Anionic Hapten-Antibody Reactions on Various Cationic Bilayer Membrane Systems

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ABSTRACT: Antigen-antibody reactions using N^{α},N^{ϵ} -bis(2,4-dinitrophenyl) L-lysine as an anionic hapten were examined in the presence of various cationic bilayer membranes. The turbidity change of the mixture provided information on the dispersion state of bilayer membranes. The degree of the turbidity change was closely related to the concentration of the haptens and the antibodies. In addition, the agglutination was remarkably dependent on the chemical structure of matrix amphiphiles, and there were two variants of the agglutination mode when the antigen-antibody reaction was examined at 35°C: one showed turbidity decrease with the hapten-antibody reaction and the other one did increase. According to DSC studies, the difference is attributable to different physical states resulting from the phase transition of matrix membranes.

KEY WORDS Amphiphile / Bilayer Membrane / Agglutination / Hapten / Immunoassay / Phase Transition /

There has been a great interest in the development of a simple, sensitive and rapid immunoassay using liposomes of phospholipids as carrier particles. This technique has two variants. The lysis technique depends on release of an indicator (enzyme, fluorescent dye, etc.) entrapped in vesicles by antigen-antibody reaction on the surface of membranes.¹⁻⁴ The passive agglutination technique uses liposomal vesicles in place of conventional latex.^{5,6} In our previous report, we described application of totally synthetic bilayer membranes using dihexadecylammonium bromide to immunoassay⁶ and this system showed possibility of applicability to high sensitive and rapid immunoassay in the antigen-antibody reaction for anti DNP-antibodies.⁶ This system has many advantages compared with liposomal vesicles, because we can select physically and chemically stable bilayer membrane systems

which possess morphologic and physicochemical properties suitable to our purpose. In addition, cationic property of carrier membranes can be utilized as a driving force to concentrate anionic samples on the membranes, although it may cause non-specific agglutination.

In this study, various cationic bilayer membranes were investigated as carrier particles to immunoassay (the chemical structures of amphiphiles used in this study are given below). It was found that the sensitivity of the immune response was remarkably dependent on the chemical structure of membrane amphiphiles. We also discuss the agglutination mechanism of matrix membranes that is associated with anionic hapten-antibody reaction.

EXPERIMENTAL

Materials

Dihexadecyldimethylammonium bromide (2C₁₆N⁺2C₁) was prepared by stepwise alkylation.⁷

Di(tetradecyl)phosphate (2C₁₄PO₄H), 2C₁₂-GluPhe-C₁₀N⁺, 2C₁₂-Glu-N⁺, 3C₁₂-tris-C₁₀N⁺, C₁₂-Azo-C₅N⁺ and 2C₁₄-gly-PC were obtained from Sogo Pharmaceutical Co., Ltd. *N*^α,*N*^ε-Bis(2,4-dinitrophenyl)-L-lysine (DNPL) and *N*-(2,4-dinitrophenyl)-glycine (DNPG) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Rabbit anti-DNP antibodies and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from Miles Ltd. (Kankakee, IL, U.S.A.) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

Assay Procedure

A typical procedure is as follows: the amphiphiles were dispersed in 10 ml of distilled water by sonicating (200W, 2 min), using an ultrasonic generator with a 4280S type vibrator (Kaijo Denki Co., Ltd.). The clear solution

(1.0 ml) was diluted to 100 ml with HEPES buffer (pH 7.2, 0.02 M). This membrane solution (0.60 ml) and phosphate buffer (0.10 M, pH 7.2, 6.0 μl) containing rabbit anti DNP-antibodies were mixed in a black quartz cell with a 10 mm path length and 2 mm path width, and shaken. After being allowed to stand at 35°C for 10 min, a hapten solution (7.0 μl) containing 140 ng of DNPL was added to the membrane solution. The turbidity change due to aggregation of bilayer membranes was spectrophotometrically followed using a JASCO UVIDEC 660. Sensitivity was indicated as ΔD_{10} in the following equation.

$$\Delta D_{10} = D_{10} - D_{10,\text{spont}} \quad (1)$$

where D_{10} and $D_{10,\text{spont}}$ are the absorbance at 500 nm after 10 min in the presence and absence of antibodies, respectively.

