

Figure 3. Effect of the route of administration and dose on immunization with naked pCMV-OVA or Man-lipoplexes. Mice were injected with 50  $\mu\text{g}$  pCMV-OVA as naked pCMV-OVA or Man-lipoplexes biweekly three times before the experiment. (a) CTL activity primed by i.p. (■), i.d. (□) and i.m. (●) administration of the Man-lipoplexes or CTL activity in the no-treatment group (○). OVA-specific cell lysis at various effector/target (E/T) ratios was calculated from the %  $^{51}\text{Cr}$  release from EL4 cells and from E.G7-OVA cells. Each value represents the mean of 4–5 experiments. (b) CTL response induced by the Man-lipoplexes (■) and naked pCMV-OVA (□) given i.p. at a dose of 50 or 100  $\mu\text{g}/\text{mouse}$ . OVA-specific  $^{51}\text{Cr}$  release at an E/T ratio of 50 : 1 was calculated from the following equation: OVA-specific  $^{51}\text{Cr}$  release = %  $^{51}\text{Cr}$  release from E.G7-OVA cells – %  $^{51}\text{Cr}$  release from EL4 cells. Each value represents the mean + S.D. (control group:  $n = 3$ , other groups:  $n = 5$ ). Statistical analysis was performed by analysis of variance (\*\* $P < 0.01$ ). (c) CTL response induced by the Man-lipoplexes given i.p. and naked pCMV-OVA given i.m. at a dose of 50 or 100  $\mu\text{g}/\text{mouse}$ . OVA-specific  $^{51}\text{Cr}$  release at an E/T ratio of 50 : 1 was calculated from the following equation: OVA-specific  $^{51}\text{Cr}$  release = %  $^{51}\text{Cr}$  release from E.G7-OVA cells – %  $^{51}\text{Cr}$  release from EL4 cells. Each value represents the mean + S.D. (control group:  $n = 3$ , other groups:  $n = 5$ ). Statistical analysis was performed by analysis of variance (\*\* $P < 0.01$ )

i.p. administration of Man-lipoplex induced a higher OVA-specific CTL response than local administration of naked pDNA. To demonstrate the antigen-specific anti-tumor effects induced by vaccination, E.G7-OVA cells (OVA expressing cells), and its parent cell line, pre-immunized mice were inoculated with EL4 cells (OVA non-expressing cells). Corresponding to the CTL response, the anti-tumor effects of the Man-lipoplex were observed only in E.G7-OVA cells and the effects of the Man-lipoplex were much greater than those following local administration of naked pDNA (Figure 6). These results suggest that the Man-lipoplex is an effective gene carrier for DNA vaccination used as cancer therapy.

To demonstrate mannose receptor-mediated gene transfection of the Man-lipoplex, its transfection

characteristics in DCs were evaluated in both *in vitro* and *in vivo* experiments. As shown in Figures 1 and 2, the Man-lipoplex showed significantly higher uptake and transfection activity than the conventional lipoplex and this was reduced in the presence of mannan, a mannose receptor ligand. These *in vitro* results suggest that the Man-lipoplex is taken up by mannose receptor-mediated endocytosis by a dendritic cell line, DC2.4 cells. This observation is in good agreement with our previous report showing that the Man-lipoplex is taken up by mannose receptor-mediated endocytosis by primary cultured mouse peritoneal macrophages [22,32]. To evaluate the importance of mannose receptor-mediated gene transfection to DCs, we also evaluated the involvement of the mannose receptor-mediated mechanism in the CTL response. The

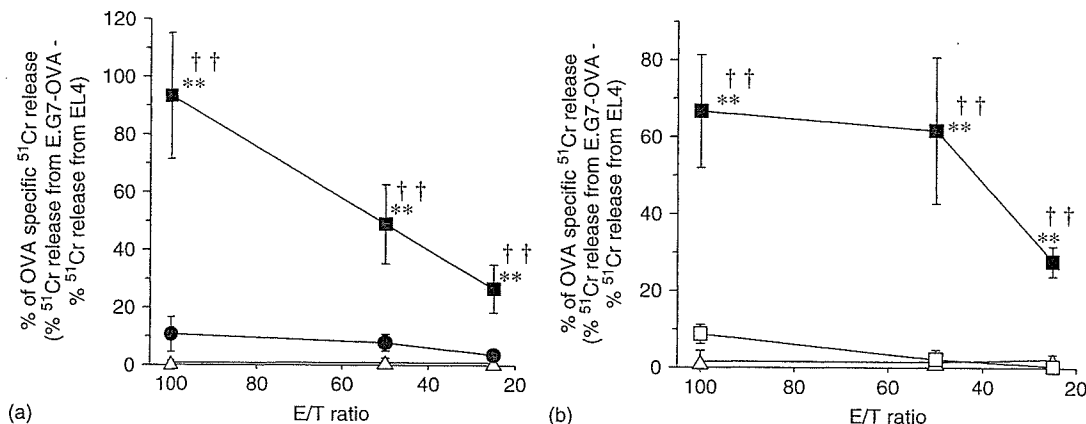


Figure 4. Effect of mannosylation of cationic liposomes on the induction of a CTL response. Mice were injected three times with naked pCMV-OVA (100 μg) or lipoplexes (pCMV-OVA; 100 μg) biweekly. (a) CTL response induced by the Man-lipoplexes (■) and the lipoplexes (●) or that of the no-treatment group (Δ). OVA-specific <sup>51</sup>Cr release was calculated from the following equation: OVA-specific <sup>51</sup>Cr release = %<sup>51</sup>Cr release from E.G7-OVA cells - %<sup>51</sup>Cr release from EL4 cells. Each value represents the mean ± S.D. (n = 4–5). (b) CTL response induced by the Man-lipoplex (■) or the Gal-lipoplex (□) or CTL response in the no-treatment group (Δ). OVA-specific <sup>51</sup>Cr release was calculated from the following equation: OVA-specific <sup>51</sup>Cr release = %<sup>51</sup>Cr release from E.G7-OVA cells - %<sup>51</sup>Cr release from EL4 cells. Each value represents the mean ± S.D. (n = 5). Statistical analysis was performed by analysis of variance. Significant difference between the no-treatment group (\*P < 0.05, \*\*P < 0.01) or pCMV-OVA given i.m. (††P < 0.01)

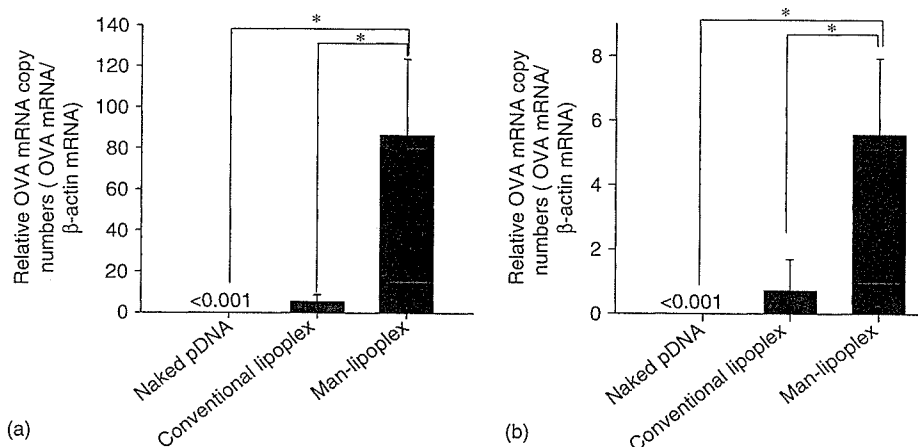


Figure 5. *In vivo* mRNA gene expression in CD11c<sup>+</sup> cells in the peritoneal cavity (a) and spleen (b) after i.p. administration to mice. Lipoplex or Man-lipoplex was prepared at a charge ratio (-/+) of 1.0:2.3 in 5% dextrose. Six hours after injection, OVA mRNA and β-actin mRNA eluted from CD11c<sup>+</sup> cells were measured by quantitative two-step RT-PCR. Each value represents the mean + S.D. (n = 3–4). Statistical analysis was performed by analysis of variance (\*P < 0.05)

Gal-lipoplex was selected because it differed only from the Man-lipoplex by a sugar moiety. In a previous study, we have already confirmed that the Gal-lipoplex was taken up by asialoglycoprotein receptor-mediated endocytosis after intraportal administration [33,34]. As shown in Figure 4b, the CTL response of the Gal-lipoplex was significantly lower than that of the Man-lipoplex. These *in vitro* and *in vivo* results suggest that the Man-lipoplex is efficiently taken up by DCs via mannose receptor-mediated endocytosis.

As control cationic liposomes, cationic liposomes composed of 3β-[N,N',N'-dimethylaminoethane]carbamoyl cholesterol hydrochloride (DC-Chol liposomes) represent a feasible formulation for clinical trials involving gene

therapy via the i.p. route [35] and it has also been reported that DC-Chol liposomes enhance DNA vaccine potency following i.p. administration in mice [36]. However, our preliminary experiment using pCMV-Luc demonstrated that DOTMA/Chol liposomes exhibited a much higher transfection activity than DC-Chol liposomes following i.p. administration (data not shown); therefore, DOTMA/Chol liposomes were selected as control liposomes for DNA vaccine therapy.

To further evaluate the effectiveness of lipoplex mannosylation, gene expression in APCs, OVA-specific CTL activity and the antitumor effect were compared with those obtained using conventional lipoplex. Since the lipid composition of the liposomes (DOTMA/Chol

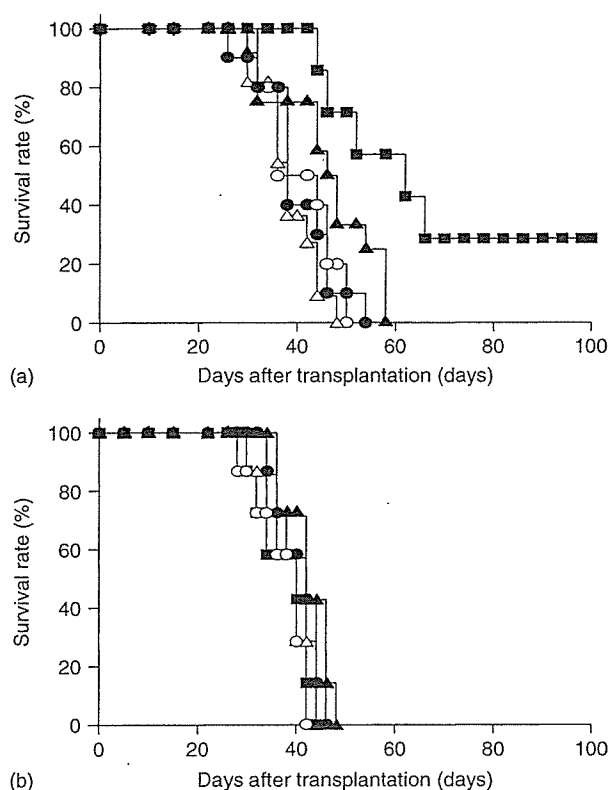


Figure 6. The anti-E.G7-OVA cell (a) or EL4 cell (b) tumor effect following pre-immunization by i.p. administration of various formulations or i.m. administration of naked pCMV-OVA solution (100  $\mu$ g). Mice were injected with naked pCMV-OVA (O), the Man-lipoplex (■), or the lipoplex (●) given i.p., naked pCMV-OVA given i.m. (▲), or no treatment ( $\Delta$ ). E.G7-OVA (a) or EL4 (b) cells were transplanted into mice 2 weeks after the last immunization and the survival rate was determined ( $n = 7$  for all groups in (b) and for Man-lipoplex in (a),  $n = 10$  for all groups but the Man-lipoplex in (a))

vs. DOTMA/Chol/Man-C4-Chol) and the physicochemical properties (zeta-potential and particle size (see Results section)) of the lipoplex and Man-lipoplex are almost the same, the effect of mannosylation of lipoplex could be investigated by such comparisons. After i.p. administration of lipoplex or Man-lipoplex, the gene expression in CD11c<sup>+</sup> cells in the spleen and peritoneal cavity of Man-lipoplex was significantly higher than that of the conventional lipoplex (Figure 5a and 5b). Furthermore, the OVA-specific CTL response (Figure 4) and anti-tumor effect (Figure 6a) of the Man-lipoplex were significantly higher than that of the conventional lipoplex. These results convinced us that lipoplex mannosylation could enhance DNA vaccine potency.

