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Effect of the Cholesterol Content on the Stability of Liposomes in the Blood Circulation

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Liposomes composed of egg phosphatidylcholine (PC) and cholesterol (Chol), of which ratios (PC/Chol) were 1/1, 1/0.6, 1/0.2 were prepared, and the stabilities of these liposomes in the blood circulation were studied by the double fluorescent labeling method described in the previous report.[1] As the result, it was proved that the liposome composed of 1/1 ratio of PC/Chol was stable enough, but with decreasing Chol contents of liposoomes, entrapped materials were leaked from liposomes. The result obtained in this study was in accordance with the previous reports[2,3] and double fluorescent labeling method[1] has been found very useful for the study on liposomes.

Keywords : liposome ; phosphatidylcholine ; cholesterol ; stability; double fluorescent labeling method ; carboxyfluorescein ; calcein

We designed a new method using carboxyfluorescein (Fig.1) and calcein (Fig.1) to compare and evaluate the transition of liposomes in the blood circulation, named it "double fluorescent labeling method" and reported.[1]

Using liposomes entrapping drugs for the drug delivery system (DDS), it should be avoided as far as possible that the drugs entrapped leak out of liposomes into the blood circulation.[2-6] As mentioned in the previous report,[1] a double fluorescent labeling method is thought to be useful for the study of liposome stability in the blood circulation. In this report, the stabilities of liposomes composed of various ratios of PC and Chol were studied by the double fluorescent labeling method.

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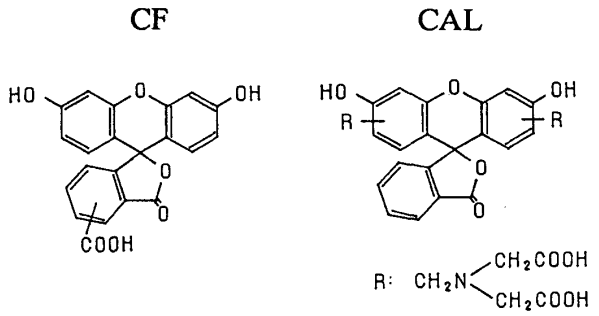

Fig. 1. Chemical Structures of Carboxyfluorescein (CF) and Calcein (CAL)

Materials and Methods

Reagents CF was purchased from Eastman Kodak Inc., and purified by the method of Ralston et al..[7] CAL was purchased from Wako Pure Chemical Industries, Ltd., and was used untreated. PC was extracted from the yolk and purified by the method of Rhodes et al.[8] and Bangham et al..[9] Chol was purchased from Wako Pure Chemical Industries, Ltd., and was used untreated. All the other reagents used were of the

highest grade commercially available.

Preparation of Liposome Containing CF and CAL As described in the previous reports,[1] the isotonic solutions, 0.1 M CF (pH 7.4) and 0.086 M CAL (pH 7.4), were used for liposome preparations. 0.64 ml of 0.1M CF solution and 1.36 ml of 0.086 M CAL solution were mingled so that CF and CAL attatined equal fluorescent intensity. A chloroform solution of PC (30μmol) and Chol (30μmol) was poured into a 50 ml of pear type flask and chloroform was distilled under reduced pressure by using a rotary evaporator to prepare the thin film. Next, 2 ml of the mixture solution of CF and CAL was added to the thin film and vigorously vortexed under nitrogen atmosphere to prepare multilamellar vesicles (MLV) according to the previous method.[1] Furthermore, MLV was sonicated for 40 min at 37 ℃ and CF and CAL which were not enclosed in the liposomes were removed on diethylaminoethyl (DEAE) Sephadex A–25 column and 3.2 ml of liposome eluted was obtained. To remove residual MLV, 3 ml of the elute sample was slowly layered on 2 ml of 10% sucrose phosphate buffer (10% sucrose, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) previously placed in a tube (5CN tube ; Hitachi Koki Co., Ltd.) and centrifuged at 105000 g for 60 min by an centrifuge (Hitachi Koki Co., Ltd., Model 65P) and swing rotor (PRS–50 type). The supernatant obtained was used for injection experiments to mice. By using 16μmol or 6μmol instead of 30μmol of Chol per 30μ mol of PC, other two kinds of liposomes were prepared according to the same procedure separately.

