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HAPTENIC ACTIVITY OF GALACTOSYL CERAMIDE AND ITS TOPOGRAPHICAL DISTRIBUTION ON LIPOSOMAL MEMBRANES

I. EFFECT OF CHOLESTEROL INCORPORATION

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The relation between the immune-reaction of phosphatidylcholine liposomes containing spin-labeled galactosyl ceramide with or without cholesterol and the topographical distribution of the glycolipid in membranes was studied. In egg yolk phosphatidylcholine liposomes, both immune agglutination and antibody binding occurred, irrespectively of the presence of cholesterol, though the motion of the fatty acyl chain of spin-labeled galactosyl ceramide was restricted by cholesterol. In dipalmitoyl phosphatidylcholine liposomes, unlike in egg yolk phosphatidylcholine liposomes, the immune-reaction depended on the cholesterol content. The electron spin resonance (ESR) spectra of spin-labeled galactosyl ceramide in dipalmitoyl phosphatidylcholine liposomes indicated that cholesterol affected the topographical distribution of spin-labeled galactosyl ceramide in the liposomes. Without cholesterol, most of the spin-labeled galactosyl ceramide was clustered on the dipalmitoyl phosphatidylcholine membrane, but with increase of cholesterol, random distribution of hapten on the membrane increased. The cholesterol-dependent change in the topographical distribution of hapten on the membranes was parallel with that of immune reactivity. 'Aggregates' composed solely of galactosyl ceramide did not show any binding activity with antibody. The findings suggest that the recognition of galactosyl ceramide by antibody depended on the topographical distribution of hapten molecules. Phosphatidylcholine and/or cholesterol may play roles as 'spacers' for the proper distribution of 'active' haptens on the membranes. The optimum density of haptens properly distributed on liposomal membranes is discussed.

Introduction

Sphingoglycolipid is an important lipid component of the surface membrane of mammalian cells and may exhibit various functions, as an annular lipid, matrix lipid, determinant of immunological specificity or receptor [1]. There is very little information about the lateral distribution and mobility of glycolipids on membranes, though the functions of glycolipids may be greatly influenced by their physical states. The ESR spectra of spin-labeled amphiphilic molecules provide useful information about local motion and the lateral distribution of the molecules on membranes. Thus, using spin-labeled galactosyl ceramide (cerebroside), we tried to determine the lateral distribution and molecular motion of galactosyl ceramide on liposomal membranes. Galactosyl ceramide, the simplest glycolipid, is a main component of myelin membranes and it has a haptenic

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property, since injection of the myelin fraction or a complex of galactosyl ceramide micelles with foreign proteins elicits antibody production against galactosyl ceramide [2]. Liposomes containing this glycolipid should provide an interesting model for use in studies on 'receptor-ligand' interaction. The relation between the immunological activity of liposomes containing haptens such as cardiolipin and 'nitroxide lipid hapten' and their physical states has recently been studied [3,4]. Brûlet and McConnell [4] reported that membrane fluidity and lateral mobility of 'nitroxide lipid hapten' molecules are important for activation of complement components. We found that membrane fluidity is important not for binding of antibody with cardiolipin but for agglutination of liposomes by antibody [3].

No information is, however, available about the dynamics of glycolipid hapten in membranes or its relation to the antigenic property. This work showed that unlike in the case of cardiolipin and nitroxide lipid hapten, recognition of galactosyl ceramide by antibody seems to depend on the topographical distribution of hapten molecules.

Materials and Methods

Chemicals. Dipalmitoyl phosphatidylcholine, cholesterol and dicetyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Egg yolk phosphatidylcholine was prepared in our laboratory as described previously [5]. Galactosyl ceramide was prepared from bovine brain as described by Carter et al. [6]. Stearic acid derivatives (12′-(*N*-oxyl-4″,4″-dimethyloxazolidine)-stearate), which have a nitroxide-containing ring in the C-12 position ((5, 10) fatty acid), were synthesized by the method of Waggoner et al. [7]. The (5,10) spin-labeled galactosyl ceramide was synthesized from bovine galactosyl ceramide by the procedure of Sharom and Grant [8]. The (5,10) spin-labeled phosphatidylcholine was synthesized from egg yolk phosphatidylcholine by the method of Hubbell and McConnell [9]. All lipid preparations gave a single spot on thin-layer chromatography.

