Influence of Cholesterol Composition on the Association of Serum Mannan-Binding Proteins with Mannosylated Liposomes

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In our previous studies, serum mannan-binding protein (MBP) accelerated the uptake by cultured macrophages. The present study was initiated to investigate the kinetics of molecular interaction between mannosylated liposomes and MBP in more details and the effects of lipid composition on the interaction. The analysis was carried out by surface plasmon spectroscopy (SPR) methods, using rabbit serum MBP isolated by affinity chromatography. In SPR studies, neither conventional liposomes nor galactosylated liposomes indicated any interaction, but each mannosylated liposomes had a high response signal corresponding to molecular interaction with immobilized MBP. Association of mannosylated liposomes to serum MBP was not dependent on the lipid composition, suggesting a diffusion-controlled association. Dissociation of the mannosylated liposomes from serum MBP was extremely slow. DSPC/Chol/Man-C4-Chol (90 : 5 : 5, molar ratio) exhibited a slower dissociation rate than DSPC/Chol/Man-C4-Chol (60 : 35 : 5). Clustering of mannose residues on liposomal surfaces might be important in determining the binding affinity of mannosylated liposomes with MBP.

Key words serum mannan-binding protein; surface plasmon resonance; mannosylated liposome; intermolecular interaction

Mannosylated liposome is a promising approach to delivering drugs,11,3 antigens,21 and genes3 in vivo mannozze receptor to macrophages, which play biologically important roles in the induction of the immune response by their capacity to ingest antigens, present them to T-cells and induce secretion of co-stimulatory molecules required for T-cell activation. Several investigators have succeeded in therapy of visceral leishmaniasis and control of cancer metastases to the liver by drugs delivered to macrophage using mannosylated liposomes. We also developed a novel mannosylated cholesterol derivative, cholest-5-ylexy-N-(4-(1-imino-2-β-D-thiomannosyethyl)amino)butyl)formamide (Man-C4-Chol), for macrophage-selective delivery of drugs and genes.8—11)

Our biodistribution study12) demonstrated that, when the liposome (Man-liposome) consisting of Man-C4-Chol, neutral distearoyl phosphatidylcholine (DSPC), and cholesterol (Chol) was injected intravenously in mice, it was rapidly eliminated from the blood circulation and taken up by the liver to a much greater extent than DSPC/Chol liposome. Moreover, it revealed that the hepatic uptake occurred mainly due to the liver nonparenchymal cells expressing mannozze receptors. On the other hand, we found that mannosylated proteins and liposomes (Man-liposomes), when injected intravenously, interact with serum mannan-binding proteins (MBP).13,14) MBP is a mannozze-specific lectin that circulates via mannozze receptor to macrophages, which play biologically important roles in the induction of the immune response by their capacity to ingest antigens, present them to T-cells and induce secretion of co-stimulatory molecules required for T-cell activation. Several investigators have succeeded in therapy of visceral leishmaniasis and control of cancer metastases to the liver by drugs delivered to macrophage using mannosylated liposomes. We also developed a novel mannosylated cholesterol derivative, cholest-5-ylexy-N-(4-(1-imino-2-β-D-thiomannosyethyl)amino)butyl)formamide (Man-C4-Chol), for macrophage-selective delivery of drugs and genes.8—11)

The present study was initiated to delineate the kinetics of binding between Man-liposomes and MBP in detail. A surface plasmon resonance (SPR) spectroscopy was used, since the method provides high-quality estimations of molecular binding kinetics, i.e., association and dissociation kinetics. It has been applied to study biomolecular interactions including receptor-ligand, DNA-protein, and protein–protein interaction.15 Lipid composition of liposomes has been known to be an important factor of phagocytosis or receptor-mediated uptake process.21) Therefore, we prepared a series of Man-liposomes varying in the cholesterol content and compared in vitro kinetic profiles among them. We report that the association kinetics of Man-liposomes with MBP might be primarily diffusion-limited and the dissociation of Man-liposomes from MBP might be affected by clustering of mannose residues, in comparative studies using Man-liposomes varying their lipid composition.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials DSPC and cholesteryl chloroformate were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Chol was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were reagent grade products obtained commercially.

