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Research paper

# Evaluation of proinflammatory cytokine production and liver injury induced by plasmid DNA/cationic liposome complexes with various mixing ratios in mice

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ABSTRACT

The purpose of this study was to investigate the cytokine production and liver injury induced by lipoplexes prepared with DOTMA/cholesterol and DOTAP/cholesterol liposomes with various mixing ratios in mice. Lipoplexes were prepared with pCMV-Luc and DOTMA/cholesterol or DOTAP/cholesterol liposomes. After intravenous administration into the mice, organ luciferase activity and serum TNFα and ALT were measured to evaluate the transfection efficacy, cytokine production and liver injury. After intravenous administration of these lipoplexes, basically the serum TNFα and ALT levels were in agreement with the transfection efficacy of the lipoplexes. The cytokine production and liver injury were markedly suppressed by reducing the pDNA dose, and achieved normal levels at a pDNA dose of 0.47 mg/kg. As far as the effects of the charge ratio at this low pDNA dose are concerned, the charge ratios of the lipoplexes affected the transfection efficacy, but not the cytokine production and liver injury. After intravenous administration of either DOTAP/cholesterol or DOTMA/cholesterol liposomes, serum TNFα and ALT levels were normal, suggesting that liver injury as well as cytokine production was caused by lipoplexes, but not by cationic liposomes. This information will be valuable for the future optimization of the preparation conditions of lipoplexes for use in clinical gene therapy.



## 1. Introduction

Intravenous administration of cationic liposomes/plasmid DNA (pDNA) complexes (lipoplexes) leads to high gene expression in the lung [1–3]; therefore, gene delivery by cationic liposomes is particularly desirable for the treatment of lung disease. Dow et al. [4] demonstrated that intravenously injected IL-2-coded pDNA complexed with cationic liposomes effectively inhibited lung metastasis in dogs. Moreover, they pointed out that side-effects, including increased body temperature and hematologic parameters, could be avoided by limiting the dose of pDNA. These results provide evidence to support the possibility of clinical application of lipoplexes under optimized conditions.

To date, many reports about the factors for transfection efficacy following the intravenous injection of lipoplexes, i.e., the mixing ratio of lipoplexes and/or the potential of cationic liposomes, have been published [5]. However, we and other group have reported that intravenously injected lipoplexes induced liver injury and proinflammatory cytokine [6–10]. Although the information about the effect of mixing ratio on cytokines response and on liver injury is essential for the safe use of lipoplexes, there are few systematic studies involving the relationship between transfection efficacy and toxicity, especially under *in vivo* conditions.

In this study, we evaluated the proinflammatory cytokine production and liver injury induced by lipoplexes with various mixing ratios in mice. Cationic liposomes, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA)/cholesterol and 1,2-Dioleoyl-3-trimethyl-ammoniopropane (DOTAP)/cholesterol liposomes, were selected because of their wide use for *in vivo* transfection [3,8,11]. Since TNFα is one of the most important immunomodulators, which is produced predominately by macrophages [12,13], and is immediately secreted following intravenous injection of lipoplexes [6,8], we measured the serum concentration of TNFα to investigate the effect of the mixing ratio on cytokine production. As an indicator of liver injury, we measured the serum level of ALT, which is used for investigation of lipoplex-induced liver injury [14,15]. For systematic study of cytokines response, serum concentration of IL12 was also measured because of its relation with liver injury.

## 2. Materials and methods

### 2.1. Materials

DOTMA was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). DOTAP was obtained from Avanti Polar Lipids, Inc. (Alabas-

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ter, AL, USA). Cholesterol was obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of the highest purity available.

### 2.2. Construction and preparation of pDNA

pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega Co., Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pDNA was amplified in the E. coli strain DH5a, isolated, and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

### 2.3. Preparation of cationic liposomes and lipoplexes

Cationic liposomes and lipoplexes were prepared as reported previously [16]. Briefly, mixtures of DOTMA or DOTAP and cholesterol were dissolved in chloroform at a molar ratio of 1:1, vacuum-desiccated, and resuspended in sterile 5% dextrose. The suspension was sonicated for 3 min, and the resulting liposomes were extruded five times through a 220 nm polycarbonate filter. Then, lipoplexes were formed by adding an equal volume of pDNA in 5% dextrose to the liposomes at various mixing ratios (−:+) and incubated at room temperature for 30 min. The particle size and zeta potential of the lipoplexes were measured using a zeta potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK).