Preparation of liposomes. Multi-lamellar liposomes were prepared in veronal buffered saline as described previously [5]. 'Aggregates' of galactosyl ceramide were prepared by dialysis of dimethyl sulfoxide solution against saline overnight.

Preparation of serum. Anti-galactosyl ceramide serum was prepared as described by Inoue et al. [10] with a minor modification. Antibody activity were found in both the IgM and the IgG fraction. Nonspecific factors, which react with liposomes without haptens, were removed by pretreatment of the serum with liposomes free from galactosyl ceramide. Goat anti-rabbit whole serum, goat anti-rabbit μ chain, and goat anti-rabbit IgG were purchased from Miles Co. (Rehovot, Israel). The complement source was fresh guinea pig serum, which was stored at −160°C until used.

Isolation of the IgG fraction from anti-serum. The IgG fraction was partially purified as follows. Sera were treated twice with 33% saturated ammonium sulfate. IgG was separated by passage of the precipitated material through a Sepharose 6B column with phosphate buffered saline (pH 7.5) as solvent. Immune diffusion of the fraction versus goat anti-rabbit whole serum, goat anti-rabbit μ chain and goat anti-rabbit IgG showed that this fraction contained IgG without any appreciable contamination of IgM.

Agglutination of liposomes containing spin-labeled galactosyl ceramide. Liposome dispersions (0.2 μmol as phospholipid) were mixed with serial 2-fold dilutions of the serum and incubated for 10 min on a glass plate with constant mechanical shaking at room temperature. Then the number and size of aggregates formed were measured. The serum titer is expressed as the logarithm (base 2) of the minimum dilution required to obtain the smallest positive results.

Complement dependent immune-damage of liposomes. Liposomes composed of phosphatidylcholine, dicetyl phosphate and galactosyl ceramide in the desired molar ratio were prepared as described previously [10]. Immune-damage of liposomes was assayed by the method of Kinsky et al. [11].

Absorption of antibody with liposomes. Undiluted anti-serum (50 μl) was incubated for 24 h at 25°C with liposomes (5 μmol as phospholipids). The mixtures were placed in a capillary (diameter, 0.8 mm) and centrifuged at $3000 \times g$ for 15 min. Residual agglutination activity in the clear supernatant thus obtained was determined, using liposomes composed of dipalmitoyl phosphatidylcholine, cholesterol, dicetyl phosphate and galactosyl ceramide (molar

ratio, 10 : 10 : 1 : 3) as test antigens.

ESR observation. Electron spin resonance spectra were measured with a JES-PE-1X, X band, 100 kHz field modulator (Jeol, Tokyo, Japan) equipped with a temperature controller. The temperature was measured with a copper-constantan thermocouple.

The simulation of ESR spectra was performed with a HITAC 8800/8700 instrument (Hitachi, Japan) and plotted on a Versatic Printer/Plotter model 1200A (Xerox, CA, U.S.A.).

Results

Haptenic activity of spin-labeled galactosyl ceramide

The reactivity of spin-labeled galactosyl ceramide with anti-galactosyl ceramide antibody was determined by measuring complement-dependent damage and agglutination of liposomes containing spin-labeled galactosyl ceramide.

Antibody-induced agglutination of liposomes composed of egg yolk phosphatidylcholine and cholesterol was dependent on the amount of spin-labeled galactosyl ceramide or galactosyl ceramide. The dose-dependence of spin-labeled galactosyl ceramide was almost the same as that of galactosyl ceramide, indicating that the antigenic property was not affected by modification of the acyl group with a nitroxide group (data not shown).

The immune-agglutination of egg yolk phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes containing spin-labeled galactosyl ceramide was measured as a function of the cholesterol content (Fig. 1). The immune-agglutination of egg yolk phosphatidylcholine liposomes was independent of the cholesterol content. However, that of dipalmitoyl phosphatidylcholine liposomes depended on the cholesterol content; with dipalmitoyl phosphatidylcholine liposomes containing a molar ratio of cholesterol to phosphatidylcholine of 0.5, a distinct reaction was observed and the reactivity of the liposomes increased with increase in the molar ratio of cholesterol to phospholipid from 0.5 to 1.0. The agglutination of liposomes containing galactosyl ceramide showed very similar cholesterol dependence to that of liposomes with spin-labeled galactosyl ceramide.

