◎原 著

Synthesis of Haptenated Phosphatidylethanolamine Derivatives Containing Different Length Spacers

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Abstract : The antigenicity of liposomes sensitized with haptenated phosphatidylethanolamine (PE) and the reactivity of the liposomes with complement depended on the length of the spacer between hapten and PE. To establish the optimal conditions for the assay, haptenated PE's with various length of spacers are required. In the previous method, hapten-spacer molecule was first synthesized to which PE was conjugated. Therefore, even different hapten molecules and different length of spacer molecules were used, every combination of hapten and spacer has to be synthesized. A new procedure for preparing hapten-spacer-PE was described here. We first prepared conjugates between PE and various length of spacer molecule, the terminal of which is an amino residue. These molecules react well with activated hapten molecules, giving a good yield of hapten-spacer-PE.

Key words : Haptenated phosphatidylethanolamine, Spacer, Liposomes

Introduction

Liposomes, in which hydrophilic or hydrophobic drugs are encapsulated, can possibly be used as drug carriers for cancer therapy[1)]. Liposomes may also be used in a field of immunodiagnosis as a target for antibody dependent complement mediated immune attack[2)]. Previously, we developed an improved immunoassay system by using antigen or antibody-loaded liposomes in which carboxyfluorescein (CF) is enclosed as a marker for leakage. The method was named as "liposome immune lysis assay (LILA)"[3,4)]. The reactivity of an antibody against a hapten incorporated in liposomes, such as TNP group, seems to be affected by the distance between the antigen and the surface of liposomes. It was reported, in fact, that the antigenicity of liposomes sensitized with haptenated phosphatidylethanolamine (PE) and the reactivity of the liposomes with complement through alternative pathway depended on the length of the spacer between hapten and PE[5~7)]. The flexibility of a hapten determinant or its lateral movability on liposomes might also be affected by the spacer length.

To establish the optimal conditions for the assay described above, haptenated PE's with various length of spacers are required.

Different hapten-PE molecules with the same length of spacer are also needed. In the previous experiments, hapten-spacer molecule was first synthesized to which PE was conjugated. Therefore, even different hapten molecules and different length of spacer molecules were used, every combination of hapten and spacer has to be synthesized. Furthermore, synthesis of hapten-spacer molecules is sometime difficult and their yield is very low. This is because conjugation of haptens with some spacers which are only soluble in water must be done using aqueous solvent although activated hapten used for the conjugation is unstable in water. In the experiments shown in this paper, we first prepared conjugates between PE and various length of spacer molecule, the terminal of which is an amino residue. These molecules react well with activated hapten molecules, giving a good yield of hapten-spacer-PE. The schematic diagram of the procedures was shown in Fig.1 (A).

