Colloid Polym Sci 274:678–684 (1996)

ORIGINAL CONTRIBUTION

S. Ando
H. Tsuge
T. Mayumi

Effect of cholesterol or phospholipid incorporation on the chemical stability of the muramyldpeptide derivative B30-MDP in mixed vesicles

Received: 21 July 1995
Accepted: 19 December 1995

Dr. S. Ando (✉) · H. Tsuge
Tokyo R & D Center
Daiichi Pharmaceutical Co., Ltd.
16-13, Kita-Kasai 1-Chome, Edogawa-ku
Tokyo 134, Japan

T. Mayumi
Faculty of Pharmaceutical Sciences
Osaka University
1-6, Yamada-oka, Suita
Osaka 565, Japan

Abstract Application of the muramyldipeptide derivative B30-MDP to liposomal vaccines will aid in the development of improved high immunogenicity vaccines. To give full play to the effectiveness of B30-MDP as a liposomal vaccine, it is important to evaluate the effect of cholesterol, dimyristoylphosphatidylcholine (DMPC) or distearoylphosphatidylcholine (DSPC) incorporation on the chemical stability of B30-MDP and physicochemical properties of B30-MDP/lipid mixed vesicles from the view point of pharmaceutics.

The observed degradation rate constants of B30-MDP by hydrolysis in B30-MDP/cholesterol mixed vesicles were increased with increasing concentration of cholesterol, however, those in B30-MDP/DMPC and B30-MDP/DSPC mixed vesicles were unchanged with increasing concentration of DMPC and DSPC. The degradation behavior of B30-MDP was then compared with physicochemical properties of B30-MDP/lipid mixed vesicles, such as membrane fluidity and particle size. It was apparent that the degradation of B30-MDP in B30-MDP/cholesterol mixed vesicles was influenced by the particle size, but not by the fluidity of the membranes. In the case of B30-MDP/phospholipid mixed vesicles, the degradation of B30-MDP was not influenced by either the membranes' fluidity or the particle size of the mixed vesicles.

It is considered that the degradation of B30-MDP in the mixed vesicles is dependent on the membrane state, and the addition of cholesterol to B30-MDP vesicle inhibits the mutual interaction of MDP regions, whereas the addition of phospholipids hardly influences the mutual interaction of MDP regions, possibly owing to phase separation between B30-MDP and phospholipids.

Key words Cholesterol – membrane fluidity – muramyldipeptide – phospholipid – stability

Introduction

The muramyldipeptide derivative 6-O-(2-tetradecylhexadecanoyl)-*N*-acetyl-muramyl-*L*-alanyl-*D*-isoglutamine (B30-MDP) was synthesized to reduce the toxicity and improve the immunoadjuvant activity of muramyldipeptide [1, 2]. B30-MDP is an amphipathic compound which has double alkyl chains consisting of 14 carbons as the lipophilic portion and a muramyldipeptide (MDP) containing a carboxylic group as the hydrophilic portion. B30-MDP is able to form vesicles by itself in aqueous solutions [3]. These characteristics suggested that B30-MDP would be useful in the development of improved

high immunogenicity vaccines. Experimentally, Nerome et al. [4] reported that the formation of liposomes with B30-MDP containing influenza hemagglutin and neuraminidase antigens enhanced the level and persistence of circulating antibody and cellular immunity in guinea-pigs and mice.

It is of importance to clarify the chemical stability of B30-MDP and the physicochemical properties of B30-MDP/lipid mixed vesicles to be used clinically as an adjuvant for liposomal vaccines [5–8]. We previously evaluated the membrane fluidity of B30-MDP vesicles containing cholesterol, dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG) [9]. The findings have shown that the membrane fluidity of B30-MDP/cholesterol mixed vesicles is slightly influenced by cholesterol concentration and temperature, and that of B30-MDP/phospholipid mixed vesicles is dependent on the phospholipid concentration and temperature. ESR spectra clearly showed the good miscibility of cholesterol with B30-MDP and the occurrence of phase separation between B30-MDP and phospholipid in the vesicle membranes. It is thus apparent that the membrane structure of B30-MDP/cholesterol vesicle differs from that of B30-MDP/phospholipid vesicle.

