Effect of Cetylmannoside Modification on the Alternative Complement Pathway Activation by Liposomes in Rat Serum

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Complement activation is important for removing foreign substances by the mononuclear phagocyte system *in vivo*. The interaction between liposomes and complement components is considered to affect the clearance of liposomes from the circulation. It has been previously demonstrated in our laboratory that multilamellar vesicles (MLV) with surfaces modified by cetylmannoside (Man) were eliminated from the circulation rapidly and showed an approximately 2-fold higher hepatic accumulation compared with control MLV (PC-MLV) (Yamashita *et al.*, *Int. J. Pharmaceut.*, 70, 225, 1991). In this study, we investigated the effect of Man-modification on complement system activation. As far as elimination from the blood is concerned, the initial values of blood liposome concentration were decreased and liposomes were removed from the circulation rapidly in accordance with the extent of the Man content into their membranes. The Man-modification also affected the organ distribution of injected liposomes and their stability in rat serum. Except for MLV containing 50 mol% Man, it was observed that the hepatic uptake of liposomes was enhanced according to the increasing Man content, whereas splenic uptake was decreased and the splenic clearance was comparable. The stability of liposomes in rat serum decreased with increasing Man content. Liposomal instability in rat serum was significantly reduced by preheating the serum at 56°C for 30 min, the treatment with anti-C3 antiserum and with EDTA but not abolished in serum treated with EGTA/MgCl₂. Thus, it is considered that the activation of the complement system through the alternative pathway is facilitated as a result of increasing the Man content in the liposomes. Furthermore, the contribution of the alternative complement pathway activation affected only the affinity of Man-modified MLV for the liver.

Key words activation; cetylmannoside-modification; complement pathway; drug carrier; liposome; stability

Liposomes have been investigated as potential drug carriers in drug delivery systems.^{1,2)} Intravenously injected liposomes become coated with serum proteins after interacting with them. It is generally known that liposomes having different surface characteristics attract different serum proteins and these differences may account for the different ways of removing liposomes from the circulation.³⁻⁵⁾ Chonn *et al.* have demonstrated an inverse relationship between the total amount of blood proteins bound to liposomes and the circulation half-lives of these liposomes.⁶⁾ However, it is not yet clear whether the serum proteins determine the *in vivo* behavior of liposomes. In our laboratory, we demonstrated that liposomes without any surface modification (PC-MLV) activated the alternative complement pathway (ACP) and complement activation resulted in the deposition of fragments of the third complement component (C3) on the surface of liposomes.⁷⁾ Furthermore, it has recently been reported that C3 fragments, deposited on the surface of PC-MLV, serve as an opsonin and participate in the uptake of PC-MLV by the liver. In addition, the extent of serum opsonization through activation of the complement system contributes to the hepatic uptake of PC-MLV.⁸⁾ These results suggest that the interaction of complement components with liposomes has important consequences for the utilization of liposomes as drug carriers and is likely to play a key role in the clearance of liposomes from the circulation by cells of the mononuclear phagocyte system (MPS) such as macrophages.

Targeting liposomes towards macrophages by the incorporating glycolipids into their membranes is an interesting approach for the potential use of liposomes as drug carriers.⁹⁾ Numerous investigators have used

mannose-modified liposomes in order to deliver liposomes to the cells of MPS *in vivo* and *in vitro*, and they showed that their liposomes were taken up by macrophages *via* the mannose receptor.^{10,11)} However, those studies have not taken into account the interaction between serum proteins and liposomes expected as a result of the targeting of mannose-modified liposomes to macrophages *in vivo*. We have demonstrated that cetylmannoside (Man)-modified liposomes (Man-MLV) are eliminated from the circulation rapidly and exhibit an approximately 2-fold higher accumulation in the liver as compared with PC-MLV.¹²⁾ Furthermore, we have recently shown that the deposition of iC3b fragments, cleaved products of C3b, on liposomes contributes to the uptake of liposomes by the liver and that the enhanced hepatic uptake of Man-MLV is mainly mediated by the complement receptor rather than the mannose receptor on the liver macrophages *in situ* as a result of enhancing ACP activation.¹³⁾ These results suggest that modification of the liposome surface with Man increases activation of the complement system and results in a higher hepatic uptake of liposomes.

In this report, we examined the effect of the Man content on the activation of the complement system by liposomes. The data we obtained in this study suggested that the degree of complement activation is gradually enhanced according to the extent of Man-modification of the liposomal surfaces.