Other Measurements

DSC (differential scanning calorimetry) thermograms of carrier membranes in HEPES buffer solutions were obtained using a heating rate of 2.0 °C min⁻¹ with Seiko I & E SSC-580/DSC-10 instruments.

The aggregation morphology was observed with a JEOL-200FX electron microscope. The sample solutions were prepared in water or HEPES buffer solutions without any other staining agent and a few drop of these solutions were applied to carbon-coated copper grids. After being dried in air at 35°C, the copper grids were contacted to aqueous solutions containing 2 wt% of uranyl acetate or ammonium molybdate for a few minute, and then dried in air for 30 min at 35°C.

RESULTS AND DISCUSSION

Courses of Turbidity Change

Aqueous bilayer membranes showed specific turbidity depending on the amphiphiles. The turbidity estimated by the absorbance increased with lowering the wavelength, because the turbidity was closely related to light-scattering with bilayer aggregates. It was observed that the turbidity in cationic membrane systems changed by addition of anionic haptens, DNPL. The maximum turbidity was observed when cationic $2C_{16}N^+2C_1$ and anionic DNPL were mixed in equimolar concentration. Therefore, the increase of turbidity in the cationic membrane systems is caused by electrostatic interaction between the membranes and the haptens.

The turbidity change accompanied with hapten-antibody reaction was spectrophotometrically followed using the absorbance at 500 nm. This wavelength was selected to reduce the contribution of the absorption due to chromophoric groups of DNPL and amphiphiles. Figure 1 shows the time courses of turbidity change in two kinds of cationic membrane systems. It is clear that there are two variants in the mode of the turbidity change: Figure 1a shows that the turbidity of $2C_{16}N^+2C_1$ solutions increased with addition of anionic hapten, DNPL, and were suppressed in the presence of anti DNP-antibodies and DNPL. Figure 1b shows that the turbidity of $3C_{12}$ -tris- $C_{10}N^+$ solutions changed slightly

Figure 1. Time courses of turbidity in cationic bilayer membrane systems in the presence of haptens and antibodies. There are two variants, which are classified as *suppression* and *acceleration* types, in the mode of the turbidity change. Conditions: amphiphiles, 0.1 mM; temp, 35°C; anti DNP-antibody, $0.14 \mu\text{g ml}^{-1}$; DNPL, $34 \mu\text{g ml}^{-1}$; 0.02 mol ml^{-1} HEPES buffer (pH 7.2). a, $2C_{16}N^+2C_1$; b, $3C_{12}$ -tris- $C_{10}N^+$.

with addition of DNPL, but increased in the presence of anti DNP-antibodies and DNPL. These two variants are classified as *suppression* and *acceleration* types in this article.

Figure 2 shows the concentration dependency ΔD_{10} as a sensitivity measure was of immune reactivity. The value of ΔD_{10} which is estimated by eq 1 stands for the degree of turbidity change after 10 min. According to Figure 2, the sensitivity is remarkably dependent on the amphiphile. $2C_{12}$ -Glu- N^+ as well as $2C_{16}N^+2C_1$ belonged to the *suppression* type, whereas $2C_{12}$ -GluPhe- $C_{10}N^+$ and C_{12} -Azo- C_5N^+ showed *acceleration* type behavior similar to that of $3C_{12}$ -tris- $C_{10}N^+$ (in the case of the measurement at 35°C).

Effect of Ionic Head-Groups of Matrix Amphiphiles

It is known that $2C_{14}PO_4H$ and $2C_{14}$ -gly-

Figure 2. Anti-DNP antibody detection using various bilayer membranes. The sensitivity (ΔD_{10}) is remarkably dependent on the amphiphile. $2C_{12}$ -Glu- N^+ and $2C_{16}N^+2C_1$ belong to the *suppression* type, whereas $3C_{12}$ -tris- $C_{10}N^+$, $2C_{12}$ -GluPhe- $C_{10}N^+$ and C_{12} -Azo- C_5N^+ show the *acceleration* type. ○, $2C_{16}N^+2C_1$; ●, $2C_{12}$ Glu- N^+ ; □, $2C_{12}$ Ph $C_{10}N^+$; ■, C_{12} Azo C_5N^+ ; △, $3C_{12}$ -tris- $C_{10}N^+$; ▲, $2C_{14}$ -gly-PC; ⊙, $2C_{14}PO_4H$. Conditions: amphiphiles, 0.1 mM; temp, 35°C; DNPL, 34 $\mu g\ ml^{-1}$; 0.02 M HEPES buffer (pH 7.2).