The effect of cholesterol on immune-damage of liposomes sensitized with spin-labeled galactosyl ceramide was next examined. Damage of dipalmitoyl phosphatidylcholine liposomes occurred only in the presence of cholesterol (data not shown). The cholesterol dependence observed here is consistent with results of agglutination experiments. Immune-agglutination by the IgG fraction showed very similar cholesterol dependence. The immune agglutination of mixtures of spin-labeled galactosyl ceramide and cholesterol could not be observed appreciably. The findings are consistent with the observation by Rapport et al. [2], who studied immune reaction of galactosyl ceramide using the absorption test.

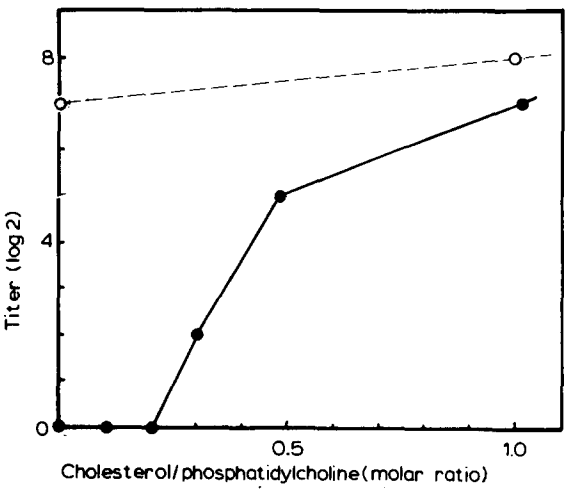

Fig. 1. Effect of cholesterol on immune agglutination of egg yolk phosphatidylcholine- and dipalmitoyl phosphatidylcholine-liposomes. Aliquots (20 μl) of liposome preparations composed of dipalmitoyl phosphatidylcholine, dicetyl phosphate, spin-labeled galactosyl ceramide (molar ratio, 10 : 1 : 1) and various amount of cholesterol (●——●) were added to serial 2-fold dilutions of serum (20 μl) and incubated for 10 min with continuous shaking at 25°C. Serum titers are expressed as the logarithms (base 2) of the minimum dilutions required to obtain the smallest positive result. Similar experiments were performed with liposomes containing egg yolk phosphatidylcholine (o- - - - -o) instead of dipalmitoyl phosphatidylcholine.

Binding of antibodies to liposomes

The binding of antibodies to liposomes was assayed by measuring the amount of antibodies remaining after absorption. The amount of remaining antibody was measured using liposomes composed of dipalmitoyl phosphatidylcholine, cholesterol,

dicetyl phosphate and galactosyl ceramide (molar ratio, 10 : 10 : 1 : 3) as test antigen. Liposomes or 'aggregates' of galactosyl ceramide were first incubated with antiserum for 24 h at 25°C. Incubation for longer than 24 h did not change the result.

Liposomes composed of egg yolk phosphatidylcholine/cholesterol/galactosyl ceramide and those composed of dipalmitoyl phosphatidylcholine/cholesterol/galactosyl ceramide both absorbed antibodies effectively (Table I). On the other hand, dipalmitoyl phosphatidylcholine/galactosyl ceramide liposomes without cholesterol were not reactive. In agreement with the observation of Rapport et al. [2], 'aggregates' composed solely of galactosyl ceramide were not reactive. All liposomes without galactosyl ceramide were not reactive, indicating that the reaction was specific for galactosyl ceramide.

Similar results were obtained when antibody activity remaining after absorption was measured using different liposomes, such as those of egg yolk phosphatidylcholine, cholesterol, dicetyl phosphate and galactosyl ceramide (10 : 10 : 1 : 3), as a test antigen.

TABLE I

ABSORPTION OF ANTIBODY ACTIVITY BY VARIOUS LIPID SUSPENSIONS

Undiluted antiserum (50 μl) was incubated for 24 h at 25°C with liposomes (5 μmol as phospholipid) or 'aggregates' of galactosyl ceramide (1.5 μmol). The titer of untreated serum is designated as 'control'. Liposomes composed of 5 μmol egg yolk phosphatidylcholine, 5 μmol cholesterol, 0.5 μmol dicetyl phosphate and 1.5 μmol galactosyl ceramide (Egg PC + Chol); 5 μmol dipalmitoyl phosphatidylcholine, 5 μmol cholesterol, 0.5 μmol dicetyl phosphate and 1.5 μmol galactosyl ceramide (DPPC + Chol) and 5 μmol dipalmitoyl phosphatidylcholine, 0.5 μmol dicetyl phosphate and 1.5 μmol galactosyl ceramide (DPPC) were used. For details of determination of residual agglutination activity, see 'Materials and Methods'.