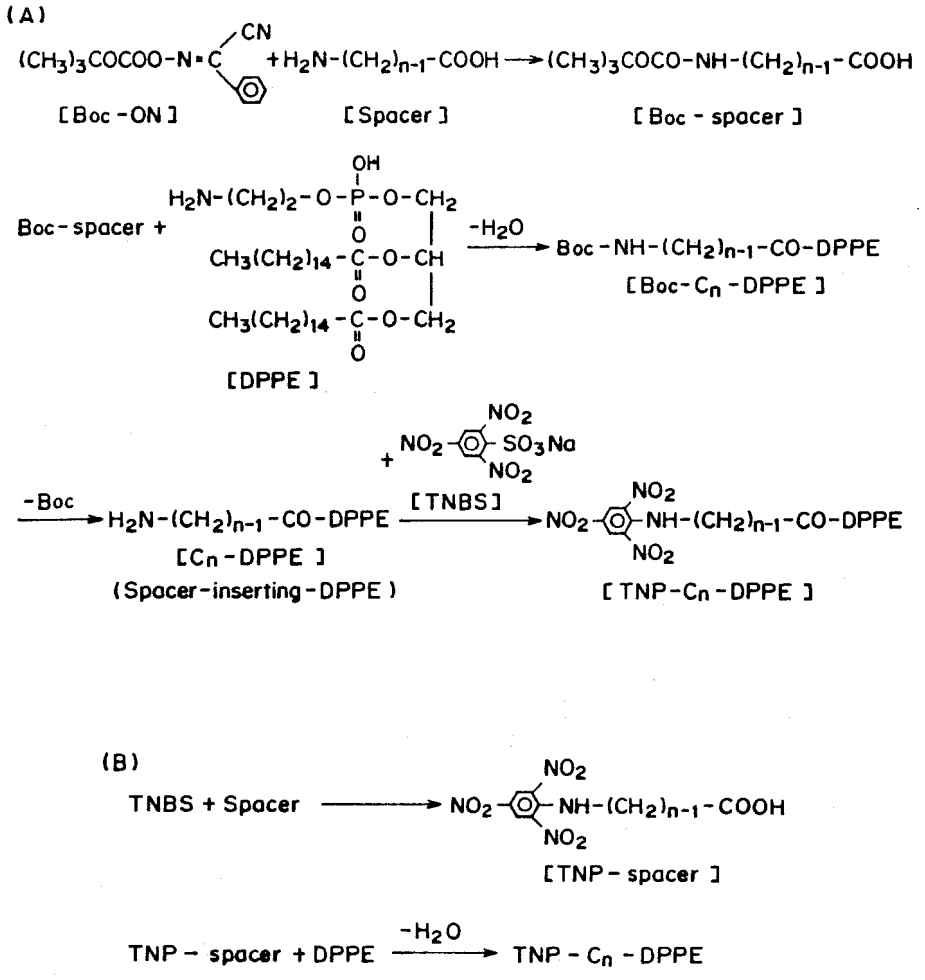

Fig. 1. Schematic diagram of reaction principle. (A) is our method and (B) is conventional one.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), and dipalmitoylphosphatidylethanolamine (DPPE) were from Sigma Chemical Co. (St. Louis, MO). Dicyclohexylcarbodiimide, hydroxysuccineimide and 2-t-butyloxycarbonyl-oxyimino-2-phenylacetonitrile (Boc-ON) were from Peptide Institute Inc. (Minoo, Osaka). Other commercially available reagents were the highest grade available and used without further purification.

DPPE-spacer molecules were synthesized as follows. We describe the procedure by employing 6-aminocaproic acid as a typical example. Essentially same procedures were used for other DPPE-spacer molecules. To protect amino group of spacers, Boc-spacers were synthesized. Two grams of 6-aminocaproic acid (Aldrich Chemical Co. Inc., Milwaukee, WI) were dissolved in 150 mℓ of dioxane/H_2O (1/1, v/v) containing 4 g of $NaHCO_3$ and pH of the mixture was adjusted to 10.5 with 5 N NaOH. This solution and 20 mℓ of dioxane were mixed and stirred at room temperature. The solution was concentrated to about 50 mℓ, and acidified with about 5 g of citric acid. The product was extracted 3 times with ethylacetate. The solvent was removed by evaporation and a pale yellow powder (4.45 g) was obtained.

To conjugate Boc-spacer with PE Boc-aminocaproic acid (100 mg), dicyclohexylcarbodiimide (310 mg), hydroxysuccineimide (359 mg) and triethylamine (150 μl) was sequentially dissolved in 60 mℓ of $CHCl_3$/CH_3OH [C/M] (10/1) containing DPPE (210 mg). The mixture was incubated for 24 h at room temperature with continuous stirring. The solvent was removed by evaporation and viscous pellet was dissolved in ethylacetate (ca. 20 mℓ). Undissolved white substance was removed by filtration, and remaining solvent was evaporated. The product, Boc-spacer-PE was obtained. To remove Boc residue, the product was dissolved in 3 mℓ of 1 N HCl/acetic acid, and incubated for 2 h at room temperature. The mixture was charged on a preparative thin layer chromatography (TLC) plate (#5717, Merck Co., Rahway, NJ) and developed by a solvent system C/M/H_2O = 65/25/4. A phosphate and ninhydrin positive layer was collected and extracted with C/M = 2/1. The yield of the product characterized as shown later was about 30%.