As far as the effect of the administration route on the CTL activity by Man-lipoplex was concerned, the CTL activity following i.p. administration was higher than that following s.c. and i.m. administration (Figure 3a). We previously reported that the transfection efficiency of the lipoplex was lower than that of naked pDNA because the lipoplex is only localized at the injection site due to its cationic and macromolecular nature [37]. Thus, the lower

CTL activity following s.c. and i.m. administration of the Man-lipoplex may be partly explained by our previous observation. In contrast, i.p. administered Man-lipoplex is considered to reach the APCs in the peritoneal cavity and lymph nodes. Even the (large sized) cancer cells could distribute to the lymph nodes from the peritoneal cavity when undergoing metastasis [38,39]. Thus, these observations strongly suggest that i.p. administration is an effective administration route for gene transfection to APCs by the (Man-)lipoplex.

In the present study, we have demonstrated the effectiveness of i.p. administration of the Man-liposome formulation. This type of infusion of not only drugs such as cisplatin [42] and paclitaxel [43] but also cationic liposome/pDNA [44] has already been performed in clinical trials for ovarian cancer therapy. Regarding the i.p. administration method, implantable infusion pumps have been developed for a number of diseases [45] and there has been remarkable progress in endoscopic and laparoscopic surgical techniques [46]. These progresses in surgical techniques and devices might make i.p. administration of the Man-liposome formulation a conventional and feasible approach for the clinical application of DNA vaccine therapy.

In this study, we demonstrated that the Man-lipoplex enhanced gene expression via a mannose receptor-mediated mechanism. Since our previous study demonstrated that the Man-lipoplex were rapidly sorted from endosomes to lysosomes after uptake via mannose receptors [47], only a small part of pDNA seems to be released from endosome/lysosome to the cytosol and enters the nucleus for gene expression. Taking these findings into consideration, further modulation of intracellular sorting with some functional device should lead to more efficient gene expression in APCs. So far, functional materials such as influenza virus hemagglutinin subunit HA-2 (mHA2) [17], fusogenic peptide, and polyhistidine [48] have been grafted to the vectors to improve the intracellular sorting of cationic carrier/pDNA complexes. Modulation by grafting such functional molecules might be effective in the further development of the Man-lipoplex.

A large number of diseases can be potentially prevented or cured by DNA vaccination [40]. Recent developments in genomics technology have identified new target antigens, not only for infectious disease pathogens, but also for a large number of tumor-associated antigens [41] and this has increased the possibility of using DNA vaccines for a variety of infectious diseases and cancer therapies. Since the pDNA that encodes a variety of antigens has almost the same physicochemical properties as a polyanion, our Man-liposomes are expected to be applicable to a range of DNA vaccine therapies to enhance the CTL response.

In conclusion, we demonstrate that the Man-lipoplex produces an extremely high antigen-specific CTL response. In addition, intraperitoneal administration is an effective route for the APCs-selective gene transfection by the Man-lipoplex. Although further optimization

is required, this information will also be valuable for the future use, design, and development of a Man-lipoplex to enhance the potential of DNA vaccine therapy.

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## Effect of the Particle Size of Galactosylated Lipoplex on Hepatocyte-Selective Gene Transfection after Intraportal Administration

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The purpose of this study was to examine the effect of the size of galactosylated cationic liposome (Gal-liposome)/plasmid DNA complex (Gal-lipoplex) on hepatocyte-selective gene transfection after intraportal administration. pCMV-Luc was selected as a model plasmid DNA. After intraportal administration of Gal-lipoplex to mice, the hepatic and intrahepatic gene expression was evaluated. To evaluate the effect of size, three different sizes of Gal-liposome were prepared. The mean particle sizes of Gal-lipoplex were about 141, 179, and 235 nm, respectively. The hepatic transfection efficacy was significantly enhanced by increasing the size of Gal-lipoplex. However, the gene expression in liver parenchymal cells (PC) of Gal-lipoplex of about 141 nm in size was significantly higher than that in liver non-parenchymal cells (NPC). In contrast, gene expression in PC of Gal-lipoplex of about 235 nm in size was significantly lower than that in NPC. These results highlight the importance of the Gal-lipoplex size for hepatocyte-selective gene transfer *in vivo*. The information in this study will be valuable for the future use, design, and development of Gal-lipoplex for *in vivo* applications.

**Key words** gene delivery; hepatocyte; targeting; galactosylated liposome; drug delivery system

Gene transfer to hepatocytes is of great therapeutic potential since hepatocytes are responsible for the synthesis of a wide variety of proteins that play important physiological roles. There has been much interest in *in vivo* gene transfer to the liver, as an alternate to *ex vivo* methods that require invasive surgery. So far, several methods involving the local administration of naked plasmid DNA (pDNA) has been tested in order to achieve gene delivery targeted to the liver.<sup>1,2)</sup> Compared with these local applications to the liver, systemic application by vascular routes could transfect the gene to a large number of cells in the whole liver. However, the highest gene expression is observed in the lung after the intravenous<sup>3–5)</sup> and intraportal<sup>6)</sup> administration of cationic liposome/pDNA complex (lipoplex).

The development of targeted gene delivery systems is a promising approach for effective and safe *in vivo* gene transfer to hepatocytes. To achieve targeted gene delivery, galactose has been shown to be a promising targeting ligand for hepatocytes (liver parenchymal cells; PC) because these cells possess a large number of asialoglycoprotein receptors that recognize the galactose units on the synthetic galactosylated carriers.<sup>7,8)</sup> Recently, we have developed Gal-liposomes containing cholesten-5-yloxy-*N*-(4-((1-imino-2-*D*-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol) for hepatocyte-selective gene transfection after intraportal administration to mice.<sup>7,9,10)</sup> However, the level of *in vivo* gene expression was not as high as that expected from the *in vitro* results.<sup>7,11)</sup> This phenomenon could explain the several barriers associated intrinsically with *in vivo* situations; therefore, these *in vivo* barriers need to be investigated to allow the successful development of an effective gene vector. However, little information is available about hepatocyte-selective gene transfer by Gal-lipoplex under *in vivo* conditions.

The passage through the sinusoids is considered an important factor for hepatocyte-selective gene transfection, since the Gal-lipoplex must pass through the endothelial cell barriers to reach the hepatocytes. In this study, therefore, we evaluated the effects of Gal-lipoplex size on hepatic transfection efficacy after intraportal administration. Once the *in vivo*

gene expression is linked with its physicochemical properties including particle size, it is then possible to design a Gal-lipoplex to enable cell-specific *in vivo* gene delivery. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA)/cholesterol (Chol)/Gal-C4-Chol liposomes were selected as a Gal-liposome for study because of its hepatocyte-selectivity after intraportal administration.<sup>7)</sup> pCMV-Luc was selected as a model pDNA for evaluating the gene expression by luciferase.

### MATERIALS AND METHODS

**Materials** *N*-(4-Aminobutyl) carbamic acid *tert*-butyl ester and DOTMA were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cholesterol (Chol) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Cholesteryl chloroformate and collagenase type IA were obtained from Sigma Chemicals, Inc. (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available.

**Animals** Female five-week-old ICR mice (20–23 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

**Construction and Preparation of pDNA (pCMV-Luc)** pCMV-Luc was constructed by subcloning the *Hind*III/*Xba*I firefly luciferase cDNA fragment from pGL3-control vector (Promega Co., Madison, WI, U.S.A.) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). pDNA was amplified in the *E. coli* strain DH5a, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the pDNA concentration was measured by UV absorption at 260 nm.

**Synthesis of Gal-C4-Chol** Gal-C4-Chol was synthesized as reported previously.<sup>11)</sup> Briefly, cholesteryl chloroformate

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mate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester were reacted in chloroform for 24 h at room temperature. A solution of trifluoroacetic acid and chloroform was added dropwise and the mixture was stirred for 4 h at 4 °C. The solvent was evaporated to obtain *N*-(4-aminobutyl)-(cholesten-5-yloxy)formamide which was then combined with 2-imino-2-methoxyethyl-1-thiogalactoside and the mixture was stirred for 24 h at 37 °C. After evaporation, the resultant material was suspended in water, dialyzed against distilled water for 48 h (12 kDa cut-off dialysis tubing), and then lyophilized.

**Preparation of Gal-liposome** Gal-liposomes were prepared as reported previously.<sup>7,10</sup> The mixtures of DOTMA, Chol, and Gal-C4-Chol were dissolved in chloroform at a molar ratio of 2 : 1 : 1 for Gal-liposomes, vacuum-desiccated, and resuspended in sterile 5% dextrose solution at a concentration of 4 mg total lipids per ml. For small sized liposomes (about 49.6 nm, see Fig. 1), the suspension was sonicated for 3 min and the resulting liposomes were extruded 5-times through 100 nm polycarbonate membrane filters. In the case of medium (about 148 nm, see Fig. 1) and large (about 197 nm, see Fig. 1) sized liposomes, the resulting liposomes were directly extruded 5-times through 200 and 400 nm polycarbonate membrane filters, respectively.

**Preparation of Gal-lipoplex** Gal-lipoplex was prepared as reported previously.<sup>7</sup> pDNA in 5% dextrose solution was mixed with an equal volume of Gal-liposomes and incubated for 30 min. The mixing ratio of liposomes and pDNA was expressed as a charge ratio, which is the molar ratio of cationic lipids to pDNA phosphate residues.<sup>12</sup> As far as the charge ratio was concerned, we selected a charge ratio (- : +) of 1.0 : 2.3 for all experiments to obtain the most effective transfection activity for receptor-mediated gene transfer.<sup>7</sup> The particle size of the Gal-lipoplex was measured using a dynamic light scattering spectrophotometer (LS-900, Otsuka Electronics Co., Ltd., Osaka, Japan). The number-fractionated mean diameter was shown.