### 2.4. In vivo gene expression experiments

Five-week-old female 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. Gene expression was measured as described previously [17]. Mice were given an intravenous injection of 300 μl lipoplex. Six hours after injection, mice were sacrificed and liver and lungs were harvested. Homogenates were prepared by adding lysis buffer (0.1 M Tris/HCl containing 0.05% Triton X-100 and 2 mM EDTA (pH 7.8)) using a homogenizer (OMNI TH, Yamato Scientific Co. Ltd., Tokyo, Japan) at 4 °C. The volume of lysis buffer added was 4 ml/mg for lung and 5 ml/mg for liver. In order to lyse the cells, the homogenates were treated with three cycles of freezing and thawing. The homogenates were centrifuged at 12,000*g* for 7 min at 4 °C, then 20 μl of each supernatant was mixed with 100 μl luciferase assay solution (Picagene, Toyo Ink Co. Ltd., Tokyo, Japan) and immediately measured using a luminometer (Lumat LB 9507, Berthold Technologies, GmbH & Co., Bad Wildbad, Germany). The protein concentration of each tissue extract was determined using a Dojindo Protein Quantification Kit (Dojindo Molecular Technologies, Inc.).

### 2.5. Measurement of inflammatory cytokines and ALT

Serum was prepared as outlined in our previous study [16]. At an indicated time (for the measurement of TNFα, IL12, and ALT), after intravenous administration of lipoplex, blood was collected from the vena cava, and then left overnight at 4 °C. Samples were centrifuged and the supernatants were collected to obtain serum. Serum TNFα and IL12 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' (Genzyme Co., Cambridge, MA, USA) instructions and the serum ALT concentrations were measured according to the manufacturers' protocol (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

### 2.6. Statistical analysis

Statistical comparisons were performed by Student's *t*-test for two groups, and one-way ANOVA was performed for multiple groups. $P < 0.05$ was considered to be indicative of statistical significance.

## 3. Results

### 3.1. Particle size and zeta potential

Fig. 1A shows the zeta potential of lipoplexes prepared from two types of cationic liposomes, DOTMA/cholesterol and DOTAP/cholesterol at a 1:1 molar ratio. The zeta potentials of DOTAP and DOTMA liposome were 58.9 ± 0.9 and 59.1 ± 1.2 mV, respectively, and those of both lipoplexes were constant (about 60 mV) at a charge ratio (−:+) of 1.0:2.3–1.0:7.0 (Fig. 1A). A similar result was obtained for the measurement of particle size (Fig. 1B), the mean particle size of both the lipoplexes was similar and almost constant (about 110–130 nm) at charge ratios (−:+) of 1.0:2.3–1.0:7.0.

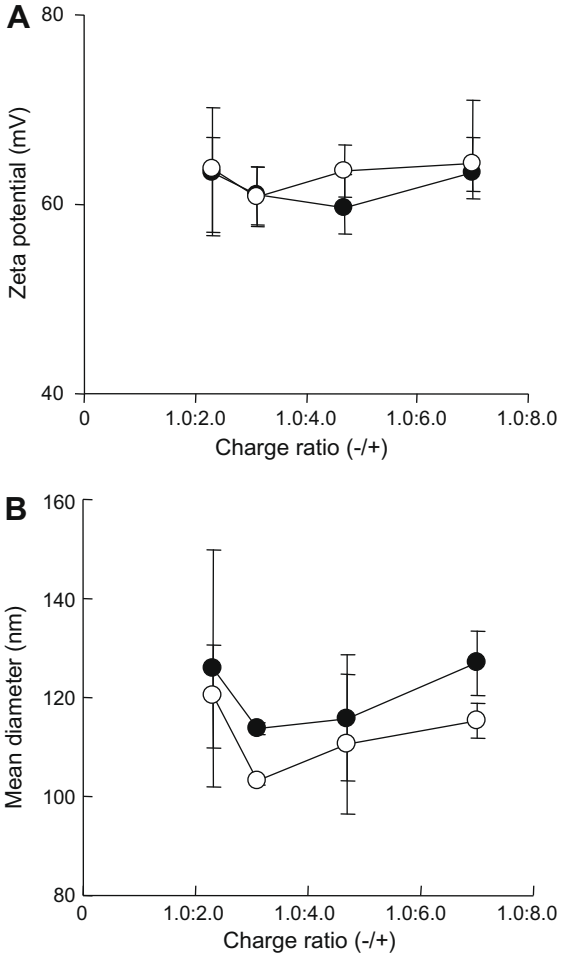


**Fig. 1.** Zeta potential (A) and mean particle size (B) of lipoplexes prepared with DOTMA/cholesterol liposomes (○) or DOTAP/cholesterol liposomes (●) at various charge ratios. Then, 30 min after preparation of the lipoplexes, their zeta potential and particle size were measured.