Lipid suspension	Titer (log 2)
Control	8
Egg PC + Chol	1
DPPC + Chol	1
DPPC	8
Galactosyl ceramide aggregates	8

Topographical distribution of spin-labeled galactosyl ceramide in egg yolk phosphatidylcholine liposomes

Various amounts of spin-labeled galactosyl ceramide were incorporated into egg yolk phosphatidylcholine liposomes. Typical ESR spectra are shown in Fig. 2. In these liposomes, spin-labeled galactosyl ceramide may be free and move laterally on the membranes. With increase in the ratio of spin-labeled galactosyl ceramide to egg yolk phosphatidylcholine, the resonance lines gradually broadened, but there was no appreciable change in the ratios of the peak heights of the three resonance lines. In Fig. 3, the width of these resonance lines in the center magnetic field (ΔH_{msl}) is plotted against the concentration of spin-labeled galactosyl ceramide and compared with that of the resonance line obtained for spin-labeled phosphatidylcholine incorporated into egg yolk phosphatidylcholine liposomes. The change in the line width of spin-labeled galactosyl ceramide resembles that of the line width of spin-labeled phosphatidylcholine. This similarity indicates that spin-labeled galactosyl ceramide diffuses rapidly in a similar way to spin-labeled phos-

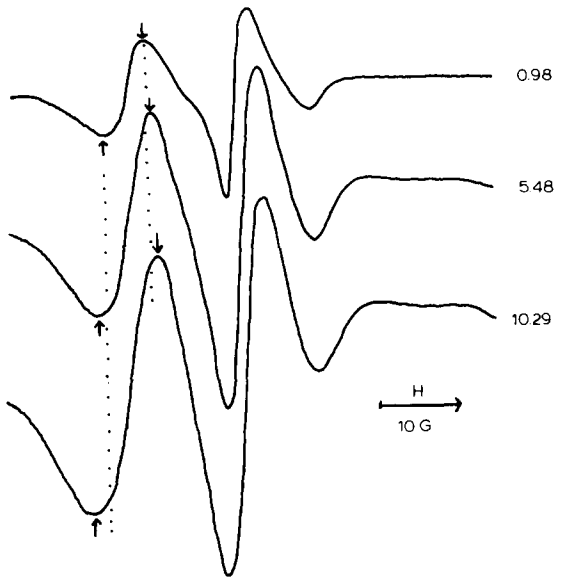

Fig. 2. Typical ESR spectra of spin-labeled galactosyl ceramide incorporated into liposomes composed of egg yolk phosphatidylcholine and dicetyl phosphate (10 : 1). Molar percentages of spin-labeled galactosyl ceramide are shown in the figure. Arrows indicate 'line widths'.

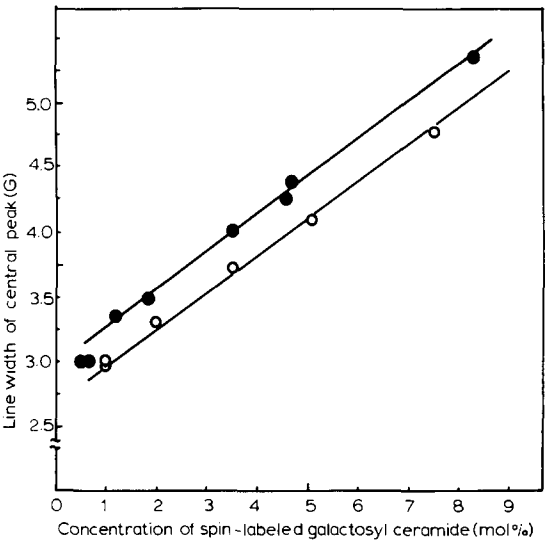

Fig. 3. Concentration-dependent change in line width of ESR signals of spin-labeled galactosyl ceramide incorporated into egg yolk phosphatidylcholine liposomes. Widths from peak to peak in the center magnetic field are plotted against molar percentages of spin-labeled galactosyl ceramide incorporated (●———●). Similar experiments were performed using spin-labeled phosphatidylcholine instead of spin-labeled galactosyl ceramide (○———○).

phatidylcholine in egg yolk phosphatidylcholine liposomal membranes.