**In Vivo Transfection Experiments** Intraportal administration was performed as reported previously.<sup>7,10</sup> Mice were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg), an incision was made in the abdomen, and the portal vein was exposed. The Gal-lipoplex was injected into the portal vein at a dose of 30  $\mu$ g, and the abdomen was closed with wound clips. Liver samples were taken 6 h after injection and each sample was homogenized with lysis buffer (0.1 M Tris/HCl containing 0.05% Triton X-

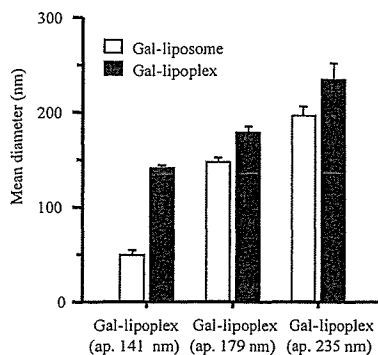


Fig. 1. The Particle Size Distribution of Gal-liposome and Gal-lipoplex

Each value represents the mean  $\pm$  S.D. of at least three experiments. ap.: approximately.

100 and 2 mM EDTA (pH 7.8)). After three cycles of freezing and thawing, the homogenates were centrifuged at 10000 *g* for 10 min at 4 °C. Twenty microliters of each supernatant was mixed with 100 ml; luciferase assay solution (Picagene, Toyo Ink Mfg. Co., Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507, Berthold Technologies, GmbH & Co., Bad Wildbad, Germany). The protein content of the samples was determined using a protein quantification kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, U.S.A.). For evaluation of the intrahepatic localization of gene expression, the luciferase activities in the liver PC and non-parenchymal cells (NPC) were independently determined after centrifugal separation of PC and NPC in collagenase-digested liver as previously described.<sup>7,9</sup>

**Statistical Analysis** Statistical comparisons were performed by Student's *t*-test for two groups, Steel-Dwassl test for multiple groups.

## RESULTS AND DISCUSSION

Recently, we developed Gal-C4-Chol with bi-functional properties of pDNA binding *via* electrostatic interaction and a high affinity for PC *via* their asialoglycoprotein receptors.<sup>11</sup> We have also demonstrated that the galactose density of Gal-liposomes is important for both effective recognition by asialoglycoprotein receptors and cell internalization *in vivo*.<sup>13</sup> Since Gal-C4-Chol possesses an imino group for binding to pDNA *via* electrostatic interaction, many galactose units could be introduced on the liposomal surface without loss of binding affinity to pDNA.<sup>11</sup> These promising properties of our Gal-lipoplex enable PC-selective gene transfer under *in vivo* conditions.<sup>7,9,10</sup>

In order to analyze the effect of Gal-lipoplex size, three different sizes of Gal-liposomes were prepared using extrusion method. Figure 1 shows the particle sizes of the Gal-liposomes and Gal-lipoplexes prepared. The mean particle sizes of Gal-liposomes prepared using 100, 200, and 400 nm polycarbonate filters were 49.6, 148, and 197 nm, respectively. The mean particle sizes of Gal-lipoplexes were 141, 179, and 235 nm, respectively. Using these Gal-lipoplexes with different particle sizes, the effect of Gal-lipoplex size on hepatic- and hepatocyte-selective gene transfection was stud-

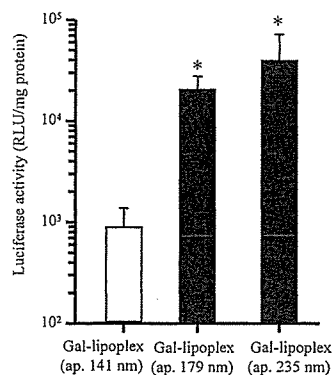


Fig. 2. Hepatic Transfection Activity of Gal-lipoplex after Intraportal Administration in Mice

Luciferase activity was determined 6 h post-injection of lipoplex. Statistically significant differences from galactosylated lipoplex (about 141 nm) (\**p* < 0.05). Each value represents the mean  $\pm$  S.D. of at least three experiments. ap.: approximately.

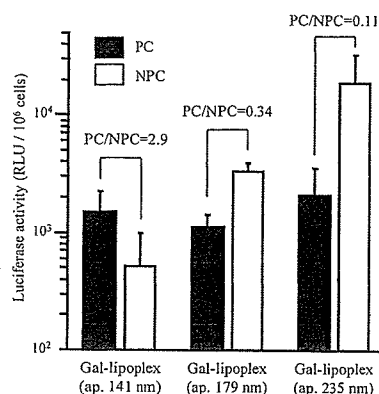


Fig. 3. Intrahepatic Transfection Activity of Gal-lipoplex after Intraportal Administration in Mice

Luciferase activity was determined 6h post-injection of lipoplex. Each value represents the mean  $\pm$  S.D. of at least three experiments. ap.: approximately.

ied.

We previously reported that gene expression in the liver was much higher than that in the lung, spleen, kidney, and heart after intraportal administration of Gal-lipoplex.<sup>7</sup> In the present study, the hepatic and intrahepatic gene expression characteristics were evaluated in relation to the size of Gal-lipoplex. As shown in Fig. 2, the hepatic transfection efficacy was significantly enhanced by an increase in the size of the Gal-lipoplex. To study the intrahepatic transfection activity, PC and NPC in the liver were separated by the collagenase perfusion technique (Fig. 3). The gene expression in PC of Gal-lipoplex about 141 nm in size was significantly higher than that in NPC. In contrast, the gene expression in PC of Gal-lipoplex about 179 and 235 nm in size was significantly lower than that in NPC. This finding might be due to the fact that the endothelial fenestrae measure 150–175 nm in diameter.<sup>14</sup>

Although we hypothesized that Gal-lipoplex over 200 nm in size has a limited ability to transfect the gene in liver because of the size limit, total hepatic gene expression was enhanced by increasing the size of the Gal-lipoplex (Fig. 2). This discrepancy may be partly explained by the presence of galactose particle receptors on Kupffer cells in NPC.<sup>15</sup> Kupffer cell would take up the Gal-lipoplex over 200 nm, which could not pass through the endothelial fenestrae. On the other hand, the uptake of the Gal-lipoplex over 200 nm by PC would be limited by the size of fenestrae. In fact, intrahepatic gene expression analysis demonstrated that Gal-lipoplex over 200 nm in size selectively transfect the gene in NPC (Fig. 3). These results highlight the importance of the Gal-lipoplex size for PC-selective gene transfer *in vivo*.

Physicochemical property of Gal-lipoplex is important concerning with PC-selective gene transfer *in vivo*.<sup>16</sup> Previously, we reported that the surface charge of Gal-lipoplex concerned with the interactions with blood components after intraportal injection of Gal-lipoplex<sup>10</sup> and charge of Gal-

lipoplex affected on the gene expression.<sup>7,17</sup> In this study, it was revealed that the size of Gal-lipoplex had effect of the hepatic distribution of gene expression. However, Goncalves et al. reported that lipoplex with different size have different gravity, therefore, such factor also might contribute on the *in vivo* gene transfer of Gal-lipoplex.<sup>18</sup>

In conclusion, we prepared three different Gal-lipoplex about 141, 179, and 235 nm in size, and demonstrated that the hepatic transfection efficacy was enhanced by increasing the size of the Gal-lipoplex after intraportal administration. However, intrahepatic transfection was altered by the size of the Gal-lipoplex, *i.e.*, PC-selectivity by Gal-lipoplex about 141 nm in size; NPC-selectivity by Gal-lipoplex about 235 nm in size. The information described in this study will be valuable for the future use, design, and development of Gal-lipoplexes for *in vivo* applications.

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## Effect of mannose density on mannose receptor-mediated cellular uptake of mannosylated O/W emulsions by macrophages

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### Abstract

Carbohydrate grafted emulsions are one of the most promising cell-specific targeting systems for lipophilic drugs. We have previously reported that mannosylated (Man-) emulsions composed of soybean oil, EggPC and cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)alkyl)formamide (Man-C4-Chol) with a ratio of 70:25:5 were significantly delivered to liver non-parenchymal cells (NPC) via mannose receptor-mediated mechanism after intravenous administration in mice. Since the efficient targeting through a receptor-mediated mechanism is largely controlled by ligand–receptor interaction, the effect of mannose density on Man-emulsions was studied with regard to both the disposition *in vivo* in mice and the uptake *in vitro*, using elicited macrophages which express a number of mannose receptors. After intravenous injection, Man-emulsions with 5.0% (Man-5.0-emulsions) and 7.5% (Man-7.5-emulsions) of Man-C4-Chol were rapidly eliminated from the blood circulation and preferentially accumulated in the liver-NPC compared with Man-emulsions with 2.5% of Man-C4-Chol (Man-2.5-emulsions) and bare emulsions (Bare-emulsions). The *in vitro* study showed increased internalization of Man-5.0- and Man-7.5-emulsions and significant inhibition of uptake in the presence of mannan. The enhanced uptake of Man-emulsions was related to the increasing of Man-C4-Chol content that corresponded to confocal microscopy study. These results suggest that the mannose density of Man-emulsions plays an important role in both cellular recognition and internalization via a mannose receptor-mediated mechanism.

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**Keywords:** Mannosylated emulsion; Macrophage; Mannose receptor; Mannose density; Targeting; Uptake

### 1. Introduction

Macrophages play a pivotal role in health and a variety of diseases such as innate and adaptive immunity [1], inflammation [2], Gaucher's disease [3] and tuberculosis [4]. A number of suitable strategies have been developed for macrophage-selective targeting systems [5]. Among them, receptor-mediated drug and gene targeting is one of the most promising approaches to achieve efficient therapy and minimize systemic toxicity [6]. One particular strategy involves the sugar recognition mechanism whereby receptors can recognize the corresponding sugars on the non-reducing terminal of sugar chains thereby mediating cellular uptake [7]. Of particular interest are mannose receptors, a 175 kDa transmembrane protein of the C-type lectin family [8], containing multiple C-

type lectin domains (CTLDs) [9], which are present on the surface of macrophages and capable of recognizing and internalizing mannose, fucose and *N*-acetylglucosamine terminated molecules [10,11].

However, the success of macrophage-selective delivery systems crucially depends on the ability to deliver drugs or agents to the intracellular site for the desired pharmacological action. Inadequate specificity for macrophages and poor internalization of drug-carrier conjugates are critical obstacles to their success. It is known that the efficiency of ligand recognition depends on the ligand–receptor interaction, multivalent ligands with a “cluster effect” showed higher binding affinity to their counter receptors than monovalent ligand [12–14]. Nevertheless, we have previously demonstrated the selective delivery of liposomes [15,16] and plasmid DNA/liposome complexes [17,18] modified with novel mannosylated cholesterol derivatives, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiomannosylethyl)amino)butyl)

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formamide (Man-C4-Chol), as a monovalent ligand to mannose receptors.

