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Involvement of serum mannan binding proteins and mannose receptors in uptake of mannosylated liposomes by macrophages

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Abstract

The roles of serum mannan binding protein (MBP) and the mannose receptor in the cellular uptake of mannosylated liposomes (Man-liposomes) by macrophages were studied. Man-liposomes were prepared by incorporating cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosylethyl)amino)butyl)formamide (Man-C4-Chol) into small unilamellar long circulating liposomes consisting of cholesterol (Chol) and distearoyl phosphatidylcholine (DSPC). In the in vitro cellular uptake study with cultured mouse peritoneal macrophages, [^{3}H]Man-liposomes were taken up to a great extent, whereas no significant uptake was observed for [^{3}H]cholesterol and DSPC liposomes without Man-C4-Chol (Bare-liposomes). The uptake of [^{3}H]Man-liposomes was dose- and temperature-dependent and inhibited by an excess of mannosylated bovine serum albumin, suggesting their specific uptake via membrane mannose receptor-mediated endocytosis. Furthermore, it was demonstrated that ^{111}In-MBP binds strongly to Man-liposomes based on the recognition of Man-C4-Chol and markedly enhanced their uptake by macrophages. These results are supported by confocal laser microscopic images. In addition, in vivo hepatic uptake of ^{111}In-MBP was enhanced by Man-liposomes. On the other hand, the uptake of Man-liposomes was significantly reduced by preincubation with serum and further with MBP-depleted serum suggesting inhibitory effects of serum proteins such as albumin on mannose receptor-mediated endocytosis. The involvement of serum-type MBP and membrane mannose receptors in the uptake of Man-liposomes is thus suggested.

Keywords: Mannosylated liposome; Peritoneal macrophage; Mannose receptor; Mannan binding protein

1. Introduction

The use of liposomes coated with ligands for receptors on cells is a promising approach for cell-specific drug delivery. Among the different receptors present on the surface of macrophages, the lectin specific for mannose residues is a promising candidate for liposomal targeting. Several investigators, including us, have reported using mannose-modified liposomes to deliver drugs, gene, antigens, and immunomodulators to macrophages [1–3] and demonstrated their uptake by macrophages via mannose receptor-mediated endocytosis. The mannose receptor is a 175 kDa type I transmembrane protein and one of the C-type lectin family containing multiple carbohydrate recognition domains. It is expressed on

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Kupffer cells [4], alveolar [5], peritoneal [6], and splenic macrophages [7], monocyte-derived dendritic cells [8], and subsets of vascular and lymphatic endothelial cells [9]. Several in vivo functions have been proposed for the mannose receptor on macrophages, e.g. endocytosis of extracellular peroxidases and hydrolysis during the resolution phase of inflammation, phagocytosis of unopsonized pathogens [10], and antigen capture for eventual presentation to T cells [11].

Besides mannose receptors, mannan binding proteins (MBPs) also bind to a variety of pathogens by recognizing D-mannose, *N*-acetyl-D-glucosamine, or L-fucose on their surface [12]. Serum-type MBP is characterized as an oligomeric serum protein of hepatic origin and belongs to the family of Ca^{2+}-dependent collagenous lectins, most of which are components of the innate immune systems of mammals [13]. It can also activate the complement system following binding to its ligands in an antibody- or C1q-independent manner (opsonin-dependent) [14].

In a series of our investigations, we have established a novel method of introducing sugar moieties directly into cholesterol derivatives and synthesized cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosylethyl)amino)alkyl)formamide (Man-C4-Chol) [15]. The novel mannosylated liposomes, consisting of Man-C4-Chol, neutral distearoyl phosphatidylcholine (DSPC), and cholesterol(Chol) (Man-liposomes), were rapidly eliminated from the blood circulation and accumulate to a great extent in the liver after intravenous administration to mice compared with DSPC/Chol liposomes [16]. It has also been suggested that uptake is mainly due to the liver nonparenchymal cells and is significantly inhibited by preadministration of mannosylated bovine serum albumin (Man-BSA). In the present study, we describe the hepatic uptake characteristics of Man-liposomes in more detail. In particular, our discussion will focus on the involvement of serum MBP and the macrophage membrane mannose receptor.

2. Materials and methods

2.1. Materials

DSPC and cholesteryl chloroformate were purchased from Sigma Chemical (St. Louis, MO, USA). Chol was obtained from Nacalai Tesque (Kyoto, Japan). [^{3}H]Cholesteryl hexadecyl ether (CHE) was purchased from NEN Life Science Products (Boston, MA, USA). Indium chloride (^{111}In) was supplied by Nihon MedioPhysics (Takarazuka, Japan). Man-BSA with an average degree of conjugation of 46 molecules per albumin molecule was synthesized as reported previously [17]. All other chemicals were reagent grade products obtained commercially. Male ICR mice and ddY mice were obtained from the Shizuoka Agriculture Cooperative Association for Laboratory Animals (Shizuoka, Japan).

2.2. Synthesis of Man-C4-Chol

Man-C4-Chol was synthesized by the method described previously [16]. 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. *N*-(4-Aminobutyl)-(cholesten-5-yloxy)formamide was obtained after evaporation of the solvent. A quantity of the resultant material was added to an excess amount of 2-imino-2-methoxyethyl-1-thiomannoside [17] in pyridine containing triethylamine. After 24 h at room temperature, the reaction mixture was evaporated, resuspended in water and dialyzed to remove any unreacted compound and, finally, lyophilized.

