Osmotic Stability of Muramyl Dipeptide-Bearing Liposomes and Molecular Miscibility in Their Membranes[a)]

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Muramyl dipeptide is the minimal and essential structural unit for the immunopotentiating activities of bacterial cell walls. The muramyl dipeptide derivative *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutamine (B30-MDP) and its amide derivative (B30-MDPA) are good basic materials for use in artificial liposome vaccines (virosomes). The osmotic stability of muramyl dipeptide-bearing liposomes composed of various binary mixtures was examined to find conditions favoring stable liposome formation. The osmotic properties of B30-MDP-bearing and B30-MDPA-bearing liposomes were significantly different from each other. Nevertheless, a good correlation between osmotic stability and virosome formation was found for both types of liposomes. Furthermore, the dynamic structures and the intermolecular interactions of phospholipids in muramyl dipeptide-bearing liposomes were investigated by solid-state ^{2}H and ^{31}P NMR. The results suggested that molecular miscibility in the liposomes is not an essential factor for osmotic stability. Negative charges on the liposome surface and flexible hydrophilic moieties were found to be the most important factors in keeping isolated liposomes osmotically stable.

The development of vaccines of high immunogenicity with minimal side effects has been an important target for decades. A new type of influenza subunit vaccine has been developed for this purpose using muramyl dipeptide-bearing liposomes.[1—3)] *N*-acetylmuramyl-L-alanyl-D-isoglutamine or muramyl dipeptide was synthesized to replace the adjuvant function of mycobacterial cells.[4,5)] The muramyl dipeptide is the minimum and essential structural unit for the immunopotentiating activities of bacterial cell walls. The immunostimulating effects of muramyl dipeptides depend highly on their chemical structures.[6—10)] The muramyl dipeptide derivative *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutamine (B30-MDP, Fig. 1) was synthesized to reduce the toxicity and to improve the immunoadjuvant activity of muramyl dipeptide.[11,12)] Since B30-MDP is an amphipathic molecule, it can, like phospholipids, form liposomes.[13)] The adjuvant activity of muramyl dipeptide was further increased when it was administrated in the form of liposomes.[10)] B30-MDP was shown to form a liposomal vaccine (virosome) with hemagglutinin and neuraminidase (HANA) molecules of the influenza virus; this virosome increased the level and persistence of circulating antibody and cellular immunity in guinea pigs and mice.[1)]

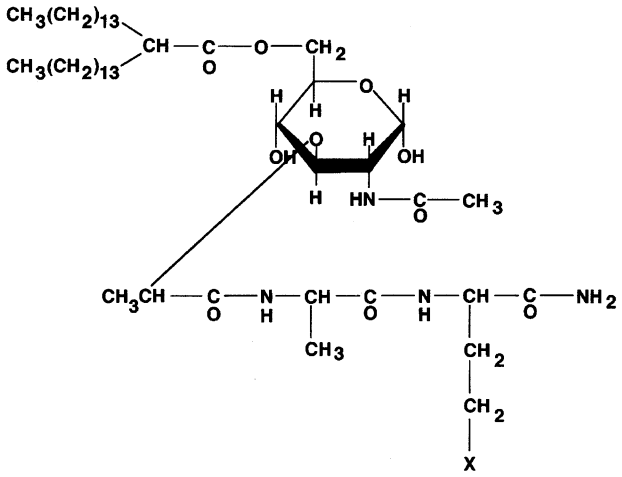

Fig. 1. Chemical structure of *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutamine (B30-MDP) and its amide derivative (B30-MDPA). X = COOH, B30-MDP; X = $CONH_2$, B30-MDPA.

Formation of stable muramyl dipeptide-liposomal vaccine is not straightforward. It still depends on trial and error. The efficiency of virosome formation has been examined for a variety of phospholipids. It has been shown that phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylinositol (PI), and a binary mixture of PC and PI (PC/PI) are fairly efficient in producing influenza virosomes.[14)] Cholesterol has

a) Abbreviations: B30-MDP, *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutamine; B30-MDPA, *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutaminamide; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PG, L-α-phosphatidylglycerol; CL, L-α-cardiolipin; Chol, cholesterol.

also stabilized liposomal vaccines.[1—3] For further improvement of liposomal vaccines, it will be important to elucidate the physicochemical basis of the stability of muramyl dipeptide-bearing liposomes. The physicochemical properties and chemical stability of MDP-bearing liposomes have been investigated using ESR, DSC, and light scattering.[13,15—17] Decrease in the phase transition temperature of a phospholipid bilayer was observed with the addition of B30-MDP. While cholesterol mixes homogeneously with B30-MDP, phase separation was observed for B30-MDP/PC and B30-MDP/PG liposomes.[13,15] Despite these studies, the physicochemical basis of the stability of muramyl dipeptide-bearing liposomes is not yet well understood.

