Communications to the Editor

[Chem. Pharm. Bull. 36(4)1589—1592(1988)]

CHARACTERIZATION OF IMMUNOLIPOSOME MEMBRANE: ESR SPECTROSCOPIC ANALYSIS OF THE EFFECT OF CHOLESTEROL DERIVATIVES IN MEMBRANE

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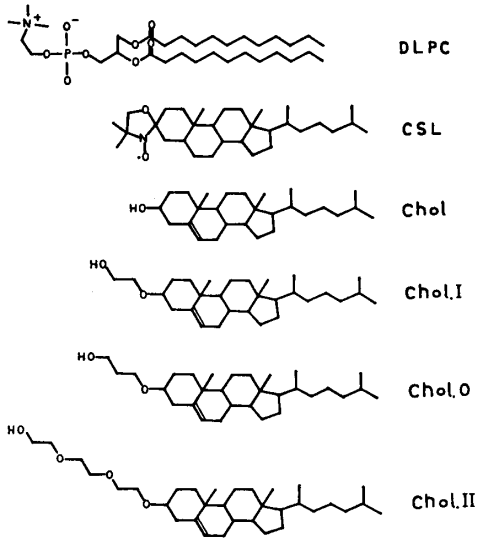
Using spin label, we have studied the effects of cholesterol derivatives with a terminal hydroxyl group at the 3-position of the cholestene nucleus on the membrane structure of dilauroylphosphatidylcholine-cholesterol liposomes. The magnitude of the empirical parameter estimated from ESR spectra progressively increased with the increasing amount of cholesterol derivatives added. However, a break appeared in the slope of the parameter versus the mole ratio of the cholesterol derivatives. This indicates that the added cholesterol derivative provides a new phase which can be related to the change in the immunolysis of liposomes.

KEYWORDS ——— dilauroylphosphatidylcholine; cholesterol derivative; immunoliposome; spin label; phase separation

We reported previously a highly sensitive and reproducible immunoliposome assay of theophylline using large unilamellar liposomes (reverse-phase evaporation vesicles).[1] The physico-chemical properties of the liposomes are significant in the sensitivity of the immunoliposome assay. In order to improve the potency of the antigen-antibody binding on the liposome membrane and to optimize the immunoliposome assay, we added cholesterol derivatives having terminal hydroxy groups at the 3-position with the result that the addition of triethoxycholesterol enhanced the immunolysis of sensitized liposomes.[2] In the assay system, calcein was used as a marker of immune lysis and the amount of marker released was dependent on the number of antigen-antibody complex formed on the liposomal membrane. It has been noted that membrane fluidity controls not only the activity of some membrane-bound enzymes but also complement mediated immune attack.[3-5] It is clear that cholesterol plays a major role in this control. To analyze the membrane property, we examined the behavior of a spin label probe affected by incorporation of cholesterol derivatives.

Dilauroylphosphatidylcholine (DLPC, C12:0) and cholesterol were used as membrane materials in liposome preparation. Cholesterol derivatives having side chains of 1-hydroxy-propyl, 1-hydroxy-ethyl, and 1-hydroxy-3,6-dioxa-octyl at the 3-position of the cholestene nucleus, named Chol.0, Chol.I, and Chol.II, respectively, were prepared by the method of Patel et al.[6] These molecules and the others used in this study are shown in Fig. 1, illustrating their relative length in the bi-



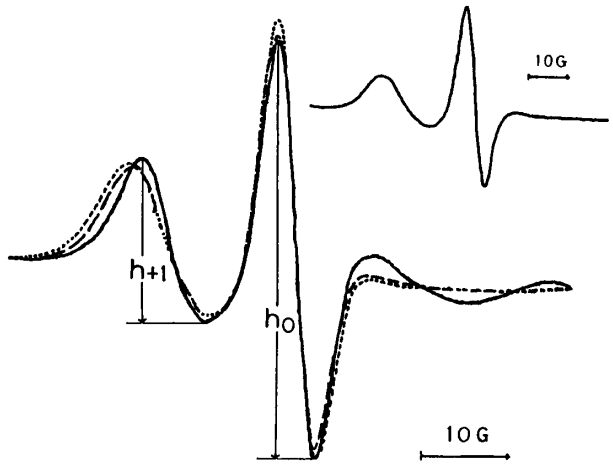


Fig. 1. (left) Schematic Representation of DLPC and Cholesterol Derivatives Used in This Study
DLPC, dilauroylphosphatidylcholine; CSL, cholestane spin-label (3-doxyl-5-cholestane); Chol, cholesterol; Chol.0 Chol.II, cholesterol derivatives.