Synthesis of Man-C4-Chol Man-C4-Chol was synthesized by the method described previously.8—11) Briefly, cholesteryl chloroformate was reacted with N-(4-aminobutyl)-carbamic acid tert-butyl ester in chloroform at room temperature for 24 h and then incubated with trifluoroacetic acid at 4 C for 4 h to obtain N-(4-aminobutyl)-(cholest-5-ylexy)formamide. After the solvent was evaporated, pyridine containing an excess amount of 2-imino-2-methoxethyl-1-thiomannoside and triethylamine was added to the resultant material. Following the reaction at room temperature for 24 h, the reaction mixture was evaporated, resuspended in

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Preparation of Liposomes DSPC/Chol (60 : 40, molar ratio), DSPC/Chol (90 : 10), DSPC/Chol/Man-C4-Chol (60 : 35 : 5), and DSPC/Chol/Man-C4-Chol (90 : 5 : 5) liposomes were prepared by the method described previously. Briefly, the lipids were dissolved in chloroform, evaporated to form a thin-layer film in a round-bottomed flask, and vacuum-desiccated. The lipid film formed was hydrated in HEPES solution (150 mM NaCl and 10 mM HEPES, pH 7.4), sonicated at 65 °C for 3 min with a bath sonicator, and extended at 65 °C each five times through 200 nm and 100 nm pore size polycarbonate membranes, respectively. Mean particle diameters were determined using a laser light scattering particle size analyzer (LS-900, Otsuka Electronics, Osaka, Japan).

Isolation of MBP from Rabbit Serum MBP was isolated from normal rabbit serum (Japan Bio-supply) using a Sepharose-Man-IgG column according to the procedure reported by Kawasaki et al. Briefly, mannosylated immunoglobulin G (Man-IgG) was coupled with N-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden) according to the instructions of the manufacturer. The rabbit serum was diluted with 1 M NaCl for 1 min each by three times. Biotinylated rabbit serum MBP was mounted onto a blank flow cell to monitor the background level of the refractive index. Liposome solutions were prepared to the solution containing 1 mM CaCl₂ just before SPR studies, in order to minimize aggregation of liposomes in the presence of Ca²⁺. The liposome solutions were adjusted to each concentration by using a running buffer (150 mM NaCl, 1 mM CaCl₂, 10 mM HEPES, pH 7.4) and flowed at 20 ml/min at 25 °C for 3 min. Each concentration was separated with 3 min and regenerated by injection of 20 µl of 20 mM EDTA solution (pH 7.4) containing 0.5% Triton X-100. All buffers were filtered and deoxygenated. The binding ability of immobilized MBP was not impaired by this regeneration procedure, since the binding of Man-liposomes was constant from beginning to end.

Real-Time SPR Spectroscopy SPR measurements were performed using a BIAcore X system with a sensor chip SA (BIAcore, Uppsala, Sweden). The sensor chip consisted of a gold surface coated with carboxymethylated dextran that was activated with streptavidin. Prior to the experiment, the streptavidin surface was first washed with 50 mM NaOH containing 1 mM NaCl for 1 min each by three times. Biotinylated rabbit serum MBP was injected manually over a single flow cell at a flow rate of 5 µl/min until a resonance unit value of approximately 2000 was obtained. An untreated sensor chip SA with MBP was mounted onto a blank flow cell to monitor the background level of the refractive index. Liposome solutions were prepared to the solution containing 1 mM CaCl₂ just before SPR studies, in order to minimize aggregation of liposomes, and was added to the rabbit serum MBP solution to yield a 1 : 10 molar ratio of rabbit serum MBP and NHS-biotin. The mixture was incubated on ice for 4 h. Unreacted materials were removed using Centricon YM-30 (Millipore, Bedford, U.S.A.). The apparent biotinylation ratio was determined by reacting biotinylated MBP with an excess of 2-(4'-hydroxyphenyl)-azobenzoic acid (HABA) and avidin (Pierce Chemical Co, Rockford, U.S.A.) by the method of Green. As HABA bound to avidin is displaced with biotin-MBP, the 500-nm absorbance associated with HABA bound to avidin is reduced.

RESULTS

Isolation and Immobilization onto a Sensor Chip of Rabbit Serum MBP The proteins isolated from rabbit serum according to the above-mentioned procedure were subjected to SDS-PAGE (Fig. 1). Only a single band (ca. 30 kDa) was detected under reducing conditions, whereas several high-molecular-mass bands were seen under non-reducing conditions. These observations were consistent with the reports of Kawasaki et al. who first identified a serum type MBP. The isolated rabbit serum MBP was biotinylated using NHS-biotin in the presence of excess mannose that protects amino residues in the carbohydrate recognition domain from being coupled with the biotinylation agent. The
apparent biotinylation ratio of the protein was approximately 1.3 mol/mol which were determined by reaction with avidin bound HABA reagent [2-(4-hydroxyphenyl)azobenzoic acid]. Biotinylated rabbit serum MBP was immobilized onto the surface of a sensor chip SA coated with streptavidin. In the surface plasmon resonance spectroscopy, nonspecific binding of an analyte and bulk refractive index change were ruled out by using a native sensor chip SA as the control.