2.3. Preparation of liposomes

DSPC/Chol (60:40, molar ratio) and DSPC/Chol/Man-C4-Chol (60:35:5, molar ratio) liposomes were prepared by the method described previously [18]. Briefly, lipids with a trace amount of [^{3}H]CHE were dissolved in chloroform and evaporated to dryness in a round-bottomed flask. For confocal microscopy, fluorescent PE-rhodamine (1 mol%) was added to the chloroform. The dried lipid film was then vacuum-desiccated for at least 4 h to remove any residual organic solvent before it was hydrated in phosphate-buffered saline (PBS, pH 7.4). The suspension was sonicated at 65°C for 3 min, then extruded five times at 65°C through 200 nm and 100 nm pore size polycarbonate membranes. The mean vesicle diame-

ters were determined using a laser light scattering particle size analyzer (LS-900, Otsuka Electronics, Osaka, Japan). The lipid compositions and mean particle sizes are shown in Table 1.

2.4. Preparation of serum and MBP-depleted serum

Blood was obtained from the vena cava of ICR mice under ether anesthesia and left at room temperature for 1 h, then kept overnight at 4°C. Serum was obtained by centrifugation at $10\,000 \times g$ for 10 min at 4°C then frozen at −20°C until required. MBP-depleted serum was obtained by applying the serum twice, at a rate of 1 drop/15 s, to an affinity column of mannan Sepharose 4B that had been equilibrated with loading buffer containing 1.25 M NaCl, 20 mM $CaCl_2$, and 20 mM imidazole. Then the column was washed with elution buffer containing 20 mM imidazole–HCl, pH 7.8, 1.25 M NaCl, and 2 mM EDTA. Liposomes were incubated with serum at 37°C for 10 min before starting the uptake experiment.

2.5. Harvesting and culture of macrophages

Resident macrophages were collected from the peritoneal cavity of male ICR mice weighing 20–25 g using balanced salt solution A. Washed cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, Irvine, UK), penicillin G (100 U/ml), and streptomycin (100 μg/ml) and then plated on 12-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) at a density of 1×10^6 cells/cm^2. After incubation for 2 h at 37°C in 5% CO_2–95% air, adherent macrophages were washed with culture medium and then cultured under the same conditions.

2.6. Cellular uptake study

Uptake studies were carried out after 48 h cultivation. Cells were washed twice with 1 ml pH 7.4 Hanks' balanced salt solution (HBSS). The liposomes were preincubated at 37°C for 10 min with buffer, MBP, serum, or MBP-depleted serum before cellular uptake studies. The opsonized liposomes were added to the cells and incubated at 37°C for the uptake experiments or incubated at 4°C for the binding experiments. At the end of the incubation period, the medium was removed and the cells were washed five times with cold HBSS. The cells were solubilized with 0.5 ml 1 N NaOH solution overnight and then neutralized with 0.1 ml 5 N HCl solution. The radioactivity of [^{3}H]CHE was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan) after addition of 5.0 ml Clear-Sol I solution. The protein content was also measured by the Bradford method using BSA as a standard [19].

2.7. Isolation of mannose binding proteins from rabbit serum and ^{111}In or FITC labeling

Serum MBP was isolated from normal rabbit serum (Japan Bio-supply) using an affinity column of Sepharose 4B–mannan, as described previously [20]. Briefly, frozen rabbit serum was thawed and diluted with an equal volume of a buffer consisting of 40 mM imidazole–HCl, pH 7.8, 40 mM $CaCl_2 \cdot 2H_2O$, and 2.5 M NaCl. The mixture was applied to the Sepharose 4B–mannan column equilibrated with loading buffer (20 mM imidazole–HCl, pH 7.8, 20 mM $CaCl_2 \cdot 2H_2O$, and 1.25 M NaCl) and the bound protein was eluted with an elution buffer (20 mM imidazole–HCl, pH 7.8, 1.25 M NaCl, and 2 mM EDTA). The eluate was applied to the second and third smaller affinity columns of Sepharose 4B–man-

Table 1
Lipid composition and mean particle size of the tested liposomes

Lipid composition (molar ratio)	Particle size (nm)[a]
DSPC/Chol (60:40) (Bare-liposomes)	89.4 ± 5.7
DSPC/Chol/Man-C4-Chol (60:35:5) (Man-liposomes)	94.5 ± 10.9

[a]The mean particle sizes of the liposomes were measured using a laser light scattering particle size analyzer. Results are expressed as the mean ± S.D. of three experiments.

nan. The final column was washed with loading buffer and eluted with more loading buffer containing 100 mM mannose. All the procedures were carried out at 4°C.

The isolated protein was subjected to SDS–PAGE (10% w/v acrylamide) under reducing and non-reducing conditions by the method of Laemmli [21]. Molecular weights were estimated by comparison with Rainbow marker proteins (Amersham Life Sciences).

MBP was radiolabeled with ^{111}In using the bifunctional chelating agent diethylenetriaminepentaacetic acid (DTPA) anhydride (Dojindo Labs, Kumamoto, Japan) by the method of Hnatowich et al. [22]. Briefly, 10 μl DTPA anhydride in dimethyl sulfoxide was added to MBP solution. The mixture was stirred for 30 min at room temperature, purified by gel filtration chromatography using a PD-10 column (Pharmacia, Uppsala, Sweden), and eluted with 50 mM HEPES buffer (pH 7.8) containing 600 mM NaCl to remove the unreacted DTPA. The fractions containing protein derivatives were collected and $^{111}InCl_3$ solution was added to the DTPA-coupled MBP. After 30 min, the mixture was purified by passing it through a PD-10 column and eluting with 50 mM HEPES buffer (pH 7.8) containing 600 mM NaCl. The fractions containing ^{111}In-MBP were collected and the protein concentration was determined by the Bradford method using BSA as a standard [19].

For the confocal microscopic study, MBP was labeled with fluorescein isothiocyanate (FITC) as described previously [23]. 200 μg MBP was incubated with 500 μg FITC in 50 mM borate buffer (pH 9.5) containing 200 mM NaCl, 20 mM $CaCl_2$, and 100 mM mannose for 18 h at 4°C. The fractions containing FITC-MBP were collected from a PD-10 column and purified by dialysis with water at 4°C overnight.