Measurement of the osmotic stability of liposomes is useful in characterizing the formation of stable liposomes.[18,19] This was used to examine the physicochemical factors regulating stability of muramyl dipeptide-bearing liposomes in this study. The molecular interactions among lipids of muramyl dipeptide-bearing liposomes was further investigated by solid-state ^{2}H and ^{31}P NMR. Solid-state ^{2}H and ^{31}P NMR provide information on the structure and interaction at atomic resolution for lipid bilayers in the liquid-crystalline state.[20,21] The amide derivative of B30-MDP (B30-MDPA) is another choice for a material with adjuvant activity. To understand the general principles of stable liposome formation, the physicochemical properties of MDPA-bearing liposomes have also been examined and compared with those of the MDP-bearing liposomes.

Materials and Methods

Materials: *N*-Acetyl-6-*O*-(2-tetradecylhexadecanoyl)-muramyl-L-alanyl-D-isoglutamine (B30-MDP) and its amide derivative (B30-MDPA) were synthesized by Daiichi Pharmaceutical Co., Ltd. Lithium tetradeuterioaluminate ($LiAl^2H_4$) was purchased from Cambridge Isotope Laboratories, Inc. (CIL). Diethyl oxomalonate was purchased from Sigma. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE), used for the osmotic experiments were extracted from egg yolk by the method of Bligh and Dyer[22] and further purified by silica-gel column chromatography. Phosphatidylglycerol (PG) was obtained from egg yolk PE by transphosphatidylation as described later. Bovine heart cardiolipin (CL) was purchased from Sigma and used for the osmotic stability experiments. The other reagents used were of special grade.

Synthesis of Perdeuterated Glycerol: 3 g of $LiAl^2H_4$ was dissolved in 100 ml of tetrahydrofuran (THF). Then, 5.5 ml of diethyl oxomalonate dissolved in 30 ml of THF was dripped slowly and refluxed under cooling with ice. After 1 h, 10 ml of water was added to decompose the remaining $LiAl^2H_4$. The product was filtered and washed with 100 ml of 0.1 M NaOH solution 1 M = 1 mol dm^{-3}. After the THF had evaporated, the product was purified by cation exchange column chromatography (Dowex 50W-X8, 50—100 mesh). The yield of deuterated glycerol was 2.1 g.

Preparation of Phospholipids Selectively Deuterated at the Glycerol Backbone: For deuteration of the glycerol backbone of phospholipids, a glycerol-requiring mutant of *E. coli* (*E. coli* K-12 GRA) was grown at 37 °C in the basal medium containing 0.01% perdeuterated glycerol. Phospholipids were extracted from the cells by the method of Bligh and Dyer.[22] PE and CL were purified by silica-gel column chromatography (Merck Silicagel 60, 230 mesh). Purity was checked by silica-gel thin-layer chromatography and ^{1}H NMR. The extent of deuteration estimated from ^{1}H NMR spectra of PE was about 80% on average.

PG selectively deuterated at the glycerol backbone was obtained from the deuterated PE by one-step transphosphatidylation catalyzed by cabbage phospholipase D in the presence of excess glycerol.[19] PC was synthesized by methylation of the purified PE by a reported method.[23] The ^{1}H NMR spectra of PG, PC, and PE were identical except for the polar group signals, suggesting that the hydrocarbon chains of PG and PC were intact.

Sample Preparation: Phospholipids were dissolved in chloroform/methanol (1 : 1, v/v) and washed with 0.5 vol. of 0.5 M Na_2SO_4 and 2.0 mM EDTA solution (pH 7.2) to remove polyvalent metal ions. The solvent was then removed by evaporation. Equal amounts of B30-MDP (or B30-MDPA) and dried lipid were dissolved in a minimal volume of chloroform. For examination of osmotic stability, the solvent was removed with a rotary evaporator to dryness and further dried under a high vacuum overnight. The lipid on the surface of the flask was suspended in an aqueous solution containing 50 mM glucose, 2 mM EDTA, and 10 mM Tris (pH 7.5) with a vortex mixer. This was used as the stock dispersion. For solid-state NMR measurements, the chloroform solution was transferred into a 5-mmϕ tube and dried to a film with nitrogen gas. It was further dried under a high vacuum. The dried lipid was dispersed in 0.1 M *N*,*N*′-piperazinebis(ethanesulfonic acid) (PIPES) and 2 mM EDTA buffer (pH 7.2) and was centrifuged at 4000 rpm and 4 °C.

Measurements of Osmotic Stability: The osmotic stability of the liposomes was examined by the reported method.[18,19] The lipids used are described in Materials. A 0.2 ml portion of the stock dispersion was diluted with 3.0 ml of 2 mM EDTA and 10 mM Tris (pH 7.5) solutions including different concentrations of glucose. Before optical measurements, each mixture was incubated for 1 h at 25 °C. The turbidity of the dispersion was measured at 435 nm and 25 °C with a Shimadzu UV-2200 UV/vis spectrophotometer with a temperature-controlled cell holder.

NMR Measurements: Solid-state ^{31}P and ^{2}H NMR measurements were done with an Otsuka Electronics CMX-400 NMR spectrometer with a CP/MAS probe. ^{31}P NMR spectra were recorded at 161.15 MHz using a single pulse under proton decoupling during acquisition. The 90° pulse width was 5 μs. ^{2}H NMR spectra were measured at 61.1 MHz, using the quadrupole echo sequence (90_x-t_1-90_y-t_2) with t_1 = 30μs, t_2 = 20μs, and 0.5 s recycle time. The 90° pulse width was 5 μs. ^{2}H NMR and ^{31}P NMR spectra were recorded for the same sample. Solution ^{1}H NMR spectra were recorded on a Bruker AM-400 NMR spectrometer.

