immunization, E.G7-OVA (1×10^6) or EL4 (1×10^6) cells were inoculated subcutaneously (s.c.) into the back of the mice. The survival of the mice was monitored up to 100 days after inoculation of the E.G7-OVA or EL4 cells.

Results

Particle sizes and zeta-potentials of Man-lipoplexes

To investigate the physicochemical properties of lipoplexes, the particle size and zeta-potential of each lipoplex were evaluated. Both lipoplexes showed a clear-cut distribution pattern and the mean particle sizes of the Man-lipoplexes and conventional lipoplex were 114 ± 7.8 and 116 ± 11.5 nm ($n = 3$), respectively. Zeta-potential analysis showed that the zeta-potential of the Man-lipoplex and the conventional lipoplex at a charge ratio (−/+) of 1.0 : 2.3 was 62.1 ± 1.85 and 64.1 ± 1.74 mV ($n = 3$), respectively. These results show that there was almost no difference in physicochemical properties between the two complexes. The galactosylated lipoplex (Gal-lipoplex) also showed a similar size distribution and zeta-potential (data not shown).

Uptake characteristics of Man-lipoplex by DC2.4 cells *in vitro*

To evaluate the potency of the Man-lipoplex in terms of targeted delivery to DCs, the uptake of the lipoplexes and subsequent transfection to cells were evaluated. In this study, we used DC2.4 cells, a cell line derived from DCs, as a model DC expressing mannose receptors [26]. The [^{32}P] Man-lipoplex was taken up by DC2.4 cells more efficiently than the conventional [^{32}P] lipoplex (Figure 1a) and this was significantly reduced in the presence of an excess of mannan (Figure 1b). In contrast, the uptake with conventional lipoplex was not significantly inhibited by an excess of mannan (Figure 1b).

Transfection characteristics of Man-lipoplex with respect to DC2.4 cells *in vitro*

We next investigated the transfection activity of the Man-lipoplex with respect to DC2.4 cells. As shown in Figure 2a, the highest gene expression was observed in the Man-lipoplex. In the presence of an excess of mannan, the gene expression of the Man-lipoplex was significantly inhibited (Figure 2b). In contrast, the gene expression with conventional lipoplex and naked pDNA was not significantly inhibited in the presence of an excess of mannan (Figure 2b).

Effect of Man-lipoplex administration routes on CTL response

To investigate the effect of the administration route of the Man-lipoplex, the induction of an OVA-specific cytotoxic response with a ^{51}Cr release assay using E.G7-OVA cells (OVA-expressing cells), and its parental cell line, EL4 cells (OVA-non-expressing cells), was examined. We found that the CTL activity induced by i.p. administration of the Man-lipoplex was higher than that induced by i.m. or i.d. administration of the Man-lipoplex (Figure 3a).

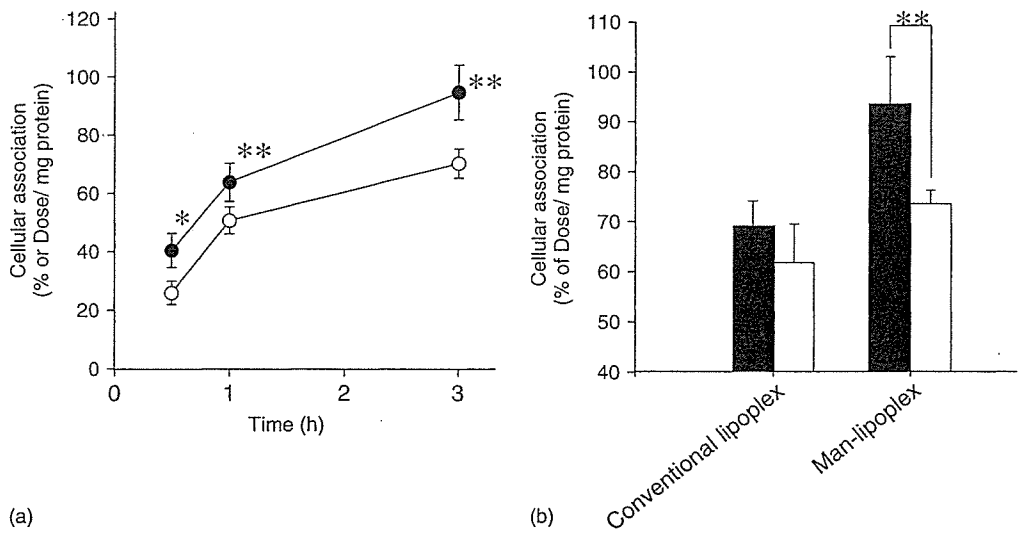

Figure 1. Cellular association of the Man-lipoplex in DC2.4 cells. (a) Cellular association time-course of ^{32}P-labeled Man-lipoplex (●) and lipoplex (○) in DC2.4 cells at 37 °C. pCMV-OVA (0.5 μg/ml) was complexed with cationic liposomes at a charge ratio (−/+) of 1.0 : 1.6. Each value represents the mean ± standard deviation (S.D.) ($n = 3$). (b) Cellular association of ^{32}P-labeled lipoplex or Man-lipoplex in the absence (■) or presence (□) of 0.125 mg/ml mannan in the culture medium. Each value represents the mean + S.D. ($n = 4$). Statistical analysis was performed by Student's *t*-test (*$P < 0.05$, **$P < 0.01$)