The ESR spectra of spin-labeled galactosyl ceramide in egg yolk phosphatidylcholine liposomes were measured in the presence and absence of cholesterol, and from the spectra, we estimated the order parameter (S values) of spin-labeled galactosyl ceramide (Table II). The order parameter increased with increase of cholesterol content, suggesting that the mobility of the fatty acyl chain of spin-labeled galactosyl ceramide was restricted by incorporation of cholesterol. These results confirm the observation by Sharom et al. [12].

TABLE II

ORDER PARAMETERS OF SPIN-LABELED GALACTOSYL CERAMIDE IN EGG YOLK PHOSPHATIDYLCHOLINE LIPOSOMES IN THE PRESENCE AND ABSENCE OF CHOLESTEROL

ESR spectra were recorded at 25°C, and the coupling constant was calibrated with Mn^{2+} (86.9 G).

Cholesterol content (mol%)	S
0	0.467 (0.477) *
17	0.566
31	0.675
50	0.700

* Value for bilayers of egg yolk phosphatidylcholine containing 1 mol% spin-labeled galactosyl ceramide reported by Sharom et al. [12].

Topographical distribution of spin-labeled galactosyl ceramide in dipalmitoyl phosphatidylcholine/cholesterol liposomes

Unlike the ESR spectra observed in egg yolk phosphatidylcholine liposomes, the spectra of spin-labeled galactosyl ceramide in dipalmitoyl phosphatidylcholine/cholesterol (molar ratio, 1 : 1) liposomes changed greatly with increase of spin-labeled galactosyl ceramide concentration (Fig. 4).

A similar dose-dependent change of the signal in synthetic nitroxide biradicals incorporated into liposomes was observed by Rey and McConnell [13]. They postulated that these nitroxide biradicals exhibit a degree of clustering in the plane

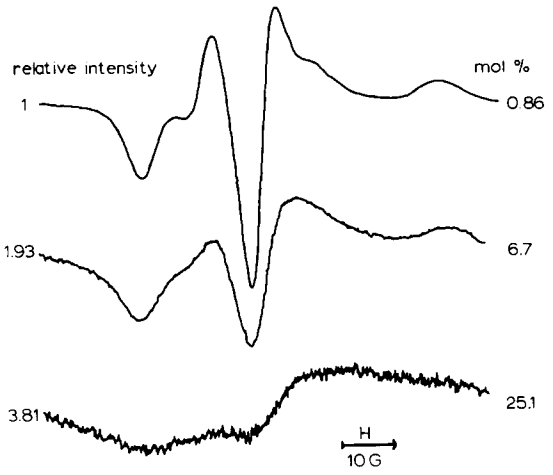

Fig. 4. Typical ESR spectra of spin-labeled galactosyl ceramide in liposomes composed of dipalmitoyl phosphatidylcholine and cholesterol (molar ratio, 1 : 1) with various concentrations of hapten. Concentrations of the hapten are indicated on the right, and relative amplitudes of signals on the left. The overall molarity of spin-labeled galactosyl ceramide was 2 mM.

of the membrane that depends on the concentration of the biradical. The single broad resonance line may be superimposed on the three resonance lines. Assuming that the experimental spectra were some normalized linear combination of various states of spin conditions, Rey and McConnell calculated the percentage of molecules of biradical clustered in the host membrane. It is quite possible that similar clustering of spin-labeled galactosyl ceramide occurs into liposomes. Therefore, we calculated the percentage of spin-labeled galactosyl ceramide clustered in the liposomes by their method. The following equation was used for the calculation,

$$I(H) = f \cdot I_1(H) + (1 - f) \cdot I_2(H)$$

where $I(H)$ is the signal intensity at the magnetic field (H), $I_1(H)$ and $I_2(H)$ are the signal intensities arising from molecules clustered and those arising from molecules distributed randomly, respectively, and f is the fraction of hapten molecules clustered on the membranes.