As far as lipophilic drug delivery systems are concerned, lipid emulsions are more appropriate than other carrier systems due to their physical stability, biocompatibility and highly solubilizing capacity [19,20]. We have previously reported the *in vivo* disposition of mannosylated emulsions (Man-emulsions) after intravenous administration in mice [21]. Man-emulsions were rapidly and mainly delivered to the liver and exhibited a high hepatic uptake clearance; nearly equal to the hepatic blood flow rate, within 10 min and were predominantly recovered in liver non-parenchymal cells (NPC) with a high NPC/parenchymal cell (PC) ratio. These results confirmed that the hepatic uptake of Man-emulsions was via mannose receptor-mediated uptake and suggested that Man-emulsions are a promising carrier for NPC targeting. We have previously demonstrated that one of the most important factors for recognition by mannose receptors is the mannose density of mannosylated proteins [22]. These observations prompted us to investigate the effect of mannose density on the uptake of Man-emulsions via mannose receptor-mediated uptake in order to achieve a more rational approach to the design of drug delivery systems.

Although *in vivo* uptake experiments have confirmed the uptake of Man-emulsions via a mannose receptor-mediated mechanism, this uptake mechanism could not be defined clearly because of complications in the *in vivo* study including interaction with endogenous substances [23,24], multispecificity of ligand recognition [10,11] and differences in the cellular localization of mannose receptors [25].

To examine the effect of the mannose density on the surface of Man-emulsions for efficient targeting to macrophages, Man-emulsions with different ratios of Man-C4-Chol were characterized in a series of *in vivo* and *in vitro* studies. The *in vivo* and *in vitro* experiments with Man-emulsions showed enhanced uptake of Man-emulsions with increasing mannose density. These results demonstrate the influence of the mannose density of Man-emulsions on their recognition and internalization by mannose receptors.

## 2. Materials and methods

### 2.1. Materials

Soybean oil was supplied from Wako Pure Chemicals Industry, Ltd. (Osaka, Japan). Egg phosphatidylcholine (EggPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesteryl chloroformate and pyridine were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). [<sup>3</sup>H]Cholesteryl hexadecyl ether (CHE) was supplied by NEN Life Science Products Inc. (Boston, MA, USA) and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cholesterol (Chol), mannan from *yeast extract* and iodoacetate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was obtained from Packard Co., Inc. (Groningen, The Netherlands). Other chemicals used were of the highest purity available.

### 2.2. Synthesis of Man-C4-Chol

Man-C4-Chol was synthesized by the method described previously [15]. Briefly, cholesteryl chloroformate was reacted with *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester in chloroform for 24 h at room temperature and then incubated with trifluoroacetic acid for 4 h at 4 °C. *N*-(4-Aminobutyl)-(cholesten-5-yloxy)formamide was obtained after evaporation. A quantity of the resulting material was added to an excess of 2-imino-2-methoxyethyl-1-thio-mannoside [26] in pyridine containing triethylamine. After 24 h incubation at room temperature, the reaction mixture was evaporated, resuspended in water and dialyzed against distilled water for 48 h using a dialysis membrane (12 kDa cutoff), and finally lyophilized.

### 2.3. Preparation of O/W emulsions

Emulsions were prepared by the method described previously [20]. Bare- or Man-emulsions consisting of soybean oil, EggPC, and Chol or Man-C4-Chol at various weight ratios were used. The lipid mixtures with a trace amount of [<sup>3</sup>H]CHE were dissolved in chloroform. For confocal microscopy study, fluorescent phosphatidylethanolamine (PE)-carboxyfluorescein (1 mol%) was added to the chloroform mixture. The dried film was then vacuum-desiccated and resuspended in pH 7.4 phosphate buffered saline (PBS). The suspensions were sonicated for 1 h (200 W) under a current of nitrogen. The particle sizes and zeta potentials of the emulsions were determined using a Zetasizer Nano ZS instrument (Malvern Instruments, Ltd., Worcestershire, UK). The concentration of the emulsions was adjusted to suitable concentrations based on the radioactivity of the [<sup>3</sup>H]CHE-labeled emulsions.

### 2.4. Animals

Four- to five-week-old male ddY and ICR mice were obtained from the Shizuoka Agriculture Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the US National Institutes of Health and the Guideline for Animal Experiments of Kyoto University.

### 2.5. *In vivo* distribution study

[<sup>3</sup>H]CHE (0.1 μCi/100 μL)-labeled emulsions at a lipid concentration of 0.5% were injected into the tail vein of male ddY mice at a dose of 25 mg/kg. At given times, mice were sacrificed, and then blood samples from the vena cava, liver, spleen, heart, lung, kidney, muscle and urine were collected and dissolved in Soluene-350 for radioactivity measurement using a liquid scintillation counter LSC6100 (Aloka Inc., Tokyo, Japan).

### 2.6. Determination of tissue uptake clearances of emulsions

Tissue distribution data were evaluated using the organ distribution clearances as reported previously [27]. Briefly,

the tissue uptake rate can be described by the following equation:

$$\frac{dX_t}{dt} = CL_{\text{uptake}} \cdot C_b \quad (1)$$

where  $X_t$  is the amount of [ $^3\text{H}$ ]CHE-labeled emulsions in the tissue at time  $t$ ,  $CL_{\text{uptake}}$  is the tissue uptake clearance and  $C_b$  is the blood concentration of [ $^3\text{H}$ ]CHE-labeled emulsions. Integration of Eq. (1) gives

$$X_t = CL_{\text{uptake}} \cdot \text{AUC}_{(0-t)} \quad (2)$$

where  $\text{AUC}_{(0-t)}$  represents the area under the blood concentration–time curve from time 0 to  $t$ . The  $CL_{\text{uptake}}$  value can be obtained from the initial slope of a plot of the amount of [ $^3\text{H}$ ]CHE-labeled emulsion in the tissue at time  $t$  ( $X_t$ ) vs. the area under the blood concentration–time curve from time 0 to  $t$  [ $\text{AUC}_{(0-t)}$ ].

### 2.7. Hepatic cellular localization of emulsions

The separation of liver-PC and NPC was performed by the collagenase perfusion method [28,29]. Briefly, mice were anesthetized with pentobarbital (40–60 mg/kg) after an intravenous injection of with 0.5% [ $^3\text{H}$ ]CHE (0.5  $\mu\text{Ci}/100 \mu\text{L}$ )-labeled emulsions at a dose 25 mg/kg. Thirty minutes later, the liver was perfused via the portal vein with perfusion medium ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free HEPES solution, pH 7.2) for 10 min and then with HEPES solution containing 5 mM  $\text{CaCl}_2$  and 0.05% (w/v) collagenase (type I) solution at pH 7.5 for 10 min. As soon as the perfusion started, the vena cava and aorta were cut and the perfusion rate was maintained at 3–4 mL/min. Following the discontinuation of perfusion, the liver was excised and its capsular membranes were removed. The cells were suspended by gentle stirring in ice-cold Hank's-HEPES buffer containing 0.1% bovine serum albumin (BSA). The dispersed cells were filtered through gauze and centrifuged at  $50\times g$  for 1 min. The pellets (PC-enriched fraction) and the supernatant (NPC-enriched fraction) were subjected to repeated low-speed differential centrifugation for cell purification [30,31]. The pellets containing the liver-PC were washed twice with Hank's-HEPES solution and then centrifuged at  $50\times g$  for 1 min for three times. The supernatant containing liver-NPC was similarly centrifuged until there was no cell pellet contamination. The resulting material was then centrifuged twice at  $200\times g$  for 2 min and then at  $400\times g$  for 2 min. PC and NPC were resuspended separately in ice-cold Hank's-HEPES solution (4 mL for PC and 2 mL for NPC). The number of viable cells was determined by the trypan blue exclusion method. Then, the radioactivity in the cells (0.5 mL) was measured using a liquid scintillation counter LSC6100 (Aloka Inc., Tokyo, Japan).

### 2.8. Harvesting and culture of macrophages

Elicited macrophages were harvested from the peritoneal cavity of male ICR mice 4 days after intraperitoneal injection of

1 mL 2.9% thioglycolate medium (Nissui Pharmaceutical, Tokyo, Japan). Cells were washed, then suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedical Inc., OH, USA), penicillin G (100 U/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ). The cells were plated on 12-well Falcon<sup>®</sup> culture plates (Becton Dickinson Labware Inc., Franklin Lakes, NJ, USA) at a density of  $1.3 \times 10^5$  cells/ $\text{cm}^2$ . After incubation for 6 h at 37 °C in 5%  $\text{CO}_2$ –95%  $\text{O}_2$ , non-adherent macrophages were washed off with culture medium and then cultured under the same conditions.

### 2.9. In vitro uptake study

Uptake experiments were carried out after 72 h cultivation [32]. Cells were washed twice with 1 mL pH 7.4 Hank's balanced salt solution (HBSS), then cultured with [ $^3\text{H}$ ]CHE-labeled emulsions at concentration of 0.12 mg/mL (0.06  $\mu\text{Ci}/\text{mL}$ ). For the inhibition study, macrophages were incubated with the mixture of emulsions and inhibitors or pre-incubated with 1 mM of iodoacetate, endocytotic inhibition, for 30 min prior to emulsions [33]. After incubation at 4 or 37 °C, the cells were washed five times with ice-cold PBS buffer. For the internalization study, the cells were washed five times with ice-cold acetate buffer pH 4.0 or 20 mM EDTA in PBS buffer to remove the emulsions bound to the cell surface. The cells were then solubilized in 0.5 mL 1 M NaOH overnight and neutralized with 0.1 mL 5 M HCl. The radioactivity was measured using a liquid scintillation counter LS2500 (Beckman Coulter, Inc., Tokyo, Japan). The protein content was determined by a Proteostain Protein Quantification Kit (Doiindo Molecular Technologies, Inc., MD, USA). The radioactivity was normalized with respect to the protein content of the cells.

### 2.10. Confocal microscopy study

The isolated macrophages were cultured at a density of  $1.3 \times 10^5$  cells/ $\text{cm}^2$  on a glass-bottom 12-well plate. After a 72 h cultivation, the cells were washed twice with 1 mL HBSS, and then incubated at 4 or 37 °C with 0.12 mg/mL of PE-carboxyfluorescein emulsions. After a 2 h incubation, the cells were washed five times with ice-cold HBSS, then fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in PBS (+) for 10 min on ice and washed three times with ice-cold HBSS. For plasma membrane staining, fixed cells were incubated with 20  $\mu\text{g}/\text{mL}$  rhodamine–concanavalin A (ConA) at room temperature for 5 min [34]. After washing twice with 1 mL ice-cold HBSS, cover glasses were mounted on slide glasses with 50% glycerol–2.5% DABCO (1,4-diazabicyclo-[2,2,2] octane) (Sigma Chemical Co., Inc., St. Louis, MO, USA) in PBS. The samples were examined by a Fluoview<sup>®</sup> confocal laser microscope (Olympus Inc., Tokyo, Japan). The objective specifications were 40 $\times$  oil immersion and numerical aperture 1.0.

### 2.11. Statistical analysis

Statistic analysis was performed using Student's paired  $t$ -test for two groups and the Turkey–Kramer test for multiple

comparisons between groups.  $P < 0.05$  was considered to be indicative of statistical significance.

### 3. Results

#### 3.1. Physicochemical properties of emulsions

The lipid composition, mean particle sizes and zeta potentials of the emulsions are shown in Table 1. Among the various ratios of Man-C4-Chol containing emulsions, the mean particle sizes were about 75–90 nm, whereas the zeta potentials were slightly increased in accordance with the content ratio of Man-C4-Chol. However, there was no significant difference in physicochemical properties among these emulsions.