2.8. Binding of ^{111}In-MBP to liposomes

The binding of ^{111}In-MBP to liposomes was tested by the sedimentation method [24]. The liposomes (500 μg) and the ^{111}In-MBP (1 μg) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 20 mg/ml BSA were separately centrifuged at $10\,000\times g$ for 10 min. In the inhibition experiment, 10 mM EDTA or 100 mM mannose was added in Tris buffer. Each liposome pellet was then suspended in 250 μl of the supernatant from the ^{111}In-MBP solution. The liposome–protein mixture was incubated for 1 h at room temperature, placed on ice, and incubated for 15 min. The mixture was centrifuged at $10\,000\times g$ for 10 min at 4°C. The supernatant was stored and the precipitate was washed once with 250 μl ice-cold Tris buffer. After centrifuging, the supernatants were combined. The amount of ^{111}In-MBP bound to the liposomes was estimated by counting the radioactivity in a well-type NaI scintillation counter (ARC-500, Aloka Co., Tokyo, Japan).

2.9. Confocal microscopy

For the confocal microscopy study, resident macrophages were cultured at a density of 1×10^5 cells/well on a glass-bottom 12-well plate and incubated at 4°C or 37°C with PE-rhodamine liposomes, with or without preincubation with FITC-MBP. After appropriate time intervals, the cells were washed five times with ice-cold HBSS and then fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in PBS(+), and stored overnight at 4°C. The cells were mounted in 2.5% DABCO, 90% glycerol, and PBS(+). The samples were examined by confocal laser microscope (ACAS 570 interactive laser cytometer, Meridian Instruments, Okemos, MI, USA).

2.10. In vivo distribution of ^{111}In-MBP

^{111}In-MBP (2 μg), with or without preincubation with Bare-liposomes or Man-liposomes (500 μg), was injected intravenously into a male ddY mouse (25–28 g) via the lateral tail vein. At predetermined time periods (1, 3, 5, 10, 30, and 60 min) after injection, blood was collected from the vena cava under ether anesthesia. The mouse was then sacrificed and its liver, spleen, kidney, and muscle were removed, rinsed with saline, weighed and their radioactivity assayed in a well-type NaI scintillation counter (ARC-500, Aloka Co., Japan).

2.11. Calculation of pharmacokinetic parameters

The distribution data of ^{111}In-MBP were analyzed in terms of the organ uptake clearance (CL_{org}) [25].

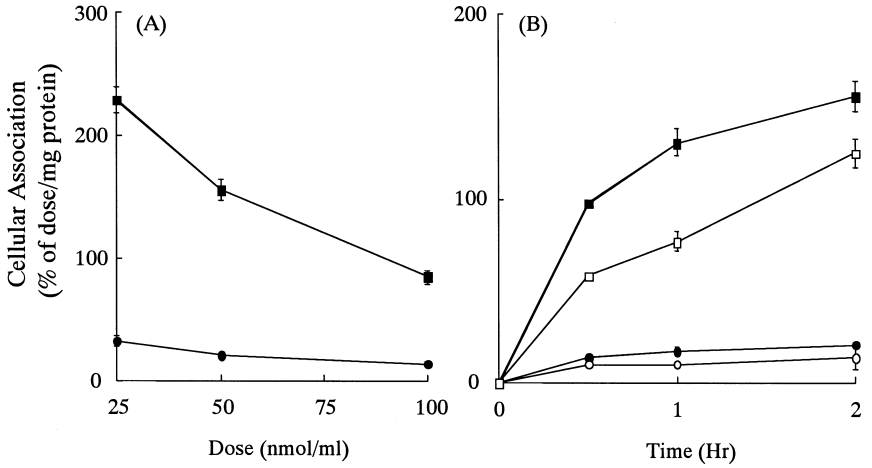

Fig. 1. (A) Dose-dependent cellular uptake of [^{3}H]Bare-liposomes (●) and [^{3}H]Man-liposomes (■). The cells were incubated with various concentrations of liposomes at 37°C for 2 h. (B) The uptake time courses of [^{3}H]Bare-liposomes (50 nmol/ml) at 37°C (●) or 4°C (○) and [^{3}H]Man-liposomes (50 nmol/ml) at 37°C (■) or 4°C (□) for 30 min, 1 h, and 2 h by cultured resident peritoneal macrophages. Results are expressed the mean ± S.D. ($n = 3$).

After injection of ^{111}In-labeled compound, the change in the amount of radioactivity in a tissue with time can be described as

$$\frac{dX_i}{dt} = CL_{org}C_p - K_{efflux,i}X_i \quad (1)$$

where X_i (normalized to % of the dose) represents the amount of radioactivity in tissue i after the administration of the ^{111}In-labeled compound; C_p (% of dose/ml) is the concentration of radioactivity in the plasma; CL_{org} (ml/h) represents the apparent tissue uptake clearance from the plasma to tissue i; and $K_{efflux,i}$ (h^{-1}) represents an efflux rate constant from tissue i. Since little efflux of ^{111}In radioactivity from tissues was observed within the overall experimental period of 1 h, the efflux process can be ignored and Eq. 1 is simplified to

$$\frac{dX_i}{dt} = CL_{org}C_p \quad (2)$$

When Eq. 2 is integrated from time 0 to t, CL_{org} can be expressed as

$$CL_{org} = \frac{X_{i,t}}{\int_0^t C_p dt} = \frac{X_{i,t}}{AUC_t} \quad (3)$$

where AUC_t (% of dose h/ml) represents the area under the plasma concentration–time curve from time 0 to t, calculated by fitting a monoexponential equation to the plasma concentration–time data of the ^{111}In radioactivity–time profile using the non-linear least-squares program MULTI [26]. Therefore, CL_{org} can be easily calculated from Eq. 3 at several time points after administration. The total body clearance (CL_{total}) can be calculated by dividing the dose by the AUC up to infinity.