Results

1. Osmotic Stability of Liposomes. The osmotic stability of multilamellar liposomes composed of a variety of lipids including B30-MDP and B30-MDPA was examined at 25°C by the turbidimetric method. The lipid bilayers examined were in the liquid-crystalline state at this temperature, due to their fatty acid composition. An osmotic gradient across the liposomal membrane was formed using glucose as described in Materials and Methods. The osmotic gradient induces a volume change in liposomes due to the uptake or release of water. When a multilamellar liposome acts as a perfect osmometer, the following relationship is satisfied:[18,19]

$$(1/A)^{3/2} = K(C_{in}/C_{out}) + K' \quad (1)$$

where A, C_{in}, and C_{out} stand for the turbidity and the glucose concentrations inside and outside of the liposomes, respectively. K and K' are constants.

The osmotic properties of the binary mixtures of B30-MDP, B30-MDPA, egg yolk phosphatidylcholine (PC), egg yolk phosphatidylethanolamine (PE), phosphatidylglycerol (PG) converted from egg yolk PE, bovine heart cardiolipin (CL), cholesterol (Chol), and dihexadecyl hydrogenphosphate (DCP) were examined. While PC and PE are zwitterionic phospholipids, PG, CL, and DCP are acidic phospholipids. B30-MDP should carry one negative charge at pH 7.5, due to the dissociation of the carboxyl group. Octadecylamine, which carries a positive charge, was also examined. However, its binary mixtures with B30-MDP and B30-MDPA did not disperse into the buffer as liposomes.

The power of $-3/2$ of turbidity of liposome dispersions was plotted in Fig. 2 as a function of the ratio of concentrations C_{in} to C_{out} for B30-MDP/PC, B30-MDP/PE, B30-MDP/PG, and B30-MDP/Chol liposomes (weight ratio 1/1). The B30-MDP/PG (Fig. 2C) and B30-MDP/Chol (Fig. 2D) liposomes exhibited linearity over wide ranges of C_{in}/C_{out}, including shrinking ($C_{in}/C_{out}<1$)and swelling ($C_{in}/C_{out}>1$) regions. Turbidity of B30-MDP/PG liposomes was much smaller than that of B30-MDP/Chol liposomes, suggesting that the liposome size is smaller for the former than for the latter. In contrast to B30-MDP/PG and B30-MDP/Chol liposomes, the plots for the B30-MDP/PE liposomes were scattered. The results for the binary combinations examined are summarized in Table 1. The B30-MDP/PC and B30-MDP/CL liposomes were found to be osmotically stable but not in a wide range of C_{in}/C_{out}. The linearity holds only in either the swelling or the shrinking region as can be seen in Fig. 2A. The B30-MDP/DCP liposomes were unstable. The result mentioned above was consistent with the osmotic stability of a ternary mixture, B30-MDP/PG/Chol (1/1/1). It forms osmotically highly stable liposomes, as shown in Fig. 3.

Since the amide derivative of B30-MDP (B30-MDPA) is another possible choice for a basic material for artificial vaccines, the osmotic properties of MDPA-bearing liposomes were also examined. The results are summarized in Table 1 as well. In contrast to B30-MDP, B30-MDPA has no charge in the hydrophilic region. Properties of MDPA-bearing liposomes were completely different from those of MDP-bearing liposomes. Binary mixtures containing B30-MDPA were hardly dispersed into the buffer. Only