### 3.2. Time course of lipoplexes prepared with DOTAP/cholesterol liposomes

To determine the time course profile, serum levels of TNFα and ALT were measured at several times after intravenous injection of lipoplexes prepared with DOTAP/cholesterol liposomes into mice. The serum levels of TNFα and ALT were enhanced by lipoplexes prepared with DOTAP/cholesterol liposomes and showed the peak at 3 h (Fig. 2A) and 24 h (Fig. 2B), respectively.

### 3.3. The effect of the charge ratio of lipoplex prepared using DOTAP/cholesterol liposomes

After intravenous injection into mice, the gene expression in the lung was about $10^7$ RLU/mg protein, which was higher than that in the liver (about $5 \times 10^4$ RLU/mg protein), and was enhanced following an increase in the charge ratio (−:+) (Fig. 3A). Depending on the increase in charge ratio (−:+) from 1.0:2.3 to 1.0:7.0, the serum level of TNFα and ALT increased (Fig. 3B and C). To confirm the effect of DOTAP/cholesterol liposomes on cytokine production and liver toxicity, serum TNFα and ALT were measured after intravenous injection of only DOTAP/cholesterol liposomes, which represent almost the same lipid dose as when we inject the maximum

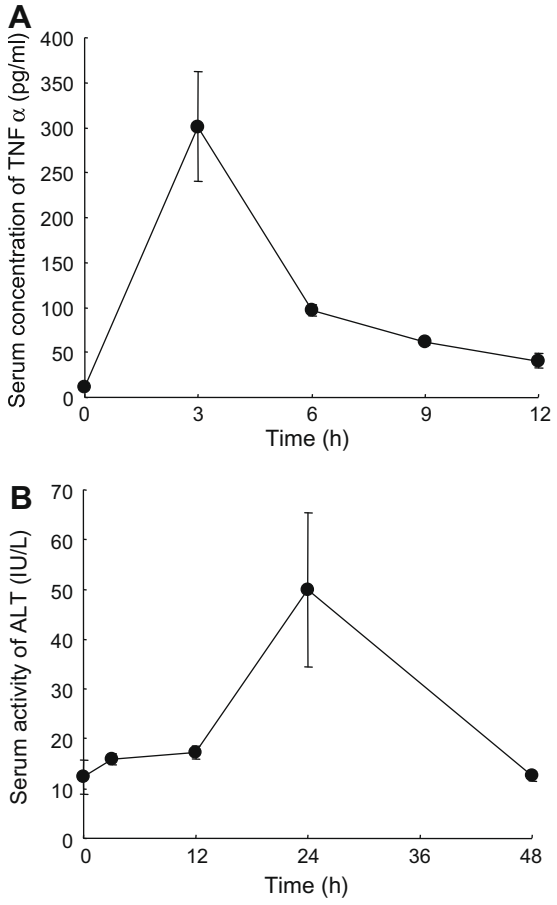


**Fig. 2.** Serum level of TNFα (A) and ALT (B) after intravenous injection of the lipoplexes prepared with DOTAP/cholesterol liposomes. DOTAP/cholesterol liposomes were mixed with pDNA at charge ratios (−:+) of 1.0:2.3. pDNA doses were fixed at 1.4 mg/kg. At 3, 6, 9, and 12 h (for TNFα) or 3, 12, 24, and 48 h (for ALT) after intravenous injection, blood was collected from the vena cava and the serum concentration of TNFα or the serum activity of ALT was measured. Each value represents the mean + S.D. of at least three mice.