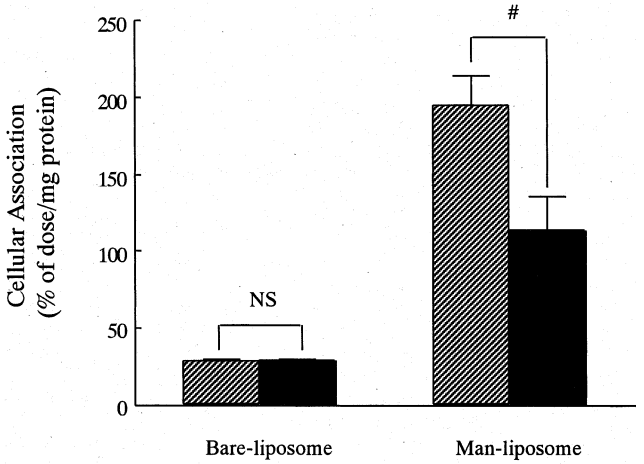

Fig. 2. The uptake of [^{3}H]Bare-liposomes and [^{3}H]Man-liposomes (50 nmol/ml) with (black bars) and without Man_{46}-BSA (2 mg/ml) (hatched bars) for 2 h by cultured resident peritoneal macrophages. Results are expressed as the mean ± S.D. ($n = 3$). $^{\#}P < 0.001$; N.S., not significant.

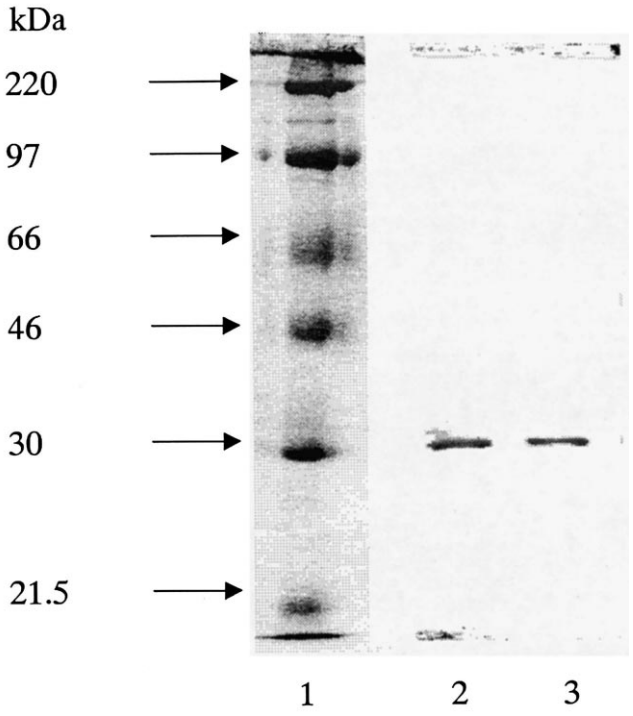

Fig. 3. SDS–PAGE of isolated mannose binding protein. Purified binding protein was subjected to 10% polyacrylamide gel electrophoresis under reducing conditions (β-mercaptoethanol). Molecular weight marker in lane 1, mannose binding protein isolated from rabbit serum in lane 2, and mouse serum in lane 3. The binding protein band has an apparent molecular weight of approx. 30 kDa.

3. Results

3.1. Uptake by cultured macrophages

Fig. 1A shows the dose-dependent in vitro uptake of ^{3}H-labeled liposomes by cultured mouse resident peritoneal macrophages at 37°C. Uptake of Man-liposomes at 2 h was approximately eight-fold greater than that of Bare-liposomes. The cellular uptake increased with time at both 37°C and 4°C (Fig. 1B) but the cellular uptake of Bare-liposomes was markedly lower than that of Man-liposomes at 37°C.

Table 2
Binding of ^{111}In-MBP to liposomes measured by the sedimentation method and the inhibitory effect of 10 mM EDTA and 100 mM mannose on the binding of ^{111}In-MBP to liposomes

Compound	Bare-liposomes (% of dose)	Man-liposomes (% of dose)
Control	16.68 ± 1.85	31.27 ± 1.07
10 mM EDTA	6.20 ± 0.63	17.37 ± 3.33
100 mM mannose	14.82 ± 2.36	12.49 ± 0.92

The results are expressed as the mean ± S.D. of three experiments.

3.2. Uptake inhibition experiment

Uptake experiments were carried out, with or without an excess amount of Man-BSA. Fig. 2 shows a significant inhibitory effect of Man-BSA on Man-liposome uptake ($P < 0.001$) by macrophages, whereas no significant uptake was observed for Bare-liposomes.

3.3. Uptake of [^{3}H]liposomes preincubated with serum-type MBP

Under reducing conditions, a single band (about 30 kDa) could be detected on the SDS–PAGE of the protein(s) purified from rabbit serum or mouse serum using an affinity column (Fig. 3). On the other hand, several high molecular weight bands were seen under non-reducing conditions. These results agreed with those of previous papers [20,27], indicating that both proteins purified from rabbit and mouse serum

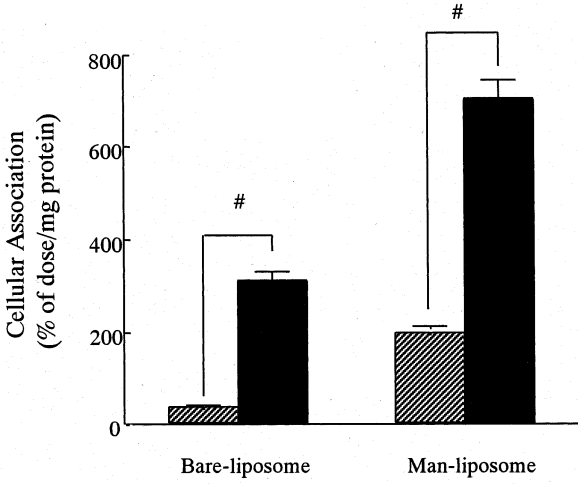

Fig. 4. Effect of MBP on the uptake of [^{3}H]Bare-liposomes and [^{3}H]Man-liposomes by cultured resident peritoneal macrophages. Both types of liposomes (50 nmol/ml) were incubated with (black bars) and without MBP (1 μg/ml) (hatched bars) at 37°C for 10 min before starting the cellular uptake experiment at 37°C for 2 h. Results are expressed as the mean ± S.D. ($n = 3$). $^{\#}P < 0.001$; N.S., not significant