MATERIALS AND METHODS

Materials Hydrogenated egg phosphatidylcholine (PC) and Man were kindly supplied by Nippon Fine Chem. Co., Ltd. (Osaka, Japan). Dicetyl phosphate (DCP) and

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cholesterol (CH) were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chem. Ind. Ltd. (Osaka, Japan), respectively. Goat Anti-Rat C3 antiserum was purchased from Organon Teknika Co. (PA, U.S.A.). 5(6)-Carboxyfluorescein (CF) was obtained from Eastman Kodak Co. (NY, U.S.A.). [^3H]Inulin and [^3H]cholesteryl hexadecyl ether were obtained from New England Nuclear Co. (MA, U.S.A.). All other reagents were of commercial analytical grade.

Preparation of Liposomes Liposomes were prepared by the method described previously.¹⁴ The total lipid concentration of liposomes in the preparation was 50 $\mu\text{mol}/\text{ml}$. The molar ratio of Man-MLV was Man/PC/DCP/CH = $X/5-X/1/4$ ($X=1, 3$, or 5 ; 10%, 30% or 50% modification, respectively), and that of PC-MLV was PC/DCP/CH = $5/1/4$. Both types of liposomes were extruded through a polycarbonate membrane (Nuclepore Co., CA, U.S.A.) of pore size 0.8 μm . CF or [^3H]inulin was used as an aqueous marker, and [^3H]cholesteryl hexadecyl ether was employed as liposomal marker. In the case of CF- or [^3H]inulin-encapsulated MLV, non-encapsulated CF and [^3H]inulin were removed by dialysis using cellulose dialyzing tubing (Wako Pure Chem. Ind. Ltd., Osaka, Japan) or a polycarbonate membrane of pore size 0.2 μm against phosphate-buffered saline (PBS).

In Vivo Study of Liposomes Male wistar rats weighing 250 ± 50 g anesthetized with ether were cannulated in the femoral vein and femoral artery. Each operated rat was placed in a Bollman's cage. After recovering from the anesthesia, liposomes were injected through the cannula into the femoral vein. The injected dose was 125 μmol lipid/kg body weight. The samples of blood were drained from the cannula inserted in the artery at 1.5, 3.0, 6.0, 15.0, 30.0, 60.0 and 120.0 min after injection. Liver, lung, spleen and kidney were subsequently removed after the blood sampling at 120 min. Radioactivities of the samples were counted using a liquid scintillation counter (Aloka LSC-602, Tokyo, Japan) as described previously.¹⁵ The area under the blood concentration-time curve (AUC) was calculated using the trapezoidal rule. Hepatic and splenic clearance were calculated by dividing the hepatic uptake and splenic uptake by AUC_{0-2h} .

Liposomal Stability Study in Vitro Normal rat serum was collected from the carotid artery and allowed to clot at room temperature for 60 min, then centrifuged at 1250 g for 10 min at 4°C. Liposomal leakage after the reaction with serum was assessed by determining the fluorescence intensity of the CF according to the method described previously.⁷ Briefly, 100 μl liposome solution (containing 32.5 $\mu\text{mol}/\text{ml}$ of total lipid) was added to 900 μl normal or treated serum and incubated at 37°C. At 0, 5, 15, 30 and 60 min after the addition of liposome solution, 10 μl reaction mixture was sampled and the fluorescence intensity was determined at excitation and emission wavelengths of 490 and 520 nm, respectively. One hundred percent leakage was measured by lysing the liposomes with Triton X-100 (Wako Pure Chem. Ind. Ltd., Osaka, Japan).

Perfusion Study The perfusion study of the effects of Man-modification on hepatic uptake of liposomes was carried out according to the single-pass method described previously in our laboratory.¹³ In brief, the liver of a

male Wistar rat was perfused *in situ* with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 6 mM glucose, which was saturated with 95% O_2 -5% CO_2 , via the portal vein at 37°C. The perfusate was circulated at a constant rate (2.5–3.0 ml/min/g liver). After 10 min pre-perfusion, liposomes were infused via the portal vein at a constant rate for 10 min. Liposomes were incubated with normal serum at a serum-to-liposome ratio of 0.62 ml serum/ μmol lipid at 37°C for 10 min just before injection into the liver. The radioactivity of the samples was counted using a liquid scintillation counter (Aloka LSC-602, Tokyo, Japan).