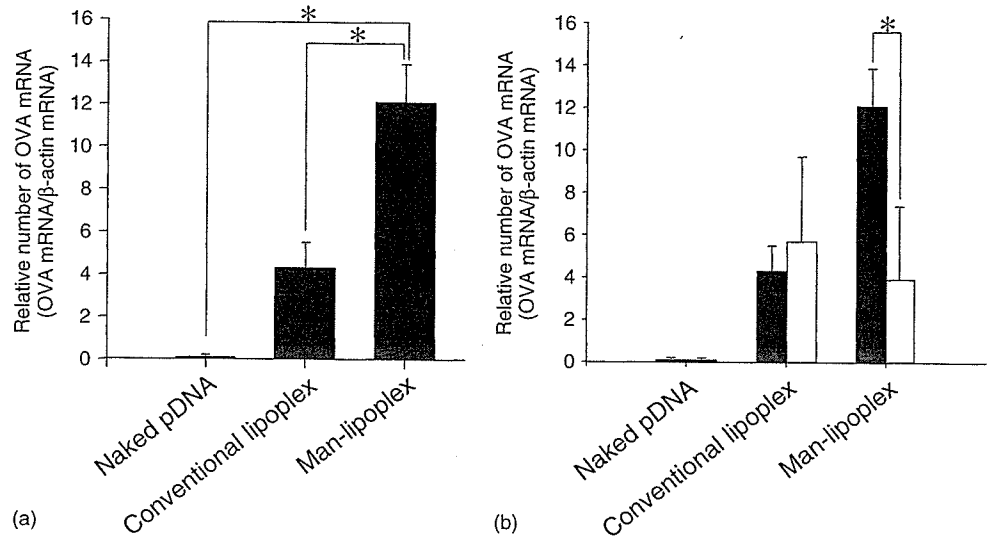

Figure 2. Transfection activity of the Man-lipoplex in DC2.4 cells. (a) Transfection activity of naked pCMV-OVA or lipoplexes in cultured DC2.4 cells. The concentration of pDNA was fixed at 0.5 μg/ml in all experiments. Each value represents the mean + S.D. (n = 3). (b) Transfection activity of naked pCMV-OVA or lipoplexes in the absence (■) or presence (□) of 0.125 mg/ml mannan. The concentration of pCMV-OVA was fixed at 0.5 μg/ml in all experiments. Each value represents the mean + S.D. values (n = 4). Statistical analysis was performed by analysis of variance (*$P < 0.05$)

Furthermore, increasing the amount of pCMV-OVA of the Man-lipoplex markedly enhanced the CTL response induced by i.p. administration (Figure 3b). Furthermore, the CTL activity of the Man-lipoplex following i.p. administration was significantly higher than that induced by i.m. administration of naked pCMV-OVA (Figure 3c).

Effect of lipoplex mannosylation on CTL response

As shown in Figure 4a, the Man-lipoplex induced a much higher CTL response than the conventional lipoplex. However, the Gal-lipoplex induced a much lower CTL response than the Man-lipoplex (Figure 4b), suggesting that the Man-lipoplex induces a strong CTL response via a mannose receptor-mediated mechanism.

Gene expression characteristics of Man-lipoplex on CD11c$^+$ cells in the spleen and peritoneal cavity after i.p. administration

To clarify the transfection activity of the Man-lipoplex with regard to DCs after i.p. administration, the OVA mRNA in the CD11c$^+$ cells in the spleen and peritoneal cavity was determined 6 h after i.p. administration of naked pCMV-OVA (100 μg) or Man-lipoplex and conventional lipoplex using quantitative RT-PCR. The relative copy number of OVA mRNA in the Man-lipoplex injected group was the highest of all in both peritoneal CD11c$^+$ cells (Figure 5a) and splenic CD11c$^+$ cells (Figure 5b).

Anti-tumor responses of Man-lipoplex after immunization

To assess the protective anti-tumor effect, EL4 and E.G7-OVA cells were transplanted into pre-immunized mice. Pre-immunization with the Man-lipoplex prolonged the survival time after transplantation of E.G7-OVA cells compared with pDNA or conventional lipoplex (Figure 6a). However, all formulations failed to prolong the survival rate after transplantation of EL4 cells (Figure 6b).

Discussion

DNA vaccine represents an exciting novel approach in vaccine development. The vaccine construct is created by insertion of a DNA encoding the desired antigen into a pDNA. The extent to which the pDNA is able to transfect cells is dependent on the application route and delivery carrier used. The encoded protein is then expressed in the transfected cells *in vivo* and, consequently, an immune response is elicited to the expressed antigen. However, to date, there have been few reports on *in vivo* gene therapy based on targeted non-viral gene delivery. In our series of experiments, we have been developing APC-selective *in vivo* gene carrier systems for use in gene therapy [22–24,32]. In the present study, we describe the Man-lipoplex given i.p. as a novel approach to enhance therapeutic potency of DNA vaccine therapy *in vivo*.