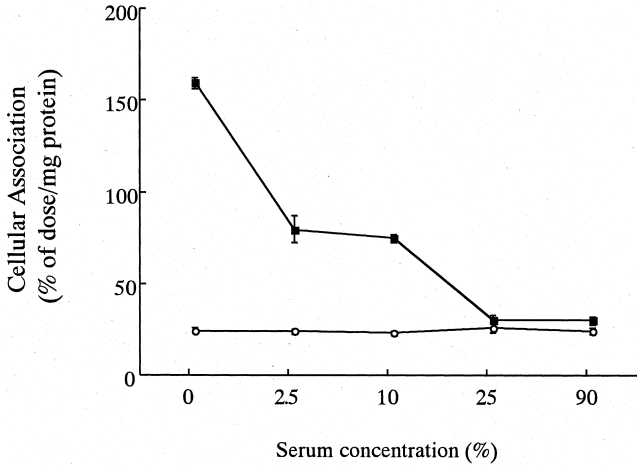

Fig. 5. Effect of preincubation with various concentrations of serum on the cellular uptake of [^{3}H]Bare-liposomes (○) and [^{3}H]Man-liposomes (■) by cultured resident peritoneal macrophages. Liposomes (50 nmol/ml) were incubated with various concentrations of normal serum at 37°C for 10 min before starting the cellular uptake experiment at 37°C for 2 h. Results are expressed as the mean ± S.D. ($n = 3$).

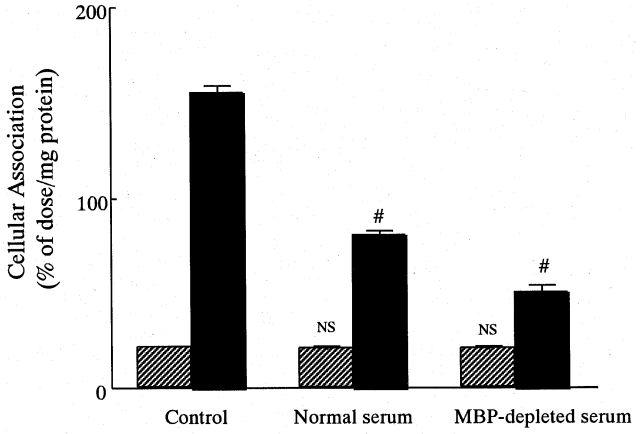

Fig. 6. Effect of serum and MBP-depleted serum on the cellular uptake of [^{3}H]Bare-liposomes (hatched bars) and Man-liposomes (black bars) by cultured resident peritoneal macrophages. Liposomes (50 nmol/ml) were incubated at 37°C for 10 min with 10% normal serum or MBP-depleted serum before starting the cellular uptake experiment at 37°C for 2 h. Control experiments were carried out without serum. Results are expressed as the mean ± S.D. ($n = 3$). $^{\#}P < 0.001$; N.S., not significant.

are serum-type MBP. To clarify the effect of MBP, [^{3}H]liposomes were preincubated with MBP at 37°C for 10 min before the uptake experiment. It is obvious from Fig. 4 that MBP markedly enhanced the uptake of both [^{3}H]Bare-liposomes and [^{3}H]Man-liposomes, but this effect was greater for [^{3}H]Man-liposomes.

3.4. Binding of ^{111}In-MBP to liposomes

The binding of ^{111}In-MBP to liposomes was determined by the sedimentation method and the results are summarized in Table 2. This shows that an approximately two-fold greater amount of ^{111}In-MBP was co-sedimented with Man-liposomes than Bare-liposomes and this binding was also inhibited by 10 mM EDTA or 100 mM mannose.

3.5. Effect of serum on the uptake

The effects of serum or its components on in vitro uptake were investigated. The results in Fig. 5 show

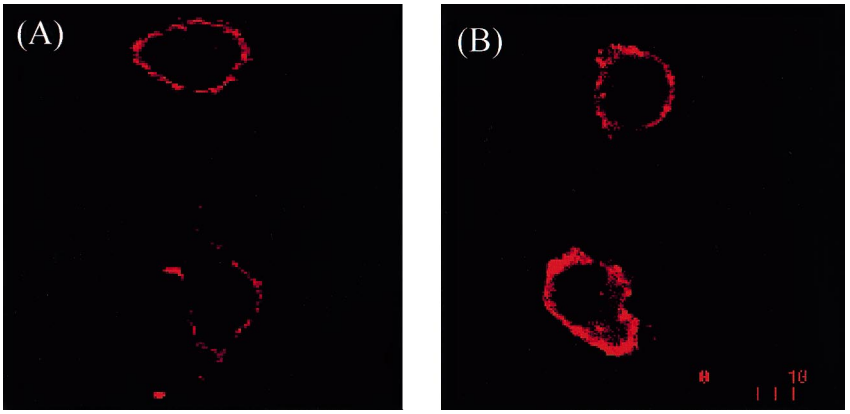

Fig. 7. Confocal microscopy images of binding and internalization of Man-liposomes by mouse peritoneal macrophages. Macrophages were incubated with PE-rhodamine Man-liposomes for 2 h at 4°C (A), or at 37°C (B). The images are typical of at least three independent experiments.