Transition of CF and CAL in Liposome within the Blood Circulation of Mice Liposome suspensions containing CF and CAL, which have equal fluorescent intensity, were injected into the tail vein of female slc:ICR mice between 26.6 g and 31.3 g of body weight by 5 ml/kg weight. At 5, 10, 15, 20. 30, 60, 120, 180, 240, and 300 min after the injection, 10μl of blood was sampled from the tail vein and fluorescent intensities of CF and CAL in it were measured according to the previous method.[1]

Results and Discussion

Kirby et al. cleverly evaluated the liposome stabilities in the blood circulation by using [^{14}C] cholesteryl oleate and CF.[2] They added CF solution to a thin film which was composed of PC, Chol and [^{14}C] cholesteryl oleate, and then sonicated it to prepare CF-containing liposomes. Consequently, the membrane of liposome was labeled by [^{14}C] cholesteryl oleate and the inner water phase was labeled by CF. These double labeled liposomes were intravenously injected into mice and the stability of the liposomes was checked by estimating the change of ^{14}C/CF ratios in the blood. In the case of the liposome composed of 7/2 ratio of PC/Chol, ^{14}C/CF ratio in the blood was increased with the time after injection. The evidence obtained suggests the leakage of CF from liposomes. On the other hand, in the case of liposomes with 1/1 ratio of PC/Chol. ^{14}C/CF ratio in the blood was always constant. These results proved that liposome composed of 1/1 ratio of PC/Chol was stable enough in the blood circulation.

Carrying out a similar examination above would be possible by employing a double fluorescent labeling method, without using tedious radioisotopes. First of all, a mixture solution of CF and CAL, which have equal fluorescent intensity as each other, was