Statistical Analysis Statistical significance was evaluated by means of Student's *t*-test and $p=0.05$ was taken as the minimum level of significance. Data were expressed as means $\pm$ S.E.

RESULTS

Effect of Man Content on the Elimination of Liposomes from Blood In order to evaluate the effect of the extent of Man-modification on the elimination of liposomes from blood after intravenous administration, liposomes incorporating various amounts of Man in their membranes were prepared and their *in vivo* behavior examined. The elimination of MLV containing 10 mol% Man (10% Man-MLV) from the circulation was almost equal to that of MLV lacking Man (PC-MLV). It was shown that MLV containing 30 mol% Man (30% Man-MLV) was cleared from the circulation rapidly as compared with PC-MLV or 10% Man-MLV. Moreover, MLV containing 50 mol% Man (50% Man-MLV) was cleared most rapidly (Fig. 1). The initial values of the blood concentrations decreased according to the increase in the amount of Man and liposomes were removed from the circulation rapidly in accordance with the extent of the Man content of their membranes (Fig. 1). Hence, the elimination of liposomes from blood is the result of enhanced hepatic uptake of liposomes and/or the increased liposomal instability *in vivo* due to Man-modification.

Effect of Man Content on Biodistribution of Liposomes

Fig. 1. Blood Elimination of Liposomes Containing Various Amounts of Man after Intravenous Administration to Rats

Liposomes consisting of Man/PC/DCP/CH at molar ratios of 0/5/1/4 (PC-MLV), 1/4/1/4/ (10% Man-MLV), 3/2/1/4 (30% Man-MLV) or 5/0/1/4 (50% Man-MLV) were injected at a dose of 125 μmol lipid/kg body weight. The radioactivity of samples was measured as described under Materials and Methods. Each value represents the mean $\pm$ S.E. ($n=3-8$). Symbols; (●) PC-MLV, (Δ) 10% Man-MLV, (○) 30% Man-MLV, (□) 50% Man-MLV.

Fig. 2. Organ Distribution of Liposomes Containing Various Amounts of Man 2 h after Intravenous Administration to Rats

Liposomes were injected at a dose of 125 μmol lipid/kg body weight. The radioactivity of samples was measured as described under Materials and Methods. Each value represents the mean $\pm$ S.E. ($n=3-8$). Symbols: (■) PC-MLV, (▨) 10% Man-MLV, (□) 30% Man-MLV, (▩) 50% Man-MLV. Significant differences from PC-MLV; *, $p < 0.05$.

The organ distribution of liposomes 2 h after intravenous administration is shown in Fig. 2. Enhanced hepatic uptake of liposomes by Man-modification was observed. The 30% Man-MLV showed the highest accumulation (51.4%) in the liver and this was approximately 2-fold higher compared with PC-MLV (Fig. 2). Thus, the rapid elimination from the circulation of Man-MLVs (Fig. 1) is due to higher accumulation in the liver. However, 50% Man-MLV showed the lowest hepatic accumulation (Fig. 2). In the spleen, the accumulation of 10% Man-MLV was comparable with that of PC-MLV, while, 30% and 50% Man-MLV showed lower accumulation compared with PC-MLV. All liposomes showed very little distribution to the lung and kidney (Fig. 2).

Effect of Man-Modification on the Stability of Liposomes

The interaction between liposomes and serum proteins affects the stability of liposomes.^{16,17} It is likely that hepatic uptake of liposomes is enhanced by Man-modification although the liposomal stability *in vivo* is decreased. Then, to confirm that liposomal stability is decreased by Man-modification in the serum, serum-mediated marker release of liposomes was measured. PC-MLV was degraded slightly in the serum after 60 min. The stability of liposomes were gradually decreased according to the increasing Man content into their membranes from 0 to 30%, with the lowest liposomal stability being observed in the case of 50% Man-MLV (Fig. 3). This lowest stability of 50% Man-MLV in the serum is responsible for the lowest hepatic uptake of 50% Man-MLV observed in Fig. 2. These results shown in Fig. 3 demonstrate that the liposomal stability in the serum is decreased by Man-modification. Thus, it seems that the liposomal stability in the serum affects the hepatic uptake of liposomes, since liposomes interact with serum proteins after intravenous administration. The liposomal instability in rat serum was significantly inhibited by preheating the serum at 56 °C for 30 min, treatment with anti-C3 antiserum, with EDTA which deactivates the classical complement pathway (CCP) and ACP (Table 1). In addition, this inhibition was observed to be more effective with the increasing amount of Man in the MLV. In