#### 3.2. In vivo distribution study and pharmacokinetic analysis

To determine the influence of mannose density on the in vivo disposition of Man-emulsions, mice were intravenously injected with 0.5% [ $^3\text{H}$ ]CHE-labeled emulsions, and then the radioactivity in blood and tissue samples was measured versus time. Bare- and Man-emulsions were rapidly eliminated from the blood circulation (Fig. 1A) and predominately recovered in the liver within the first 10 min (Fig. 1B). Man-5.0- and Man-7.5-emulsions exhibited significant accumulation in the liver, accounting for 70% and 80% of the dose, respectively. In contrast, Man-2.5-emulsions had a similar liver accumulation, about 50% of the dose, to that of Bare-emulsions.

The tissue uptake clearance was analyzed in the early phase up to 10 min to avoid the tissue uptake efflux of [ $^3\text{H}$ ]CHE. Table 2 summarizes the pharmacokinetic parameters including the AUC and the uptake clearances of Bare- and Man-emulsions. The uptake clearances were determined for liver ( $\text{CL}_{\text{liver}}$ ), spleen ( $\text{CL}_{\text{spleen}}$ ), heart ( $\text{CL}_{\text{heart}}$ ), lung ( $\text{CL}_{\text{lung}}$ ), kidney ( $\text{CL}_{\text{kidney}}$ ), muscle ( $\text{CL}_{\text{muscle}}$ ) and urine ( $\text{CL}_{\text{urine}}$ ). This pharmacokinetic analysis provided information about the uptake clearance characteristics of these emulsions with a small AUC and a large hepatic uptake clearance. Although their hepatic uptake clearance was the highest among the tissues examined, the hepatic uptake clearances of Man-5.0- and Man-7.5-

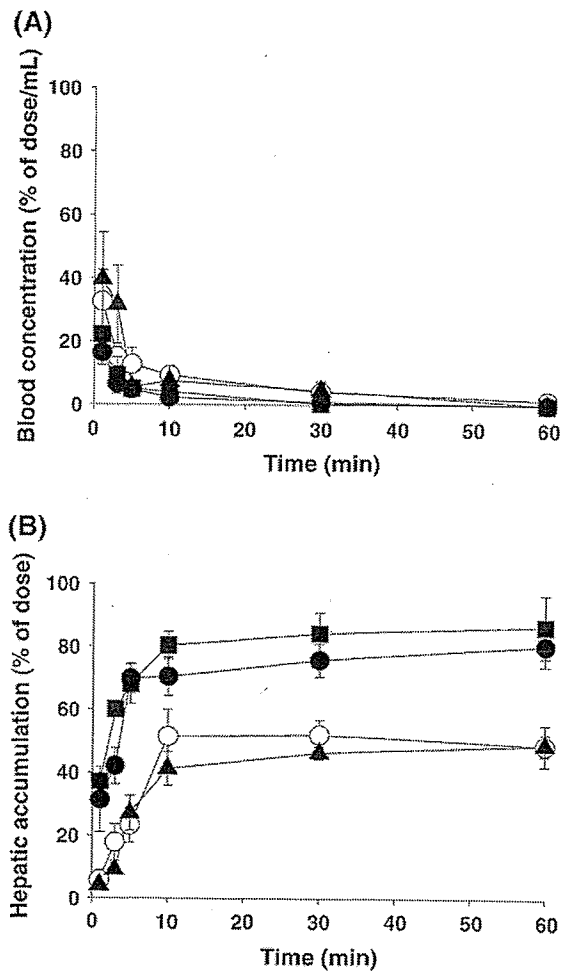


Fig. 1. (A) Blood concentration and (B) hepatic accumulation of Bare- (○), Man-2.5- (▲), Man-5.0- (●) and Man-7.5- (■) emulsions following intravenous injection in mice. Each value represents the mean  $\pm$  S.D. of three experiments.

emulsions were similarly high and about 3.5 times greater than those of Bare- and Man-2.5-emulsions. Bare- and Man-2.5-emulsions had identical AUC and hepatic uptake clearance values.

#### 3.3. Hepatic cellular localization

To examine the cell-selective targeting ability of Man-emulsions, the hepatic cellular distribution of 0.5% [ $^3\text{H}$ ]CHE-labeled emulsions 30 min post-administration was studied by the collagenase method (Fig. 2). The distribution in NPC and PC was determined by the NPC/PC ratio after normalization of cell numbers. The affinity of these emulsions for NPC was increased in parallel with the Man-C4-Chol content. Man-5.0 and Man-7.5-emulsions were clearly preferentially localized in NPC with a high NPC/PC ratio, 2.0 and 13.7, respectively ( $P < 0.001$ ) with a similar degree of uptake. However, Man-2.5-emulsions were equally localized in NPC and PC, whereas Bare-emulsions were predominately localized in PC with a low NPC/PC ratio, 0.4 ( $P < 0.05$ ).

Table 1

The lipid composition, mean particle sizes and zeta potentials of emulsions

Emulsions (lipid composition, weight ratio)	Mean particle size (nm)	Zeta potential (mV)
Bare-emulsions (soybean oil/EggPC/Chol, 70:25:5.0)	85 $\pm$ 5.1	-1.1 $\pm$ 1.5
Man-2.5-emulsions (soybean oil/EggPC/Man-C4-Chol, 70:27.5:2.5)	90 $\pm$ 13	-0.2 $\pm$ 0.3
Man-5.0-emulsions (soybean oil/EggPC/Man-C4-Chol, 70:25:5.0)	75 $\pm$ 3.0	2.2 $\pm$ 3.4
Man-7.5-emulsions (soybean oil/EggPC/Man-C4-Chol, 70:22.5:7.5)	80 $\pm$ 1.7	7.4 $\pm$ 3.0

Each value represents the mean  $\pm$  S.D. values ( $n=3$ ).

Table 2

Area under the blood concentration–time curve (AUC) and tissue uptake clearance of [ $^3\text{H}$ ]CHE-labeled emulsions after intravenous injection into mice<sup>a</sup>

Emulsions	AUC <sup>a</sup>	Tissue uptake clearance ( $\mu\text{L}/\text{h}$ )						
	(% of dose h/mL)	CL <sub>liver</sub>	CL <sub>spleen</sub>	CL <sub>heart</sub>	CL <sub>lung</sub>	CL <sub>kidney</sub>	CL <sub>muscle</sub>	CL <sub>urine</sub>
Bare-emulsions	2.8	18,300	724	1067	161	129	26	12
Man-2.5-emulsions	2.8	14,800	390	2060	145	140	32	15
Man-5.0-emulsions	1.2	60,500	4493	699	621	138	13	12
Man-7.5-emulsions	1.4	56,000	3521	272	1086	716	–	56

<sup>a</sup> The AUC and tissue uptake clearances were calculated for periods up to 10 min after intravenous injection. An average of three experiments is shown.

### 3.4. In vitro uptake study

In order to define the uptake characteristics of Man-emulsions, the uptake conditions were studied in terms of the dose, temperature and incubation time. Fig. 3A shows the dose-dependent in vitro uptake of Bare- and Man-5.0-emulsions by elicited macrophages at 37 °C for 2 h. The uptake of Man-5.0-emulsions markedly depended on the dose compared with that of Bare-emulsions. The uptake time courses both at 4 and 37 °C are shown in Fig. 3B. Man-5.0-emulsions were increasingly taken up over the incubation time at 37 °C compared with Bare-emulsions ( $P < 0.01$ ), although the uptake of both Man-5.0- and Bare-emulsions was very low at 4 °C.

### 3.5. Uptake inhibition study

To verify the uptake mechanism of Man-emulsions in a mannose receptor-mediated manner, competitive uptake experiments on [ $^3\text{H}$ ]CHE-labeled emulsions were performed with or without an excess of mannan, a known ligand for mannose receptors, and unlabeled bare-emulsions (Fig. 4). The highest cellular uptake was observed in Man-7.5-emulsions, followed by Man-5.0-emulsions, Man-2.5-emulsions and uptake was considerably lower by Bare-emulsions. The uptake by Man-emulsions with different Man-C4-Chol ratios was significantly inhibited in the presence of 1 mg/mL mannan ( $P < 0.05$  and 0.01) but not by bare-emulsions. On the other hand, the uptake

of Bare-emulsions was markedly suppressed by only co-incubation with bare-emulsions ( $P < 0.01$ ).

To confirm whether mannose residues trigger the binding and internalization of Man-emulsions into macrophages, the amount of surface binding and internalization of emulsions in macrophages were determined by pre-incubation with 1 mM

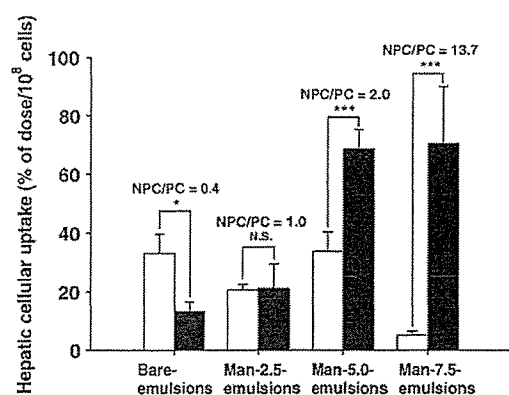


Fig. 2. Hepatic cellular localization of emulsions after intravenous injection in mice. Radioactivity was determined 30 min post-injection in PC (□) and NPC (■). Each value represents the mean  $\pm$  S.D. of three experiments. Statistically significant differences ( $*P < 0.05$ ,  $***P < 0.001$ ) between PC and NPC in each group; N.S., not significant.

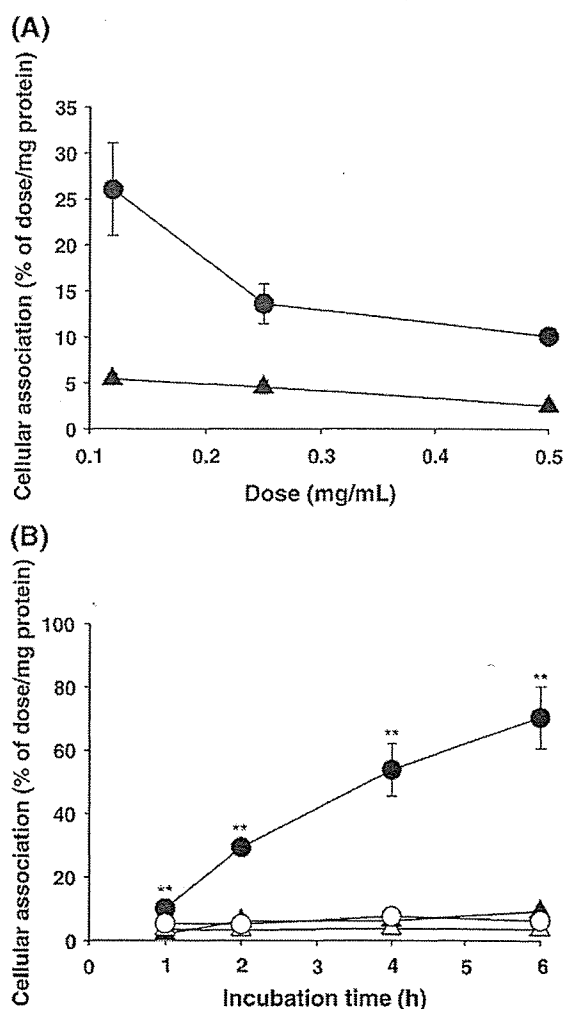


Fig. 3. (A) Dose-dependent cellular uptake of Bare-emulsions (▲) and Man-5.0-emulsions (●). Elicited macrophages were incubated with different concentrations of emulsions at 37 °C for 2 h. (B) The uptake time-course of 0.12 mg/mL of Bare-emulsions (▲, △) and Man-5.0-emulsions (●, ○) at 4 (opened) or 37 °C (filled) for given incubation time. Results are expressed the mean  $\pm$  S.D. of three experiments. Statistically significant differences ( $**P < 0.01$ ) from Bare-emulsions at each time point.