Since some clinical trials have involved the local administration of naked pDNA [7–9], we evaluated the OVA-specific CTL response following the local administration of naked pDNA. As shown in Figure 3c,

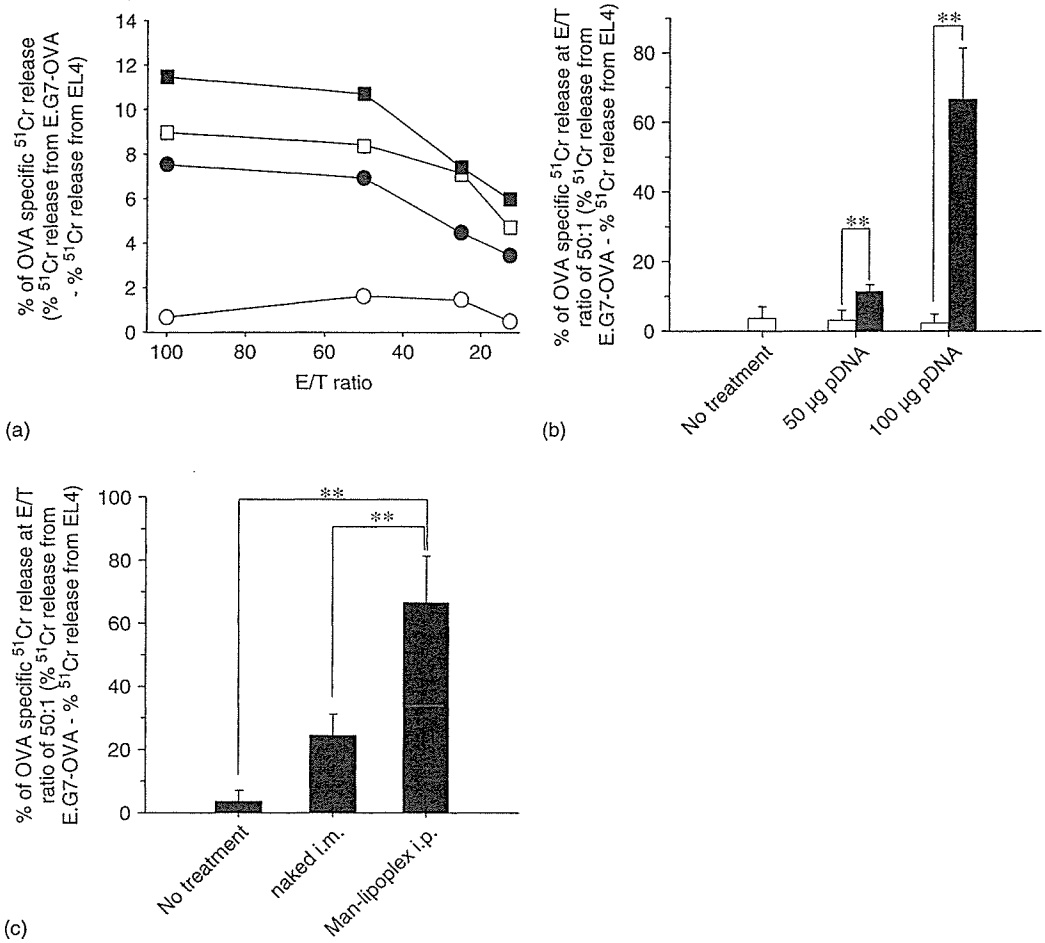

Figure 3. Effect of the route of administration and dose on immunization with naked pCMV-OVA or Man-lipoplexes. Mice were injected with 50 μg pCMV-OVA as naked pCMV-OVA or Man-lipoplexes biweekly three times before the experiment. (a) CTL activity primed by i.p. (■), i.d. (□) and i.m. (●) administration 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}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 μg/mouse. OVA-specific ^{51}Cr release at an E/T ratio of 50 : 1 was calculated from the following equation: OVA-specific ^{51}Cr release = %^{51}Cr release from E.G7-OVA cells −%^{51}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 μg/mouse. OVA-specific ^{51}Cr release at an E/T ratio of 50 : 1 was calculated from the following equation: OVA-specific ^{51}Cr release = %^{51}Cr release from E.G7-OVA cells −%^{51}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