With increase in incorporation of spin-labeled galactosyl ceramide, the fraction of clustered hapten molecules increased (Fig. 5).

Eighty percent of the spin-labeled galactosyl ceramide molecules were clustered in dipalmitoyl phosphatidylcholine membranes when 6.7 mol% of the spin-labeled compound was incorporated into the membranes, whereas only 20% of the molecules were clustered in membranes containing 0.86 mol% of the compound.

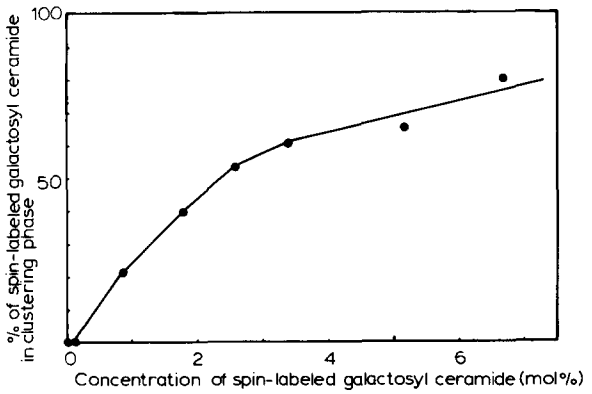

Fig. 5. Percentage of spin-labeled galactosyl ceramide clustered in liposomes of dipalmitoyl phosphatidylcholine and cholesterol (molar ratio, 1 : 1). Assay temperature, 25°C. For details of determination of the percentage of clustered molecules, see the text.

Effect of cholesterol on the topographical distribution of spin-labeled galactosyl ceramide in dipalmitoyl phosphatidylcholine

Various amounts of cholesterol were incorporated into liposomes containing a certain amount of spin-labeled galactosyl ceramide. When spin-labeled galactosyl ceramide was incorporated into dipalmitoyl phosphatidylcholine liposomes without cholesterol, only very broad spectra were observed (Fig. 6A). On incorporation of increasing amounts of cholesterol into the liposomes, the single broad line decreased with concomitant increase of the three lines. Application of the simulation developed by Rey and McConnell [13] to the present system suggested that in spectra A, B, C and D, 2%, 7%, 16% and 35% of spin-labeled galactosyl ceramide may be in the phase of random distribution.

In conclusion, cholesterol affected the topographical distribution of spin-labeled galactosyl

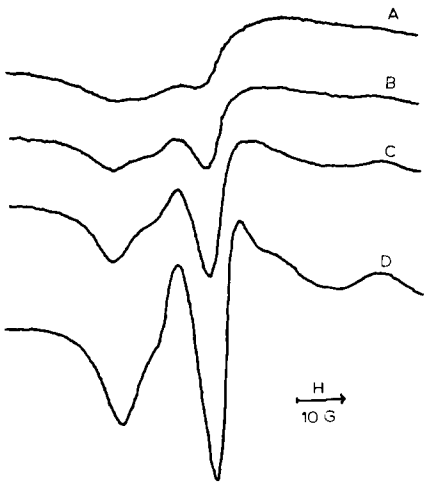

Fig. 6. ESR spectra of spin-labeled galactosyl ceramide in liposomes composed of dipalmitoyl phosphatidylcholine and various amounts of cholesterol. Spectra A, B, C and D are for liposomes containing molar ratios of cholesterol to phospholipid of 0, 0.1, 0.3 and 0.96, respectively. All liposomes contained 10.2 mol% of spin-labeled galactosyl ceramide.

ceramide; with increase of the cholesterol content from 0% to 50%, the percentage of randomly distributed spin-labeled galactosyl ceramide on the membrane increased from 2% to 35%.

Discussion

Immune-agglutination of egg yolk phosphatidylcholine liposomes was independent of the cholesterol content at 25°C. At this temperature egg yolk phosphatidylcholine should be in a liquid crystalline state ($T_c = -10$ to 0°C). ESR study indicated that all the spin-labeled galactosyl ceramide molecules in egg yolk phosphatidylcholine liposomes were randomly distributed on the membrane, irrespective of the cholesterol content. But cholesterol affected the mobility of hydrocarbon chains of spin-labeled galactosyl ceramide in the liposomes; with increase of the cholesterol content, the mobility of spin-labeled galactosyl ceramide was restricted. The haptenic activity of spin-labeled galactosyl ceramide, however, was not changed significantly by cholesterol incorporation. Thus it can be concluded that restriction of the hydrocarbon chain did not have any significant influence on the immune agglutinability of liposomes with spin-labeled galactosyl ceramide.