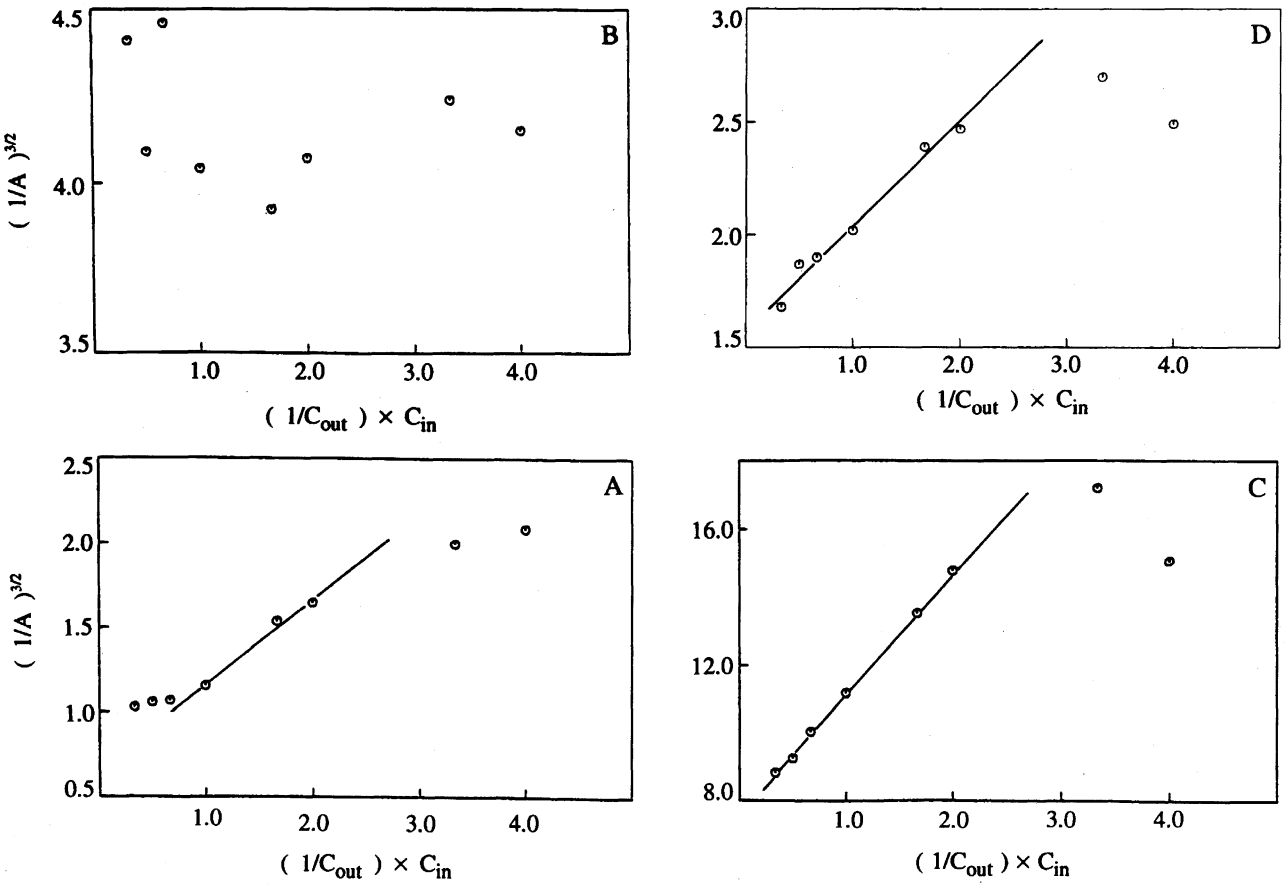

Fig. 2. Osmotically induced turbidity changes for muramyl dipeptide-bearing liposomes at 25 °C.
Liposomes were composed of the following binary mixtures: A, B30-MDP and phosphatidylcholine; B, B30-MDP and phosphatidylethanolamine; C, B30-MDP and phosphatidylglycerol; and D, B30-MDP and cholesterol. Weight ratios were one to one. C_{in} and C_{out} are glucose concentrations inside and outside of liposomes, respectively. A is the turbidity at 435 nm. The phospholipids were obtained from egg yolk.

Table 1. Osmotic Stability of Liposomes of Binary Mixtures (1 : 1 by weight)
B30-MDP, *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutamine; B30-MDPA, *N*-acetyl-6-*O*-(2-tetradecylhexadecanol)muramyl-L-alanyl-D-isoglutaminamide; PC, egg yolk phosphatidylcholine; PE, egg yolk phosphatidylethanolamine; PG, phosphatidylglycerol converted from egg yolk PE; CL, bovine heart cardiolipin; Chol, cholesterol; DCP, dihexadecyl hydrogenphosphate.

	B30-MDP	B30-MDPA	PC	PE	PG	CL	Chol	DCP
B30-MDPA	×		+	×	++	+	++	×
B30-MDPA		××	×	××	−	++	××	××
PC	+	×	××	×	++	++	××	
PE	×	××	×	××	++	×		
PG	++	−	++	++	+	++		
CL	+	++	++	×	++	+		
Chol	++	××	××					
DCP	×	××						

++, osmotically stable over a wide range of C_{in}/C_{out}. +, osmotically stable over a narrow range of C_{in}/C_{out}. ×, osmotically unstable. ××, could not be dispersed into buffer. −, optical density change could not be detected due to small size.

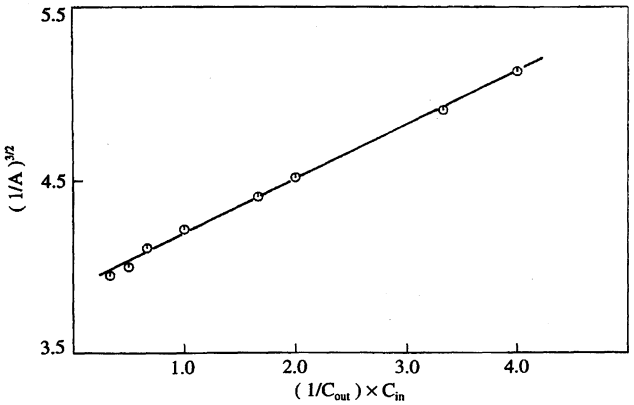

Fig. 3. Osmotically induced turbidity changes for B30-MDP/phosphatidylglycerol/cholesterol liposomes at 25 °C. The weight ratio was one to one to one. C_{in} and C_{out} are glucose concentrations inside and outside of liposomes, respectively. A is the turbidity at 435 nm. The phospholipids were obtained from egg yolk.

the B30-MDPA/CL combination yielded osmotically stable liposomes. The B30-MDPA/PG liposomes were too small to detect turbidity changes. However, the addition of a small amount of PC (one tenth of that of PG) made the B30-MDPA/PG liposomes larger. The resultant liposomes were osmotically stable. Another remarkable finding seen in Table 1 is the role of PG in osmotic stability. PG stabilized the liposomes for all combinations examined except B30-MDPA/PG.