PC can form anionic and zwitter ionic bilayer membranes in aqueous solutions.⁸ No turbidity change due to hapten-antibody reaction was detected in the presence of either an anionic $2C_{14}PO_4H$ and a zwitterionic $2C_{14}$ -gly-PC at temperature range of 30–70°C (Figure 2). The UV spectra showed no significant spectral change of DNPL (355 nm) in the presence of $2C_{14}PO_4H$ and $2C_{14}$ -gly-PC lipid bilayers, although slight λ_{max} shift to 353 nm with decrease of the absorbance at 370–420 nm was observed in cationic $2C_{16}N^+2C_1$ membrane systems. This indicates that DNPL does not interact with $2C_{14}PO_4H$ and $2C_{14}$ -gly-PC, and that cationic property of carrier membranes is essential for the turbidity change.

Aggregation Morphology of Carrier Particles

It is known that the amphiphiles used in this study can form bilayer membrane aggregates and the morphology is remarkably dependent on the chemical structure of amphiphiles.⁹ The aggregation form in a HEPES solution was observed by electron microscopy with a negative staining method and the morphology was classified according to previous articles by Kunitake.⁹ The staining procedure was carried out after drying the sample solution in order to reduce perturbation of the staining agent. The vesicle form was recognized as a globular form. As summarized in Table I, the aggregation forms determined in this study agreed almost completely with the morphology reported previously.⁹ According to Table I, it seems that the agglutination mode in the hapten-antibody reaction is independent of the morphology of cationic carrier membranes.

Phase Transition of Carrier Membranes

Table I summarizes the value of T_C for other carrier membranes. All the values were determined in the same buffer solution as used for the measurement of immune reactivity. As is evident from Table I, the aqueous aggregates from $2C_{12}$ -Glu- N^+ and $2C_{16}N^+2C_1$ (*suppression* types) have lower T_C than the measurement temperature (35°C): both aggregates are in liquid crystalline states at 35°C. On the contrary, the other cationic membranes (*acceleration* types) have higher T_C : the aggregates are in gel states. This indicates that the mode of agglutination is closely related to the physical states of carrier membranes. As supported this estimation, we reported briefly that sensitivity of immune reactivity was influenced by temperature, and the $2C_{16}N^+2C_1$ system provided an inflection point near 28°C in the plots of temperature vs. ΔD_{10} (Figure 3). This inflection point agrees with the endothermic peak (peak top, T_C) of the aqueous aggregate determined by DSC measurement. Figure 3 shows that the mode of agglutination changes from “suppression” to “acceleration” when

Table I. Agglutination mode of various bilayer membranes

Membranes ^a	Charge of lipid	Agglutination mode ^b at 35°C	T_c^c	Morphology	
			°C	Buffer ^d	Water
2C ₁₂ -Glu-N ⁺	Cationic	<i>Suppression</i>	27	Globules	Vesicles ^f
2C ₁₆ N ⁺ 2C ₁	Cationic	<i>Suppression</i>	28	Globules	Vesicles ^g
(2C ₁₆ N ⁺ 2C ₁) ^e		(<i>Acceleration</i>) ^e			
3C ₁₂ -tris-C ₁₀ N ⁺	Cationic	<i>Acceleration</i>	39	Globules	Tubules ^h
2C ₁₂ GluPhe-C ₁₀ N ⁺	Cationic	<i>Acceleration</i>	46	Tubules	Rods ⁱ
C ₁₂ -Azo-C ₅ N ⁺	Cationic	<i>Acceleration</i>	76	Disks	Globules ^j
2C ₁₄ -gly-PC	Zwitterionic	No response	37	Globules	—
2C ₁₄ PO ₄ H	Anionic	No response	62	Globules	Vesicles ^k

