglycolipid membranes by the incorporation of cholesterol, with which macrophages could distinguish active liposomes from inactive ones. The effects of the cholesterol incorporation on the physical properties of the glyceroglycolipid membranes were poorly investigated [6], though those of the phospholipid membranes were extensively studied [7,8]. Regarding the physical properties of glyceroglycolipid membranes, several preliminary studies have been done by X-ray diffraction [9,10], ^{2}H-NMR [10] and monolayer techniques [11,12]. Since the materials examined in these studies were natural glyceroglycolipids, which contained a mixture of fatty acyl components, this has hampered detailed analysis of the physical properties of the membrane. The structure

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Abbreviations: Cel-DAG, 1,2-dipalmityl[β-cellobiosyl-(1′ → 3)]glycerol; SL-GC, spin-labeled galactosyl ceramide; SL-6-DPPA, dipalmitoylglycerophospho-*N*-[[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)carbamoyl]methyl]ethanolamine; DPPC, dipalmitoylglycerophosphocholine; DCP, dicetyl phosphate.

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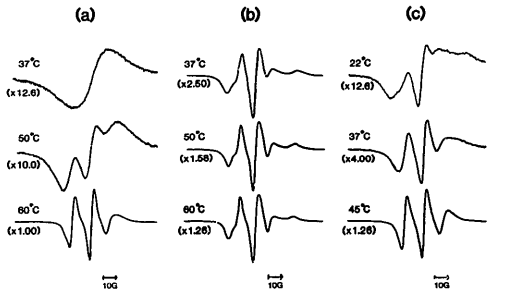

Fig. 1. Temperature-dependent change of ESR spectra of SL-GC in Cel-DAG or DPPC liposomes. 2 mol% of SL-GC was incorporated into liposomes composed of Cel-DAG/DCP (1 : 0.1) (a), Cel-DAG/DCP/cholesterol (1 : 0.1 : 1) (b), and DPPC/DCP (1 : 0.1) (c). The ESR spectra were recorded at the temperatures shown in the figure. Relative amplitudes of signals are presented in parentheses.

and dynamics of synthetic glyceroglycolipids having monosaccharides have been extensively studied by ^{2}H-NMR [13–15]. Since relatively minor changes in glyceroglycolipid headgroups lead to dramatic differences in the physical properties of glycolipid membranes [15], it is likely that the physical properties of monoglycosylglycerolipid membranes are rather different from those of diglycosyl ones. Monoglycosylglycerolipids, for instance, undergo a transition from a lamellar to a hexagonal structure [13,15], whereas diglycosyl glycerolipids do not [9,10].

In the present study, in order to characterize the physical properties of the synthetic glyceroglycolipid membranes and the effect of the incorporation of cholesterol into the glyceroglycolipid membranes on the physical properties, we carried out ESR spectroscopic studies with two species of spin-labeled lipids in the Cel-DAG liposomal membranes and also studied the thermal properties of the Cel-DAG liposomal membranes. The results suggest that the Cel-DAG molecules are packed tightly and restricted in motion in the membranes without cholesterol, and that the cholesterol incorporation into the Cel-DAG liposomal membranes disturbs the tightly packed structure of the Cel-DAG membranes and increases the molecular motions of the Cel-DAG.

Materials and Methods

Lipids. Cel-DAG [4], SL-GC [16], and SL-6-DPPA [17] were synthesized as described previously. DPPC, DCP and cholesterol were obtained from Sigma.

Preparation of Liposomes. Multilamellar vesicles were prepared in 0.3 M aqueous glucose solution as described previously [18]. In most cases, liposomes contained DCP as a charged material at a molar ratio of 1 : 0.1 in order to cause electrostatic repulsion between liposomes to avoid spontaneous aggregation of liposomes. The final concentration of liposomes was 10 mM as the Cel-DAG or the DPPC. The diameter of the Cel-DAG liposomes ranged from 300 to 1000 nm.

ESR measurement. ESR spectra were recorded with a JEOL JES-PE-1X spectrometer (X-band, 100 kHz field modulation, 0.25 mT modulation width) equipped with a temperature controller. The order parameter of SL-GC in liposomal membranes was evaluated as described previously [19]. The rotational correlation time ratio (τ_B/τ_C) and the apparent rotational correlation time (τ_R) were calculated as described previously [17,20,21].

Scanning calorimetry. Calorimetric experiments were performed in a Daini Seikosha SSC-544 apparatus as described previously [19]. The concentration of the sample was 50 mM Cel-DAG.

Results

ESR spectra of SL-GC in the Cel-DAG liposomes.