2. ^{31}P NMR Spectra of Muramyl Dipeptide-Bearing Liposomes. To monitor the polymorphic structures of the binary lipid samples and the effects of B30-MDP and B30-MDPA on the dynamic structure of the polar head groups of phospholipids, solid-state ^{31}P NMR spectra were measured. The phosphorus chemical shift anisotropy ($\Delta\sigma = \sigma_{//} - \sigma_{\perp}$) obtained from a powder pattern spectrum provides such information. ^{31}P NMR powder pattern spectra of the single phospholipid component and its binary mixture with B30-MDP or with B30-MDPA were measured at 30, 35, 40, 45, and 50 °C for *E. coli* PE and CL, and for PG and PC converted from *E. coli* PE. The spectra gave rise to typical axially symmetric powder patterns, indicating that all of them took on bilayer structures and were in the liquid-crystalline state. Typically, the chemical shift anisotropies of PE/B30-MDP, CL/B30-MDP, PG/B30-MDP, and PC/B30-MDP bilayers are −39.3, −30.0, −36.6, and −45.2 ppm, respectively, at 40 °C. The observed $|\Delta\sigma|$ for the bilayer containing B30-MDP was smaller than that for the single component bilayer, indicating that a conformational change had occurred at the polar head group of phospholipid, or that fluctuation of the head group in the bilayer had been increased. This was also the case for the bilayer containing B30-MDPA. The percent reduction of $|\Delta\sigma|$ for the bilayer containing muramyl dipeptide lipid (B30-MDP and B30-MDPA) relative to that of the single-component phospholipid bilayer is presented as a function of temperature in Fig. 4. The addition of muramyl dipeptide lipid to the CL bilayer induced a larger change in the chemical shift anisotropy than with other liposomes. The effect of B30-MDP on the extent of the induced change was larger than that of B30-MDPA in the CL bilayers. In contrast to osmotic stability, however, the effects of B30-MDP and B30-MDPA on the phosphorus chemical shift anisotropy of phospholipid bilayers were similar.

3. ^{2}H NMR Spectra of Muramyl Dipeptide Liposomes. The effects of B30-MDP and B30-MDPA on the dynamic structures of the glycerol moieties of phospholipids in the bilayers of binary mixtures were examined by solid-state ^{2}H NMR. The phospholipids specifically deuterated in the glycerol moieties (labeled by *) were used in these measurements. A ^{2}H NMR spectrum of B30-MDP/CL* liposomes at 50 °C is presented in Fig. 5. The assignment of the major peaks is given in Fig. 5 on the basis of the reported one[24] along with nomenclature for the deuterons. Both ^{2}H and ^{31}P NMR spectra were recorded for the same sample. The peak separation indicated in Fig. 5 gives the residual deuterium quadrupolar splitting ($\Delta\nu_Q$). The quadrupolar splitting can be related to the conformation and fluctuation of the glycerol backbone of a phospholipid molecule quantitatively

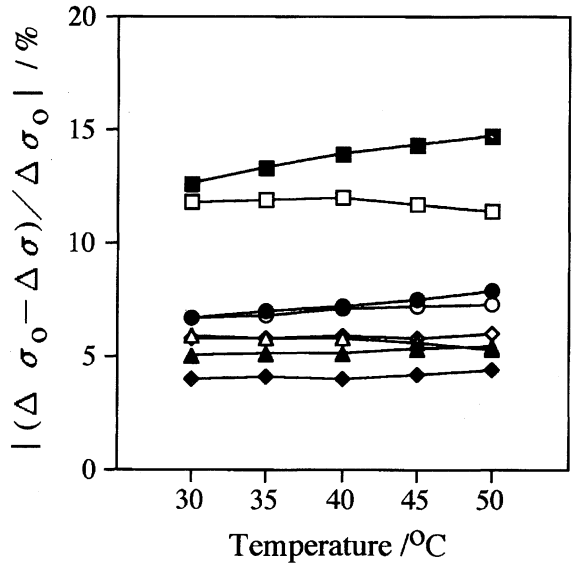

Fig. 4. Percent reduction of phosphorus chemical shift anisotropy of phospholipid in muramyl dipeptide-bearing liposomes as a function of temperature.
$\Delta\sigma_o$, the phosphorus chemical shift anisotropy of the single-component phospholipid bilayer; $\Delta\sigma$, the phosphorus chemical shift anisotropy of the binary mixture. The chemical shift anisotropy was determined from the ^{31}P NMR spectra. Closed and open symbols represent mixtures with B30-MDP and B30-MDPA, respectively. Circles, phosphatidylethanolamine; diamonds, phosphatidylcholine; triangles, phosphatidylglycerol; and squares, cardiolipin. Weight ratios for the binary mixtures were one to one. Phospholipids were obtained from *E. coli* cells.