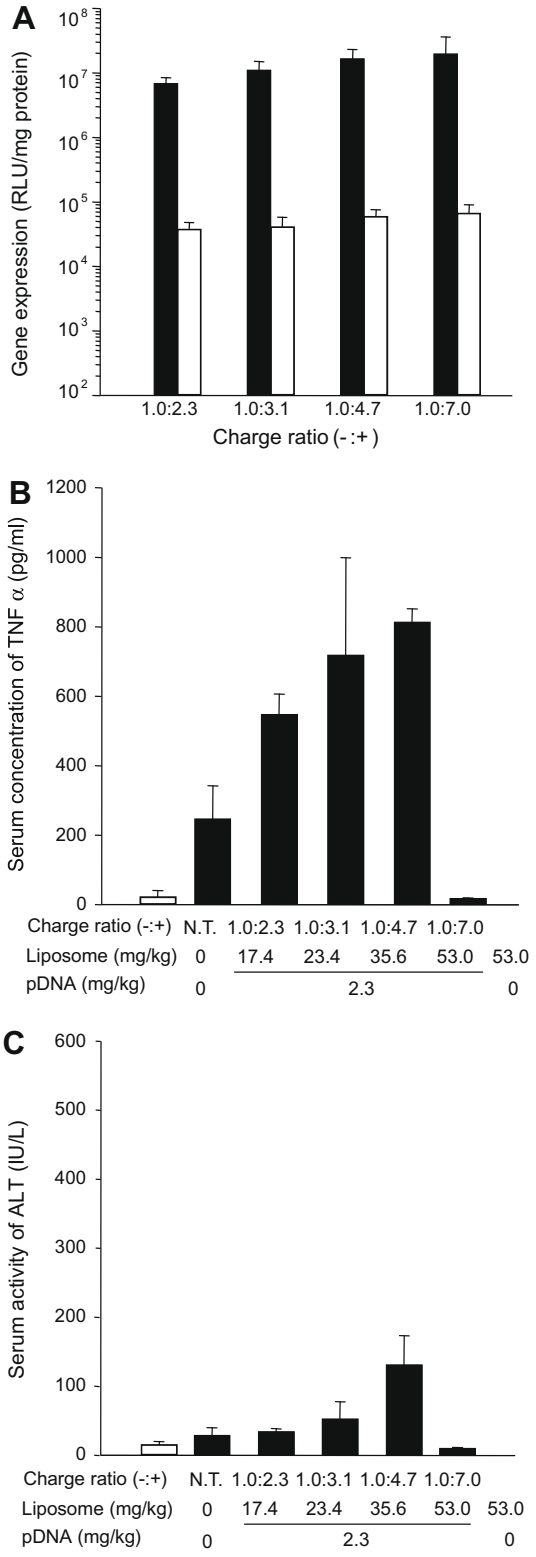


**Fig. 3.** Effect of the charge ratio of the lipoplexes prepared with DOTAP/cholesterol liposomes on gene expression in the lung (■) or liver (□) (A), serum TNFα concentration (B) and serum ALT activity (C) after intravenous injection. DOTAP/cholesterol liposomes were mixed with pDNA at charge ratios (−:+) of 1.0:2.3, 1.0:3.1, 1.0:4.7, and 1.0:7.0. pDNA doses were fixed at 2.3 mg/kg. Six hours after intravenous injection, mice were sacrificed and the luciferase activity was measured. Three hours (for TNFα) or 24 h (for ALT) after intravenous injection, blood was collected from the vena cava and the serum concentration of TNFα or the serum activity of ALT was measured. Each value represents the mean + S.D. of at least three mice.

dose of lipoplexes in this study. The serum levels of TNFα and ALT were comparable with those of normal mice.

### 3.4. The effect of the charge ratio of lipoplex prepared by DOTMA/cholesterol liposomes

After intravenous injection into mice, the gene expression in lung was $10^7$ RLU/mg protein, which was higher than that in liver (about $5 \times 10^4$ RLU/mg protein), and was enhanced by the increase in charge ratio (−:+) (Fig. 4A). Depending on the increase in charge ratio (−:+) from 1.0:2.3 to 1.0:7.0, the serum level of TNFα and ALT increased (Fig. 4B and C). To confirm the effect of DOTMA/cholesterol liposomes on cytokine production and liver toxicity, serum TNFα and ALT were measured after intravenous injection of only DOTMA/cholesterol liposomes, which represent almost the same lipid dose as when we inject the maximum dose of lipoplexes in this study. The serum levels of TNFα and ALT were comparable with those of normal mice.