Furthermore, we also have described the effect of octylglucoside (OG) incorporation on the chemical stability of B30-MDP in vesicle forming process [8]. Degradation of B30-MDP in both vesicle and B30-MDP/OG mixed micellar solutions occurred by a pseudo first-order reaction at 313, 323 and 333 K. The shelf-life of B30-MDP mixed micellar solution supplemented with OG was approximately one-seventh that of B30-MDP alone in the vesicle. The observed changes in degradation rate constant (k_{obs}) of B30-MDP correlated well with those in membrane fluidity induced by OG incorporation. It was clarified that the increase in membrane fluidity labilizes B30-MDP in the vesicle-forming process and that the increase of k_{obs} value of B30-MDP is dependent on a rise in the frequency of its contact with water molecules, resulting from the increase in membrane fluidity. We thus arrived at the conclusion that the hydrophilic region of B30-MDP plays an important role in determining the relationship between the chemical stability of B30-MDP and the physicochemical properties of the vesicle.

In the present study, we evaluated the effect of cholesterol, dimyristoylphosphatidylcholine (DMPC) or distearoylphosphatidylcholine (DSPC) incorporation on the chemical stability of B30-MDP in mixed vesicle as determined by high-performance liquid chromatography (HPLC). We next evaluated the membrane fluidity of mixed vesicles as determined by the electron spin resonance (ESR) method and particle size as determined by quasi-elastic laser light scattering (QELS) method, and discussed the correlation between the degradation kinetics of B30-MDP and membrane fluidity or particle size of B30-MDP/lipid mixed vesicles.

Materials and methods

Chemicals

The muramyldipeptide derivative 6-O-(2-tetradecylhexadecanoyl)-*N*-acetyl-muramyl-*L*-alanyl-*D*-isoglutamine (B30-MDP) (purity not less than 99%) synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan) was used [1]. The phospholipids dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) (purity not less than 99%; Nichiyu Liposome Co., Ltd., Tokyo, Japan) were used without further purification. Cholesterol (purity not less than 99%) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The spin-labeled reagent 5-doxyl-stearic acid (5NS) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wl., USA). All other chemicals used were of the highest grade commercially available.

Preparation of vesicle samples

Mixed vesicle samples were prepared by the Bangham method [10]. Briefly, B30-MDP, cholesterol and phospholipids were dissolved separately in chloroform and the solutions were mixed in a 20 ml round-bottomed flask. The cholesterol and phospholipid content ranged from 0% to 75% (molar basis) against B30-MDP. In addition, the content of 5NS was set at 0.5% (molar basis) of the total, where necessary. The sample solutions were evaporated to dryness for making lipid film. The films were hydrated at a temperature above 333 K with phosphate-buffered saline (PBS: pH 7.4, $\mu = 0.17$) and sonicated with an ultrasonic cleaner (model 5200, Branson Ultrasonics, Japan) for 30 min to obtain mixed vesicles. The prepared mixed vesicles were filtrated using a membrane filter (Millipore filter, pore size 0.45 μm) and PBS was then added to bring the B30-MDP concentration to 100 μmol/dm^3. No degradation of B30-MDP during the sonicating procedure was detected by HPLC.

Kinetic measurement

Aliquots of the sample solutions were placed in 2 ml ampules in a nitrogen atmosphere and sealed, and then allowed to stand in constant temperature cabinets set at

313 and 333 K (accuracy ± 0.3 K). The maximum storage periods of sample solutions were set for 16 weeks at 313 K and 14 days at 333 K, respectively. The B30-MDP concentration in sample solutions was determined by HPLC analysis as described previously [8]. In view of the necessity for use of liposomal vaccines to maintain the stability of antigens in the clinical setting, kinetic measurements of B30-MDP in the mixed vesicles were carried out at a constant pH (pH = 7.4) and ionic strength ($\mu = 0.17$) since antigens generally tend to be aggregated and denatured by changes in pH, especially in acidic solutions [11–13].