Fig. 3. CF Release from Liposomes Containing Various Amounts of Man after Being π Incubated with Rat Serum

Liposomes (3.25 μmol lipid/ml, total lipid conc.) were incubated with normal rat serum (90%, v/v) at 37 °C. CF leakage from the liposomes was measured as described under Materials and Methods. Each value represents the mean $\pm$ S.E. ($n=3$). Symbols: (●) PC-MLV, (Δ) 10% Man-MLV, ($\circ$) 30% Man-MLV, ($\square$) 50% Man-MLV.

Table 1. The Alternative Complement Pathway-Mediated Instability of Liposomes in Rat Serum

Treatment	Release (%)			
	PC-MLV	10% Man-MLV	30% Man-MLV	50% Man-MLV
Normal	4.5 $\pm$ 1.3	8.2 $\pm$ 1.6	13.3 $\pm$ 1.1 ^{a)}	49.8 $\pm$ 2.2 ^{a)}
Heating (56 °C/30 min)	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0	0.5 $\pm$ 0.1
Anti-C3 antiserum	0.2 $\pm$ 0.1	0.4 $\pm$ 0.0	1.0 $\pm$ 0.0	10.6 $\pm$ 0.3
EDTA (10 mM)	0.1 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	0.5 $\pm$ 0.2
EGTA/MgCl ₂ (10 mM)	4.6 $\pm$ 0.5	9.9 $\pm$ 0.8	22.1 $\pm$ 1.0 ^{a)}	32.4 $\pm$ 1.4 ^{a)}

Liposomes were incubated with normal or treated rat serum (80%, v/v) at 37 °C for 60 min. CF leakage was determined as described under Materials and Methods. Each value represents the mean $\pm$ S.E. ($n=3$). ^{a)} Significant differences from PC-MLV at $p < 0.01$.

addition it was not abolished in serum containing EGTA and MgCl₂, a condition in which CCP, but not ACP, was inhibited (Table 1). It is generally known that the complement activity of CCP needs both Ca²⁺ and Mg²⁺, while ACP requires only Mg²⁺.¹⁸ These results indicate that the surface modification of liposomes by Man causes potent activation of the complement system through ACP, and ACP activation by the liposomes is effectively proceeds according to the increase in the amount of Man in the liposomes.

Effect of Man-Modification on the Hepatic Accumulation *in Situ* We have previously demonstrated the contribution of complement activation to the hepatic uptake of Man-MLV by perfused rat liver.¹³ Then, the effect of Man content on the hepatic uptake of liposomes was examined using a perfusion technique. [³H]Cholesteryl hexadecyl ether was used as a lipid marker in this study because the uptake of carrier vehicle could be investigated. The hepatic uptake of 10% Man-MLV was comparable with that of PC-MLV. However, 30% and 50% Man-MLV showed a higher hepatic uptake compared with PC-MLV, and the highest hepatic uptake of liposomes was observed with 50% Man-MLV (Fig. 4). As shown in Fig. 3 and Table 1, ACP activation effectively proceeds according to the increasing amount of Man in the liposomes. These results support the considerations mentioned above and the complement-mediated hepatic

Fig. 4. The Effect of Man Content on the Uptake of Pre-opsonized Liposomes by Perfused Rat Liver

Liposomes were opsonized with normal serum at 37°C for 10 min before the perfusion study. The serum-to-liposome ratio was 0.62 ml/ μ mol lipid. The radioactivity of the liver after 10 min perfusion was measured as described in Materials and Methods. Each value represents the mean $\pm$ S.E. ($n=3$).

uptake of liposomes increases with increasing Man content in the liposomes.

DISCUSSION

We have previously demonstrated that 30% Man-MLV showed an approximately 2-fold higher accumulation in the liver as compared with PC-MLV and we suspected that the enhanced hepatic uptake of 30% Man-MLV mainly involved Kupffer cells, mediated by opsonization with plasma protein(s).¹²⁾ Furthermore, the contribution of the increased deposition of iC3b fragments on the 30% Man-MLV, as a result of facilitating ACP activation, to the enhanced hepatic uptake of 30% Man-MLV was demonstrated recently.¹³⁾ These results led us to consider that modification of the liposome surface with Man increases the activation of the complement system *via* ACP and the hepatic uptake of liposomes increases with increasing Man content. Then, we investigated the effect of Man content on the hepatic uptake of liposomes and on the activation of the complement system by liposomes.