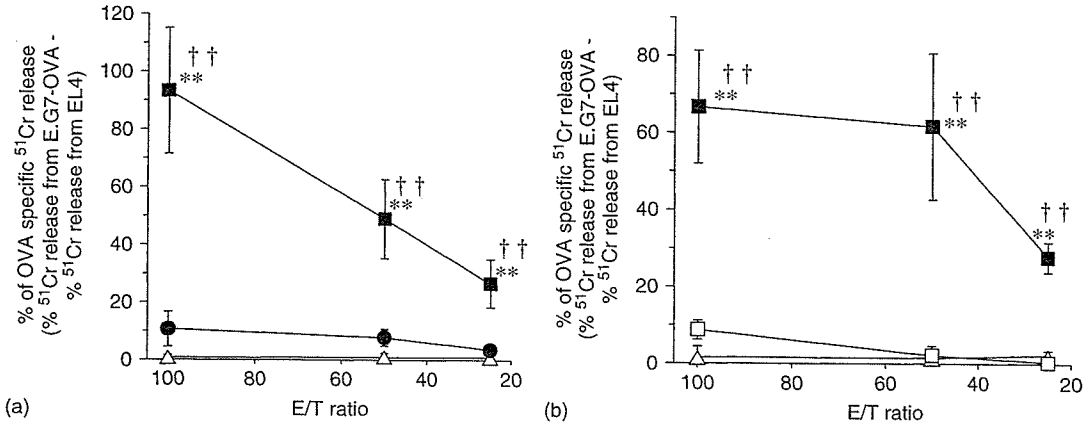

Figure 4. Effect of mannosylation of cationic liposomes on the induction of a CTL response. Mice were injected three times with naked pCMV-OVA (100 μg) or lipoplexes (pCMV-OVA; 100 μg) biweekly. (a) CTL response induced by the Man-lipoplexes (■) and the lipoplexes (●) or that of the no-treatment group (△). OVA-specific ^{51}Cr release was calculated from the following equation: OVA-specific ^{51}Cr release = %^{51}Cr release from E.G7-OVA cells −%^{51}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 (△). OVA-specific ^{51}Cr release was calculated from the following equation: OVA-specific ^{51}Cr release = %^{51}Cr release from E.G7-OVA cells −%^{51}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. (††$P < 0.01$)

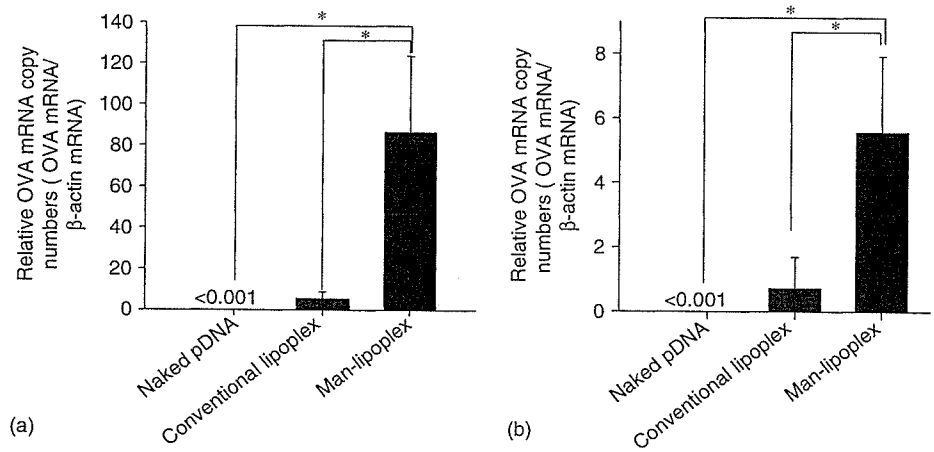

Figure 5. *In vivo* mRNA gene expression in CD11c$^+$ cells in the peritoneal cavity (a) and spleen (b) after i.p. administration to mice. Lipoplex or Man-lipoplex was prepared at a charge ratio (−/+) of 1.0 : 2.3 in 5% dextrose. Six hours after injection, OVA mRNA and β-actin mRNA eluted from CD11c$^+$ 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

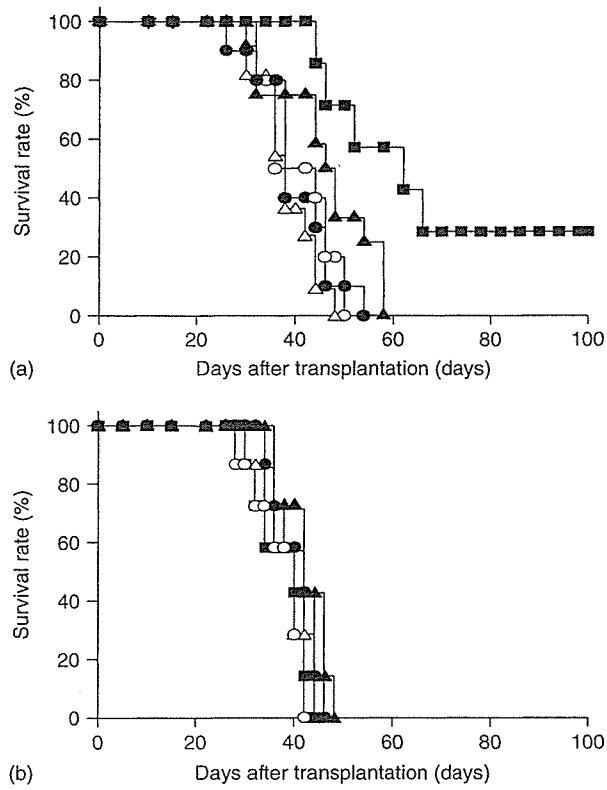

Figure 6. The anti-E.G7-OVA cell (a) or EL4 cell (b) tumor effect following pre-immunization by i.p. administration of various formulations or i.m. administration of naked pCMV-OVA solution (100 μg). Mice were injected with naked pCMV-OVA (○), the Man-lipoplex (■), or the lipoplex (●) given i.p., naked pCMV-OVA given i.m. (▲), or no treatment (△). 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 $CD11c^+$ 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. administrated 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], fusigenic 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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Suppression of TNFα production in LPS induced liver failure in mice after intravenous injection of cationic liposomes/NFκB decoy complex