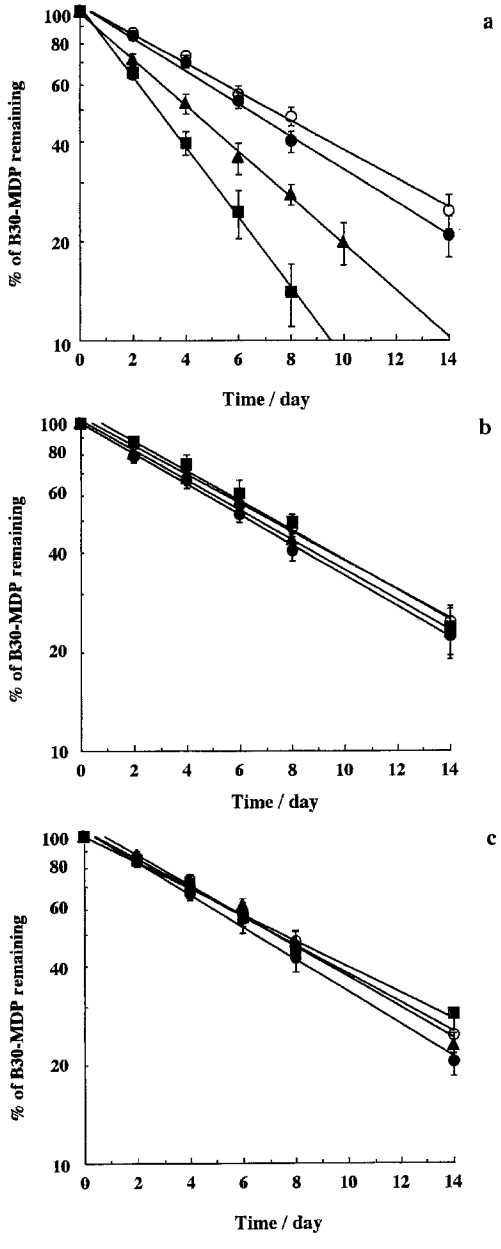

Fig. 1 Plots of the degradation kinetics of B30-MDP in mixed vesicles at 333 K. ○, B30-MDP alone vesicle. Additives concentration (molar basis): ●, 25%; ▲, 50% and ■, 75%. a; B30-MDP/cholesterol mixed vesicles, b; B30-MDP/DMPC mixed vesicles, c; B30-MDP/DSPC mixed vesicles

ESR measurement

ESR spectra were recorded at the X-band according to experimental conditions described previously [3]. ESR measurements were performed to determine the membrane fluidity of B30-MDP with cholesterol, DMPC or DSPC mixed vesicles at 313 and 333 K. Membrane fluidity of the mixed vesicles was expressed in A_{max} value, thus an increase in fluidity is signified by a decrease in A_{max} value. The A_{max} value, or outer hyperfine splitting constant, was obtained from the separation between a lower-field maximum and a higher-field minimum peaks corresponding to $2A_{max}$ in experiments using 5NS as described by McConnell et al. [14]. We previously reported the effect of cholesterol, DPPC or DPPG incorporation on membrane fluidity of B30-MDP mixed vesicles [9]. The membrane fluidity was evaluated by the use of 5NS, 12-doxyl-stearic acid (12NS), and 2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPO). The changes in membrane fluidity detected by A_{max} were essentially in good agreement with those detected by TEMPO parameter, and it was considered that A_{max} values reflected the total membrane fluidity of the membrane surface. Thus, we used 5NS for the evaluation of membrane fluidity of mixed vesicles in this study.

Particle size measurement

The particle size of the mixed vesicles was measured by the QELS method with a dynamic light scattering spectrophotometer (model DLS-700 Ar, laser power 75 mW; Otsuka Electronics Co., Ltd., Osaka, Japan) at 293 K. Average diameter was measured by scattered intensity, and the distribution of particle size was determined using histograms [15].

Results and discussion

Chemical stability of B30-MDP in mixed vesicles

The effects of cholesterol, DMPC or DSPC incorporation on chemical stability of B30-MDP in vesicles at 333 K are shown in Fig. 1. The semi-logarithmic plots of residual B30-MDP in vesicles versus time showed linearity (correlation coefficients, $|r| > 0.99$). In addition, the results of chemical stability of B30-MDP at 313 K also indicated

