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Fluorescence quenching measurements of the membrane bound lipid haptens with different length spacers

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Dansyl lipid haptens with three different length (short, intermediate and long) spacers have been incorporated into DMPC or DPPC liposomes. Anti-DNS-IgG bound most efficiently to these liposomes containing lipid haptens with an intermediate length spacer, although the binding efficiency became more increased when liposomes were made of a mixture of phospholipid (DMPC or DPPC) and cholesterol. To explain these results we have measured the accessibility of dansyl lipid haptens in liposomal membranes by the fluorescence quenching method. It was found that the dansyl haptens located on the surfaces of DMPC (or DPPC) membranes and fluorescence quenchers (iodide ions) possessed almost similar accessibility for the dansyl haptens with different length spacers. However, in the DMPC (or DPPC) membranes with 50% cholesterol, a part of the dansyl haptens became buried into the interior of the liposomal membranes depending on the length of the spacer and another part removed into the aqueous solution with greater affinity for antibody. These results were explained well by our recent model for antibody binding to the membrane-bound lipid haptens.

Introduction

Various studies on the immune responses using liposomal membranes have suggested that the affinity of antibody binding to lipid haptens can be affected by several factors. They are lipid composition, chemical and physical nature of lipids, hapten (epitope) density, and spacer lengths of lipid haptens [1–7]. However, few physical experiments have been done to explain the effects of factors on antigen recognition. Clarifying the nature of these factors is important to understand the general problem of cell surface recognition in immunology and in membrane biochemistry.

Then, we have tried here to study these problems by measuring the accessibilities of the membrane-bound lipid haptens with different length spacers. In our previous paper we have determined the location of trinitrophenylated haptens (TNP haptens) with different length spacers by using the fluorescence energy transfer method. In that experiment we determined the vertical distance between TNP haptens (acceptors) and a pyrene fluorophore (donor) which was embedded in the middle of lipid membranes [7]. However, it should be important to determine whether the previous findings for TNP lipid haptens are applicable to other lipid haptens. And it is also important to confirm whether the results obtained by the fluorescence energy transfer method are compatible with those obtained by different methods.

Thus, in this paper we have taken an advantage of fluorescence lipid haptens (dansyl haptens) to study the accessibilities of the membrane-bound haptens with different length spacers. Here, we have measured the

Abbreviations; DNS, 5-dimethylaminonaphthalene-1-sulfonyl; PE, phosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PBS, phosphate-buffered saline; DNS-C_6-PE, *N*-(DNS)-dipalmitoyl-L-α-phosphatidylethanolamine; DNS-C_6-PE, *N*-(DNS)-aminohexanoyl-dipamitoyl-L-α-phosphatidylethanolamine; DNS-C_{12}-PE, *N*-(DNS)-aminododecanoyldipalmitoyl-L-α-phosphatidylethanolamine.

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accessibilities of the dansyl haptens by the fluorescence quenching method [6]. The present results demonstrated clearly how the spacer-length and the membrane composition can control antigen recognition on the membrane surfaces.

Materials and Methods

Materials

DMPC and DPPC were purchased from Avanti (Birmingham, AL). *N*-Dansyl-dipalmitoyl-L-α-phosphatidylethanolamine (DNS-C_6-PE), *N*-dansyl-aminohexanoyl-dipalmitoyl-L-α-phosphatidylethanolamine (DNA-C_6-PE) and *N*-dansyl-aminododecanoyl-dipalmitoyl-L-α-phosphatidylethanolamine (DNS-C_{12}-PE) were prepared by the previous method [8,9]. Dansyl glycine and dansyl cadaverine were purchased from Sigma (St. Louis, MO). Dansylaminocaproylic acid (DNS-C_6) and dansylaminolaurylic acid (DNS-C_{12}) were prepared by the previous methods [8]. Potassium iodide and potassium chloride were purchased from Wako Pure Chem (Osaka, Japan).