### 3.5. The effect of the pDNA dose of lipoplex prepared by DOTMA/cholesterol liposomes

Since liver injury induced by lipoplexes prepared by DOTMA is more severe than that by DOTAP/cholesterol liposomes with different charge ratios in mice, the effect of the pDNA dose was evaluated using only DOTMA/cholesterol liposomes. As shown in Fig. 5A, as the dose of pDNA increased from 0.47 to 3.7 mg/kg, the gene expression in lung and liver increased about $10^4$ -fold. Similarly, the serum TNFα concentration and ALT activity were also increased depending on the increase in the pDNA dose from 0.47 to 3.7 mg/kg (Fig. 5B and C). At a low dose of pDNA (0.47 mg/kg), the increase in charge ratio enhanced gene expression (Fig. 6A), but not the serum TNFα, IL12 (Fig. 6B and C) and ALT activity (Fig. 6D).

## 4. Discussion

Although transfection efficacy of lipoplexes could be affected by the mixing ratio of the lipoplexes and/or by the potentials of the cationic liposomes, little attention has been paid to the factors associated with cytokine production and liver injury. For the clinical application of lipoplexes, a detailed evaluation of the relationship between transfection efficacy and side-effects is required. Since many factors are involved in the side-effects, including the types of gene carrier themselves [18], recognition of the CpG motif in pDNA [19], and the cellular [20] or intracellular distribution [21] of gene carriers, a systematic *in vivo* study of the cytokine production and liver injury is very important. The purpose of this study was to investigate the cytokine production and liver injury induced by lipoplexes prepared with DOTMA/cholesterol and DOTAP/cholesterol liposomes, which are the commonly used cationic liposomes for *in vivo* application, with various mixing ratios in mice.

After intravenous administration of lipoplex under different conditions, the transfection efficacy in lung was much higher than that in liver (Figs. 3A, 4A, and 5A). As far as the types of cationic lipids are concerned, the transfection efficacies of DOTAP and DOTMA/cholesterol liposomes in lung and liver were comparable (Figs. 3A and 4A). The increase in the charge ratio and pDNA dose enhanced gene expression in lung (Figs. 4A and 5A). These observations about the transfection characteristics of lipoplexes prepared by DOTAP/cholesterol and/or DOTMA/cholesterol liposomes are in good agreement with the previous reports [7,9,22–24].

To clarify the relationship between gene expression, cytokine production and liver injury, serum TNFα, and ALT levels were investigated with regard to the gene expression at several charge