^a Membrane, 0.1 mM.^b DNPL, 34 $\mu\text{g ml}^{-1}$; anti DNP-antibody, 0.14 $\mu\text{g ml}^{-1}$.^c Gel-to-liquid crystalline phase transition temperature.^d 0.02 M HEPES buffer (pH 7.2).^e Measurement temperature, 15°C.^f Ref 12. ^g Ref 13. ^h Ref 14. ⁱ Ref 15. ^j Ref 16. ^k Ref 17. **Figure 3.** Temperature dependence of sensitivity (ΔD_{10}). Conditions: 2C₁₆N⁺2C₁, 0.1 mM; DNPL, 34 $\mu\text{g ml}^{-1}$; anti DNP-antibody, 140 ng ml^{-1} ; 0.02 M HEPES buffer (pH 7.2).

the measurement temperature alters from 40°C to 15°C.

Mechanisms of Immune Reactivity

A turbidity change was observed only in cationic membrane systems. This indicates that an ionic interaction is an important factor

Figure 4. Electron micrographs of 2C₁₆N⁺2C₁ alone (a) and 2C₁₆N⁺2C₁ containing DNPL (b). Addition of DNPL to the solution induces membrane fusions to cause the formation of larger aggregates. Conditions: 2C₁₆N⁺2C₁ and DNPL, 0.1 mM; temp, 35°C; 0.02 M HEPES buffer (pH 7.2); scale bars, 1000 Å.

for immune reactivity. According to our previous study,⁶ a light scattering measurement indicated that increase of turbidity in the cationic membranes from 2C₁₆N⁺2C₁ with anionic hapten, DNPL included morphological transitions of matrix membranes to larger aggregates. In the present study, the transition was detected by electron microscopy. Figure 4a showed that 2C₁₆N⁺2C₁ was dispersed in a HEPES buffer solution as globular aggregates (vesicles) with diameter

Figure 5. DSC thermograms of membrane solutions. The transitions (Figure 5a) indicate that the addition of DNPL and anti DNP-antibodies induce the change of the ordered structures of the $2C_{16}N^+2C_1$ membranes. On the contrary, DNPL by itself provides only small perturbation to the ordered structures of the $3C_{12}$ -tris- $C_{10}N^+$ membranes (Figure 5b). Conditions: amphiphiles, 0.01 M; molar ratio, amphiphiles: DNPL = 1 : 1; heating rate, $2.0^\circ\text{C min}^{-1}$; 0.02 M HEPES buffer (pH 7.2).

150–600 Å. Furthermore, addition of DNPL to this solution induced membrane fusions to cause the formation of larger aggregates as shown in Figure 4b. No similar fusion was observed in either $3C_{12}$ -tris- $C_{10}N^+$ (*acceleration* type) membrane systems or in $2C_{14}PO_4H$ and $2C_{14}$ -gly-PC membrane systems. In order to understand this difference, calorimetric behaviors of matrix membranes were examined in the systems containing DNPL and anti DNP-antibodies. Figure 5a shows DSC thermograms in the $2C_{16}N^+2C_1$ systems. The aqueous solution containing $2C_{16}N^+2C_1$ alone had a single endothermic transition (T_C , 28°C). This transition due to a gel-to-liquid crystalline transition. When DNPL was added to the $2C_{16}N^+2C_1$ solution, the peak-top temperature shifted to 43°C . The subsequent addition of anti DNP-antibodies to this solution lowered the peak-top temperature from 43 to 30°C . These transitions indicate that the addition of DNPL and anti DNP-antibodies induce the change of the ordered structures of $2C_{16}N^+2C_1$ membranes. On the other hand, without addition of DNPL the $3C_{12}$ -tris-

$C_{10}N^+$ membranes showed a higher phase transition temperature (T_C , 39°C) (Figure 5b). Interestingly, addition of DNPL to the solution induced only a slight shift of T_C from 39 to 38°C . This indicates that DNPL by itself provides only small perturbation to the ordered structures of $3C_{12}$ -tris- $C_{10}N^+$.