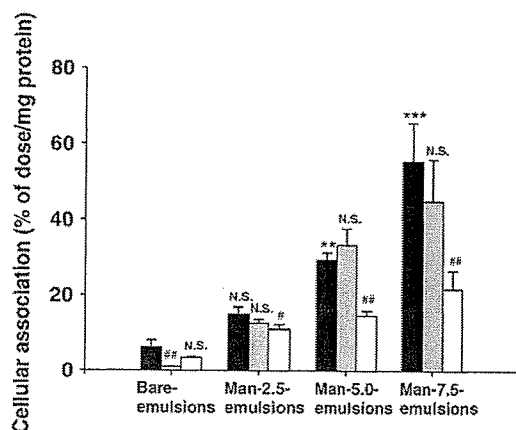


Fig. 4. The uptake of 0.12 mg/mL of emulsions by elicited macrophages. Macrophages were incubated with each emulsions alone (black) or with 1 mg/mL of bare-emulsions (grey) or 1 mg/mL of mannan (white). The amount of emulsions associated with the cells was measured following a 2 h incubation at 37 °C. Each value represents the mean  $\pm$  S.D. of three experiments. Statistically significant differences (\*\* $P$ <0.01, \*\*\* $P$ <0.001) from control of Bare-emulsions (# $P$ <0.05, ## $P$ <0.01) from control of each group; N.S., not significant.

iodoacetate and washing with acetate buffer pH 4 or 20 mM EDTA in PBS, respectively (Fig. 5). Since the binding of mannose residues to mannose receptors has been reported to be sensitive to the environmental pH and calcium ions [35,36], these methods could be applied to determine the amount of internalized Man-emulsions. The amount of surface-bound and internalized emulsions obtained by these different methods was identical. Bare-emulsions underwent hardly any binding to the surface of macrophages, and then were internalized into the cells to a minor extent. Although Man-2.5-emulsions were bound to the cell surface to some extent, they underwent scarcely any internalization into the cells. In contrast, Man-5.0- ( $P$ <0.01) and Man-7.5-emulsions ( $P$ <0.001) showed a significantly increase in surface binding and exhibited

extensive uptake into the cells compared with Bare-emulsions. The uptake amount was reduced when cells were pretreated with iodoacetate. These inhibition studies also demonstrated the influence of acid and ions on the binding and internalization of Man-emulsions.

### 3.6. Confocal microscopy study

The cellular association of fluorescent emulsions after a 2 h incubation at 4 and 37 °C is shown in Fig. 6. Confocal microscopy images revealed a temperature-dependent binding (4 °C) and internalization (37 °C) of emulsions. In addition, the surface binding and internalization of Man-emulsions were increased with regard to the Man-C4-Chol content in a manner similar to that found in the in vitro uptake experiments.

## 4. Discussion

The internalization of drug-carrier conjugates into target cells is an important method of obtaining therapeutic efficacy. In order to evaluate the influence of the mannose density on the internalization of Man-emulsions in vivo, mice were intravenously injected with Man-emulsions containing different ratios of Man-C4-Chol. After intravenous administration, Man-5.0- and Man-7.5-emulsions were largely taken up by the liver (Fig. 1) accompanied by a high hepatic uptake clearance (Table 2) that was nearly equal to the hepatic plasma flow rate (66,000  $\mu$ L/h) [37]. Furthermore, Man-5.0- and Man-7.5-emulsions selectively distributed into NPC, rather than PC, with high NPC/PC ratio (based on cell number) compared with Bare- and Man-2.5-emulsions (Fig. 2). However, the whole liver uptake of Man-7.5-emulsions was predominantly contributed by NPC, whereas that of Man-5.0-emulsions was equally involved by NPC and PC. These results indicate that Man-emulsions exhibit efficient and selective targeting to NPC in accordance with the mannose residues. These present results are

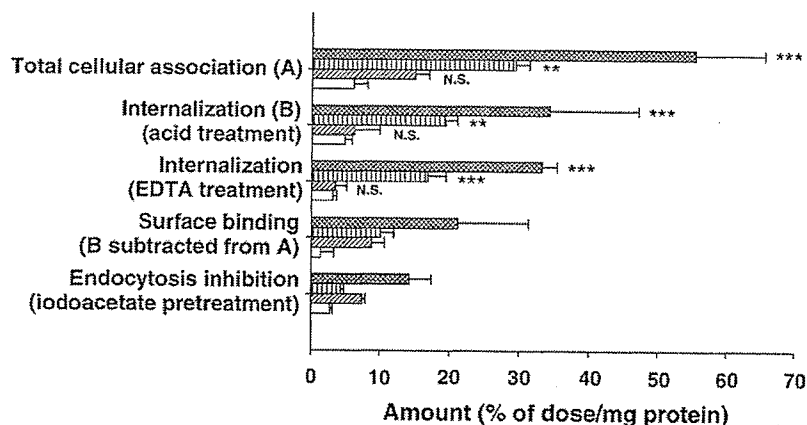


Fig. 5. Amount of emulsions associated with macrophages. Macrophages were incubated with Bare- (□), Man-2.5- (▨), Man-5.0- (▧) or Man-7.5- (▩) emulsions. After a 2 h incubation, the cells were washed with ice-cold PBS (total association), acetate pH 4 buffer or 20 mM EDTA in PBS in separate experiments. The difference in cellular association between EDTA or acid treatment and ice-cold PBS treatment was regarded as the amount associated with the cell surface. In another group, the cells were pre-incubated with HBSS containing 1 mM iodoacetate for 30 min prior to exposure to emulsions. Each value represents the mean  $\pm$  S.D. of three experiments. Statistically significant differences (\*\* $P$ <0.01, \*\*\* $P$ <0.001) from Bare-emulsions in each group; N.S., not significant.

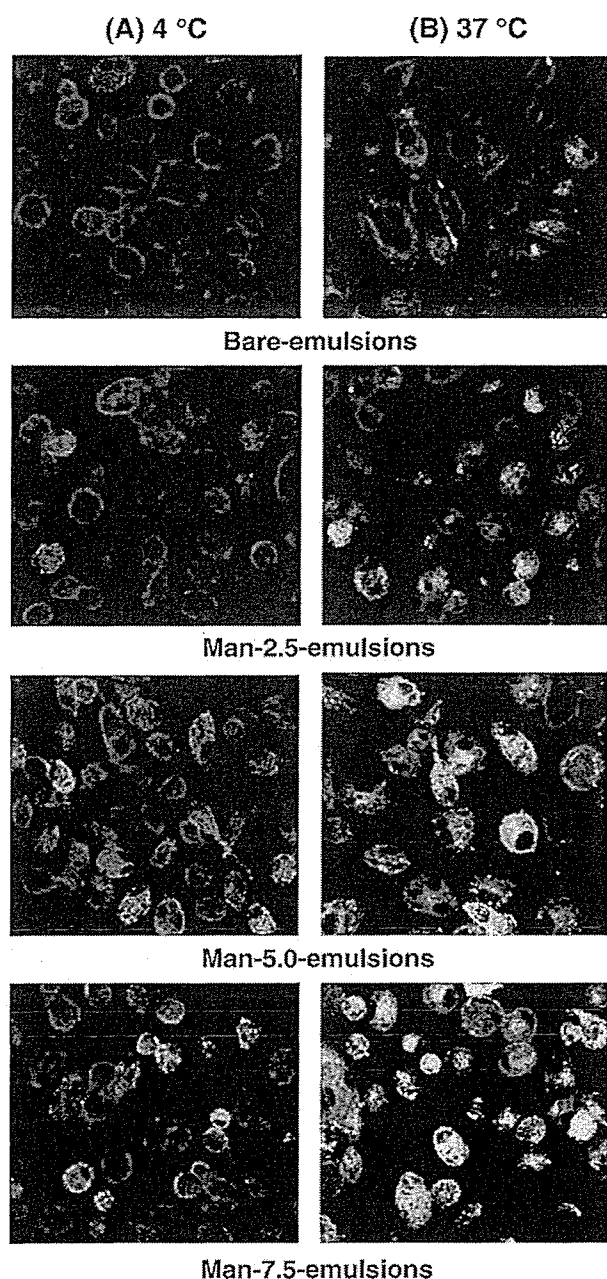


Fig. 6. Confocal microscopy images of binding and internalization of fluorescent PE-carboxyfluorescein emulsions by elicited macrophages. Macrophages were incubated with 0.12 mg/mL of emulsions at (A) 4 or (B) 37 °C for 2 h. The images are typical of at least three independent experiments.

similar to those of our previous study using galactosylated liposomes, which showed that the hepatic uptake was increased in proportion to the galactose density on the liposome surface via asialoglycoprotein receptors on PC after intravenous injection in mice [38].

To verify the uptake of Man-emulsions through mannose receptor recognition with respect to mannose density, *in vitro* uptake was examined in elicited macrophages which express mannose receptors on their cell surface. The uptake of Man-emulsions was considerably enhanced by increasing the number

of Man-C4-Chol residues and significantly inhibited by an excess of mannan (Fig. 4) suggesting mannose receptor-mediated uptake which is in agreement with our previous studies that the *in vivo* uptake of mannose-modified emulsions and liposomes were significantly inhibited in the presence of Man-BSA [16,21]. The enhanced uptake of Man-emulsions corresponded to the results in the confocal microscopy study (Fig. 6). The *in vivo* and *in vitro* uptake experiments suggest that the mannose residues on the surface of Man-5.0- and Man-7.5-emulsions are enough to interact with mannose receptors, and trigger internalization. This trend agrees with our previous study involving galactosylated emulsions which showed that the uptake of galactosylated emulsions by HepG2 cells was enhanced on increasing the galactose density on the emulsions via an asialoglycoprotein receptor-mediated mechanism [39].