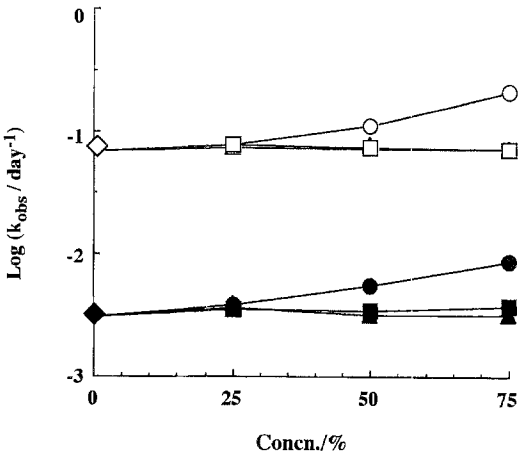

Fig. 2 Effect of the concentration of cholesterol, DMPC or DSPC on degradation rate constant of B30-MDP in the mixed vesicles. ◇ and ◆: B30-MDP alone vesicle, ○ and ●: B30-MDP/cholesterol mixed vesicle, □ and ■: B30-MDP/DMPC mixed vesicle, △ and ▲: B30-MDP/DSPC mixed vesicle. Open symbols: 333 K, Closed symbols: 313 K

linearity (correlation coefficients, $|r| > 0.99$) against the semi-logarithmic plots of residual B30-MDP in vesicles versus time (data not shown). These results suggest that the degradation of B30-MDP occurs by a pseudo first-order reaction in the vesicles. We previously reported that the degradation of B30-MDP in B30-MDP single component vesicle (B30-MDP alone vesicle) occurred via a pseudo first-order reaction [8]. We then concluded that the degradation occurred at the MDP region where the hydrophilic portion of the B30-MDP membrane is situated and that the degradation depended on the hydrolysis reaction [8]. Accordingly, it was inferred that the hydrolysis of B30-MDP in the B30-MDP/cholesterol or B30-MDP/phospholipid mixed vesicles occurred at the MDP region in the present study.

The effect of cholesterol, DMPC or DSPC incorporation on k_{obs} values of B30-MDP in the mixed vesicles at 313 and 333 K is shown in Fig. 2. In the B30-MDP/cholesterol mixed vesicles, k_{obs} values of B30-MDP increased with increasing concentration of cholesterol. In contrast, k_{obs} values of B30-MDP in B30-MDP/DMPC and B30-MDP/DSPC mixed vesicles were scarcely influenced by changes in the concentration of DMPC and DSPC. The degradation of B30-MDP in the mixed vesicles was calculated in accordance with the Arrhenius equation; and the apparent activation energy virtually did not differ among B30-MDP/cholesterol, B30-MDP/DMPC and B30-MDP/DSPC mixed vesicles (about 130 kJ/mol, data not shown). From a theoretical point of view, this is consistent with the assumption that the degradation processes of B30-MDP in the mixed vesicles are the same [8, 16], and it suggests that the acceleration of B30-MDP degradation occurs independently of the catalytic action of cholesterol. Accordingly, it was suspected that the increase in the degradation rate of B30-MDP might depend on the changes in frequency of contact of water molecules with the MDP region of B30-MDP, and the frequency might relate to physicochemical properties such as the membrane fluidity and particle size of the mixed vesicles [8].

Effect of cholesterol, DMPC or DSPC incorporation on membrane fluidity and particle size of mixed vesicles

Figure 3 shows the effect of cholesterol, DMPC or DSPC incorporation on the membrane fluidity of mixed vesicles at 313 and 333 K. The fluidity of the B30-MDP/cholesterol mixed membrane was similar to that of B30-MDP alone membrane at 313 K, and was slightly decreased with increasing concentration of cholesterol at 333 K. The fluidity of B30-MDP/DMPC membrane evidently increased with increasing concentration of DMPC at 313 and 333 K. With increasing concentration of DSPC, the membrane fluidity of B30-MDP/DSPC mixed vesicles was slightly increased at 313 and 333 K. The membrane fluidity of B30-MDP/cholesterol mixed vesicles was slightly influenced by cholesterol concentration and temperature. Conversely, the membrane fluidity of B30-MDP/phospholipid mixed vesicles was dependent on temperature and phospholipid concentration. In addition, DMPC alone membrane is in a liquid crystalline phase at 313 and 333 K, and DSPC alone membrane is in a gel phase at 313 K and a liquid crystalline phase at 333 K [17, 18]. Furthermore, it was apparent that good miscibility of cholesterol with B30-MDP and the occurrence of phase separation between B30-MDP, DMPC and DSPC were recognized in the ESR spectrum of the mixed vesicles (data not shown) as described previously [9]. Therefore, it was reconfirmed that the effect of DMPC and DSPC on the fluidity and miscibility of B30-MDP mixed membrane were in agreement with those of DPPG and DPPC on these properties of B30-MDP mixed membrane [9].