Preparations of anti-dansyl IgG were described in the previous paper [10]. Rabbit anti-DNS-IgG was prepared by immunizing anti-dansyl-BSA into a rabbit with Freund's complete adjuvant.

Liposomes

Small unilamellar liposomes were prepared by the injection of an ethanolic solution of a mixture of phospholipid (DMPC or DPPC), cholesterol and one of dansyl lipid haptens into phosphate-buffered saline (PBS, pH 7.2) at 70°C [7,11,12]. Multilamellar liposomes were prepared by co-dissolving phospholipid, cholesterol and one of the dansyl lipid haptens in chloroform, and drying onto the slide of flasks. Then, PBS was added to the flasks which were kept at 70°C for 15 min and were shaken to give a liposome suspension.

Antibody binding experiments

Antibody binding affinity to the dansyl lipid haptens with different length spacers was measured by the inhibition assay experiment of complement fixation [13]. In this experiment, we used two kinds of liposomes. One was the target liposome which was made of 49.5% DMPC, 49.5% cholesterol and 1% DNS-C_6-PE. It contained carboxyfluorescein as a maker of the liposome lysis. The other was the inhibitor liposome which was made of 99% DMPC and 1% of one of the dansyl lipid haptens with different length spacers (or 49.5% DMPC, 49.5% cholesterol and 1% of the dansyl lipid haptens). This inhibitor liposome did not contain carboxyfluorescein.

For the measurements, 25 μl of the inhibitor liposomes (50 μM of lipids) and 25 μl of the diluted solution (1/10000) of anti-dansyl rabbit serum were added to 5 μl of the target liposome solution (2.5 μM of lipids). Then fresh guinea pig serum as complement (1/140 dilution) was added to the solution. After the addition of complement, the mixture was incubated for 60 min at 37°C. The extent of the inhibition on the liposome lysis was determined by the following equation.

$$\text{Percentages of inhibition} = \frac{F_0 - F}{F_0} \times 100 \qquad (1)$$

where F is the fluorescence intensity of released carboxyfluorescein from the target liposomes after incubation. F_0 is the fluorescence intensity of released carboxyfluorescein from the target liposomes when we used a control liposome for the inhibitor liposome. The control liposome (50% DMPC + 50% cholesterol) did not contain any dansyl lipid haptens. The fluorescence intensity was measured at 520 nm (excitation wavelength at 490 nm).

Fluorescence measurements

Fluorescence spectra were observed with a Hitachi model 650-60 fluorescence spectrophotometer. A decay of fluorescence intensity after pulsed excitation was measured with a single photon counting apparatus as described previously [14]. Excitation wavelength was 350 nm and the emission above 460 nm was collected at 30°.

Accessibility of dansyl lipid haptens

Accessibility of dansyl haptens in liposomal membranes was measured by the fluorescence quenching method, using iodide ions as fluorescence quenchers. According to the theory of the fluorescence quenching, the lifetime of a fluorophore decreases in the presence of a quencher by the following Stern-Volmer equation [6,15–17],

$$\frac{1}{t} = \frac{1}{t_0} + k_q[Q] \qquad (2)$$

where t and t_0 are lifetimes of fluorophores in the presence and in the absence of quenchers, respectively. [Q] is the concentration of quenchers (potassium iodide). k_q is a bimolecular quenching rate constant which should represent the accessibility of a fluorophore by quenchers [6,15–17]. We calculated the percentages of the accessibilities of the dansyl haptens by the following equation.

$$\text{Percentage of the accessibility} = \frac{k_q}{k_q(0)} \times 100 \qquad (3)$$

where we estimated $k_q(0)$ from a quenching rate constant of dansylaminocaproylic acid (or dansylcadaverine) by iodide ions in solution.