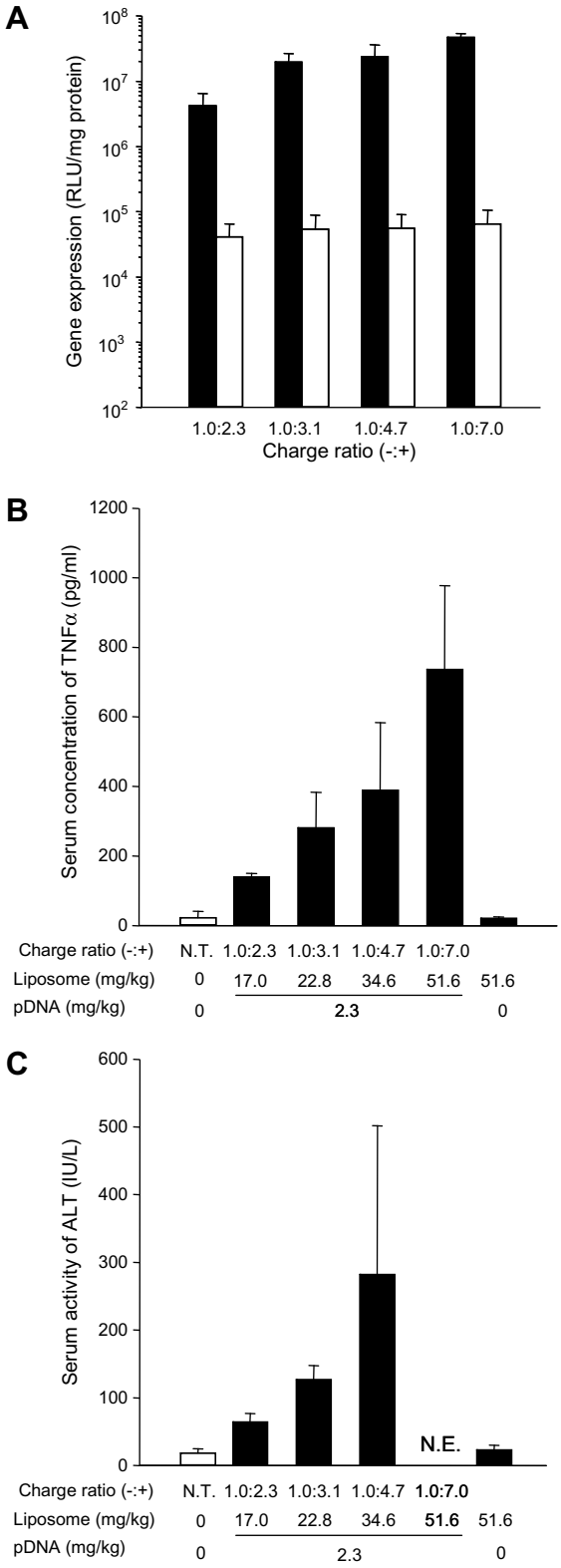


**Fig. 4.** Effect of the charge ratio of the lipoplexes prepared with DOTMA/cholesterol liposomes on gene expression in the lung (■) or liver (□) (A), serum TNFα concentration (B) and serum ALT activity (C) after intravenous injection. DOTAP/cholesterol liposomes were mixed with pDNA at charge ratios (−:+) of 1.0:2.3, 1.0:3.1, 1.0:4.7, and 1.0:7.0. pDNA doses were fixed at 2.3 mg/kg. Six hours after intravenous injection, mice were sacrificed and the luciferase activity was measured. Three hours (for TNFα) or 24 h (for ALT) after intravenous injection, blood was collected from the vena cava and the serum concentration of TNFα or the serum activity of ALT was measured. Each value represents the mean + S.D. of at least three mice.

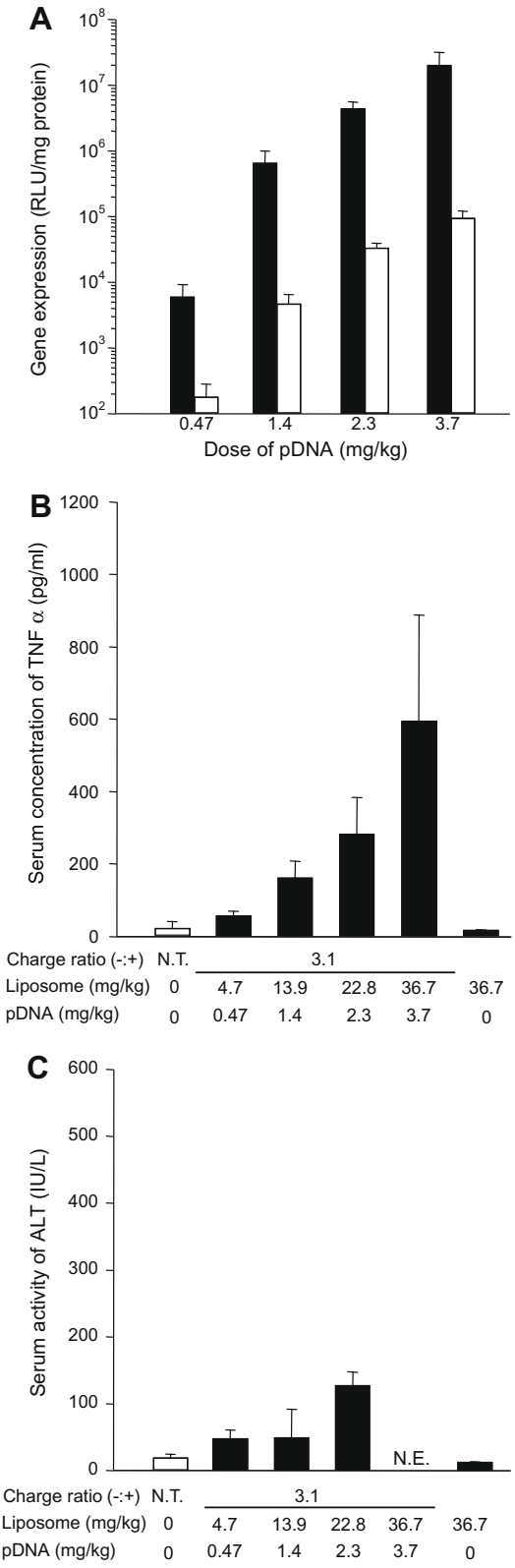


**Fig. 5.** Effect of the pDNA dose of lipoplex prepared with DOTMA/cholesterol liposomes on the gene expression in the lung (■) or liver (□) (A), serum TNFα concentration (B), and serum ALT activity (C) after intravenous injection. DOTMA/cholesterol liposomes were mixed with pDNA at a charge ratio of 1.0:3.1(−:+) and different doses of pDNA. Six hours after intravenous injection, mice were sacrificed and the luciferase activity was measured. Three hours (for TNFα) or 24 h (for ALT) after intravenous injection, blood was collected from the vena cava and the serum concentration of TNFα or the serum activity of ALT was measured. Each value represents the mean + S.D. of at least three mice.