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NFκB decoy, double stranded oligonucleotides containing NFκB binding sequences, inhibits NFκB-mediated production of inflammatory cytokines, and therefore NFκB decoy has been applied to several diseases. However, naked NFκB decoy, which is quickly cleared from the circulation in mice after intravenous injection, is readily absorbed into the systemic circulation. In order to deliver enough NFκB decoy for a therapeutic effect, it is necessary to develop a carrier, which enables much more NFκB decoy to transfer to the target cells. In this study, using *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA)/cholesterol (1 : 1) liposomes, the therapeutic effect of NFκB decoy was investigated in an LPS induced acute hepatitis model mice. The mean diameter of the cationic liposomes/NFκB decoy complex was about 70.9 nm and the zeta potential of complex was about 37.4 mV. Tissue distribution was determined by measuring the radioactivity of a cationic liposomes/[^{32}P] NFκB decoy complex after intravenous injection. The cationic liposomes/[^{32}P] NFκB decoy complex was rapidly accumulated in the lung and gradually moved to the liver. The therapeutic effect was determined by the serum concentration of TNFα in LPS treated mice. The production of TNFα was significantly inhibited by cationic liposomes/NFκB decoy complex but not by cationic liposomes/random decoy complex or naked NFκB decoy. These results suggested that NFκB decoy therapy could be achieved using cationic liposomes. This information is of great value for the design of NFκB decoy carrier systems.

1. Introduction

Endotoxin syndrome is a particularly serious complication because bacteriologically proven infection occurs in up to 80% of patients with hepatic failure (Rolando et al. 1990, 2000). Binding of lipopolysaccharide (LPS) by toll-like receptor 4 expressed on macrophages causes the activation of transcriptional factor nuclear factor kappa B (NFκB), triggering the rapid release of cytokines TNFα, IL-1, IL-2, IL-6, IL-8, IL-12, and IFN-β, etc. by macrophages (Morrison et al. 1987; Heinzel, 1990; Dinarello et al. 1993; Essani et al. 1996; Pahl et al. 1999; Han et al. 2002). Therefore, prevention of NFκB activation would be a critical therapeutic goal of fetal liver injury caused by the endotoxin syndrome. Several recent reports indicate that NFκB decoy, double stranded oligonucleotides containing NFκB binding sequences, inhibits NFκB-mediated production of inflammatory cytokines (Morishita et al. 1997, Tomita et al. 2000a); therefore, NFκB decoy could be used in various diseases (Tomita et al. 2000, Yoshimura et al. 2001, Azuma et al. 2003). Considering that naked NFκB decoy is easily digested by DNase in serum and hardly taken up by cells, it is necessary to develop a non-invasive form of NFκB decoy delivery, which would be safe for repeated use and provide reproducible therapeutic effects, for wider clinical application.

In spite of the high transfection efficiency of viral vectors, safety concerns have been raised in clinical trials because of their highly toxic nature; therefore, the use of non-viral vectors has attracted great interest for *in vivo* gene delivery because they are free from some of the risks inherent in these systems (Mahato et al. 1997; Kawakami et al. 2002; Hashida et al. 2005). We previously reported that rapid clearance of pDNA from the circulation was observed with extensive accumulation in the lung and liver after intravenous injection of pDNA complexed with cationic liposomes (Mahato et al. 1995a, 1995b). As regards intrahepatic distribution, pDNA complexed with cationic liposomes was predominantly taken up by liver non-parenchymal cell (NPC), composed of Kupffer cells, sinusoidal endothelial cells etc., via a phagocytic process. NFκB decoy is a double stranded oligonucleotide which could interact with cationic liposomes. Because NPC is a main target for NFκB decoy inhibition of NFκB-mediated production of inflammatory cytokines, it would be expected that NFκB decoy delivery to liver NPC by cationic liposomes would efficiently suppress cytokine production by the prevention of NFκB activation. However, little information is available on the use of cationic liposomes/NFκB decoy complex against inflammatory disease after intravenous administration.

In this study, the physicochemical properties and distribution characteristics of a NFκB decoy/cationic liposomes complex were evaluated. In mice with LPS induced hepatitis, TNFα concentration was measured to evaluate the prevention of NFκB activation after intravenous administration of NFκB decoy/cationic liposomes complex. *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA)/cholesterol (1 : 1) liposomes were selected as the cationic liposomes because of the many reports about their use for *in vivo* pDNA delivery with high transfection efficacy (Song et al. 1997; Kawakami et al. 2000, 2001). Results were compared with naked NFκB decoy.