Ghosh and Bachhawat showed that the hepatic uptake of their α -mannose liposomes after intravenous injection in mice was higher than that of control liposomes.¹⁹⁾ Moreover, Strik and Baldeschwieler have reported that the density of mannoside in the liposomes is one of the determinant factors on the uptake of liposomes by mouse peritoneal macrophages. In their study, the uptake of MLV by mouse peritoneal macrophages increased according to the increase in the amount of mannoside in the MLV and MLV containing 20 mol% of 6-amino- α -D-mannose showing the highest accumulation.²⁰⁾ Here in this report, we have demonstrated that the hepatic uptake of liposomes increased with increasing Man content from 0 to 30 mol% and liposomes bearing 30 mol% Man showed the maximum value (Fig. 2). On the other hand, the hepatic uptake of 50% Man-MLV decreased on the account of its instability in serum (Fig. 3), although 50% Man-MLV showed the highest uptake by perfused rat liver (Fig. 4). The discrepancy in this liposomal distribution to the liver between *in vivo* and *in situ* is still unclear even now.

However, [3 H]inulin, an aqueous marker, was employed *in vivo* to determine the distribution of drugs encapsulated in liposomes, while [3 H]cholesteryl hexadecyl ether, a lipid marker, was used *in situ* to determine the behavior of liposomes. In addition, hepatic accumulation of liposomes *in vivo* was determined 2 h after intravenous administration, while that *in situ* was measured 10 min after pre-incubation with normal rat serum. It is possible that these differences affect the uptake of 50% Man-MLV by the liver. Hence, taking into account the usefulness as a drug carrier and the liposomal stability of Man-modified liposomes, we believe that 30 mol% is the most appropriate Man content for targeting the liver by liposomes *in vivo*.

It was observed that the blood elimination of liposomes from the circulation was rapid and the initial values of the blood concentrations decreased according to the increasing Man content (Fig. 1). Moreover, the hepatic uptake of liposomes was enhanced by Man-modification except in the case of 50% Man-MLV (Fig. 2). We consider that the elimination of liposomes from blood is result of enhanced hepatic uptake of liposomes and/or in increased liposomal instability *in vivo* produced by Man-modification. Das *et al.* have reported that α -mannoside-modified liposomes (mannoside liposome) are cleared from the circulation rapidly and showed an enhanced hepatic uptake compared with nonglycosylated liposomes.²¹⁾ They considered that the rapid disappearance and enhanced hepatic uptake of mannoside liposomes depended to their uptake by Kupffer cells as a consequence of the interaction of liposomal mannose residues with mannose receptors. However, they did not take into account the interaction between mannoside-modified liposomes and serum proteins. The interaction of liposomes with serum proteins affect the stability of liposomes^{16,17)} and may affect the clearance of liposomes by cells of MPS *in vivo*. The hepatic uptake of PC-MLV, without any surface modification, was shown to be mediated by complement-mediated phagocytosis.²²⁾ So, the interaction between complement components and Man-modified liposomes is considered one of the most important factors on the hepatic uptake of them *in vivo*. Actually, it was observed that the ACP-mediated lysis of Man-MLV was enhanced with increasing Man content from 0 to 50 mol% in the liposomes (Fig. 3, Table 1). Simultaneously, the hepatic clearance of Man-MLV was increased from 0.135 to 0.619 ml/min when the amount of Man in the liposomes was increased from 0 to 30 mol%, although the 50% Man-MLV showed a low hepatic clearance (0.292 ml/min). In the spleen, the accumulation of injected liposomes decreased with increasing Man content although splenic macrophages probably have mannose receptors on their surfaces (Fig. 2). This low splenic accumulation of Man-modified liposomes was, however, not due to the low affinity of Man-modified liposomes for the spleen since the splenic clearance of PC-MLV, 10% Man-MLV and 30% Man-MLV was 0.068, 0.060 and 0.068 ml/min, respectively. In addition, the splenic clearance of 50% Man-MLV was low (0.042 ml/min). These results demonstrate that Man-modification of liposomal surfaces enhances only the

hepatic clearance and not the splenic clearance. It has been reported that phagocytosis by liver macrophages is mainly mediated by complement receptors, whereas that of splenic macrophages is by Fc receptors.²³⁾ Moreover, we have recently demonstrated that the increased deposition of iC3b fragments on the surface of 30% Man-MLV through ACP activation is responsible for their higher hepatic uptake.¹³⁾ Thus, it seems that the ACP activation *in vivo* is facilitated as a result of increasing the Man content in the liposomes and it contributes only to the uptake of Man-MLV by the liver.