Binding affinity of dansyl-spacers to the bilayer membranes

Binding affinity of dansyl-spacers (without phospholipids) to the bilayer membranes was measured by the following procedure. Dansylaminoglycine (DNS-C_2), dansylaminocaproylic acid (DNS-C_6), or dansylaminolaurylic acid (DNS-C_{12}) was incubated with preformed multilamellar liposomes at 30 °C for 15 min. Then the liposomes were collected by centrifugation for 7 min at 3000 rpm. Liposome pellets were dissolved into an ethanol solution. Amounts of the membrane-bound dansyl-spacers were calculated from fluorescence intesities of dansyl haptens in ethanol solutions.

Results

Antibody binding to dansyl lipid haptens with different length spacers

We first checked the binding affinity of anti-dansyl antibody to the dansyl-lipid haptens in multilamellar liposomes with different length spacers (Fig. 1). Here, we measured the efficiencies of antibody binding to dansyl haptens from the inhibition assay experiment of complement fixation (see materials and methods). Fig. 1A shows that the lysis of target liposomes was more efficiently inhibited by DNS-C_6-PE liposomes (99% DMPC and 1% DNS-C_6-PE) than by DNS-C_0-PE or DNS-C_{12}-PE liposomes. This result indicated that anti-dansyl IgG bound more efficiently to DNS-C_6-PE liposomes than DNS-C_0-PE or DNS-C_{12}-PE liposomes. In the existence of cholesterol, antibody binding to the dansyl lipid haptens was more enhanced for any length spacers (Fig. 1B). Here again, however, anti-dansyl IgG bound more efficiently to DNS-C_6-PE liposomes (49.5% DMPC, 49.5% cholesterol and 1% DNS-C_0-PE) than DNS-C_0-PE or DNS-C_{12}-PE liposomes with cholesterol.

Microenvironments of dansyl haptens as revealed by fluorescence spectrum

It is well known that fluorescence spectrum of dansyl residues are very sensitive to the microenviroments of the dansyl fluorophores. Fig. 2a shows fluorescence spectra of DNS-C_6-PE in DMPC liposomes. In DMPC/cholesterol liposomes (1 : 1) fluorescence spectrum of DNS-C_6-PE decreased drastically in intensity (Fig. 2b). Fluorescence spectra of DNS-C_0-PE in DMPC and DMPC/cholesterol liposomes showed almost similar patterns as those of DNS-C_6-PE in DMPC and DMPC/cholesterol liposomes, respectively.

However, the fluorescence spectrum of DNA-C_{12}-PE in DMPC/cholesterol (1 : 1) liposomes shifted drastically to the shorter wavelength and increased in intensity (Fig. 2c), although the fluorescence spectrum of DNS-C_{12}-PE in DMPC liposomes closely resembled that of DNS-C_6-PE in DMPC liposomes (Fig. 2a). This indicated that the microenvironments of the dansyl residues of DNS-C_{12}-PE in DMPC/cholesterol liposomes became more hydrophobic than those in DMPC liposomes. Similar results were also observed for the dansyl haptens in DPPC/cholesterol liposomes.

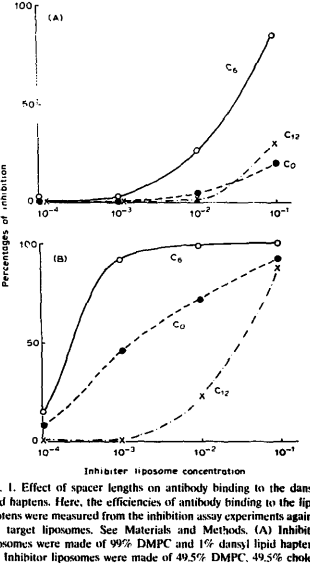

Fig. 1. Effect of spacer lengths on antibody binding to the dansyl lipid haptens. Here, the efficiencies of antibody binding to the lipid haptens were measured from the inhibition assay experiments against the target liposomes. See Materials and Methods. (A) Inhibitor liposomes were made of 99% DMPC and 1% dansyl lipid haptens. (B) Inhibitor liposomes were made of 49.5% DMPC, 49.5% cholesterol and 1% dansyl lipid haptens.