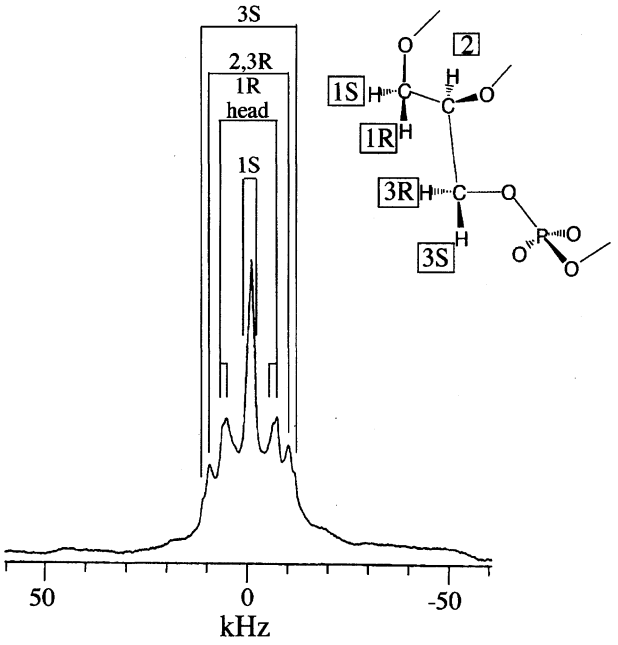

Fig. 5. A ^{2}H NMR spectrum of B30-MDP/cardiolipin liposomes at 50 °C. Assignment of the peaks and nomenclature for the deuterons in the glycerol backbone are shown. The peak separation indicated in connection with the assignment yields the residual deuterium quadrupolar splitting ($\Delta\nu_Q$).

through the order parameter as follows:[20]

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD}^{obsd}, \quad (2)$$

where (e^2qQ/h) and S_{CD}^{obsd} are the static quadrupolar coupling constant and the orientational order parameter of the C–^{2}H bond with respect to the director (perpendicular to the membrane surface). When the motions of the molecular axis and the intramolecular segment are independent,

$$S_{CD}^{obsd} = S_{mol} \times S_{CD}, \quad (3)$$

where S_{mol} and S_{CD} are the order parameter of the molecular axis with respect to the director and the orientational order parameter of the C–^{2}H bond with respect to the molecular axis. S_{mol} is one for the completely ordered state and is zero for the completely disordered state. S_{CD} includes the contribution of conformational parameters of the C–^{2}H bond. Only S_{CD} differs for different deuterons in a rigid molecule.

The percent reduction of $\Delta\nu_Q$ of phospholipid bilayers containing muramyl dipeptide lipid is plotted as a function of temperature in Fig. 6 for PC*, PG*, and CL*. The result for PE* is not included because the percent reduction of $\Delta\nu_Q$ for PE was similar to that for PC*. Addition of B30-MDP to a phospholipid bilayer reduced $\Delta\nu_Q$, indicating that either a change in the dynamic conformation of the glycerol backbone (S_{CD}) or an increase of molecular fluctuation ($1/S_{mol}$) had been induced. This was also the case with B30-MDPA. A similar effect was observed for the phosphorus chemical shift anisotropy of the same sample, as mentioned above. Since the phase transition temperature of lipid bilayers decreases with the addition of B30-MDP,[13] the contribution of the molecular fluctuation should be dominant. The magnitude of the change in $\Delta\nu_Q$ was greater than 10%, which is twice as large as the effect induced by the temperature change from 30 to 50 °C. If an increase of molecular fluctuation is the sole reason for the reduction of $\Delta\nu_Q$, the effect of B30-MDP would be similar for all deuterons of the glycerol backbone. It is actually observed for PC* and PE*. In contrast, the extents of change in $\Delta\nu_Q$ for CL* and PG* differed depending on deuteron position, as can be seen in Figs. 6B and 6C. This strongly suggests that the addition of B30-MDP induces changes in both conformation and fluctuation for CL* and PG*, but induces mainly an increase of molecular fluctuation for PC* and PE*. As for the phosphorus chemical shift anisotropy, the effects of B30-MDP and B30-MDPA on the deuterium quadrupolar splitting were more or less similar. Nevertheless, the effect of B30-MDPA on $\Delta\nu_Q$ was larger than that of B30-MDP for the PG bilayer. Thus, the molecular order parameter S_{mol} of PG should be smaller in the B30-MDPA/PG* bilayer, suggesting that the mode of interaction between PG and B30-MDPA differs from that between PG and B30-MDP. This should have something to do with formation of much smaller liposomes for B30-MDPA/PG than for B30-MDP/PG binary mixtures.