Fig. 2. (right) ESR Spectra of DLPC-Cholesterol Liposomes Containing Various Amounts of Chol.II
Inset: distearoylphosphatidylcholine-cholesterol (1:1 mole ratio) liposomes
———, DLPC-cholesterol (1:1); – – – , DLPC-cholesterol-Chol.II (1:1:1);
·····, DLPC-cholesterol-Chol.II (1:1:2).
3-Doxyl-5-cholestane of 2 mol% related to DLPC were used for ESR measurement.

layer.[7] Sensitized liposomes, containing a small amount of 8-(3-carboxypropyl)-theophylline-phosphatidylethanolamine conjugate as a sensitizer, were prepared by the same method described previously in buffer free from calcein. The ESR spectra were measured at room temperature on a JES-FE spectrometer (JEOL Ltd., Japan) by using 3-doxyl-5 -cholestane as a spin label.

Since 3-doxyl-5-cholestane is a cholesterol-like molecule, this probe is expected to provide information about cholesterol-rich regions in the bilayer. ESR spectra of the probe incorporated into DLPC-cholesterol liposomes in the absence and presence of Chol.II are shown in Fig. 2. As the amount of Chol.II increased, the peak height of the low field line, h_{+1}, decreased with increasing peak-to-peak line width. The asymmetry of the peaks at higher field became prominant. As the concentration of Chol.II increased, the spectral patterns become similar to that of distearoylphosphatidylcholine liposomes as shown in Fig. 2 (inset). This is generally explained by the decrease of the molecular motions in the bilayers and condensing or rigidifying effect on the bilayers. The ratio of peak heights, h_0/h_{+1}, can be used as an empirical parameter for the membrane fluidity. An increase of this parameter reflects a decrease in the mobility of the radicals. The variation in the parameter as a function of cholesterol derivatives molar ratio to DLPC-cholesterol mixtures is illustrated in Fig. 3. The amplitude of the parameter progressively increased with increasing concentration of the cholesterol derivatives.

In Fig. 3, there are two remarkable features: (1) The curve obtained from the Chol.II system is different from those from the Chol.0 and Chol.I systems, showing a rather complicating feature. As the amount of Chol.I or Chol.0 increases, the mobil-

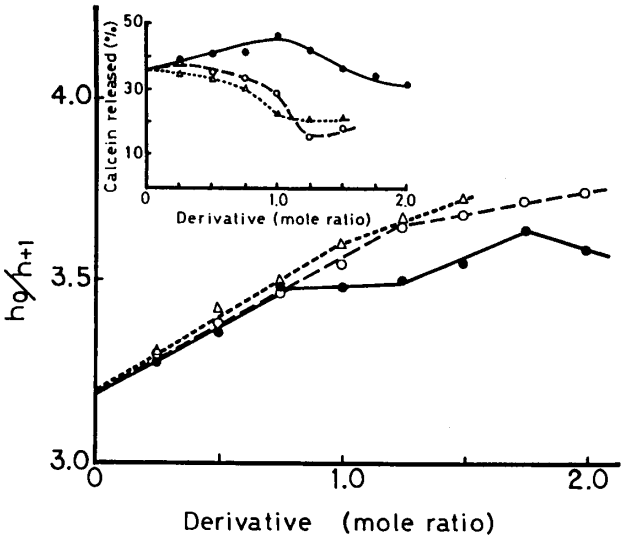


Fig. 3. The Ratio of Peak Height as a Function of the Mole Ratio of Cholesterol Derivatives in DLPC-Cholesterol Liposomes
Inset: effect of cholesterol derivatives on the immunolysis of liposomes
···△···, Chol.I ; –○–, Chol.0 ;
—●—, Chol.II.
Each point was the mean of two or three samples for Chol.0 and Chol.I. For Chol.II it was the mean of three or four samples.