Accessibility of dansyl haptens as revealed by the fluorescence quenching method

To confirm further the microenvironments of the dansyl haptens, we have studied the fluorescence quenching measurements of the dansyl hapens using potassium iodide as a quencher. Fluorescence intensities of the dansyl residues in DMPC liposomes were weaker in KI solution than in KCl solution. This suggested that the quenchers (iodode ions) were accessible to the dansyl haptens in the bilayer membranes.

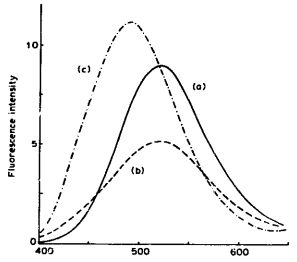

Fig. 2. Fluorescence spectrum of dansyl lipid haptens in the bilayer membranes. The bilayer membranes contained 1% dansyl lipid haptens. Excited at 340 nm. (a) Fluorescence spectrum of DNS-C_6-PE in the DMPC liposomes. (b) Fluorescence spectrum of DNS-C_6-PE in the DMPC/cholesterol (1:1) liposomes. (c) Fluorescence spectrum of DNS-C_{12}-PE in the DMPC/cholesterol liposomes.

Then, we have measured quantitatively the accessibility of the dansyl lipid haptens in the liposomes using by the nanosecond time-dependent fluorescence spectroscopy. Fig. 3 shows the time-dependent fluorescence decay curves of DNS-C_6-PE in DMPC liposomes in 0.5 M KCl solution (0.5 M KCl + PBS) and in 0.5 M KI

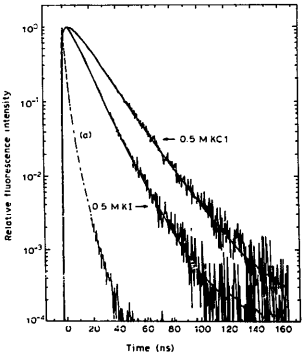

Fig. 3. Time-dependent fluorescence decay curves of DNS-C_6-PE in DMPC liposomes in the 0.5 M KI solution and in the 0.5 M KCl solution. DMPC liposomes contained 1% DNS-C_6-PE. A curve (a) is the excitation pulse profile.

TABLE I

Summary of the fluorescence life-times of the dansyl lipid haptens with different length spacers in the liposomes at 30 °C

Membranes	Solution	Spacer length		
		C_0	C_6	C_{12}
DMPC	0.5 M KCl	15.0±0.3	15.2±0.8	15.5±0.2[n.s.]
	0.5 M KI	9.3±0.1	9.7±0.4	9.5±0.2
DMPC/cholesterol	0.5 M KCl	10.9±0.3	13.3±0.4	18.5±0.2
	0.5 M KI	8.9±0.4	10.8±0.6	15.6±0.8
DPPC	0.5 M KCl	16.0±0.3	15.8±0.3	15.5±0.0
	0.5 M KI	10.0±0.4	9.7±0.2	9.1±0.1
DPPC/cholesterol	0.5 M KCl	10.9±0.1	12.7±0.7	19.8±1.6
	0.5 M KI	8.5±0.1	10.1±0.8	16.8±0.7

solution (0.5 M KI + PBS). A fluorescence lifetime of DNS-C_6-PE of DMPC liposomes was shorter in 0.5 M KI solution than in 0.5 M KCl solution. Table I shows the summary of the lifetimes of the dansyl lipid haptens with different length spacers in 0.5 M KI and 0.5 M KCl solution. From the results in Table I, the fluorescence quenching rate constants (k_q) were calculated using Eqn. 2. Regardless of the spacer lengths, the quenching rate constants for the dansyl haptens in DMPC liposomes were almost similar to one another. This suggested that the dansyl residues of lipid haptens in the DMPC membranes located at the similar positions on the bilayer surfaces even if they had different length spacers.