To investigate the uptake mechanism of Man-emulsions through a mannose receptor-mediated process in more detail, the cellular association of the emulsions was analyzed under different conditions. The inhibition of uptake of Man-emulsions by pretreatment with iodoacetate (Fig. 4) and reducing the temperature to 4 °C (Fig. 3B) suggests an energy-dependent endocytosis mechanism. After washing with acid and EDTA buffer, the total cellular association of Man-emulsions was reduced by both treatments suggesting calcium-dependent binding to mannose receptors which is in agreement with our previous study involving the hepatic uptake of Man-BSA after single-pass perfusion in rats [40]. The EDTA study showed that the amount of surface-bound and internalized Man-emulsions was similar to that in the acid treatment study. These identical results demonstrated that the amount of internalized Man-emulsions was significantly increased in proportion to the Man-C4-Chol content, while the corresponding surface-bound emulsions were slightly enhanced. The difference in the amount internalized could be explained by the effect of the mannose density on the surface of Man-emulsions that may be associated with the relative affinity of Man-emulsions for mannose receptors. These results lead us to believe that the improved internalization of Man-emulsions is, at least partly, due to the increased mannose density on their surface.

In this present study, the mean particle sizes of these emulsions were maintained at around 100 nm (Table 1), since the particle size appears to alter the physical stability during storage [21] and biodistribution [41,42]. The previous studies of the *in vivo* disposition of Bare-emulsions with a small particle size demonstrated that Bare-emulsions are largely taken up by PC after intravenous administration [41–43]. Consistent with previous reports, we observed that Bare-emulsions accumulated in the liver to a modest degree (Fig. 1) and were mainly localized in PC (hepatocytes) (Fig. 2), suggesting the selective uptake of Bare-emulsions by PC.

The uptake specificity of Bare-emulsions was examined in primary cultured peritoneal mouse macrophages. As shown in Fig. 3B, the low uptake of Bare-emulsions by macrophages at 37 °C agreed well with previous studies [44,45]. Granot et al. [44] and Sakurai et al. [45] investigated the cellular uptake of Bare-emulsions (with 60–80 nm in diameter) and showed that they were hardly taken up by J774 macrophage cell lines, which

express mannose receptors, in the absence of apo-E. These observations support the relatively low uptake of Bare-emulsions with small particle size by macrophages.

It is worthwhile using carriers with improved therapeutic properties for either systemic or local administration. The Kupffer cells, resident macrophages in the liver-NPC, secrete large amounts of proinflammatory cytokines after activation leading to cytokine-related diseases such as liver inflammation [46]. Since Man-emulsions selectively and highly accumulate in the liver-NPC, Man-emulsions containing anti-inflammatory drugs might improve low-dose treatment with less undesirable effects given by intravenous injection. Recently, the incidence of pulmonary tuberculosis has increased particularly with the AIDS pandemic, which has resulted in a huge number of deaths worldwide [47]. Besides the undesirable effects associated with long-term treatment, one problem associated with treatment is the failure of anti-tubercular drugs to penetrate into infected macrophages [48]. Accordingly, much effort has been directed toward the development of macrophage-selective targeting systems using a variety of carriers; however, their efficiency and specificity need further investigation. In this study, we have shown that Man-emulsions with 5.0% or 7.5% Man-C4-Chol are preferentially delivered to macrophages and effectively internalized into cells; therefore, they might be of use as a drug carrier for lipophilic drugs in the treatment of macrophage-related diseases.

In this present study, we evaluated the uptake characteristics of Man-emulsions by primary cultured mouse peritoneal macrophages. Previously, Muller and Schuber studied the uptake characteristics of neo-mannosylated liposomes composed of Egg phosphatidylcholine and cholesterol with mouse Kupffer cells and resident peritoneal macrophages and reported that neo-mannosylated liposomes were taken up by Kupffer cells and peritoneal macrophages in the same manner with nearly similar amount [49]. Although further studies are needed, this report might partly support the uptake characteristics of Man-emulsions by primary cultured peritoneal macrophages corresponding to that by primary cultured Kupffer cells.

## 5. Conclusion

Man-emulsions preferentially accumulated in liver-NPC after intravenous administration in mice based on the mannose density. Furthermore, Man-emulsions with a high content of mannose residues exhibited a higher binding affinity for macrophages and are extensively internalized compared with emulsions with a lower mannose content. These results strongly suggest that the mannose density of Man-emulsions play an important role in both efficient cellular recognition and internalization. These observations provide valuable information for the rational drug design of mannosylated carrier systems for efficient macrophage-targeting.

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## Evaluation of Proinflammatory Cytokine Production Induced by Linear and Branched Polyethylenimine/Plasmid DNA Complexes in Mice

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### ABSTRACT

The purpose of this study was to evaluate the cytokine response induced by linear and branched polyethylenimine (PEI)/plasmid DNA (pDNA) complex (polyplex) in relation to the ratio of PEI nitrogen and DNA phosphate (N/P ratio) of the polyplex, dose of pDNA, and structure and molecular weight of PEI, which are important for transfection efficacy of PEI polyplex. As a control, a *N*-[1-(2, 3-dioleyloxy) propyl]-*n,n,n*-trimethylammonium chloride/cholesterol liposome/pDNA complex (lipoplex) was selected for its high transfection efficacy *in vivo*. The concentration of proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  were much lower after the administration of polyplex than lipoplex irrespective of the N/P ratio, dose of

pDNA, or structure and molecular weight of PEI, although these factors affected the transfection efficacy *in vivo*. We demonstrated that the amount of activated nuclear factor- $\kappa$ B, which contributes substantially to the production of cytokines, was comparable with the control (no treatment) level, and significantly less than that obtained with lipoplex. Although the production of proinflammatory cytokines (TNF- $\alpha$ , interferon- $\gamma$ , and interleukin-12) was reduced on the administration of the linear PEI polyplex, serum alanine aminotransferase levels were significantly enhanced by pDNA in a dose-dependent manner, suggesting that such hepatic damage is not induced by proinflammatory cytokines.

The success of gene therapy largely depends upon the development of delivery vehicles or vectors, which can selectively and efficiently deliver therapeutic genes to target cells with minimal toxicity (Ross et al., 1996). Viral vectors, although highly efficient, have inherent drawbacks such as immunogenicity; therefore, nonviral vectors have increasingly been receiving attention (Yang et al., 1994; Knowles et al., 1995). Of the various types of nonviral vectors, polyethylenimine (PEI) and cationic liposomes are most effective vectors in transfecting pDNA into target cells *in vivo* (Boussif et al., 1995; Boletta et al., 1997; Goula et al., 1998; Tranchant et al., 2004; Neu et al., 2005). PEI and cationic liposome-mediated gene transfer efficiently delivers a gene to the pulmonary endothelium after an *i.v.* administration. The

factors that enhance the transfection efficacy have been well studied, but it is also important to analyze the side effects of nonviral vectors for clinical applications.

As for the cationic liposome/pDNA complex (lipoplex), side effects have been documented. The CpG motifs in the pDNA sequence up-regulate the expression of transcription factors such as nuclear factor (NF)- $\kappa$ B, which contributes substantially to the production of cytokines (Krieg et al., 1995; David et al., 2000; Klinman, 2004). Consequently, lipoplex could induce the production of large quantities of proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$ , and interleukin (IL)-12 (Freimark et al., 1998; Li et al., 1999; Yew et al., 1999; Sakurai et al., 2002). It was suggested that these cytokines cause liver damage (Tan et al., 2001). In addition, these cytokines cause gene inactivation-inducing transient gene expression after a single injection and a refractory period on repeated dosing (Li et al., 1999; Tan et al., 2001). Therefore, these studies demonstrated that the immune response could influence the hepatic toxicity as well as the gene expression period.

To date, the transfection efficacy of the PEI/pDNA complex

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**ABBREVIATIONS:** PEI, polyethylenimine; pDNA, plasmid DNA; lipoplex, cationic liposome/pDNA complex; NF $\kappa$ B, nuclear factor  $\kappa$ B; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; N/P ratio, the ratio of PEI nitrogen and DNA phosphate; polyplex, PEI/pDNA complex; DOTMA, *N*-[1-(2, 3-dioleyloxy) propyl]-*n,n,n*-trimethylammonium chloride; -:+, charge ratio; ALT, alanine aminotransferase.

(polyplex) has been determined based on the N/P ratio of PEI polyplex, the dose of pDNA, and the structure and molecular weight of PEI (Goula et al., 1998; Bragonzi et al., 2000; Zou et al., 2000; Wightman et al., 2001). In contrast, not much has been done to clarify the cytokine response to PEI polyplex after its i.v. administration. Therefore, these three factors need to be related with cytokine levels for the optimization of gene therapy using PEI polyplex.

In this study, the transfection efficacy, production of cytokines, and toxicity in the liver after the i.v. administration of linear and branched PEI polyplexes were evaluated in relation to the N/P ratio of PEI polyplex, the dose of pDNA, and the structure and molecular weight of PEI. As a control, a *N*-[1-(2, 3-dioleoyloxy) propyl]-*n,n,n*-trimethylammonium chloride (DOTMA)/cholesterol liposome-based lipoplex was selected because of its high transfection efficacy *in vivo* (Song et al., 1997; Sakurai et al., 2001).

## Materials and Methods

**Materials.** DOTMA was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The branched PEI (10, 25, and 70 kDa) and linear PEI (25 kDa) were obtained from Polysciences, Inc. (Warrington, PA). Cholesterol and Clear-sol I were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Soluene-350 was purchased from PerkinElmer, Inc. (Wellesley, MA). [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) was obtained from Amersham Biosciences Co. (Piscataway, NJ). QIAGEN Endofree Plasmid Giga Kit was purchased from QIAGEN GmbH (Hilden, Germany). All other chemicals were of the highest purity available.

**Animals.** Five-week-old female ICR mice (20–23 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Kyoto University.

**Preparation of pDNA.** pCMV-Luc was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from a pGL3-control vector (Promega Co., Madison, WI) into the polylinker of a pcDNA3 vector (Invitrogen, Co., Carlsbad, CA). pDNA was amplified in the *Escherichia coli* strain DH5 $\alpha$ , isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit. The concentration of DNA was determined by measuring UV absorption at 260 nm. The pDNA was labeled with [ $\alpha$ - $^{32}$ P]dCTP by nick translation.

**Preparation of PEI Polyplex.** PEI polyplex was prepared as reported (Morimoto et al., 2003). In brief, linear PEI or branched PEI was dissolved in a 5% dextrose solution and adjusted to pH 7.4. PEI polyplex was formed by adding an equal volume of PEI to pDNA in 5% dextrose at various ratios and left at 37 °C for 30 min. The ratio of PEI to pDNA was expressed as the N/P ratio, which is the molar ratio of PEI nitrogen to DNA phosphate (Goula et al., 1998).