TNP-C_6-DPPE was synthesized by following procedures. Ten μmoles of C_6-DPPE and 12 μmoles of trinitrobenzenesulfonic acid [TNBS] (Na salt; Tokyo Chemical Industry, Tokyo) supplemented with triethylamine (10 μl) were incubated at room temperature for 1 h and spotted on TLC plate. The plate was developed using the same solvent system as described above and yellow layer containing phosphate was obtained.

Results and Discussion

The Rf values of various products were listed on the table below. A TLC plate (#5752, Merck Co.) was used in a solvent mixture of C/M/H_2O [65/25/4]. DPPE; 0.57, Boc-C_6-DPPE; 0.65, Boc-C_{11}-DPPE; 0.68, C_6-DPPE; 0.68, C_{11}-DPPE; 0.71, TNP-DPPE; 0.60, TNP-C_6-DPPE; 0.70, TNP-C_{11}-DPPE; 0.73.

The authors confirmed the product based on both the disappearance of Boc-group and the appearance of amino group on H^+-NMR

(JEOL, model 90Q) spectra. The mass spectroscopy data of C_6-DPPE were as follows : A mass spectrometer (JEOL model JMS-DX 300), fitted with a FAB (fast atom bombardment) gun operated with xenon, was used. A mass spectrum was recorded at a scanning speed of 10 sec over a mass range of 1 to 1,000 at 3 kV accelerating voltage. The expected mass peak at $M/Z = 805$ corresponding to $[M+H]^+$ was detected. The spectrum also exhibited characteristic fragments at $M/Z=157$, 255, 295 and 551, each of which corresponded to [6-aminocaproylethanolamine-H]$^+$, $[C_{15}H_{31}COO]^+$, $[M-2x\ C_{15}H_{31}COO+H]^+$ and [dipalmitoylglycerol-OH]$^+$, as shown in Fig. 2.

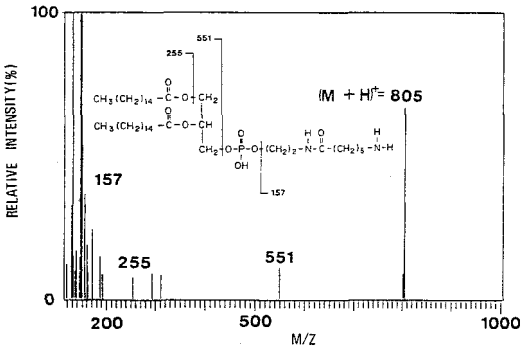

Fig. 2. Mass spectrum of 6-aminocaproyldipalmitoylphosphatidylethanolamine.

We studied the effect of the spacer length in haptenized DPPE on the immunoresponse using liposomes which were contained DPPC, Chol and TNP-, TNP-C_6-, or TNP-C_{11} DPPE [molar ratio ; 1 : 1 : 0.05, respectively] and encapsulated carboxyfluorescein (CF) as a release marker. TNP-DPPE was prepared according to a previous paper[6]. The procedure of the liposome preparation and the assay system were mentioned in the previous paper[3]. Diluted SPF guinea pig serum (1.4 CH_{50}) was used as a complement source. Fig. 3 shows the titration curve of rabbit anti-TNP-BSA serum against liposomes containing TNP-DPPE, TNP-C_6-DPPE, and TNP-C_{11}-DPPE. The TNP-C_6-DPPE-liposomes provided a larger response to antiserum than those containing TNP-DPPE. This would be because of preventing from steric hindrance of liposomal surface in TNP-C_6-DPPE-liposomes. The response of the TNP-C_6-DPPE-liposomes prepared here was the same as that of the liposomes containing TNP-C_6-DPPE made by another method[6]. The TNP-C_{11}-DPPE-liposomes, however, showed a different response from TNP-DPPE- or TNP-C_6-DPPE liposomes. An optimal spacer length might exist for an effective reaction of haptenated liposomes to antibody.