Discussion

MDP-bearing and MDPA-bearing liposomes have com-

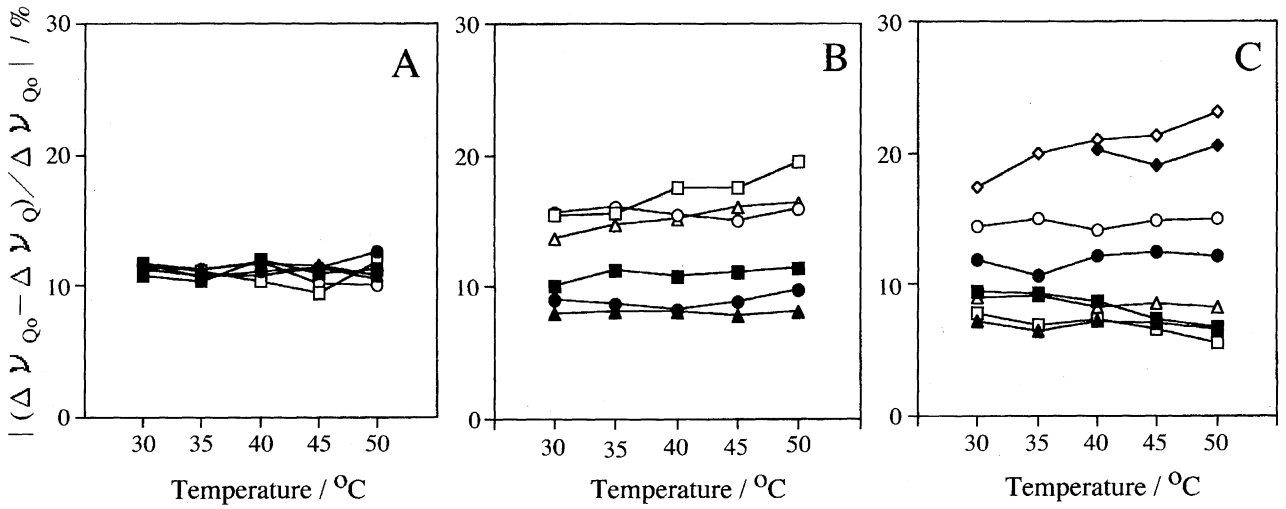

Fig. 6. Percent reduction of residual deuterium quadrupolar splitting of the glycerol backbone of the phospholipid in muramyl dipeptide-bearing liposomes as a function of temperature. $\Delta\nu_{Q_0}$, the residual quadrupolar splitting of the single-component phospholipid bilayer; $\Delta\nu_Q$, the residual quadrupolar splitting of the binary mixture. A, mixture with phosphatidylcholine; B, mixture with phosphatidylglycerol; and C, mixture with cardiolipin. Phospholipids are selectively deuterated in the glycerol moieties. Closed and open symbols represent mixtures with B30-MDP and B30-MDPA, respectively. Squares, 1R deuteron of the glycerol backbone; circles, 2 and 3R deuterons; triangles, 3S deuteron; and diamonds, the deuterons of the cardiolipin head group. Weight ratios of the binary mixtures were one to one. Phospholipids were obtained from *E. coli* cells.

pletely different favorite partners in liposome formation. Although B30-MDP form an osmotically stable liposome with PC, CL, PG, and Chol, B30-MDPA can do so only with CL. This difference is due to the charge in the hydrophilic region of the muramyl dipeptide lipid. While B30-MDP has one negative charge, B30-MDPA has no charge in the hydrophilic region. Since B30-MDPA could form osmotically stable liposomes only with CL, this negative charge should be important for the formation of osmotically stable liposomes. However, DCP could not form stable liposomes with B30-MDP and B30-MDPA. Therefore, thepresence of negative charge is just not enough.

It has been known that the binary mixture of B30-MDP/Chol is a good basic material for an influenza liposomal vaccine.[1—3] B30-MDP/PG was also found to be a good combination for formation of influenza liposomal vaccines [Tsuge et al., unpublished data]. The binary mixtures of B30-MDP/Chol and B30-MDP/PG formed highly stable liposomes as shown in Table 1. Therefore, a good correlation between osmotic stability and liposomal vaccine formation for B30-MDP/lipid liposomes is suggested. This correlation was also confirmed for B30-MDPA/CL liposomes [Tsuge et al., unpublished data] in spite of the properties of B30-MDPA being significantly different from those of B30-MDP. Thus, all binary mixtures forming highly stable liposomes in Table 1 were found to be good materials for influenza liposomal vaccines. Given this correlation, the turbidimetric method should be useful in finding good candidates for materials for artificial vaccines.

The osmotic stability of liposomes cannot be ascribed only to the expansion or shrinkage of the lipid membrane.[25,26] To be osmotically stable in a wide C_{out}/C_{in} range, liposomes must change their shapes as well. Therefore, lipid bilayers should be flexible enough to accommodate changes in local curvature. This flexibility would also be associated with the ability to accept antigen membrane proteins. On the other hand, osmotic stability should also have important effects during circulation of the liposome vaccine in a human body.