ratios (Figs. 3 and 4) and pDNA doses (Fig. 5). After intravenous administration of lipoplexes prepared with DOTAP/cholesterol liposomes, the peaks of the serum level of TNFα and ALT was observed at 3 h and 24 h, respectively (Fig. 2). When lipoplexes prepared with DOTMA/cholesterol liposomes were administrated, the peak times of serum level of TNFα and ALT was similar (data not shown). These characteristics of peak time of serum level of TNFα and ALT was consistent with the previous reports [8,25]. Therefore, we selected 3 and 24 h for measuring TNFα and ALT, respectively. Lipoplexes were prepared with DOTAP (Fig. 3) or DOTMA/cholesterol liposomes (Figs. 4 and 5). Serum TNFα (Figs. 3B, 4B and 5B) and serum ALT activity (Figs. 3C, 4C and 5C) were enhanced by an increase in the pDNA dose or charge ratio of the lipoplexes. Considering the relationship with gene expression, serum TNFα and ALT levels increased depending on the increase in gene expression by lipoplex prepared with DOTAP/cholesterol (Fig. 3) and DOTMA/cholesterol liposomes (Figs. 4 and 5). These observations provide evidence that basically cytokine production and liver injury characteristics are in accord with the transfection efficacy of the lipoplexes.

The cytokine production and liver injury were markedly suppressed by reducing the pDNA dose (Fig. 5), and reached to normal levels at a pDNA dose of 0.47 mg/kg. Therefore, the effect of charge ratio (−: +) on transfection efficacy, cytokine production and liver injury was also evaluated at a pDNA dose of 0.47 mg/kg. Although the serum TNFα (Fig. 6B) and IL12 (Fig. 6C) and ALT (Fig. 6D) levels were normal, the transfection efficacy was enhanced on increasing the charge ratio (Fig. 6A). In this study, these results suggest that the increase of charge ratio of lipoplexes enhances transfection efficacy, but not cytokine production and liver injury at a low pDNA dose (< 0.47 mg/kg). This observation also partly supports the report by Dow et al., showing that intravenous administration of lipoplex including IL-2-encoded pDNA inhibits lung metastasis at a pDNA dose of 0.02 mg/kg and a charge ratio (−: +) of 1.0: 6.7 without producing any significant side-effects [4]. Since the CpG motif in pDNA is expected to affect cytokine production and subsequent liver injury, either reducing the pDNA dose or reducing the CpG motif number [26] would be an effective method of suppressing the side-effects of lipoplexes.

To date, it is still unclear whether lipoplexes or cationic liposomes cause liver injury as well as cytokine production. Therefore, cationic liposomes alone were administered to mice at the highest doses in this study to identify the contribution of cationic liposomes to liver injury and cytokine production. After intravenous administration of DOTAP/cholesterol liposomes, serum TNFα (Fig. 3B) and ALT (Fig. 3C) levels were normal. Similar results for serum TNFα (Fig. 4B) and ALT (Fig. 4C) levels following administration of DOTMA/cholesterol liposomes were also obtained. These observations provide evidence that liver injury as well as cytokine production is caused by lipoplexes, but not by cationic liposomes.

## 5. Conclusion

In conclusion, we have demonstrated that basically cytokine production and liver injury characteristics are in accord with the transfection efficacy of the lipoplexes. However, at a low pDNA dose (0.47 mg/kg), transfection efficacy is affected by the charge ratio (−: +), but this did not affect cytokine production and liver injury. These may lend support to the view that both increasing charge ratio and keeping the pDNA dose low might realize safe gene therapy. In addition, liver injury as well as cytokine production appears to be caused by lipoplexes, but not by cationic liposomes. This information will be valuable for the future optimization of the preparation conditions of lipoplexes for use in clinical gene therapy.