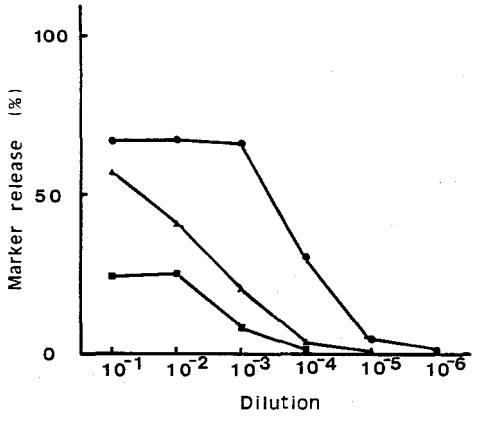

Fig. 3. Dose responses of liposomes containing three kinds of TNP-haptenized-DPPEs against rabbit anti-TNP-BSA antiserum.
The preparation of liposomes and the analytical procedure are referred to the text. (●) ; TNP-C_6-DPPE, (▲) ; TNP-C_{11}-DPPE, (■) ; TNP-DPPE.

The authors prepared DPPE introduced various kinds of hydrocarbon spacer (C_3, C_4, C_5, C_8 and C_{12}) by a similar technique mentioned above. Other haptenated DEEP having a spacer (C_6) could be obtained from a coupling reaction between C_6-DPPE and dansyl chloride or fluorescein isothiocyanate. Spacer-introduced-DPPE can supply an easy preparation of lipids introduced by various haptens to study the effects of spacer on the interaction of haptens and antibodies, and so on. We will expand these kinds of modified DPPE to the protein antigen measuring system in LILA, such as α-fetoprotein and so forth. We will also apply a series of spacer-interposed-DPPE for investigating the interaction of various haptens on liposomes, antibody and complement.

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Rererences

1. "Liposomes in Biological Systems", (G. Gregoriadis and A. C. Allison, eds.), John Wiley & Sons Ltd., Chichester, UK 1980.
2. Yasuda, T. : Liposomes as immunodiagnostic tools-Liposome immune lysis assay (LILA) method for the determination of glycolipid and protein antigens. In "Medical application of liposomes",(K. Yagi ed.), Japan Sci. Soc. Press, Tokyo and Karger, Basel, 1986.
3. Ishimori, Y., Yasuda, T., Tsumita, Notsuki, M., Koyama, M. and Tadakuma, T. : Liposome immune lysis assay (LILA) : a simple method to measure anti-protein antibody using protein antigenbearing liposomes. J. Immunol. Methods, 75 : 351－360, 1984.
4. Umeda, M., Ishimori, Y., Yoshikawa, K., Takada, M. and Yasuda, T. : Homogeneous determination of C-reactive protein in serum using liposome immune lysis assay (LILA). Jpn. J. Exp. Med., 56 : 35—42, 1986.
5. Dancey, G. F., Iaskson, P. C. and Kinsky, S. C. : Immunogenicity of liposomal model membranes sensitized with dinitrophenylated phosphatidylethanolamine derivatives containing different length spacers. J,Immunol., 122, 638 — 642 1979.
6. Okada, N., Yasuda, T., Tsumita, T. and Okada, H. : Activation of the alternative complement pathway of guinea-pig by liposomes incorporated with trinitrophenylatedphosphatidylethanolamine. Immunology, 45 : 115－124, 1982.
7. Okada, N., Yasuda, T., Tsumita, T. and Okada, H. : Differing reactivity of human and guinea pig complement on haptenized liposomes via the alteynative pathway. Mol. Immunol., 20 : 857－864, 1983.

種々の長さのスペーサーをもつハプテン化ホスファチジルエタノールアミンの新しい合成法

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人工脂質膜であるリポソームにハプテン化ホスファチジルエタノールアミン（PE）を挿入することで，リポソーム膜上での免疫反応の研究が進んでいる。いろいろな因子のなかでリポソーム表面とハプテン基の間のスペーサーの長さも重要な因子であることが判明してきた。このスペーサーの役割を研究するためには汎用性のある合成法の開発が望まれている。これまでのハプテン基 - スペーサー分子を結合する方法は種類の異なるハプテン基をもつ分子群を合成するには煩雑である。そこで種々のスペーサーをもつPEを先に合成することで種類の違うハプテン基をもち，異なるスペーサーをもつハプテン化脂質抗原の合成法を開発した。

キーワード：ハプテン化ホスファチジルエタノールアミン，スペーサー，リポソーム