We measured the ESR spectra of SL-GC in the Cel-DAG liposomes without cholesterol at various temperatures (Fig. 1a). At 37 °C a single broad line was observed, but at 60 °C, which is higher than the phase-transition temperature of the Cel-DAG (56 °C), triplet lines appeared instead of the single broad line. In the spectra observed between 45 °C and 55 °C, the triplet lines seemed to be superimposed on the single line. As the single broad line arises from clustered SL-GC molecules and the triplet line from randomly distributed ones [16,19], almost all SL-GC molecules exist in a clustered phase at 37 °C and in a phase of random distribution at 60 °C. In the ESR spectra of SL-GC in

the Cel-DAG liposomes containing equimolar cholesterol, the triplet lines were observed even at 37 °C and the peak height of the triplet lines increased with an increase in the temperature (Fig. 1b). The peak height at 37 °C was almost half of that at 60 °C, suggesting that approx. 50% of the SL-GC molecules are present in the random distribution phase compared with those at 60 °C. Fig. 1c shows the ESR spectra of SL-GC in the DPPC liposomes without cholesterol. The triplet lines superimposed on a single broad line could be seen even at 22 °C, which was 20 C° lower than the phase-transition temperature of the membrane composed of DPPC/DCP (1 : 0.1) [17]. It should be noted here that the spectrum in the Cel-DAG liposomes without cholesterol at 37 °C, which was also about 20 C° lower than the phase transition temperature of the Cel-DAG, was in principle a single broad line. The SL-GC molecules in the Cel-DAG liposomes tend to form clusters more than those in the DPPC liposomes.

We next estimated order parameters of SL-GC in the Cel-DAG liposomes containing various amounts of cholesterol at various temperatures (Fig. 2). At and above 55 °C the order parameter increased with an increase in cholesterol content, indicating that the mobility of the fatty acyl chain of the SL-GC was suppressed by cholesterol (condensing effect). Conversely, below 50 °C the order parameter decreased with the increase in cholesterol content, indicating the enhanced mobility (fluidizing effect).

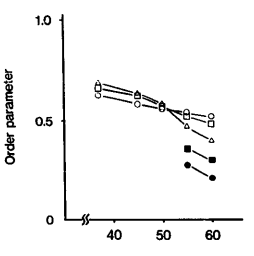

Fig. 2. Order parameter of SL-GC in Cel-DAG liposome. containing various amount of cholesterol as a function of temperature. 2 mol% of SL-GC was incorporated into Cel-DAG liposomes containing various molar ratio of cholesterol; (●), 0; (■), 0.1; (△), 0.3; (□), 0.5; and (○), 1. Order parameters were calculated from ESR spectra shown in Fig. 1, as described under 'Materials and Methods'. The order parameters could not be calculated in the spectra of Cel-DAG liposomes containing less than 10% cholesterol at below 50 °C, because the relative intensity of the triplet resonance lines was too weak to evaluate order parameters.

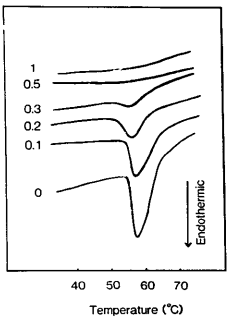

Fig. 3. Differential scanning calorimetry heating curves of Cel-DAG liposomes containing various amount of cholesterol. The samples contained 2.5 μmol of Cel-DAG in 50 μl of distilled water. Cholesterol content (molar ratio) is shown in the figure.

Thermal properties of the Cel-DAG liposomes. To obtain further information about the physical properties of the Cel-DAG liposomal membranes, a different set of experiments was done using differential scanning calorimetry (Fig. 3). The liposomes composed of the Cel-DAG alone showed a thermotropic phase transition ($T_c = 56$ °C) as described previously [4]. The incorporation of cholesterol into the Cel-DAG liposomal membranes suppressed the phase transition of the Cel-DAG in a dose dependent manner. When 0.5 molar ratio of cholesterol was incorporated into the Cel-DAG liposomes, the apparent thermotropic phase transition was completely suppressed.

ESR spectra of SL-6-DPPA in the Cel-DAG liposomes. In order to study further the motional properties of the surface segment of the Cel-DAG liposomal membranes, ESR spectra were also observed with headgroup spin-labeled SL-6-DPPA (Fig. 4). The spectrum of SL-6-DPPA in the Cel-DAG liposomes without cholesterol at 60 °C consisted of three narrow lines, suggesting that the spin-label moiety in the molecule exhibits a rapid tumbling motion in the liposomal membranes. The low-field peak height ($h(+1)$) was higher than the central one ($h(0)$), and the high-field peak height ($h(-1)$) was the lowest. The spectrum of SL-6-DPPA at 37 °C was quite different from the spectrum at 60 °C. The central peak was the highest and each resonance line at 37 °C was broader than the corresponding line at 60 °C. This suggests that the spin-label moiety in SL-6-DPPA should be relatively restricted in motion at 37 °C.