2. Investigations, results and discussion

The table summarizes data on mean diameter and zeta potential of cationic liposomes and the cationic liposomes/NFκB decoy complex. It has been reported that mixing complexes at low ion strength prevents aggregation, although large complexes resulting from aggregation showed high transfection efficiency *in vitro* in the case of DNA/transferring-PEI complexes (Ogris et al. 1998). Having regard to this report, the cationic liposomes/NFκB decoy complexes were prepared with 5% dextrose solution. The mean diameter of the cationic liposomes or cationic liposomes/NFκB decoy complex was about 73.8 and 70.9 nm, respectively. There was no significant difference in the mean diameter of empty liposomes and complex.

The zeta potential of cationic liposomes or cationic liposomes/NFκB decoy complex was about 56.7 and 37.4 mV, respectively. The zeta potential of the cationic liposomes/NFκB decoy complex was slightly smaller than that of empty liposomes. This result suggested that NFκB decoy would form a complex with cationic liposomes because attaching NFκB decoy with anionic charge to cationic liposomes would decrease the zeta potential of the cationic liposomes.

The liver and lung accumulation of the cationic liposomes/[^{32}P] NFκB decoy complex was compared with that of naked [^{32}P] NFκB decoy (Fig. 1). Both liver and lung accumulation of the cationic liposomes/[^{32}P] NFκB decoy complex were significantly higher than for naked [^{32}P] NFκB decoy at 1, 10, and 60 min after injection. Focusing on the cationic liposomes/[^{32}P] NFκB decoy complex, the complex accumulated initially in the lung at 1 min after injection, then moved gradually to the liver. At 60 min, 60% of the dose of the cationic liposomes/[^{32}P] NFκB decoy complex had accumulated in the liver (Fig. 1B). In our previous study, we demonstrated that [^{32}P] pDNA complexed with DOTMA/cholesterol liposomes rapidly accumulated in the lung and gradually accumulated in the liver (Mahato et al. 1995a, 1995b). Our findings concur that distribution of the cationic liposomes/NFκB decoy complex agrees with the distribution of the cationic liposomes/pDNA complex.

In order to determine the therapeutic effect of NFκB decoy on acute hepatitis *in vivo*, the serum concentration of TNFα in LPS treated mice was measured after intravenous injection of NFκB decoy (Fig. 2). Cationic liposomes/NFκB decoy complex suppressed the increase of TNFα in the serum, although naked NFκB decoy did not show an inhibitory effect on the production of TNFα. In endotoxin-induced liver failure, it is known that Kupffer cells, resident macrophages in the liver, play a major role in producing inflammatory cytokines (Arai et al. 1993; Iimuro et al. 1994; Mochida et al. 1996). Considering that the liver accumulation of the cationic liposomes/NFκB decoy complex was significantly higher than that of naked NFκB decoy (Fig. 1), this raises the possibility that some part of the NFκB decoy taken up by Kupffer cell might suppress NFκB mediated TNFα production induced by LPS.

To examine whether the inhibitory effect of NFκB decoy depended on the sequence of NFκB binding site, a similar size of double stranded oligonucleotide without NFκB binding site and also without any effective sequence, which was called random decoy, was designed and the inhibitory effect of its complex with cationic liposomes on TNFα production investigated. As shown in Fig. 2, the cationic liposomes/random decoy complex had no effect on TNFα production; therefore, the inhibitory effect on TNFα production was observed to depend on the sequence of NFκB decoy.

To determine the effect of dose of NFκB decoy, the inhibitory effect on LPS induced TNFα production in serum was investigated at different doses of NFκB decoy com-

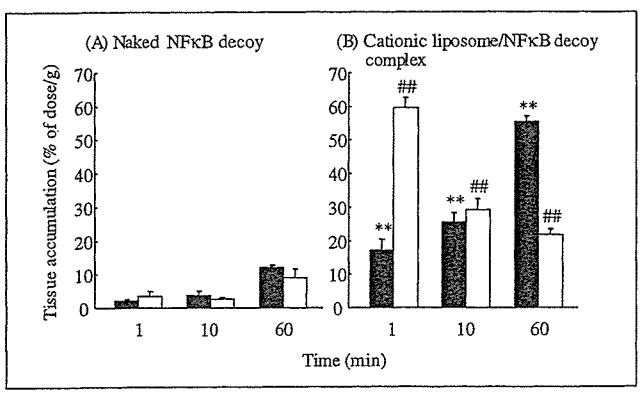

Fig. 1: Tissue accumulation of naked [^{32}P] NFκB decoy (A) or cationic liposomes/[^{32}P] NFκB decoy complex (B) after intravenous injection into mice. Radioactivity was determined in liver (■) and lung (□). Each value represents the mean + S.D. (n = 3). Significant difference ** $P < 0.01$ vs. liver accumulation of naked NFκB decoy and ## $P < 0.01$ vs. lung accumulation of naked NFκB decoy