On the basis of these results, a course of turbidity changes in the *suppression* type membrane systems is proposed as shown in Figure 6a: $2C_{16}N^+2C_1$ membranes as a typical example lie in liquid crystalline states at 35°C . DNPL anionic haptens act as counter ions and the electrostatic binding on the $2C_{16}N^+2C_1$ membranes induces morphological transition including the transition of physical states from liquid crystal to gel. On the other hand, anti DNP-antibodies suppress the electrostatic interaction of DNPL to the matrix membranes because of preference of hapten-antibodies reactions. In this case, hapten-anti DNP-antibody reaction does not promote the agglutination of $2C_{16}N^+2C_1$ membranes at 35°C . This indicates that it is too fluid to induce crosslinking of carrier

Figure 6. Schematic representation of agglutination at 35°C. 2C₁₆N⁺2C₁ membranes as a typical example lie in liquid crystalline states at 35°C. DNPL anionic haptens act as counter ions and the electrostatic binding on the 2C₁₆N⁺2C₁ membranes induces morphological transition including the transition of physical states from liquid crystal to gel. On the other hand, anti DNP-antibodies suppress the electrostatic interaction of DNPL to the matrix membranes because of preference of hapten-antibodies reactions. 3C₁₂-tris-C₁₀N⁺ membranes as a typical example lie in gel states at 35°C. Addition of DNPL induces no significant changes on the morphology and physical state of the membranes. Increase of turbidity change occurs both in the presence of DNPL and anti-DNP antibodies.

membranes because 2C₁₆N⁺2C₁ membranes lie in the liquid crystal state.

A second course is proposed for the *acceleration* type (Figure 6b). 3C₁₂-tris-C₁₀N⁺ membranes as a typical example lie in gel states at 35°C. Addition of DNPL induces no significant changes on the morphology and physical state of the membranes. The difference between this and the 2C₁₆N⁺2C₁ membrane system is attributable to the difference in membrane stability based on the physical state. Increase of turbidity change, that is formation of larger aggregates, occurs both in the presence of DNPL and anti DNP-antibodies. This agglutination mechanism is very similar to that of agglutination using conventional polystyrene latex beads.¹⁰ Presumably, DNPL are concentrated on the

Figure 7. Comparison of turbidity change between DNPL and DNPG in 3C₁₂-tris-C₁₀N⁺ membrane systems. Conditions: 3C₁₂-tris-C₁₀N⁺, 0.1 mM; temp, 35°C; haptens (○, DNPL; ●, DNPG), 7.1 × 10⁻⁵ M; HEPES buffer (pH 7.2).

membranes by the electrostatic force and the anti DNP-antibodies are combined to the membranes through the hapten-anti DNP-antibody reaction. In addition, the hapten-antibody reaction promotes crosslinking of carrier membranes. As supported this assumption, no increase of turbidity were observed when mono DNP-substituted hapten (*N*-(2,4 dinitrophenyl)-glycine, DNPG) was added in the mixture of $3C_{12}$ -tris- $C_{10}N^+$ and anti DNP-antibodies instead of DNPL (Figure 7). This indicates that the agglutination of the membranes is induced through bifunctional haptens.

CONCLUSIONS

In the present study, various cationic bilayer membranes were examined for detection of immune reactivity. The hapten-antibody reaction is varied as a function of turbidity change accompanying the dispersion states of matrix membranes, and two variants (*suppression* and *acceleration* types) in the agglutination mode was apparently observed. The difference between the two variants is closely related to that of the physical states of the matrix membranes at the measurement temperature. This is consistent with the observation that the $2C_{16}N^+2C_1$ membrane systems provided an inflection point at around 28°C (corresponding to T_C) in the plots of the temperature *vs.* ΔD_{10}^{60} and that the agglutination mode changed from the *suppression* to the *acceleration* type. Similar inflection points have been often observed for biomembrane systems.¹¹ Therefore, investigation of immune reactivity on these synthetic, simple bilayer membrane systems is not only important for development

of new immunoassay, but also for understanding antigen-antibody reactions on biomembranes.

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