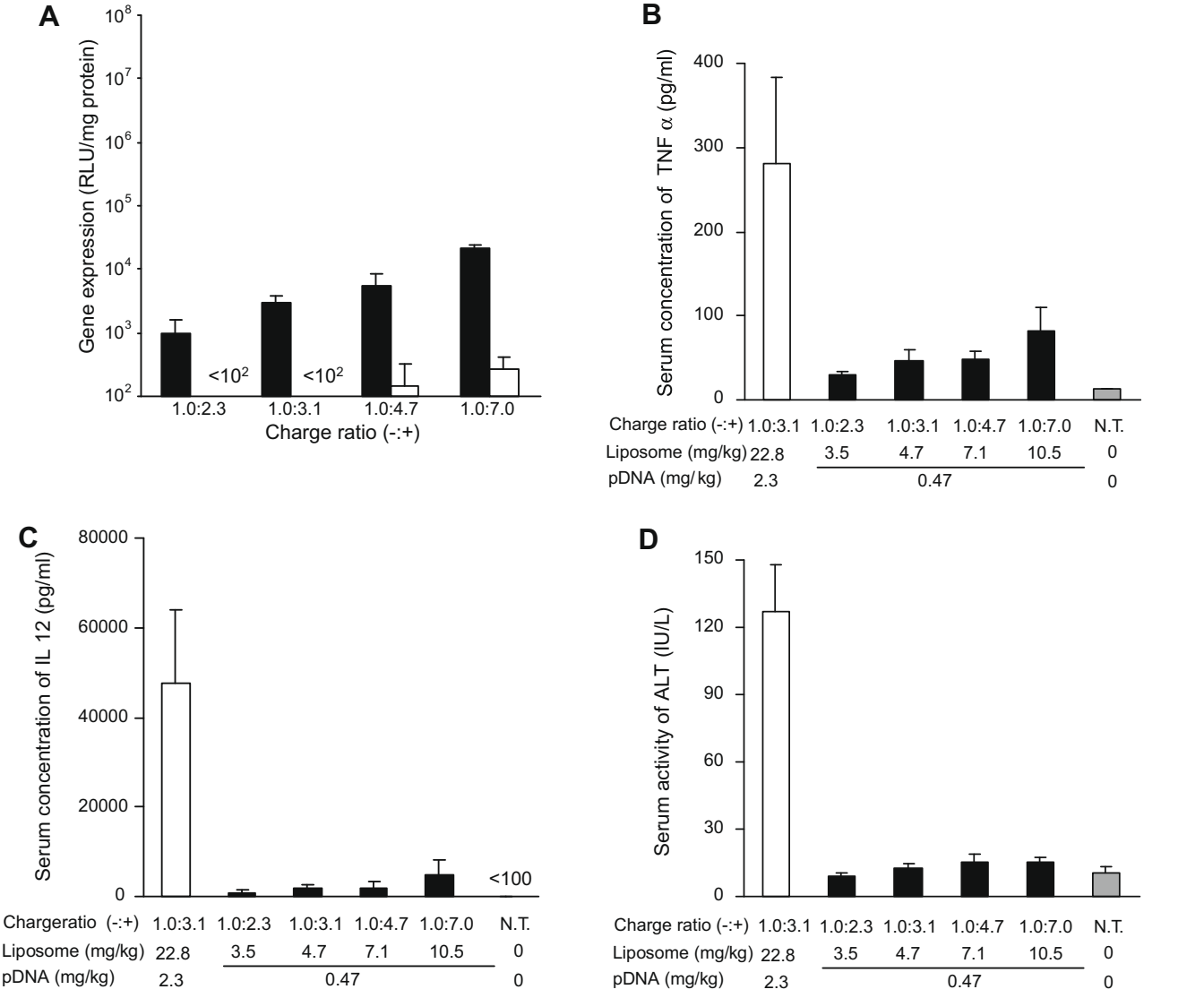


**Fig. 6.** Effect of the charge ratio of the lipoplexes prepared with DOTMA/cholesterol liposomes on gene expression in the lung (■) or liver (□) (A), serum TNFα concentration (B), IL12 concentration (C) and serum ALT activity (D) after intravenous injection. DOTMA/cholesterol liposomes were mixed with pDNA at charge ratios (−:+) of 1.0:2.3, 1.0:3.1, 1.0:4.7, and 1.0:7.0. pDNA doses were fixed at 0.47 mg/kg. Six hours after intravenous injection, mice were sacrificed and the luciferase activity was measured. Three hours (for TNFα), 6 h (for IL12) or 24 h (for ALT) after intravenous injection, blood was collected from the vena cava and the serum concentration of TNFα, IL12 or the serum activity of ALT was measured. Each value represents the mean + S.D. of at least three mice.

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