The particle size of mixed vesicles is shown in Table 1. The average diameters of the mixed vesicles were estimated to be about 90 to 180 nm, and the size of B30-MDP/cholesterol, B30-MDP/DMPC and B30-MDP/DSPC mixed vesicles tended to increase with increasing cholesterol, DMPC and DSPC concentration. The size distribution of the vesicles prepared by the Bangham method [10] was increased by increasing the average diameter and was wider than that of the vesicles made by detergent removal method [9]. The magnitude of increase

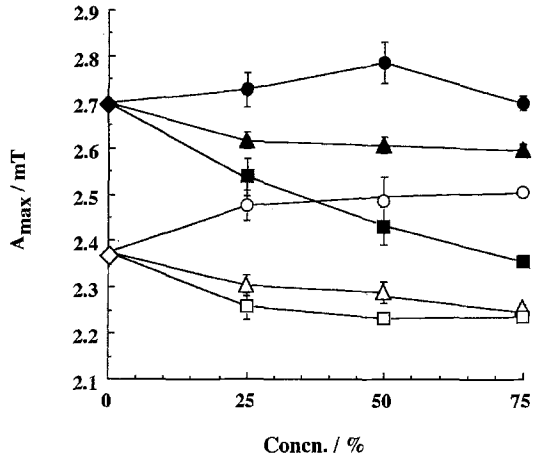

Fig. 3 Effect of cholesterol, DMPC or DSPC incorporation on the A_{max} values of the mixed vesicles. ◇ and ◆: B30-MDP alone vesicle, ○ and ●: B30-MDP/cholesterol mixed vesicle, □ and ■: B30-MDP/DMPC mixed vesicle, △ and ▲: B30-MDP/DSPC mixed vesicle. Open symbols: 333 K, Closed symbols: 313 K Vertical bars denote S.D. for a series of three separate determinations

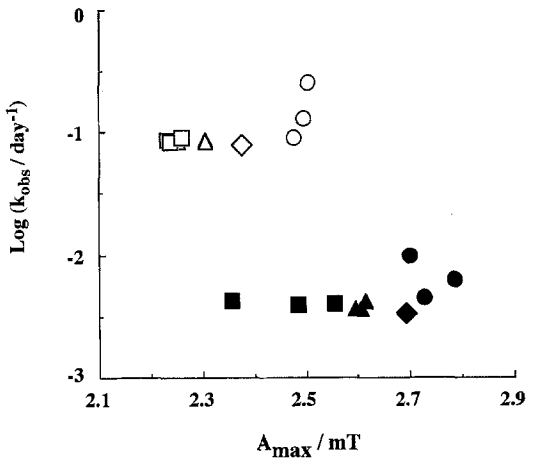

Fig. 4 Correlations between the A_{max} values and the observed degradation rate constants of B30-MDP in mixed vesicles. ◇ and ◆: B30-MDP alone vesicle, ○ and ●: B30-MDP/cholesterol mixed vesicle, □ and ■: B30-MDP/DMPC mixed vesicle, △ and ▲: B30-MDP/DSPC mixed vesicle Open symbols: 333 K, Closed symbols: 313 K

Table 1 Effect of cholesterol, DMPC and DSPC on the particle size of B30-MDP vesicle

Formulation of vesicles	Additives concentration (molar basis; %)	Average diameter (nm)	Standard deviation (nm)
B30-MDP alone	0	135	38
B30-MDP/cholesterol	25	110	25
	50	140	32
	75	178	43
B30-MDP/DMPC	25	104	26
	50	135	44
	75	178	39
B30-MDP/DSPC	25	89	19
	50	113	41
	75	159	44