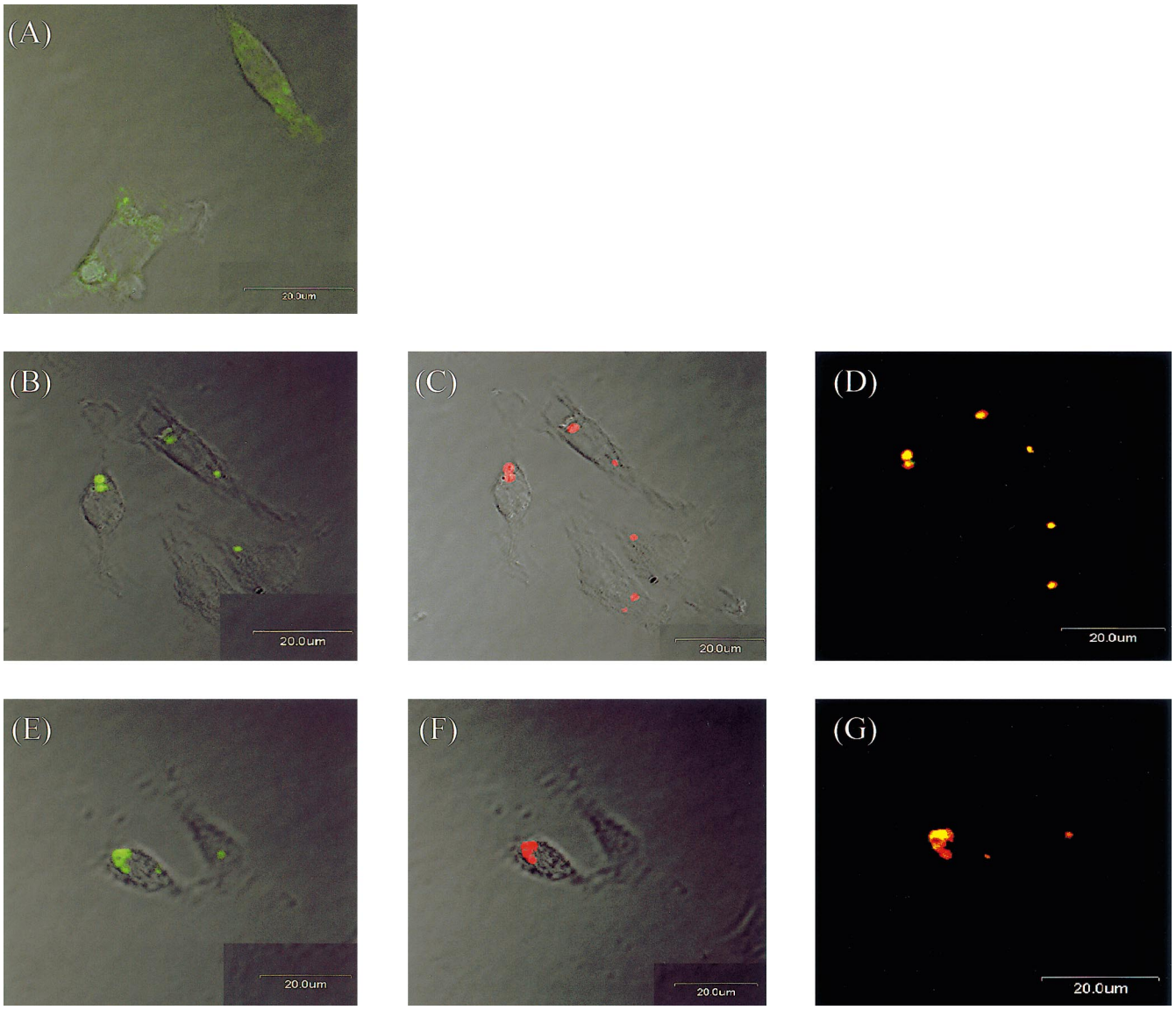

Fig. 8. Confocal microscopy of FITC-MBP preincubated with PE-rhodamine Bare-liposomes and PE-rhodamine Man-liposomes in the culture of mouse peritoneal macrophages. Macrophages incubated with FITC-MBP at 37°C for 2 h are shown by the green color of FITC (A). Macrophages incubated with a mixture of FITC-MBP and PE-rhodamine Bare-liposomes at 37°C for 2 h are displayed in three panels with green (FITC) (B), red (PE-rhodamine) (C), and yellow (depicting colocalization) (D) signals. Macrophages incubated with a mixture of FITC-MBP and PE-rhodamine Man-liposomes at 37°C for 2 h are displayed in three panels with green (FITC) (E), red (PE-rhodamine) (F), and yellow (depicting colocalization) (G) signals. Typical images of the distribution pattern from at least three independent experiments are shown.

that the uptake of Man-liposomes incubated with serum significantly decreased on increasing the concentration of serum. The uptake of liposomes by macrophages in the presence of serum, with or without MBP, is compared in Fig. 6. The uptake of Bare-liposomes by macrophages was unaffected by both serum samples. On the other hand, serum drastically suppressed the uptake of Man-liposomes by macrophages, but the uptake was still greater than that for Bare-liposomes. Man-liposomes preincubated with MBP-depleted serum showed a significantly lower uptake than with normal serum indicating involvement of MBP in the uptake of Man-liposomes by macrophages.

3.6. Confocal microscopy

The results of confocal microscopy are shown in Fig. 7 and these suggest close agreement with those obtained in the in vitro uptake experiment. Confocal microscopy images revealed time-dependent binding at 4°C (Fig. 7A) and internalization at 37°C (Fig. 7B) of Man-liposomes by macrophages. In addition, about 50% of the bound Man-liposomes were internalized in 30 min (data not shown). Bare-liposomes showed a similar pattern of binding and internalization, but the number of cells exhibiting binding and internalization was less than 10% of the total cells, whereas the figure was more than 90% for Man-liposomes.