However, the quenching rate constants for the dansyl haptens in DMPC/cholesterol (1:1) liposomes were apparently smaller than those in DMPC liposomes. Especially, a quenching rate constant for DNS-C_{12}-PE in DMPC/cholesterol liposomes was much smaller than those for DNS-C_0-PE and DNS-C_6-PE in DMPC/cholesterol liposomes. These results suggested that the dansyl residues of DNS-C_{12}-PE in DMPC/cholesterol liposomes became buried into the interior of the bilayer membranes The results were well consistent with the above-mentioned results that the fluorescence spectrum of DNS-C_{12}-PE shifted to the shorter wavelength and increased in intensity in DMPC/cholesterol liposomes (see Fig. 2).

To evaluate quantitatively the accessibility of the dansyl haptens in the bilayer membranes, we calculated percentages of the accessibilities of the dansyl haptens using Eqn. 3. As for $k_q(0)$, we used a quenching rate constant for dansylaminocaproylic acid (or dansylcadaverine) in solution. Lifetimes of dansylaminocaproic acid and dansylcadaverine were following; 3.42 ns in 0.5 M KCl and 3.06 ns in 0.5 M KI for dansylaminocaproic acid, and 3.15 ns in 0.5 M KCl and 2.84 ns in 0.5 M KI for dansylcadaverine. Then, we calculated percentages of the accessibilities of the dansyl haptens on the bilayer membranes using eqns. 2

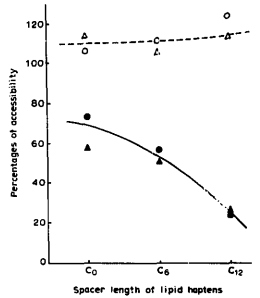

Fig. 4. The percentages of the accessibilities of dansyl lipid haptens with different length spacers on the bilayer membranes. Open circles for the DMPC membranes, closed circles for the DMPC/cholesterol membranes, open triangles for the DPPC membranes and closed triangles for the DPPC/cholesterol membranes.

and 3. The results are shown in Fig. 4. Fig. 4 shows that the dansyl haptens with different length spacers located on the similar positions of DMPC (or DPPC) membranes and that iodide ions were fully accessible to the dansyl haptens. However, in DMPC/cholesterol (or DPPC/cholesterol) membranes, a part of the dansyl haptens became buried into the interior of the bilayer membranes depending on the lengths of the spacer. Especially, the dansyl haptens with longer spacers became buried deeper into the bilayer membranes.

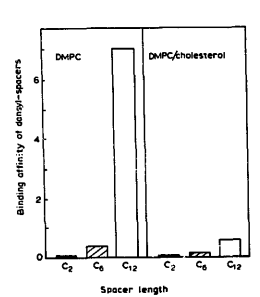

Fig. 5. Binding affinity of dansyl-spacers to the bilayer membranes. (A) Binding to the DMPC liposomes. (B) Binding to the DMPC/cholesterol liposomes.

Binding affinity of dansyl-spacers to the membrane surfaces

Lastly, we measured the binding affinity of model compounds of dansyl-spacers (without phospholipids) to the bilayer membranes. Dansylaminoglycine (DNS-C_2), dansylaminocaproylic acid (DNS-C_6), or dansylaminolaurylic acid (DNS-C_{12}) was incubated with the preformed DMPC (or DMPC/cholesterol) multilamellar liposomes at 30 °C for 5 min. After that, we measured the amounts of DNS-spacers bound to the liposomes. DNS-C_{12} bound much greater to the DMPC membranes than DNS-C_6 and DNS-C_2 did. In addition, all of the dansyl-spacers less bound to the DMPC/cholesterol membranes than to the membranes with DMPC alone as shown in Fig. 5.

Discussion

By the fluorescence quenching method it was shown that the dansyl lipid haptens in liposomes without cholesterol located at the surface of the bilayer. Their locations were independent of the lengths of the spacer. This result was well consistent with our previous findings of trinitrophenyl (TNP) lipid haptens in DMPC (or DPPC) liposomes, where we determined the location of TNP haptens by the fluorescence energy transfer method.