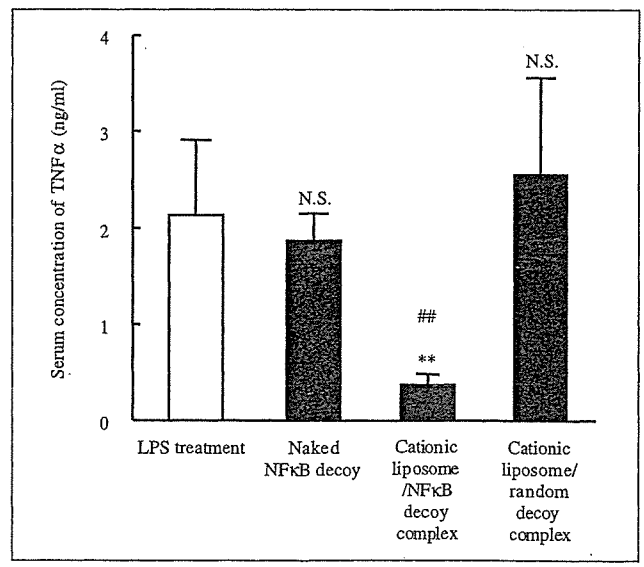

Fig. 2: Effect of complex formation and sequence of NFκB decoy on prevention of the TNFα production after intravenous injection of naked NFκB decoy, cationic liposomes/NFκB decoy complex or cationic liposomes/random decoy complex into mice. Each value represents the mean + S.D. (n = 3). Significant difference ** $P < 0.01$ vs. naked NFκB decoy, ## $P < 0.01$ vs. random decoy complex and N.S. not significant

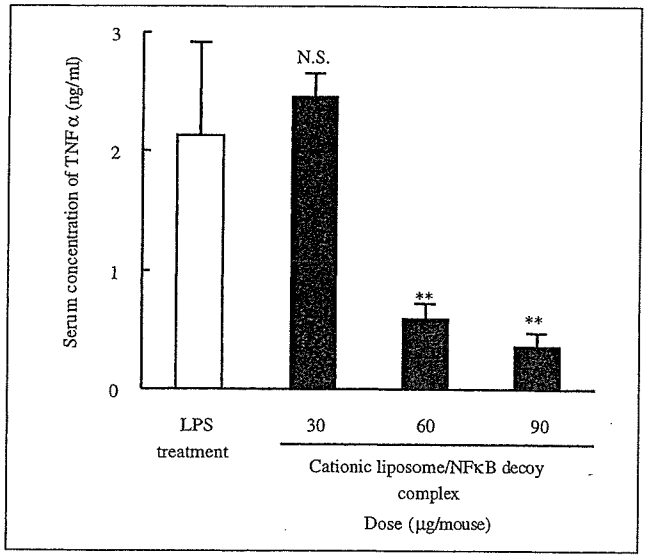

Fig. 3: Effect of dose on the prevention of TNFα production after intravenous injection of cationic liposomes/NFκB decoy complex into mice. Each value represents the mean + S.D. (n = 3). Significant difference ** $P < 0.01$ vs LPS treatment and N.S. not significant

plexed with cationic liposomes (Fig. 3). At doses of 60 and 90 μg/mouse, the production of TNFα was significantly inhibited but not at 30 μg/mouse.

To date, several kinds of non-viral carriers have been applied to *in vivo* gene delivery. As far as NFκB decoy delivery targeting to the liver is concerned, Ogushi et al. (2003) have also demonstrated the inhibitory effect of NFκB decoy on the production of cytokines using HVJ liposomes given by intraportal injection. HVJ liposomes are well known as a gene carrier, which could rapidly fuse to cells and transfer the incorporated gene to the cells. However, HVJ liposomes are not suitable for intravenous injection because intravenously injected HVJ liposomes accumulate in the lung, spleen, kidney and liver; therefore, several researchers have reported that they could not achieve enough therapeutic effect or transfection efficiency (Hirano et al. 1998; Morishita et al. 2000; Yoshida et al. 2002; Ogushi et al. 2003). Conversely, we report here that intravenously injected cationic liposomes/NFκB decoy complex accumulates in the liver (Fig. 1).

Cationic liposome-based systemic pDNA delivery has been reported as a novel technology, however the uptake of cationic liposomes/pDNA by Kupffer cells triggers production of inflammatory cytokines and disturbs gene transfer efficiency, which is largely due to unmethylated CpG motif in the pDNA (Sakurai et al. 2002). Recently, Tan et al. have reported that NFκB decoy could inhibit TNFα induction by a cationic liposomes (DOTAP/cholesterol, 1 : 1)/pDNA complex, which was prepared by mixing liposome solution and DNA solution containing pDNA and NFκB decoy (Tan et al. 2002). In this study, we demonstrate that LPS induced TNFa production could be inhibited by cationic liposomes/NFκB decoy complex (Fig. 2). This result leads us to believe that the cationic liposomes/NFκB decoy complex in liver is mainly distributed in Kupffer cells after intravenous injection.

In this study, we demonstrate that intravenously injected cationic liposomes/NFκB decoy complex effectively inhibits TNFα production in LPS induced acute hepatitis in mice. This result will give useful information on NFκB decoy therapy using cationic liposomes. With more study and development of cellular targeting with cationic liposomes, it will be possible to establish NFκB therapy by intravenous injection.