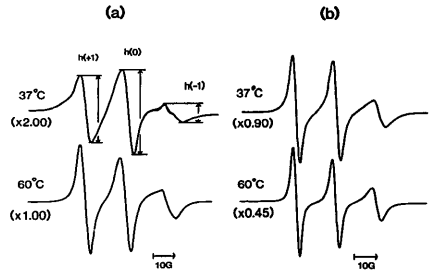

Fig. 4. Typical ESR spectra of SL-6-DPPA in Cel-DAG liposomes. 2 mol% of SL-6-DPPA was incorporated into liposomes composed of Cel-DAG/DCP (1:0.1) (a) or Cel-DAG/DCP/cholesterol (1:0.1:1) (b). The ESR spectra were recorded at the temperatures shown in the figure. Relative amplitudes of signals were presented in parentheses.

The spectra in the Cel-DAG liposomes containing equimolar cholesterol at 37°C showed much sharper resonance lines than those without cholesterol, and the low-field peak was higher than the central one. At 60°C, the line shape did not change drastically but narrowed by the addition of cholesterol.

To elucidate the motional mode of the spin-label moiety, we calculated the apparent rotational correlation time (τ_R) and the ratio of two different rotational correlation times (τ_B/τ_C). These parameters for the spectra of SL-6-DPPA in the DPPC liposomes were analyzed in detail previously [17]. We compared these parameters in the Cel-DAG liposomes with those in the DPPC liposomes and also analyzed the effect of the cholesterol incorporation. The τ_R of SL-6-DPPA in the Cel-DAG liposomes without cholesterol showed remarkably large values relative to those in the other three liposomes, and the value decreased with an increase in temperature (Fig. 5a). As the τ_R should be correlated to the rate of the tumbling motion of the spin-label moiety, the large values of the τ_R in the Cel-DAG liposomes at the low temperature suggest the slow rate of tumbling motion in the Cel-DAG membranes. The incorporation of cholesterol into the Cel-DAG or DPPC membranes lowered the τ_R values, resulting in the increase in the rate of the tumbling motion.

The τ_B/τ_C values, on the other hand, could be a good parameter for the degree of anisotropy of the motion and the direction of the preferred axis [17]. In the DPPC liposomes, the ratio was larger than 1.0 even

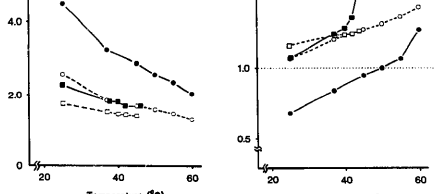

Fig. 5. Temperature dependence of (a) the apparent rotational correlation time (τ_R) and (b) the rotational correlation time ratio (τ_B/τ_C) of SL-6-DPPA in Cel-DAG or DPPC liposomes. The τ_R and the τ_B/τ_C values were calculated from the ESR spectra of SL-6-DPPA shown in Fig. 4, as described under 'Materials and Methods' and were plotted against the temperature. ●, Cel-DAG/DCP (1:0.1); ○, Cel-DAG/DCP/cholesterol (1:0.1:1); ■, DPPC/DCP (1:0.1); □, DPPC/DCP/cholesterol (1:0.1:1).

at 25°C (Fig. 5b), indicating that the spin-label moiety of SL-6-DPPA rotates predominantly about the x-axis (the direction of N-O bond, which aligns along the long axis of the molecule). The ratio in the Cel-DAG liposomes at 25°C was smaller than 1.0, which suggested that the axial rotation about the x-axis in the Cel-DAG membranes was restricted and that the segmental motion of the spin-label moiety became isotropic. In both liposomes the ratio increased with an increase in temperature and the steep increases around the phase transition temperatures were observed. Above 55°C, the ratio in the Cel-DAG liposomes became larger than 1.0. The ratios in the DPPC or Cel-DAG liposomes containing equimolar cholesterol were always larger than 1.0 above 25°C. In both cases the temperature dependent increase was also observed, whereas the steep increase could not be found.

Discussion

We previously reported that glyceroglycolipids including the Cel-DAG formed liposomes as did phospholipids [4]. The fundamental properties of the glyceroglycolipid membranes were quite similar to those of the phospholipid membranes, though a higher phase-transition temperature [4,22], a higher permeation rate of water [4], and lower fluidity at the surface [22] were demonstrated. We also reported that the liposomes composed of the glyceroglycolipids enhanced a protective immunity against transplantable tumor cells [5]. In order to characterize further the properties of the glyceroglycolipid membranes, ESR spectroscopic studies were carried out with two species of spin-labeled lipids in the Cel-DAG and DPPC liposomes.