In conclusion, the activation of the complement system through ACP mainly contributes to the hepatic uptake of Man-modified liposomes and complement component C3, serve as opsonin, plays a key role on the enhanced uptake of Man-modified liposomes by the liver. Furthermore, the activation of the complement system through ACP was affected by the extent of Man-modification. We also demonstrated that the activation of the complement system through ACP influences both the uptake of liposomes by the liver and their stability *in vivo*. Considering their usefulness as a drug carrier and the liposomal stability of Man-modified liposomes, we believe that 30 mol% Man is the most appropriate Man content for targeting the liver by liposomes *in vivo*. In this study, it was shown that in spite of the liposomes having surfaces modified by mannoside residues for effective delivery of liposomes to macrophages, mannoside-modified liposomes were taken up by macrophages through the complement system, an important self-defence system *in vivo*, but not taken up simply by mannose receptors on the surface of macrophages. Thus, the interaction between mannoside-modified liposomes and serum proteins is great important when using them as a drug carriers *in vivo*.

REFERENCES

- 1) Gregoriadis G., Florence A. T., *Drugs*, **45**, 15 (1993).
- 2) Juliano R. L., Akhtar S., *Antisense Res. Dev.*, **2**, 165 (1992).
- 3) Patel H. M., *Crit. Rev. Ther. Drug Carrier Syst.*, **9**, 39 (1992).
- 4) Juliano R. L., "Liposomes," ed. by Ostro M. J., Marcel Dekker, Inc., New York, 1983, pp. 53—86.
- 5) Bonte F., Juliano R. L., *Chem. Phys. Lipids*, **40**, 359 (1986).
- 6) Chonn A., Semple S. C., Cullis P. R., *J. Biol. Chem.*, **267**, 18759 (1992).
- 7) Funato K., Yoda R., Kiwada H., *Biochim. Biophys. Acta*, **1103**, 198 (1992).
- 8) Harashima H., Sakata K., Kiwada H., *Pharm. Res.*, **10**, 606 (1992).
- 9) Jones M. N., *Adv. Drug Del. Rev.*, **13**, 215 (1994).
- 10) Moonis M., Ahmad I., Bachhawat B. K., *J. Drug Targeting*, **1**, 147 (1993).
- 11) Muller C. D., Schuber F., *Biochim. Biophys. Acta*, **986**, 97 (1989).
- 12) Yamashita C., Matsuo H., Akiyama K., Kiwada H., *Int. J. Pharmaceut.*, **70**, 225 (1991).
- 13) Matsuo H., Funato K., Harashima H., Kiwada H., *J. Drug Targeting*, **2**, 141 (1994).
- 14) Kiwada H., Niimura H., Fujisaka Y., Yamada S., Kato Y., *Chem. Pharm. Bull.*, **33**, 753 (1985).
- 15) Sato Y., Kiwada H., Kato Y., *Chem. Pharm. Bull.*, **34**, 4244 (1986).
- 16) Hernandez-Caselles T., Villalain J., Gomez-Fernandez J. C., *Mol. Cell. Biochem.*, **120**, 119 (1993).
- 17) Jones M. N., Nicholas A. R., *Biochim. Biophys. Acta*, **1065**, 145 (1991).
- 18) Okada N., Yasuda T., Tsumita T., Okada H., *Immunology*, **45**, 115 (1982).
- 19) Ghosh P., Bachhawat B. K., *Biochim. Biophys. Acta*, **632**, 562 (1980).
- 20) Stirk A. H., Baldeschwieler J. D., "Medical Application of Liposomes," ed. by Yagi K., Japan Scientific Societies, Tokyo, 1986, pp. 31—41.
- 21) Das P. K., Murray G. J., Zirzow G. C., Brandy R. O., Barranger J. A., *Biochem. Med.*, **33**, 124 (1985).
- 22) Harashima H., Sakata K., Funato K., Kiwada H., *Pharm. Res.*, **11**, 402 (1994).
- 23) Juliano R. L., *Adv. Drug Del. Rev.*, **2**, 31 (1988).