In contrast, both agglutination and complement-mediated damage of dipalmitoyl phosphatidylcholine liposomes were dependent on the cholesterol content. In dipalmitoyl phosphatidylcholine liposomes without cholesterol, which are in the gel state at 25°C ($T_c = 41$°C), no immune agglutination could be observed. Agglutination was observed in liposomes containing a molar ratio of cholesterol to dipalmitoyl phosphatidylcholine of more than 0.2. It is noteworthy that the minimum concentration of cholesterol to obtain full agglutinability is close to that of cholesterol required for fluidization of bilayers in the gel state [14]. These findings are similar to our previous results with cardiolipin [3] and those obtained by Brûlet and McConnell [4] who carried out a similar study on the complement fixation reaction of liposomes containing nitroxide lipid hapten. The cholesterol dependence of agglutination may result from the dependence of antibody binding to haptens on membranes, since liposomes containing equimolar amounts of cholesterol and phospholipid could absorb antibody, whereas those without cholesterol could not. All these findings indicate that the fluid state of host lattice is required for the reactions. The other possibility that cholesterol affects the 'gel-liquid crystal transition' of galactosyl ceramide ($T_c = 61$°C), which may be responsible for the immune reactivity, could be excluded, since cholesterol was not required for the reaction of egg yolk phosphatidylcholine liposomes with antibody.

The ESR signals observed indicated that cholesterol affected the topographical distribution of spin-labeled galactosyl ceramide in bilayer membranes. With increase in the cholesterol content, the proportion of clustered spin-labeled galactosyl ceramide decreased with increase in the amount of randomly distributed molecules (Fig. 7). The change in topographical distribution of spin-labeled galactosyl ceramide upon addition of cholesterol was parallel to the change of haptenic activity. Thus, the haptenic activity of spin-labeled galactosyl ceramide may depend on its topographical distribution (Fig. 8). It is noteworthy that not all the spin-labeled galactosyl ceramide in dipalmitoyl phosphatidylcholine bilayers is in a free state even in the presence of equimolar amounts of cholesterol and phospholipid. The topographical distribution was also affected by the amount of hapten molecules incorporated.

The amount of haptens added may, therefore, not simply reflect the density of 'active' hapten, which should be distributed randomly on the membranes. To obtain the density of 'active' hapten, it is necessary to measure the fraction of hapten molecules that is randomly distributed (Fig. 5). This was calculated by the following equation.

$$\rho = f \cdot C$$

where ρ is the density of hapten molecules, C is the amount of hapten added, and f is the fraction of hapten molecules distributed randomly.

It was calculated from the results in Fig. 7C that the density of 'active' haptens required for liposomes prepared with 10.2 mol% of haptens to show the minimum immune reaction was 0.9 mol%. The minimum density of 'active' haptens required (0.7–0.8 mol%), which was obtained in liposomes prepared with 2.9 mol% and 5.8 mol% of haptens, is similar to that obtained in liposomes prepared with