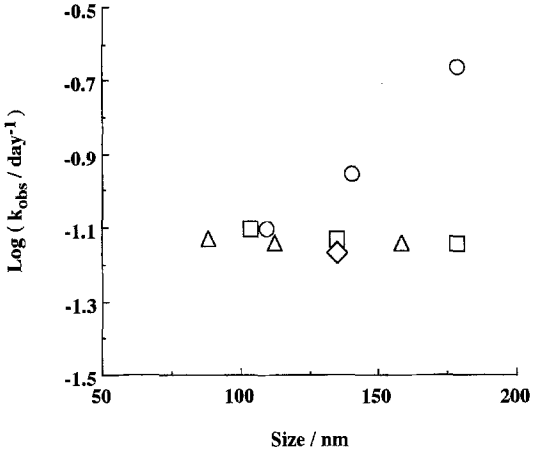

Fig. 5 Correlation between the particle size and the observed degradation rate constants of B30-MDP in mixed vesicles. ◇: B30-MDP alone vesicle, ○: B30-MDP/cholesterol mixed vesicle, □: B30-MDP/DMPC mixed vesicle, △: B30-MDP/DSPC mixed vesicle at 333 K

in average diameter induced by increasing the additive concentration was essentially the same for the three additives cholesterol, DMPC and DSPC.

Relationship between the degradation kinetics of B30-MDP in mixed vesicles and the membrane fluidity or particle size vesicles

Figure 4 shows the relation between A_{max} values and k_{obs} values at 313 and 333 K, and Fig. 5 shows the relation between particle size and k_{obs} values at 313 K. In the B30-MDP/cholesterol mixed vesicles, increase in cholesterol concentration labilized B30-MDP and slightly decreased the membrane fluidity. Correlation between the k_{obs} values of B30-MDP and the particle size of B30-MDP/cholesterol mixed vesicles was recognized ($r = 0.877$), indicating that an increase of particle size labilizes B30-MDP in the mixed vesicles. On the other hand, k_{obs} values of B30-MDP in B30-MDP/DMPC and B30-MDP/DSPC mixed vesicles were scarcely influenced by changes in the concentration of DMPC and DSPC, while membrane fluidity and particle size were increased by the addition of phospholipids.

In the case of B30-MDP/cholesterol mixed vesicles, an increase in the concentration of cholesterol in the vesicle membrane increased the k_{obs} value of B30-MDP (Fig. 2). Fluidity of the B30-MDP/cholesterol mixed membrane was similar to that of B30-MDP alone membrane at 313 K, and slightly decreased with increasing concentration of cholesterol at 333 K. In addition, the good miscibility of cholesterol with B30-MDP was recognized in the mixed vesicles. We previously reported that the membrane fluidity of B30-MDP in a liquid crystalline phase was similar to that of phospholipids in a gel phase [3, 9], and that the k_{obs} values of B30-MDP in vesicle formation were much lower than those of other hydrophilic MDP derivatives [8, 19], while the degradation occurred at the membrane surface. Accordingly, the degradation of B30-MDP seemed to relate to the decrease in formation of hydrogen bonding in the mutual MDP regions at the membrane surface [3, 8, 9]. If the distance between the MDP regions of B30-MDPs reacting with each other is increased by increasing the concentration of cholesterol in the mixed vesicle, the mutual interaction at the MDP regions of B30-MDPs, which might inhibit the degradation of B30-MDP in the mixed vesicle, would be diminished. It is thus inferred that the membrane fluidity of B30-MDP/cholesterol mixed vesicle does not influence the degradation of B30-MDP. The particle size of B30-MDP/cholesterol mixed vesicles increased with increasing concentration of cholesterol. It is considered that the distance between the mutual B30-MDPs is increased by existence of cholesterol in the membrane. In view of the effect of particle size of B30-MDP/cholesterol mixed vesicles, it appears likely that the distance between the mutually interacting MDP regions of B30-MDPs is increased by increasing the particle size and, eventually the interaction at the MDP region of B30-MDPs diminished. Accordingly, an increase of particle size labilizes B30-MDP in the B30-MDP/cholesterol mixed vesicles.

In addition, Zaslavsky et al. [20] reported that the relative hydrophilicity of the phospholipid membrane surface is increased by the addition of cholesterol via division of the phospholipid head group. On the basis of their report, it was inferred that the relative hydrophilicity of the B30-MDP membrane surface was increased by the addition of cholesterol via division of MDP region and that the increased hydrophilicity resulted in an increase in frequency of contact of water with the MDP region when cholesterol was added to the B30-MDP membrane. Thus, the changes in the frequency of contact of water with the MDP region is related to the state of the interaction at the MDP region of B30-MDPs. It is considered that the increase of cholesterol in B30-MDP mixed vesicles increases the frequency of contact between water and the MDP region in the mixed vesicles.