The spectrum of SL-GC in the Cel-DAG liposomes at 37°C was a single broad line, indicating that the SL-GC molecules formed clusters in Cel-DAG membranes. On the other hand, the spectrum in the DPPC liposomes showed the triplet lines superimposed on the single broad line, even at 22°C, which indicated that some SL-GC molecules were present in random distribution phase. When higher concentrations of SL-GC were incorporated into the DPPC liposomes, the spectra became to nearly a single broad line because of the formation of the SL-GC rich domains as described previously [16,19]. Therefore the SL-GC molecules in the Cel-DAG membranes are segregated more completely than those in the DPPC membranes. The Cel-DAG molecules might interact with each other more strongly and exclude other lipids more completely than do the DPPC molecules.

The spectra of the other spin-labeled lipid, SL-6-DPPA, in the Cel-DAG liposomes were very interesting. The rotational correlation time ratios (τ_B/τ_C) of the SL-6-DPPA in the Cel-DAG membranes above 55°C were greater than 1.0, indicating that the rate of x-axis rotation of the spin-label moiety was more predominant than that of the tumbling motion of the rotational axis. On the other hand, below 50°C, the ratios were less than 1.0, and the apparent rotational correlation time (τ_R) was remarkably great at lower temperature. These facts indicate that the motion of the spin-label moiety became isotropic and greatly restricted in the Cel-DAG membrane at the low temperature.

It is very interesting that the shape of the spectrum, the τ_B/τ_C ratio and the τ_R value of SL-6-DPPA in the Cel-DAG liposomes at 37°C are quite similar to those in the DPPC liposomes at 2°C [17]. As the difference in diameter between liposomes does not influence these parameters under the present experimental conditions, the state of the Cel-DAG membranes at 37°C could resemble the rigid packed structure of the DPPC membranes in the Lc phase at 2°C. Taken together, we assume that the Cel-DAG molecules are packed tightly and restricted in motion in the membrane at 37°C.

We previously reported that the haptenic activity of globoside incorporated into glyceroglycolipid liposomes was reduced as compared to that in phospholipid liposomes, whereas the haptenic activity of cardiolipin was not changed [23]. We also reported that the haptenic activity of Forssman glycolipid was affected by glycophorin, and the interaction of the glycophorin with lectins was also affected by the glycolipids when they were incorporated into the same phosphatidylcholine liposomes [24]. The interaction between ganglioside headgroups has been suggested by using spin-labeled gangliosides [25]. Furthermore it was suggested that the interaction between headgroups of glyceroglycolipids is stronger than that in the DPPC by X-ray diffraction [10], monolayer [11] and fluorescence studies [22]. These observations suggest the presence of 'carbohydrate–carbohydrate interaction' between sugar residues of the molecules described above. Thus, carbohydrate–carbohydrate interaction between sugar residues of the Cel-DAG molecules might exist, which results in the complete exclusion of the SL-GC and the motional restriction of the SL-6-DPPA in the Cel-DAG membranes, and higher phase-transition temperature than the DPPC.

When cholesterol was incorporated into the Cel-DAG liposomes, the motional state of the Cel-DAG was drastically changed. The amount of SL-GC in the random distribution phase in the Cel-DAG membranes increased with an increase in cholesterol content. The order parameter analysis suggests that the molecular motion of acyl moiety of SL-GC is increased at 37°C with the increase in cholesterol content, although the order parameter in the Cel-DAG liposomes without cholesterol could not be evaluated. This effect is confirmed by the calorimetric study. The apparent thermotropic phase transition was suppressed with the increase in cholesterol content in the Cel-DAG liposomes. The ESR spectra of SL-6-DPPA in the Cel-DAG liposomes

were also influenced by the incorporation of cholesterol. In the Cel-DAG liposomes, the τ_R value of SL-6-DPPA was reduced and the τ_B/τ_C ratio was increased, and they became to similar values to the corresponding ones in the DPPC liposomes at 37°C. These observations suggest that not only fatty acyl chains but also carbohydrate domain of the Cel-DAG membranes are re-mobilized and the tightly packed structure of the Cel-DAG membranes are disturbed by the incorporation of cholesterol. Since the activation of macrophages was observed with cholesterol-free Cel-DAG liposomes but not with phospholipid liposomes or cholesterol-containing Cel-DAG liposomes, the state of the carbohydrate residues of the Cel-DAG liposomes could be important for the activities [5]. We assume that macrophages can recognize only the cholesterol-free Cel-DAG liposomes with the rigid-packed regularly arranged array of carbohydrate residues which are extremely restricted in motion on the surface of the membranes, resulting in the activation to the tumoricidal state. The Cel-DAG liposomes containing an equimolar cholesterol would not be recognized by macrophages because such liposomes does not have the rigid-packed carbohydrate residues on the surface and the molecular motion of the carbohydrate residues are greatly increased.

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