To clarify the effect of MBP on the uptake of Man-liposomes, colocalization images were examined (Fig. 8). The photographs show that FITC-MBP (Fig. 8A) was internalized into macrophages, but the fluorescence signal was rather weaker than that of the complex with PE-rhodamine Bare-liposomes (Fig. 8B) and PE-rhodamine Man-liposomes (Fig. 8E). FITC-MBP preincubated with PE-rhodamine Bare-liposomes (Fig. 8D) and with PE-rhodamine Man-liposomes(Fig. 8G) is internalized into macrophages. As shown by the yellow signal depicting colocalization, FITC-MBP exhibited increased colocalization with PE-rhodamine Man-liposomes and PE-rhodamine Bare-liposomes. The number of cells exhibiting colocalization of PE-rhodamine Bare-liposomes with FITC-MBP was less than 10% of the total cells, whereas more than 90% of cells showed colocalization for Man-liposomes.

3.7. In vivo distribution of ^{111}In-MBP

Fig. 9 shows the plasma concentration and liver accumulation time courses of ^{111}In-MBP after intravenous injection in mice, with or without preincubation with Bare-liposomes and Man-liposomes. Although ^{111}In-MBP was mainly recovered in the liver in all cases, the hepatic uptake of ^{111}In-MBP preincubated with Man-liposomes was the greatest. Table 3 summarizes the AUC, CL_{total}, hepatic uptake clearance (CL_{liver}), and tissue uptake rate index of ^{111}In-MBP after intravenous injection. The CL_{liver} made a major contribution to the CL_{total}, suggesting that the liver is the main organ for ^{111}In-MBP uptake. The results confirmed that binding to Man-liposomes enhanced the hepatic uptake of ^{111}In-MBP.

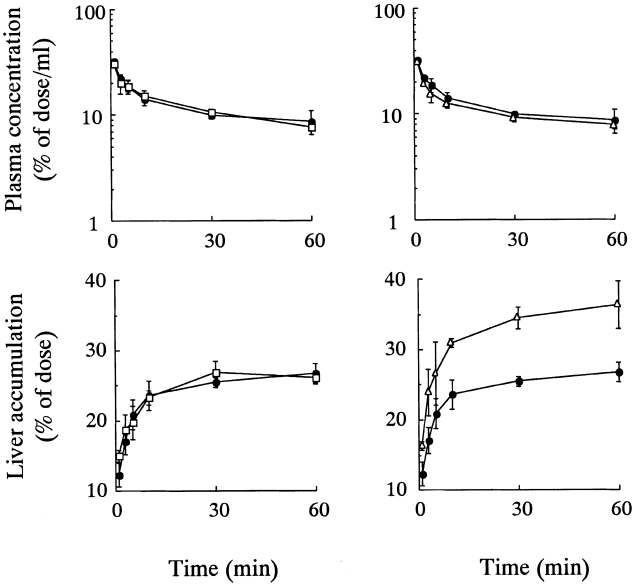

Fig. 9. Time courses of the plasma concentration (upper) and liver accumulation (lower) of ^{111}In-MBP preincubated with Bare-liposomes (left: □) and Man-liposomes (right: △) or without preincubation (●) after intravenous injection into mice. Results are expressed as the mean ± S.D. of three mice.

4. Discussion

Cell-specific targeting systems using carbohydrate

Table 3
AUC, clearances, and tissue uptake rate index of ^{111}In-MBP, ^{111}In-MBP preincubated with Bare-liposomes, and ^{111}In-MBP preincubated with Man-liposomes after intravenous injection in mice

Compound	AUC (% of dose h/ml)	Clearance (ml/h)		Tissue uptake rate index (ml/h/g)			
		Cl_{total}	Cl_{liver}	Liver	Kidney	Spleen	Muscle
In-MBP	16.56	6.03	2.15	1.3	1.4	0.9	0.001
In-MBP+Bare-liposomes	16.43	5.73	2.04	1.2	1.6	0.8	0.003
In-MBP+Man-liposomes	15.34	6.52	3.23	2.3	2.1	2.4	0.003

recognition mechanisms have attracted great interest as far as drug and gene delivery are concerned, and we have reported developments of various polymeric or particulate carriers in this context [28–30]. However, in order to achieve highly specific cellular targeting via receptor-mediated endocytosis, we need to pay great attention to interactions of carriers with biological components such as serum binding protein. For example, Kawasaki et al. [31] reported that MBP, mannose-specific lectin, circulates in serum, binds to pathogens having mannose units on their surface, and functions directly as an opsonin recognized by the collectin receptor on macrophages [32,33] and complement activation [34,35]. In our series of investigations, we have described the effect of specific binding to MBP on the targeting potential of mannosylated polymer carriers in mice. Dose-dependent reiteration of cell-specific hepatic uptake due to complex formation was confirmed by a pharmacokinetic analysis (submitted): serum MBP retarded hepatic uptake of mannosylated polymer at lower doses of 0.05 and 0.1 mg/kg. At doses between 0.1 and 1 mg/kg, the binding to MBP was saturated and excess ligands existing in a free form were recognized by hepatic mannose receptors resulting in rapid hepatic uptake. At doses over 1 mg/kg, the mannose receptors were also saturated with ligands and the hepatic uptake was retarded again. A similar phenomenon would be suspected for particulate carriers such as mannosylated liposomes, but little information has been obtained about the effects of MBP on the in vivo behavior of Man-liposomes. Therefore, in this investigation, the roles of MBP and mannose receptor in the uptake of Man-liposomes by macrophages were studied in detail.

In general, liposome composed of DSPC and Chol and small in size, such as < 100 nm, are known to have a long half-life in the blood circulation due to their ability to avoid reticuloendothelial systems (RES) trapping [36], failure to undergo complement activation [37], and a lower susceptibility to destruction by serum components [38]. Therefore, in our approach, we chose DSPC/Chol liposomes as Bare-liposomes for modification with Man-C4-Chol to obtain mannosylated targeting liposomes. The in vitro uptake experiment demonstrated the rapid attachment and internalization of Man-liposomes by peritoneal macrophages (Fig. 1) and similar results were obtained by confocal microscopy observations (Fig. 7). Furthermore, the uptake was dose-dependent (Fig. 1) and inhibited by Man-BSA (Fig. 2). These results suggest that the mannose residues on the surface of Man-liposomes present a conformational mobility which allows interaction with the mannose receptors of the macrophages under serum-free conditions. However, this may not be the case in vivo since administered liposomes would be exposed to serum components including MBP.