ity of the probe undergoes biphasic change. (2) The degree of the parameter increase is inversely proportional to the increase in the length of the side-chain of derivatives. From these results, it appears that the long head group of Chol.II influences not only the mobility of cholesterol but also the surface character of the liposome membrane. This may account for the fact that only the addition of Chol.II enhances the immunolysis and forms stable liposome.[8] According to Shimshick and McConnell,[9] the change in the slope usually indicates the intersection of another phase. As cholesterol is progressively added to the phospholipids in a phosphatidylcholine-cholesterol system above transition temperature, the system changes from one which is entirely in the fluid phases to a mixed phase composed of fluid and solid phases, and eventually to an all solid phase. At sufficiently high cholesterol concentrations, the lateral compressibility of the membrane would be expected to be enhanced, because the sterols are intercalated between the hydrocarbon chains of the phospholipid molecules, and a phase separation should be obtained. As can be seen from Fig. 3, the ESR parameter generally increase as the amount of derivatives increase. This indicates the homogeneous incorporation of the derivatives to the bilayer of liposomal membrane.[10] However, with the further addition of the derivatives, a phase separation was obtained as observed by a break in the slope of the plots of the parameter versus the derivative molar ratio.

Previously we examined the effect of incorporating cholesterol derivatives in sensitized liposomes on immunolysis.[2] In that study, when only Chol.II was added, an enhancement in the immunolysis was observed, as shown in Fig. 3(inset). This indicates an increase of complement fixation. It is noted that the profile of Chol.II is different from that of Chol.I and Chol.0. This may be related to the difference in their ESR data. Concerning Chol.II, the large hydrophilic groups may play an important role on immune reaction. Among the derivatives, only Chol.II can form stable liposomes in the absence of any phospholipids.[8] Liposome formation is a consequence of the cooperative nature of the hydrophilic side-chain and the lipophilic cholesteryl molecule. As the result, the addition of Chol.II can provide a characteristic property for the membrane surface of the DLPC-cholesterol-Chol.II liposomes, which may be different from that induced by Chol.0 or Chol. I.

The maximum enhancement of the immunolysis was observed at the molar ratio of about 1:1:1 (DLPC:cholesterol:Chol.II) (Fig. 3 (inset)). The ESR data in Fig. 3,

however, showed little change in parameter at a molar ratio around 1:1:1. This discrepancy can be interpreted as follows: The planar sterol ring region of Chol.II is an essential feature in the interaction between sterol and phospholipid. By incorporating Chol.II, the interaction causes the membrane to increase in internal viscosity, which is reflected in the enhancement of the parameter observed in the present study. On the other hand, it appears that the addition of Chol.II was accompanied by changes in the properties of the liposome surface because of the large hydrophilic groups. At lower concentration of Chol.II (less than 0.75 molar ratio), Chol.II molecules are homogeneously incorporated in the DLPC-cholesterol membrane. At this Chol.II concentration, the molecular motion of the spin probe is restricted. At an intermediate concentration of Chol.II (0.75<molar ratio<1.25), the head group of Chol.II affects the chain interaction of lipids. This perturbation should be accompanied by a disturbance of the arrangement of phosphate groups of DLPC. As a result, the enhancement in a complement fixation or hapten exposure is provided, which increases the immunolysis.[11] At this concentration, the condensing effect of the cholesteryl group of Chol.II in phosphatidyl bilayer and the disturbance effect of head groups of Chol.II on phosphorylcholines on the membrane surface coexist at equilibrium, resulting in a constant value of the ESR parameter. At higher concentrations of Chol.II (above 1.25 molar ratio), a phase separation may occur with formation of a portion rich in sterol. At this concentration, the condensing effect of Chol.II is predominant and the disturbance effect is reduced, because the hydrophilic groups of Chol.II are in the minimum condition of free energy of molecules as a result of maximizing both van der Waals interactions and the configurational entropy of the flexible molecules.[12] This leads to a decrease of the immunolysis. On the other hand, although Chol.0 and Chol.I have short head groups, their features are rather close to cholesterol itself. Thus, their incorporation resulted in a simple phase separation as seen in Fig. 3.

We can say that Chol.II has two distinct effects on membrane structure. One is a condensing effect, which increases the organization and rigidity of the cholesterol region. Second is a modulation effect on the phosphorylcholine groups, which can control a membrane surface orientation by its large hydrophilic group. From these considerations, it appears that an addition of appropriate amphiphilic molecules, which can form stable liposomes in the absence of an phospholipids, can enhance a complement fixing activity in liposomes. Our results in the improvement of liposomal membrane is applicable in many fields of artificial membrane research.

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(Received January 25, 1988)