Although the effects of B30-MDP and B30-MDPA on the osmotic stability of lipid liposomes were significantly different from each other, those on the phosphorus chemical shift anisotropy and deuterium quadrupolar splitting of phospholipid were more or less similar. Namely, addition of B30-MDP or B30-MDPA to each phospholipid decreased $|\Delta\sigma|$ and $\Delta\nu_Q$ in all cases. In general, this is consistent with the observation that the phase transition temperature of a lipid bilayer decreases on addition of B30-MDP.[13] This suggests that phospholipids mixed with B30-MDP and B30-MDPA to a certain extent. The effects of muramyl dipeptide lipids differed depending on phospholipid species. The effect was most significant for the liposomes containing CL. In addition, the percent reduction of $\Delta\nu_Q$ was different for different deuterons for both B30-MDP/CL and B30-MDPA/CL liposomes, suggesting that the dynamic conformation of the glycerol backbone of CL changed on interacting with B30-MDP and B30-MDPA, respectively. This change should be induced by direct interaction between CL and B30-MDP (or B30-MDPA). In contrast to CL-containing liposomes, those containing PC or PE showed similar percent reductions of $\Delta\nu_Q$ for all deuterons of the glycerol backbone. This suggests that fluctuation of the glycerol backbone changed on addition of B30-MDP (or B30-MDPA) to the PC (or PE) bilayer with little change in conformation. The intermolecular interaction among PC or PE in the single-component bilayer should therefore be retained. It can be deduced from these observations that CL is more miscible with B30-MDP and B30-MDPA at the molecular level than PC and PE. Judging from the percent reduction of $\Delta\nu_Q$, miscibility of PG with

B30-MDP and B30-MDPA was in-between those of CL and PC (PE). This is consistent with the observation by EPR that B30-MDP/PC and B30-MDP/PG bilayers show phase separation, while cholesterol is completely miscible with B30-MDP.[15] Now, the similar effects of B30-MDP and B30-MDPA on ^{31}P and ^{2}H NMR spectra can be ascribed to the similar degrees of miscibility of B30-MDP and B30-MDPA with each phospholipid. Therefore, it can be concluded that the molecular miscibility of lipids is not an essential factor in regulation of the osmotic stability of isolated muramyl dipeptide-bearing liposomes.

Molecular miscibility among phospholipids and its effect on lipid-protein interactions have been extensively investigated.[21,27,28] While PE can mix with CL and PG completely, PC cannot mix well with PE, CL, or PG.[21] In the latter case, formation of microdomains was suggested. Since the quadrupole splittings of glycerol deuterons of PC an PE did not change on mixing with each other, the conformations and molecular fluctuations were thought to be the same in each microdomain as in the single component bilayer. In contrast, $\Delta\nu_Q$ of PC and PE became smaller on mixing with B30-MDP or B30-MDPA. Heterogeneous backbones of lipids (glycerol for phospholipids, *N*-acetylglucosamine for B30-MDP and B30-MDPA) should be responsible for the decrease of $\Delta\nu_Q$. This means that the domain sizes of PC and PE are not large enough to neglect the effect of B30-MDP and B30-MDPA. That is, they should also form microdomains in muramyl dipeptide-bearing liposomes. This is consistent with the observation that the osmotic properties of B30-MDP and B30-MDPA bilayers have changed on mixing with other lipids.

Since liposomes are to change shape in response to osmotic pressure, their membranes should be flexible. With given fatty acid compositions, the hydrophilic moieties should play the most important role in keeping membranes flexible. In the PE domain, the polar groups are not flexible because of the strong intermolecular interactions, including hydrogen bonding. This explains the osmotic instability of the B30-MDP/PE liposomes. On the other hand, the polar head group of PC in the PC domain is flexible because of weak intermolecular interactions,[21] explaining the formation of the relatively stable liposomes. The polar head group of PG in the PG microdomain may be relatively flexible because of negative charge repulsion, despite its ability to form hydrogen bonds. The head groups of Chol and CL are small and have little flexibility. However, they can function as molecular spacers for B30-MDP and B30-MDPA because of their good miscibility. The expanded space provides greater flexibility for the muramyl dipeptide groups. This explains the osmotic stability of B30-MDP/Chol liposomes, but B30-MDP/CL liposomes were less stable. The strong negative charge density of CL would restrict the movable space of the dipeptide because of the electrostatic repulsion. In contrast to this, the neutral hydrophilic moiety of B30-MDPA has more movable space in B30-MDPA/CL liposomes. When the dipeptide turns back to the membrane surface, the hydrophilic moiety becomes bulky. But, when it extends into the water, the hydrophilic moiety becomes slim. This flexibility may make the B30-MDPA/CL liposomes osmotically stable. The B30-MDPA/Chol liposomes were unstable because they lack negative charges. The negative charge on the membrane surface is required for formation of stable isolated muramyl dipeptide-bearing liposomes as has been indicated for other liposomes.

Concerning the flexibility of the PG head group, it should be noted that PG was the most efficient component in formation of osmotically stable two-component liposomes as can be seen in Table 1. The essential role of PG in keeping *E. coli* cells osmotically stable was also previously reported.[19] Therefore, this should be a general feature of PG, indicating that the flexibility of the head group is a unique property of the PG molecule.

In conclusion, MDP-bearing and MDPA-bearing liposomes showed significantly different osmotic behaviors although the molecular miscibility with phospholipids were similar to each other. Flexibility of the hydrophilic moieties and presence of negative charges on a membrane account for the formation of an osmotically stable liposome. In spite of the significantly different properties of B30-MDP and B30-MDPA, a lipid mixture with B30-MDPA, which generates osmotically stable liposomes, was found to be good basic materials for the liposomal vaccines just like those with B30-MDP. Therefore, osmotic stability observed by the turbidimetric method seems to be a good criterion in the screening of basic materials for a variety of liposomal vaccines.

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