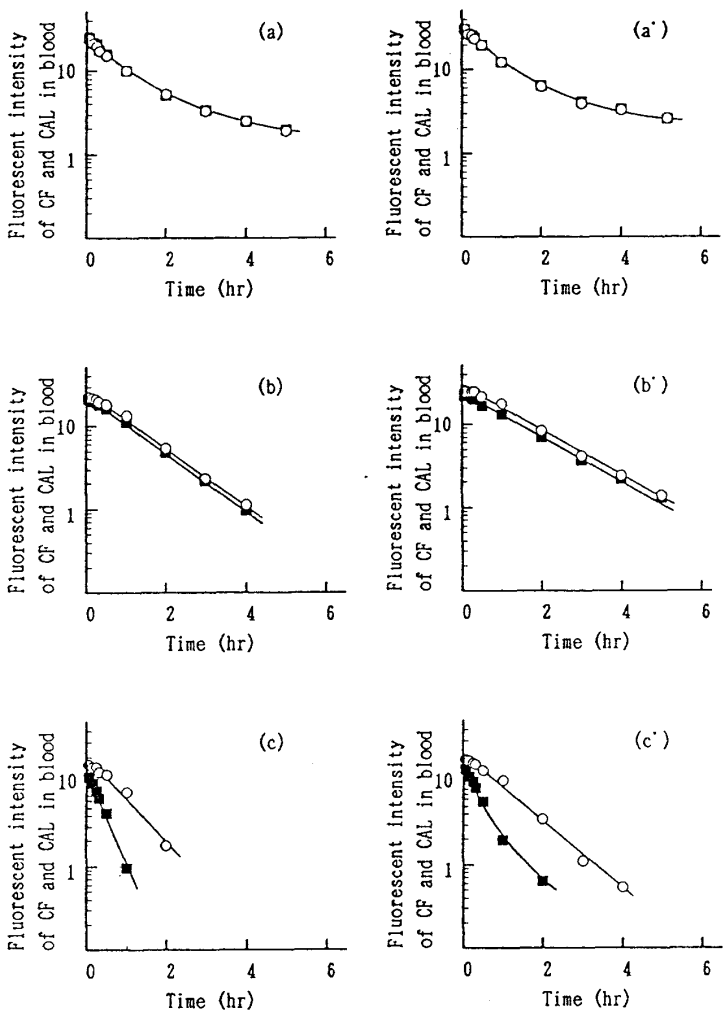

Fig. 2. Effect of Cholesterol Contents on Leakage of CF (■) and CAL (○) from Liposomes on the Circulation of Mice.
The liposomes composed of 1/1 ratio of PC/Chol were administered to mice in (a) and (a') experiments, that of 1/0.6 ratio of PC/Chol to mice in (b) and (b'), that of 1/0.2 ratio of PC/Chol to mice in (c) and (c').

prepared, and second, three kinds of liposomes were constructed by the use of the solution and three kinds of lipids of which the composition of PC/Chol ratios are 1/1, 1/0.6 and 1/0.2. These liposomes were intravenously injected into the individual mice, respectively. The ratio of CF to CAL in the blood have been subsequently determined by a fractional assay. Fig. 2 shows the time course of CF/CAL ratio in the blood after the injection of liposomes. Experiments were carried out twice for each liposome. (a) and (a') in Fig. 2 show that CF and CAL entrapped in the liposome composed of 1/1 ratio of PC/Chol had exactly the same elimination rate. The half-life times of CF and/or CAL in (a) and (a') mouse were 62 min and 75 min, respectively. There was the difference of half-life times between (a) and (a') mouse, but the CF/CAL ratio showed 1 throughout observation in experiments. Evidently, the results agreed with the report of Kirby et al.,[2] In the case of the liposomes with 1/0.6 ratio of PC/Chol (Fig. 1 (b) and (b')), CF/CAL ratio in the blood was decreased slightly at the beginning after injection, but the CF/CAL ratio became constant immediately. The results represent that the liposome are relatively stable in the blood circulation. We considered that the initial decrease of CF is due to the leakage of the CF molecule contaminated in the surface of the liposome membrane, and CAL would not leak because CAL has larger molecular weight and a complex molecular shape in comparison with CF (Fig. 1). The half-life times of (b) and (b') mouse were 55 min and 69 min, respectively. They are relatively shorter than those of (a) and (a') mouse. However, it should be noted that the stabilities of both liposomes in the blood are not able to compare precisely only the difference of half-life times, because both the liposomes may give a different distribution in the mice. In the case of the liposomes with 1/0.2 ratio of PC/Chol (Fig. 2 (c) and (c')), CF/CAL ratio was decreased rapidly after injection. The half-life times of CF and CAL in (c) mouse were 16 min and 39 min and those in (c') mouse were 21 min and 47 min respectively.

Each mouse has a rapid elimination rate of both CF and CAL, and CF/CAL ratio rapidly deceases with time as well. These results suggested that CF as well as CAL is leaked from the liposome composed of 1/0.2 ratio of PC/Chol, and because CF has a smaller molecular weight and relatively simple molecular shape (Fig. 1), CF leaked from the liposome faster than CAL.

In conclusion, it was proved that the liposome with 1/1 ratio of PC/Chol was stable enough in the blood circulation, and the liposome with 1/0.6 ratio of PC/Chol was relatively stable, but in the case of the liposome with 1/0.2 ratio of PC/Chol entrapped materials were obviously leaked from the liposome.

It was suggested that the double fluorescent labeling method[1)] can efficiently evaluate a stability of liposomes in the blood circulation without the troublesome treatment of radioisotopes. And we proved that leakage of the drug entrapped in the liposome depends on the molecular weight of the drug and property of its own.

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