When mannosylated ligands interact with serum, their biodistribution becomes more complicated. The serum components that can recognize the mannose moiety or simply be adsorbed on Man-liposomes may accelerate (opsonins) or suppress (disopsonins) the mannose-specific uptake of Man-liposomes, respectively. Thus, the apparent hepatic disposition feather would be observed in the balance of the acceleration and suppression. In Fig. 4 purified MBP enhanced the uptake of both Bare-liposomes and Man-liposomes but the increase was greater for Man-liposomes. These results are in good agreement with the results of confocal microscopy in that the number of cells exhibiting colocalization of FITC-MBP and PE-rhodamine-labeled liposomes for Bare-liposomes was less than 10% of total cells, whereas it was more than 90% for Man-liposomes. The enhancement of the uptake of Bare-liposomes by MBP might be provoked by the non-specific adsorption on the liposomal surface or the specific binding of MBP to phospholipids since binding of MBP to phospholipids and liposomes in addition to mannose moieties was also reported in previous studies [39]. In vivo experiments also demonstrated that only Man-liposomes enhanced the hepatic uptake of ^{111}In-MBP (Fig. 9) suggesting effective uptake of the complex of MBP and Man-liposomes by macrophages. Moreover, [^{3}H]CHE-labeled Man-liposomes complexed with ^{111}In-MBP showed greater uptake than [^{3}H]liposomes administered alone (data not show). Serum albumin, the major serum protein, has been reported to reduce the hepatic uptake of polystyrene colloidal particles by coating the hydrophobic surface of particles [40]. In this study, the uptake experiments demonstrated that serum components present on the surface of Man-liposomes suppressed its cellular uptake (Fig. 5), probably due to disruption of the specific interaction between the mannose moiety

and the receptor on the macrophages. A similar effect was observed for serum heated at 56°C for 30 min (decomplemented serum) (data not shown). These results suggest that heat-stable serum components such as albumin might coat the liposomal surface and inhibit recognition. This result seems to be inconsistent with the previous report of Matsuo et al. [41] who demonstrated stimulatory and inhibitory effects of serum and heated serum, respectively, on the uptake of multilamellar vesicles with cetylmannoside in the in situ hepatic uptake experiments. They suggested the involvement of complement but not the mannose receptor. This discrepancy could be due to differences in the size and types of the mannose moieties as well as lipid composition of the liposomes. On the other hand, MBP-depleted serum showed a significantly higher uptake inhibition for Man-liposomes than normal serum, suggesting involvement of MBP in uptake by macrophages (Fig. 6). Further study is necessary to clarify the detailed role of serum components in the suppression of cellular uptake of liposomes.

In a previous study, we have found that the soluble mannosylated polymer complexed with MBP loses its ability to be recognized by macrophages (submitted). Although the structure, functions, and manner of binding to simple sugar of MBP have been extensively studied [13–15], no study has so far shown the relationship between the characteristics of the mannosylated ligands and the uptake efficiency of its MBP complex. The critical features of the ligands, such as their size and shape and the mechanisms of inhibition or stimulation of MBP on the uptake of mannosylated ligands, remain to be elucidated. It has been reported that MBP can be recognized by collectin receptors expressed on macrophages, monocytes, and neutrophils [42] and the collagen-like N-terminal region of MBP is responsible for this interaction. It has been hypothesized that the collagenous region of the MBP motif is normally masked by the MBP-associated serine protease (MASP). However, following complement activation, MASP is removed to reveal the collectin receptor binding sites. Alternatively, binding of mannosylated ligands to the carbohydrate recognition domain region might result in a conformational change in the collagenous region with exposure of a previously hidden site, leading to binding and internalization of the MBP/mannosylated ligand complex into the macrophages by collectin receptors [43]. In the case of soluble mannosylated polymers, MBP hides mannose moieties while retaining its soluble form. Although the interaction between MBP and mannosylated polymeric ligands leads to tight and specific binding through multivalent interaction, it could not produce a conformational change in the collagenous region in MBP. These differences could be explained in terms of the size and shape of the ligands, rather than the clustering effect of the multivalent interaction. Thus, the characteristics of mannosylated ligands are crucial as far as exposure of the binding sites of the collagenous region is concerned.

Native and recombinant MBP have been shown to bind to virulent *S. montevideo* expressing a mannose-rich O-polysaccharide which results in attachment, uptake, and killing of the coated bacteria by phagocytes, providing the first evidence that MBP can act as an opsonin [44]. Thus, it has been presumed that this direct opsonin activity is mediated by a collectin-specific receptor expressed on phagocytic cell surfaces, although the physiological importance of the MBP interaction with the collectin receptor(s) remains unclear due to the absence of data on receptor kinetics and expression. On the other hand, Tenner et al. [45] recently showed that MBP does not associate or bind to the target being ingested. Rather, cellular interaction with MBP alone induces, as yet unknown, intracellular responses, possibly binding of a target to independent recognition receptors like FcR or CR1. The exact mechanism involved needs further investigation.

In summary, the novel mannosylated liposomes developed for liver non-parenchymal cell selective targeting were confirmed to be recognized by the mannose receptors of macrophages with a high degree of specificity. In addition to the mannose receptor, serum MBP specifically associates at the surface of Man-liposomes and enhances its binding and internalization by macrophages. On the other hand, some components in serum simply interfere with the recognition by coating the surface of liposomes. The characterization of these interactions between Man-liposomes and the macrophage receptors or MBP would not only provide fundamental information for the rational design of cell-specific carriers, but also suggest a role of these interactions in infec-

tion and other pathological conditions involving macrophages.

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