In the case of B30-MDP/DMPC and B30-MDP/DSPC mixed vesicles, k_{obs} values of B30-MDP were virtually not influenced by changes in membrane fluidity and particle size. We previously determined that phase separation occurred in a B30-MDP/DPPC mixed membrane [3, 9], and the phase separation also occurred in both B30-MDP/DMPC and B30-MDP/DSPC mixed membranes. Regarding the occurrence of phase separation in the mixed membranes, interaction at the MDP region would be maintained by the formation of B30-MDP clusters. It is considered, therefore, that the cluster formation of B30-MDP was maintained despite changes in the membrane fluidity and particle size, and there were no appreciable changes in k_{obs} values of B30-MDP/DMPC and B30-MDP/DSPC mixed membranes possibly owing to the cluster formation.

We concluded that the degradation behavior of B30-MDP in the vesicles is dependent on the state of mutual interaction at the MDP regions of B30-MDPs.

Conclusion

The observed degradation rate constant of B30-MDP in the mixed vesicles was increased with increasing concentration of cholesterol, but was unchanged with increasing concentration of DMPC or DSPC. We then discussed the relation between the degradation of B30-MDP and physicochemical properties such as membrane fluidity and particle size of B30-MDP mixed vesicles. It was apparent that the degradation of B30-MDP/cholesterol mixed vesicles is influenced by the particle size, but not by the fluidity of the membranes. In the case of B30-MDP/phospholipid mixed vesicles, the degradation of B30-MDP was not influenced by either the membrane fluidity or the particle size of B30-MDP mixed vesicles.

It is considered that the addition of cholesterol inhibits mutual interaction at the MDP regions, whereas the addition of phospholipids having membrane-forming ability had little or no influence upon the mutual interaction of MDP regions via phase separation between B30-MDP and phospholipids in the mixed vesicles. We thus conclude that the degradation behavior of B30-MDP in the vesicles is dependent on the state of mutual interaction at the MDP regions of B30-MDPs, which relates to the frequency of contact of water with the MDP region.

References

1. Kusumoto S, Inage M, Shiba T, Azuma I, Yamamura Y (1978) Tetrahedron Lett 49:4899–4902
2. Adam A, Lederer E (1984) Med Res Rev 4:111–152
3. Ando S, Tsuge H, Miwa A (1994) Coll Poly Sci 272:1281–1288
4. Nerome K, Yoshioka Y, Ishida M, Oka T, Kataoka T, Inoue A, Oya A (1990) Vaccine 8:503–509
5. Kinsky SC, Yasuda T, Tadakuma T (1982) Immunol Today 3:308–310
6. Davis D, Gregoriadis G (1987) Immunology 61:229–234
7. Bakouche Q, David F, Gerlier D (1987) Eur J Immunol 17:1839–1842
8. Ando S, Tsuge H, Miwa A (1995) Coll Poly Sci 273:399–404
9. Ando S, Tsuge H, Mayumi T (1996) Coll Poly Sci, in press
10. Bangham AD, Standish MM, Watkins JC (1965) J Mol Biol 13:238–244
11. Maeda T, Onishi S (1980) FEBS Lett 122:283–287
12. Webster RG, Brown LE, Jackson DC (1983) Virology 126:587–599
13. Yoden S, Kida H, Kuwabara M, Yanagawa R, Webster RG (1986) Virus Res 4:251–261
14. McConnell HM, Wright KL, McFarland BG (1972) Biochim Biophys Res Commun 47:273–281
15. Eshuis A, Harbers G, Doornink DJ, Mijnlieff PF (1985) Langmuir 1:289–294
16. Grit M, Underberg WJM, Crommelin DJA (1993) J Pharm Sci 83:362–366
17. Mabrey S (1981) In: Knight CG (ed) Liposomes: From physical structure to therapeutic applications, Elsevier, Amsterdam pp 3–7
18. Cevc G (1993) In: Cevc G (ed) Phospholipids handbook, Marcel Dekker Inc, New York pp. 939–956
19. Powell MF, Foster LC, Becher AR, Lee W (1988) Pharm Res 5:528–532
20. Zaslavsky BY, Borovskaya AA, Rogozhin SV (1984) Mol Cell Biochem 60:131–136