**Size and Stability of Liposomes** Table 1 summarizes the lipid composition and particle size of the liposomes prepared. All liposomes were similar in size (an average diameter of approximately 100 nm). When the liposomes were preserved in HEPES solution (150 mM NaCl and 10 mM HEPES, pH 7.4) at 4 °C, the size of the liposomes remained constant for more than one month. Although the liposomal solution was adjusted to 1 mM Ca²⁺ solution just before binding studies, aggregation of liposomes were not detected during the experiments.

### Table 1. Lipid Composition and Mean Particle Size of Liposomes Investigated

<table>
<thead>
<tr>
<th>Lipid composition (molar ratio)</th>
<th>Particle size (nm)</th>
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<tbody>
<tr>
<td>DSPC/Chol (60 : 40)</td>
<td>106.3 ± 5.8</td>
</tr>
<tr>
<td>DSPC/Chol (90 : 10)</td>
<td>101.9 ± 8.3</td>
</tr>
<tr>
<td>SPC/Chol/Gal-C4-Chol (60 : 35 : 5)</td>
<td>103.5 ± 7.7</td>
</tr>
<tr>
<td>DSPC/Chol/Man-C4-Chol (60 : 35 : 5)</td>
<td>110.3 ± 5.9</td>
</tr>
<tr>
<td>DSPC/Chol/Man-C4-Chol (90 : 5 : 5)</td>
<td>100.9 ± 3.8</td>
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</table>

**Specific Binding of Man-Liposomes to Immobilized Rabbit Serum MBP**

To determine whether Man-liposomes bind to immobilized rabbit MBP in a specific manner, competitive inhibition experiments with mannose were performed (Fig. 2A). After infusion of DSPC/Chol/Man-C4-Chol (90 : 5 : 5) was started, the SPR response signal increased in a time-dependent manner. Three minutes later, the infusion solution was switched back to the running buffer that did not contain the liposome. The decrease in the response signal was very slow, suggesting that the Man-liposome was tightly associated with MBP. When 1 mM mannose was infused with the liposome, the response signal was less than half of the control (Fig. 2A). On the other hand, the liposomes lacking mannose residues, such as DSPC/Chol (60 : 40), DSPC/Chol (90 : 10), and DSPC/Chol/Gal-C4-Chol (65 : 35 : 5) showed no detectable response signal (Fig. 2B). These results indicated that the Man-liposome specifically interacted with MBP via mannose residues on the surface.

**Effect of Cholesterol on the Interaction between Man-Liposomes and Rabbit Serum MBP**

Figure 3 shows the binding patterns of DSPC/Chol/Man-C4-Chol (60 : 35 : 5) and DSPC/Chol/Man-C4-Chol (90 : 5 : 5) to rabbit MBP. Man-liposomes were infused at various concentrations (100, 150, 200, 250, 300 μM). The association of Man-liposomes with a sensor chip increased in a time- and concentration-dependent manner. DSPC/Chol/Man-C4-Chol (90 : 5 : 5) exhibited a larger intensity of the response signal than DSPC/Chol/Man-C4-Chol (60 : 35 : 5). When test solutions containing Man-liposomes were switched to the running buffer at 3 min, dissociation of Man-liposomes from the sensor chip occurred. However, the dissociation rate was extremely slow, especially for DSPC/Chol/Man-C4-Chol (90 : 5 : 5).

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**References**

Purified binding protein was subjected to 12.5% polyacrylamide gel electrophoresis under reducing condition (β-mercaptoethanol). Molecular weight marker in lane 1, MBP from rabbit serum in lane 2. The binding protein band has an apparent molecular weight of approx. 32 kDa.

Three hundred micromolars liposomes were injected over immobilized rabbit MBP. Each sensorgram was overlaid and zeroed on the y-axis to the average baseline. The start injection time for each sample was set to zero on x-axis.

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Each sensorgram was analyzed by globally fitting using a 1:1 (Langmuir) binding model-derived equation (Table 2). The association-rate constant ($k_a$) was similar in both Man-liposomes, suggesting that the association process be diffusion-limited. On the other hand, the dissociation-rate constant ($k_d$) of DSPC/Chol/Man-C4-Chol (60 : 35 : 5) was three-time larger than that of DSPC/Chol/Man-C4-Chol (90 : 5 : 5). These kinetic analysis revealed that larger binding of DSPC/Chol/Man-C4-Chol (90 : 5 : 5) to immobilized serum MBP was primarily due to slower dissociation as compared to DSPC/Chol/Man-C4-Chol (60 : 35 : 5). The dissociation constant of DSPC/Chol/Man-C4-Chol (90 : 5 : 5) and DSPC/Chol/Man-C4-Chol (60 : 35 : 5) was 5.37×10^{-6} and 20.5×10^{-6} M, respectively.