In liposomes with cholesterol, however, the accessibilities of the dansyl haptens decreased, especially for DNS-C_{12}-PE. Most dansyl haptens of DNS-C_{12}-PE in DMPC/cholesterol (or DPPC/cholesterol) liposomes seemed to be located inside the bilayers. For DNS-C_6-PE and DNS-C_6-PE in DMPC/cholesterol liposomes, it seemed that a part of hapten residues became buried into the interior of the bilayers. If so, why antibody molecules bound more preferably to the haptens in DMPC/cholesterol (or DPPC/cholesterol) liposomes than those in DMPC (or DPPC) liposomes?

The above results are most simply accounted for if, in the absence of antibody, there exists an equilibrium between almost two states for hapten residues, H_m and H_s in the DMPC (DPPC) membranes [4,5,7].

$$H_m \rightleftharpoons H_s \qquad (4)$$

Here, H_m represents a physical state of the dansyl group which interacts with the surfaces of the bilayers and unavailable for antibody binding. H_s represents a physical state of the dansyl group which is exposed in aqueous solution and available for antibody binding. In addition, most dansyl groups which are covalently bonded phosphatidylethanolamine are considered to exist in the state of H_m [7]. Schematic representation of the dansyl groups in the states of the H_m and H_2 is shown in Fig. 6. In the H_m state, the hydrophobic interaction between a longer spacer and the lipid bilay-

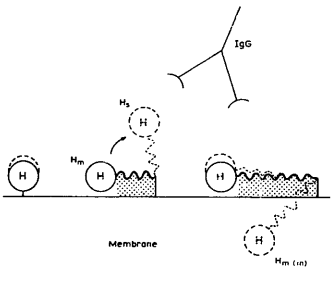

Fig. 6. Schematic representation of the dansyl haptens with (a) a shorter, (b) an intermediate and (c) a longer spacer in the states of H_m, H_s and $H_{m(in)}$ of the Eqn. 5. Dotted lines show schematically the H_s and $H_{m(in)}$ states.

ers were much stronger than that of a shorter spacer or that of an intermediate spacer. Thus, the dansyl groups in DNS-C_{12}-PE are more difficult to transfer from the H_m state (bound to the membranes) to the H_s state (free in solution) than dansyl groups in DNS-C_6-PE. That is, the dansyl groups with longer spacers are less efficient for antibody binding, although the dansyl haptens with different length spacers located at the similar position on the membrane surfaces.

In DMPC/cholesterol (or DPPC/cholesterol) liposomes, we must consider another state for the dansyl haptens in the membranes. We thought that there exists an equilibrium between three states for hapten residues, H_m, H_s and $H_{m(in)}$ as follows,

$$H_{m(in)} \rightleftharpoons H_m \rightleftharpoons H_s \qquad (5)$$

Here H_m and H_s represent the physical states of the dansyl groups as described in Eqn. 4. In the state of $H_{m(in)}$ the dansyl groups are buried inside the bilayers and they are no longer available for antibody binding. We supposed that the existence of the $H_{m(in)}$ state in the membranes with cholesterol is due to the decreased hydrophobicity on the membrane surfaces. This model is partly supported by the findings that the integration of cholesterol into phospholipid bilayers causes a marginal increase in the freedom of motion of the lipid headgroup terminal moiety and subsequently increases in hydration of the bilayer [18]. Thus, in the membranes with cholesterol, the hydrophobic interaction between the hapten-spacers and the bilayer surfaces becomes much weaker, and most of the dansyl haptens are no longer stay on the bilayer surfaces. Then, a part of the dansyl haptens tends to remove from the bilayer surfaces into aqueous solution (H_s state) and another part tends to move inside the bilayers, keeping sufficient amount of the hydrophobic interaction between the hapten-spacers and the interior of bilayers ($H_{m(in)}$ state). And the dansyl haptens with the longer spacer (DNS-C_{12}-PE) become buried more deeply into the bilayers. This explanation was consistent with the experimental results for antibody binding to the dansyl haptens in the liposomes with an without cholesterol.

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