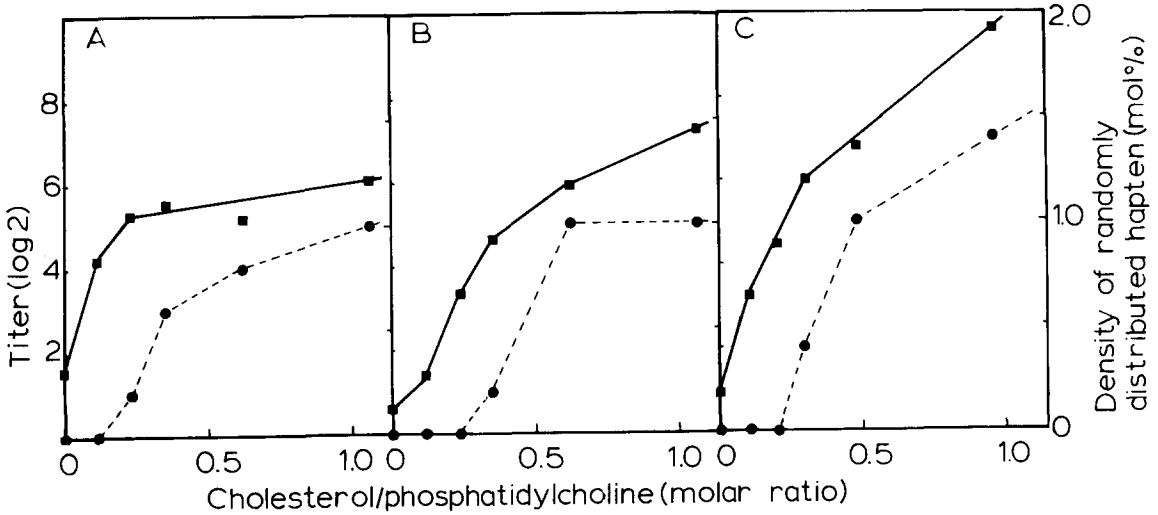

Fig. 7. Effect of cholesterol on immune-agglutinability of liposomes and the density of hapten molecules distributed randomly on the membranes. Liposomes composed of dipalmitoyl phosphatidylcholine and 2.9 mol% (A), 5.8 mol% (B) or 10.2 mol% (C) of spin-labeled galactosyl ceramide with various amounts of cholesterol were used. The immune-agglutinability of liposomes (•) was determined by incubating liposomes (0.2 μmol as phospholipid) with 20 μl antiserum for 10 min at 25°C. The density of haptens distributed randomly on liposomal membranes (▪) was estimated from the amounts of hapten added and the proportion of molecules distributed randomly shown in Fig. 5.

10.2 mol% haptens (Fig. 7A and B). This calculated value for the minimum density of haptens was rather different from that obtained in egg yolk phosphatidylcholine liposomes. In the latter, the haptenic density can be simply obtained from the amount of haptens originally added, since all galactosyl ceramides were randomly distributed under the present experimental conditions. The minimum hapten density in liposomes without cholesterol was calculated as 0.1 mol% (data not shown). This value is much less than that for dipalmitoyl phosphatidylcholine liposomes, probably due to a difference in the lateral diffusion rate of hapten molecules on the liposomal membranes. In fact, it was reported the lipid diffusion coefficient in the gel phase was slower than that in the fluid phase by a factor of 10^2 [15,16].

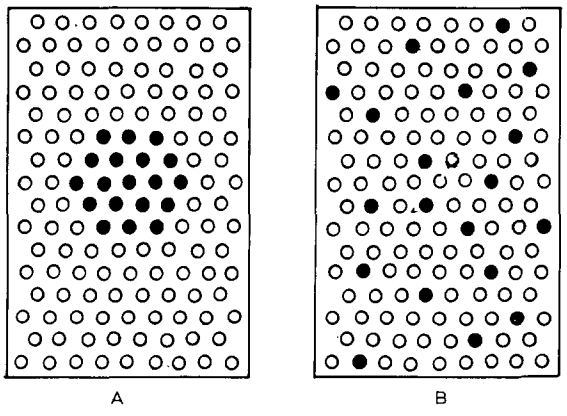

Fig. 8. Schematic representation of topographical distribution of galactosyl ceramide on phospholipids membranes. Closed circles represent glycolipids and the open circles other lipids. Glycolipids are clustered in the membrane in (A), and distributed randomly in (B).

The enhancing effect of cholesterol could result from increase in exposure of haptens and in the accessibility of their sugar group to antibody, as suggested previously by Alving et al. [17], and Brûlet and McConnell [18]. In our case, however, this possibility is rather unlikely, since preliminary experiments showed that increase of the incubation temperature also enhanced the haptenic activity in dipalmitoyl phosphatidylcholine liposomes even without cholesterol, and increase of temperature in unlikely to increase hapten exposure.

No line broadening brought about by spin-spin

interaction was observed with nitroxide lipid hapten, even in dipalmitoyl phosphatidylcholine liposomes [4]. Findings were different with spin-labeled galactosyl ceramide. This difference may depend on differences in structure of hapten molecules. The molecular mechanism of clustering of galactosyl ceramide is not yet known: possibly self association of galactosyl ceramide is due to hydrogen bond formation between molecules. It is noteworthy that Neuringer et al., using the deuterium NMR technique, recently observed self association of galactosyl ceramide in lipid bilayers [19].

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