### Table 2. Rate Constants and Dissociation Constant for the Interaction between Immobilized Rabbit MBP and Mannosylated Liposomes

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Rate constants ($k_a$ (s^{-1} s^{-1})</th>
<th>$k_d$×10^6 (s^{-1})</th>
<th>$K_d$×10^6 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC/Chol/Man-C4-Chol (60 : 35 : 5)</td>
<td>36.9±2.31</td>
<td>7.51±0.78</td>
<td>20.5±3.11</td>
</tr>
<tr>
<td>DSPC/Chol/Man-C4-Chol (90 : 5 : 5)</td>
<td>41.5±8.86</td>
<td>2.36±1.48</td>
<td>5.37±2.49</td>
</tr>
</tbody>
</table>

$k_a$ and $k_d$ represent association- and dissociation-rate constants, respectively. $k_a$ and $k_d$ were calculated based upon the mole number of total lipids. Results are expressed as the mean±S.D. of three replicates.

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### DISCUSSION

Serum MBP is known to be a member of the innate immune system and initiate complement desposition on relevant surfaces. It discriminates between self and non-self, by recognizing certain patterns of carbohydrate structures. Serum MBP is an oligomer of polypeptide chains that are composed of a collagen-like region and carbohydrate recognition domain (CRD). The monomer of MBP (32 kDa) contains only one carbohydrate recognition domain (CRD) at the COOH-terminus, three monomers are held together by interaction with the α-helical neck region, and two to six sets of the trimer form the bouquet-like structure of MBP. Thus, the assembly of CRDs have the ability of react with patterns of carbohydrates. MBP does not selectively recognize only mannose or its multimers; and the CRDs also bind to N-acetyl-d-glucosamine and L-fucose.

It has been postulated that MBP functions directly as an opsonin and the MBP/pathogen complexes are phagocytosed by the collectin receptor following conformational change of MBP. In our previous study, when 111In-labeled MBP was injected intravenously into mice after preincubation with Man-liposomes, in vivo hepatic uptake of the protein was larger than that of non-treated MBP. It was also revealed that the presence of MBP accelerated the uptake of Man-liposomes by cultured mouse peritoneal macrophages. These data suggested that MBP be complexed with Man-liposome and taken up by macrophages.

**Fig. 3.** SPR Sensorgrams of Binding with Isolated Rabbit Serum Mannan Binding Protein (MBP) for DSPC/Chol/Man-C4-Chol (60 : 35 : 5) and DSPC/Chol/Man-C4-Chol (90 : 5 : 5) Liposomes

Varying concentrations (100, 150, 200, 250, 300 μM) of the liposomes were injected over immobilized rabbit MBP. As the concentration increased, the response signal was greater.

**Fig. 4.** Differential Scanning Calorimetry Analysis of (A) DSPC/Chol/Man-C4-Chol (60 : 35 : 5), (B) DSPC/Chol/Man-C4-Chol (90 : 5 : 5), and (C) DSPC Liposomes

**Fig. 5.** DSC thermograms of DSPC/Chol/Man-C4-Chol (60 : 35 : 5), DSPC/Chol/Man-C4-Chol (90 : 5 : 5), and DSPC liposomes.
The present study using SPR spectroscopy demonstrated that Man-liposome directly binds to MBP in a mannose-specific manner (Fig. 2), and the dissociation constant (Total lipids) was calculated (Table 2). When the dissociation constant was evaluated using the apparent concentration of mannoside (Man-liposome) was calculated (Table 2). When the dissociation constant was determined basically by diffusion across an unstirred layer formed at the vicinity of the sensor chip surface. Since these liposomes were similar in size (Table 1), it would be reasonable that they gave similar association-rate constants. In contrast, the dissociation rate would be determined by a ligand/receptor binding energy. Since these Man-liposomes contained the same amount of Man-Chol, the probability of immobilized MBP to meet with a mannoside residue would be essentially the same. Therefore, the difference in the dissociation rate between the two is rather complex.

MBP is an oligomeric protein that reacts with patterns of carbohydrates. As suggested by the dissociation constant of binding between Man-liposomes and MBP, their binding would also be multivalent. Therefore, a possible explanation for the difference in binding characteristics between DSPC/Chol/Man-C4-Chol (60 : 5 : 5) and DSPC/Chol/Man-C4-Chol (60 : 5 : 5), whereas both liposomes gave similar association-rate constants. The association rate would be determined using the endothermic peak was observed for cholesterol component.32,33) In fact, the DSC analysis for cholesterol component.32,33) In fact, the DSC analysis for cholesterol composition of Man-liposomes might cause the distribution and mobility of Man-C4-Chol on the surface and thereby have a great effect on the binding kinetics and affinity with serum MBP.

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