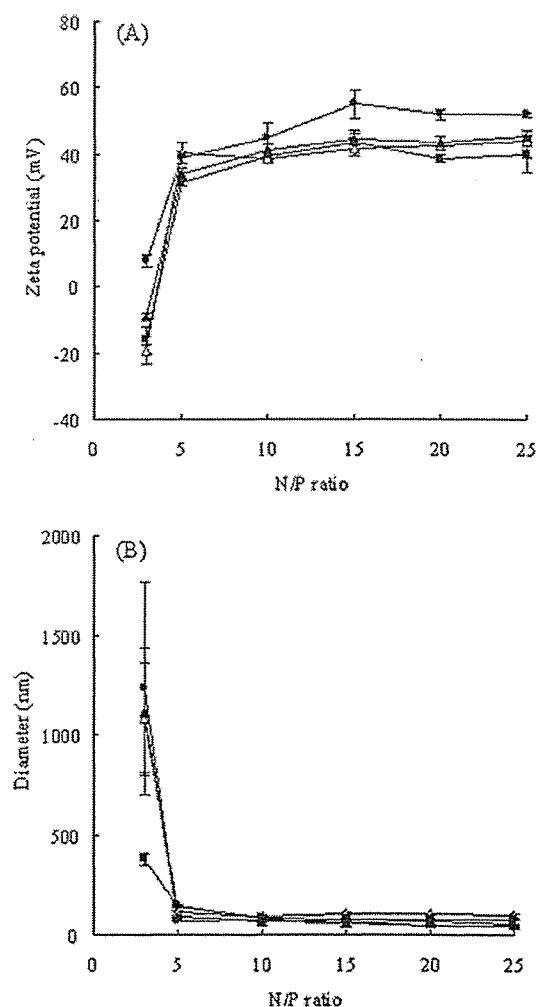
**Preparation of Cationic Liposomes.** DOTMA/cholesterol liposomes were prepared as reported (Kawakami et al., 2000a). DOTMA and cholesterol were dissolved in chloroform at a molar ratio of 1:1. The mixture was vacuum-desiccated and resuspended in 5% dextrose. After hydration, the suspension was sonicated on ice for 3 min, and the resulting liposomes were extruded through a 220-nm polycarbonate filter.

**Preparation of Lipoplex.** Lipoplex was prepared as reported (Kawakami et al., 2000a,b; Sakurai et al., 2001). In brief, it was formed by adding an equal volume of cationic liposomes to pDNA in 5% dextrose at a mixing ratio (–:+) of 1.0:3.1 and stored at room temperature for 30 min. The ratio of liposomes to pDNA was expressed as a charge ratio (–:+) which is the molar ratio of cationic lipids to DNA phosphate (Yang and Huang, 1997).

**Measurement of  $\zeta$  Potential and Particle Size.** PEI and pDNA were mixed in 5% dextrose as above and concentrated for i.v. administration. After 30 min, the  $\zeta$  potential and size of PEI polyplex were measured using Nano ZS (Malvern Instruments, Ltd., Malvern, Worcestershire, UK).

**Gene Expression Experiments.** Gene expression was measured as described previously (Liu et al., 1997; Kawakami et al., 2000a,b). Mice were administered i.v. with 300  $\mu$ l of lipoplex or PEI polyplex. At specific time points, mice were sacrificed, the lung and liver were harvested, and homogenates were prepared by adding lysis buffer (0.05% Triton X-1000, 2 mM EDTA, and 0.1 M Tris, pH 7.8) using homogenizer (OMNI TH; Yamato Scientific Co. Ltd., Tokyo, Japan) at 4°C. The volume of lysis buffer added was 4  $\mu$ l/mg for lung and 5  $\mu$ l/mg for liver. To lyse cells, the homogenates were treated with three cycles of freezing and thawing. The homogenates were centrifuged at 12,000g for 7 min at 4°C. Twenty microliters of each supernatant was analyzed for luciferase activity with 100  $\mu$ l of luciferase assay buffer (Picagene; Toyo Ink Mfg. Co. Ltd., Tokyo, Japan), using a luminometer (Lumat LB 9507; Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany).

**Measurements of Cytokines and ALT.** Serum was prepared as outlined in our previous study (Sakurai et al., 2001). At specific time points after the i.v. administration of PEI polyplex and lipoplex,



**Fig. 1.**  $\zeta$  Potential (A) and particle size (B) of PEI polyplex at various N/P ratios. Linear PEI<sub>25</sub> polyplex (open triangles), branched PEI<sub>10</sub> polyplex (filled squares), branched PEI<sub>25</sub> polyplex (filled triangles), and branched PEI<sub>70</sub> polyplex (filled circles) were mixed with pDNA in 5% dextrose for 30 min at room temperature. Results are the mean  $\pm$  S.D. of three measurements.

blood was collected from the vena cava and left to stand for 3 h at 37°C and then overnight at 4°C. Samples were centrifuged, and the supernatants were collected for serum. Serum TNF- $\alpha$ , IFN- $\gamma$ , and IL-12 concentrations were determined with enzyme-linked immunosorbent assay kits according to the manufacturer's (Genzyme Co., Cambridge, MA) instructions. The serum ALT concentration was measured with kits using the UV-Rate method according to the manufacturer's direction (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Measurement of NF $\kappa$ B.** Three hours after the administration of PEI polyplex and lipoplex, mice were sacrificed, and their livers were collected. A nuclear extract of liver cells was prepared using the Nuclear/Cytosol Fractionation Kit (BioVision, Inc., CA). To analyze the extract, protein concentrations were prepared at 0.25  $\mu$ g/ $\mu$ l. The amount of activated NF $\kappa$ B was measured by using an Enzyme Immunoassay for NF $\kappa$ B (human, mouse and rat) (Oxford Biomedical Research, Inc., Oxford, MI).

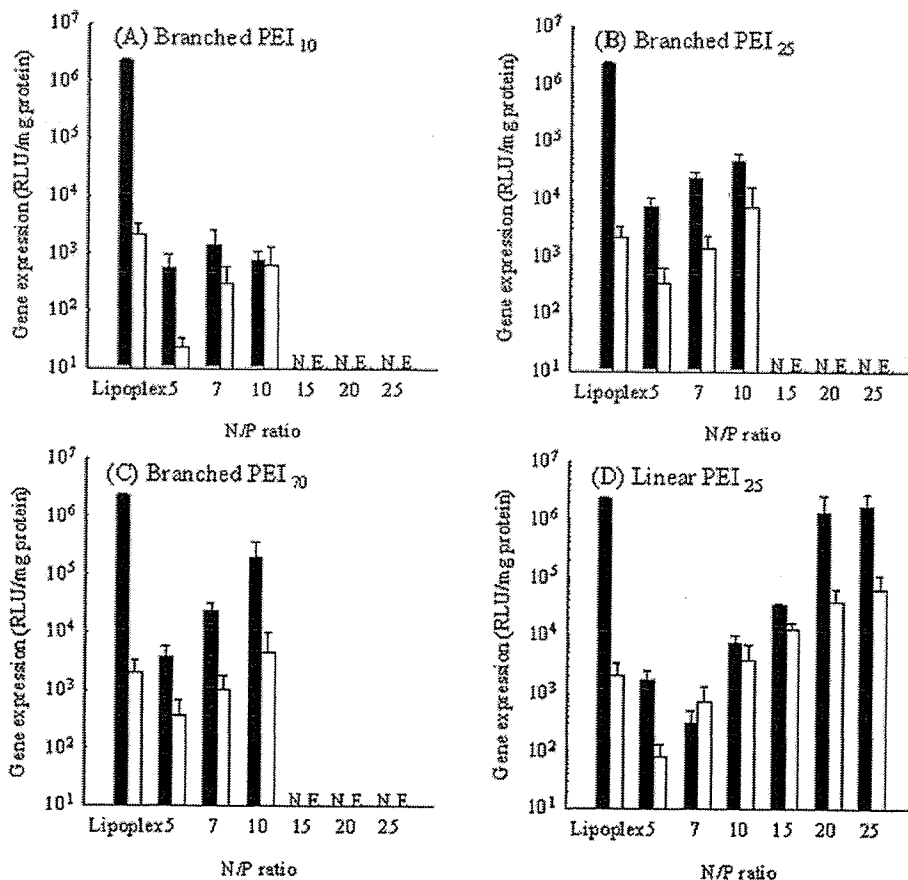
**Experiments on in Vivo Distribution.** Radioactivity was measured as reported previously (Kawakami et al., 2000a). Mice received an i.v. injection of 10 kBq [ $^{32}$ P]pDNA and pDNA (30  $\mu$ g) in a complex with linear PEI $_{25}$  or cationic liposomes in 5% dextrose (300  $\mu$ l) and were killed at a given time point. The liver and lung were removed, washed with saline, blotted dry, and weighed. Ten microliters of blood and 20 to 30 mg of each tissue were digested with 700  $\mu$ l of Soluene-350 by incubation overnight at 45 °C. After the digestion, 200  $\mu$ l of isopropanol, 200  $\mu$ l of 30% hydrogen peroxide, 100  $\mu$ l of 5 N HCl, and 5.0 ml of Clear-sol I were added. The samples were stored overnight, and radioactivity was measured in a scintillation counter (LSA-500; Beckman, Tokyo, Japan).

**Statistical Analysis.** Statistical analysis was performed using Student's paired *t* test for two groups. Multiple comparisons among different groups were performed with the Turkey-Kramer test. *P* < 0.05 was considered to be indicative of statistical significance.

## Results

**$\zeta$  Potential and Particle Size.** Figure 1A shows the  $\zeta$  potential of the linear and branched PEI with various molecular weights and N/P ratios. PEI polyplex was negatively charged at an N/P ratio of 3 except the branched PEI $_{70}$  polyplex which was positive, and increasing the N/P ratio from 5 resulted in a positive charge for all polyplexes (approximately 30–40 mV). Increasing the N/P ratio between 5 and 25 hardly changed the  $\zeta$  potential of PEI polyplex (approximately 30–50 mV). As for the size of particles, it was approximately 400 nm at an N/P ratio of 3, except for the branched PEI $_{10}$  polyplex (Fig. 1B). At an N/P ratio of from 5 to 25, the size of all PEI polyplexes appeared to be reduced to approximately 80 to 90 nm, and this value was constant as the N/P ratio increased. In the present study, the  $\zeta$  potential and particle size of lipoplex (-: + of 1.0:3.1) were  $60 \pm 3.1$  mV (*n* = 3) and  $103 \pm 0.78$  nm (*n* = 3), respectively.

**Effect of the N/P Ratio of Polyplex and Structure and Molecular Weight of PEI on Cytokines.** After the i.v. administration of PEI polyplex in mice, the gene expression was much stronger in lung than liver, heart, spleen, or kidney (data not shown). Figure 2 shows the gene expression in lung and liver after the i.v. administration of the PEI polyplex and lipoplex preparations. The higher the N/P ratio of the branched PEI polyplex and molecular weight, the higher the transfection efficiency observed (Fig. 2, A–C). As far as the branched PEI polyplex is concerned, an N/P ratio of 10 yielded the highest level of expression in lung and liver, but



**Fig. 2.** Effect of N/P ratio of PEI polyplex and molecular weight of PEI on gene expression after the i.v. administration of branched and linear PEI polyplexes delivering 30  $\mu$ g of pDNA per mouse. Branched PEI $_{10}$  (A), PEI $_{25}$  (B), and PEI $_{70}$  (C), and linear PEI $_{25}$  (D) were used to produce polyplexes with an N/P ratio of 5, 7, 10, 15, 20, and 25, respectively. The lipoplex had a -: + of 1.0:3.1 and delivered 30  $\mu$ g of pDNA per mouse. Mice were sacrificed at 6 h after the injection, and levels of luciferase activity in the lung (filled bars) and liver (open bars) were measured. N.E., not evaluated. Each value represents the mean + S.D. for at least three mice.