Table: Mean particle sizes and zeta potentials of cationic liposomes and cationic liposomes/NFκB decoy complexes

	Particle size (nm)	Zeta potential (mV)
Cationic liposome	73.8 ± 5.46	56.7 ± 1.53
Cationic liposome/NFκB decoy complex	70.9 ± 1.07	37.4 ± 2.84

Results are expressed as the mean ± SD of three experiments

3. Experimental

3.1. Materials

NFκB decoy and control oligonucleotides used in this study are phosphodiester double stranded oligonucleotides. Their sequences are as follows: 20 mer NFκB decoy 5′-AGTTGAGGGGACTTTCCCAGGC-3′ 5′-TCAACTCCCCTGAAAGGGTCCG-3′ (B), control oligonucleotides 5′-TTGCCGTACCTGACTTAGCC-3′ 5′-AACGGCATGGACTGAATCGG-5′. These oligonucleotides were purchased from Operon Biotechnologies, Inc. (Tokyo, Japan). DOTMA was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cholesterol, Clear-Sol I and Soluen 350 were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Lipopolysaccharide (LPS) from Salmonella Minnesota Re 595 (Re mutant) was purchased from Sigma Chemicals Inc. (St. Louis, MO, USA). [γ-^{32}P] ATP and NAP 5^{TM} columns were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA). MEGALABELTM 5′-End Labeling Kit was purchased from Takara Bio Inc. (Shiga, Japan). OptiEIA^{TM} enzyme-linked immuno-sorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA). Enzyme Immunoassay for NFκB Product No.TF 01 was purchased from Oxford Biomedical Research, Inc. (Oxford, MI, USA). All other chemicals were of the highest purity available.

3.2. Preparation of liposomes

DOTMA was mixed with cholesterol, each in chloroform at a molar ratio of 1 : 1 and the mixture was dried, vacuum desiccated, and resuspended in sterile 5% dextrose. After hydration, the dispersion was sonicated for 10 min in a bath sonicator and then for 3 min in a tip sonicator to form liposomes. The preparation method for liposomes/NFκB decoy complexes for *in vivo* use has been reported previously (Kawakami et al., 2000b, 2004). Briefly, equal volumes of NFκB decoy and stock liposome solution diluted with 5% dextrose to produce various ratios of liposomes/NFκB decoy were mixed in 1.5 ml tubes at room temperature. Then, the NFκB decoy solution was added rapidly to the liposomes solution using a Pipetman pipet and the mixture was agitated rapidly by pumping it up and down twice in the pipet tip. The mixing ratio of liposomes and NFκB decoy was expressed as a +/− charge ratio, which is the molar ratio of cationic lipids to NFκB decoy phosphate residue (Huang et al. 1997).

3.3. Measurement of particle size and zeta potentials

The mean diameters of cationic liposomes or cationic liposomes/NFκB decoy complex were measured by dynamic light-scattering spectrophotometric methods using an LS-900 (Otsuka Electronics, Osaka, Japan). The zeta potentials of cationic liposomes or cationic liposomes/NFκB decoy complex were measured by laser-Doppler electrophoresis using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK).

3.4. Radiophosphorylation of decoy oligonucleotides

Oligonucleotides were labeled with [γ-^{32}P] ATP using a MEGALABELTM 5′-End Labeling Kit. Briefly, oligonucleotides, [γ-^{32}P] ATP and T4 polynucleotide kinase were mixed in phosphorylation buffer. After 30 min incubation at 37 °C, the mixture was incubated for 10 min at 70 °C in order to inactivate T4 polynucleotide kinase. Then, the mixture was purified by gel chromatography using a NAP 5^{TM} column and eluted with 10 mM Tris-Cl and 1 mM EDTA (pH 8.0). The fractions containing derivatives were selected on the basis of their radioactivity.

3.5. In vivo distribution

[^{32}P] NFκB (20 μg) decoy complexed with liposomes in 300 μl of 5% dextrose solution was injected intravenously to female five-week-old ICR mice (19–22 g). The mice were killed at 1, 10 and 60 min. Liver and lung were removed, washed with saline, blotted dry, and weighed. A small amount of each tissue was digested with 0.7 ml of Soluene-350 by incuba-

tion overnight at 54 °C. Following digestion, 0.2 ml isopropanol, 0.2 ml 30% hydrogen peroxide, 0.1 ml 5 N HCl, and 5.0 ml Clear-Sol I were added. The samples were stored overnight, and radioactivity was measured in a scintillation counter (LSA-500, Beckman, Tokyo, Japan).

3.6. *Cytokine secretion*

Mice were purchased from 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. The indicated amount of NFκB decoy or random decoy complexed with liposomes or the indicated amount of naked NFκB decoy in 300 µl of 5% dextrose solution was intravenously injected to the mice. Blood was collected from the mice 1 h after intravenous injection of LPS. The blood was allowed to coagulate for 3 h at 4 °C and serum was isolated as the supernatant fraction following centrifugation at 17000 × g for 20 min. The serum samples were immediately stored at −80 °C. The amounts of TNFα were analyzed using an OptiEIA™ ELISA kit according to the manufacturer's protocol.

3.7. *Statistical analysis*

Statistical comparisons were performed by Student's t test for two groups, one-way ANOVA for multiple groups, and Scheffe's post hoc test after ANOVA.

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