

**Figure 10** X-gal staining of the liver of mice receiving  $\beta$ -galactosidase-expressing pDNA. (a) Naked pDNA was injected directly into the left lateral lobe. (b, c) Naked pDNA was injected into the tail vein and electric pulses (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) were applied to the left lateral lobe at 30 s after injection. (d) Hepatocytes of the left lateral lobe were isolated by collagenase perfusion and stained with X-gal as described in Materials and methods. More than 100 individual cells per sample were observed under a microscope, and the number of blue cells, that is,  $\beta$ -galactosidase-expressing cells, was counted. Results are expressed as the mean  $\pm$  s.d. of three measurements of each sample from two mice per group.

The number of hepatocytes expressing  $\beta$ -galactosidase using vascularly administered naked pDNA followed by electroporation was significantly ( $P < 0.01$ ) and about 25-fold greater than that obtained by local injection of naked pDNA.

### Discussion

Naked pDNA injection is the simplest method of the nonviral gene delivery approaches. However, no significant gene expression can be detected in any tissue when naked pDNA is intravenously injected by a normal, conventional technique because of its rapid degradation and low membrane permeability. Therefore, pDNA needs to be protected from nuclease-mediated degradation by complex formation with a vector such as cationic polymer or cationic liposome.<sup>1</sup> Cationic vectors can increase transfection efficiency by pDNA, but the expression level seems to be still low as far as therapeutic applications are concerned. As a different challenge involving *in vivo* gene transfer, electroporation has been applied to various tissues after local injection of naked pDNA, and great improvements in transgene expression have been reported.<sup>9</sup> However, direct tissue injection of pDNA has the disadvantage of the limited distribution of transfected cells,<sup>16,22</sup> which would greatly restrict the efficacy of gene replacement therapy of intracellular protein.<sup>23</sup> These two different approaches, that is, pDNA

delivery via the intravascular route and *in vivo* electroporation, could be used to achieve significant transgene expression in a large number of target cells. In a pioneer work by Liu and Huang,<sup>17</sup> this approach was found to be effective as far as achieving transgene expression in a greater number of liver cells was concerned. However, it was not clear how electroporation alters the distribution of pDNA within the liver. Transgene expression occurs only in the cells that take up intact pDNA into the cytoplasm. Therefore, in the present study, we focused on the changes in the distribution of pDNA and examined the option involving liver-targeted gene transfer by injecting naked or hepatocyte-targeted pDNA followed by electroporation to the organ.

As hepatocytes uniquely express asialoglycoprotein receptors on their sinusoidal surface, asialoglycoproteins or galactosylated polymers have been used to deliver a variety of pharmaceutical agents ranging from anticancer drugs to genes. Attempts to deliver pDNA to hepatocytes by conjugating or mixing it with galactose-containing vectors, however, sometimes resulted in failure because of problems in tissue distribution. We have been trying to overcome these problems by controlling the physicochemical properties of the pDNA/galactosylated vector complex.<sup>2,3,20</sup> In these studies, we successfully delivered pDNA to the liver, especially to hepatocytes, in amounts up to about 60% of the injected dose of pDNA. Although the pDNA complex taken up by asialoglycoprotein receptor-mediated endocytosis undergoes lysosomal degradation, a fraction is believed to be released from the endosome/lysosome pathway into the cytoplasm, then being transported into the nucleus. Some structural features have been introduced onto vectors to increase gene transfer by facilitating the release of pDNA into the cytoplasm. They are fusogenic peptides, which create pores on the plasma and/or endosomal membrane,<sup>2,24</sup> and polymers having buffering capacity like PEI.<sup>25</sup> In the present study, pDNA/galactosylated vector complexes showed greater transgene expression in the liver than naked pDNA. Of the complexes used, Gal-PEI was superior to Gal-PLL in terms of transfection efficiency to mouse liver, reflecting the buffering ability of PEI. Electroporation of a set of fixed parameters (250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz) increased the expression in the electroporated lobe of the liver after intravenous injection of both pDNA/Gal-PLL and pDNA/Gal-PEI complexes. As the tissue distribution profile of <sup>32</sup>P-pDNA was hardly altered by electroporation (Figure 2), the enhanced expression would be mediated by changes in the intrahepatic and/or intracellular distribution of pDNA. Assuming that electroporation creates pores on biological membranes,<sup>9</sup> it is reasonable to accept that it can increase the amount of pDNA delivered to the cytoplasm prior to degradation. The pDNA/galactosylated vector complex is believed to bind to cell-surface receptors, captured in endosomes and then transported into lysosomes where it is degraded. In the present study, electric pulses were applied to a mouse at 30 s after injection of the pDNA/vector complex. However, the interval between the injection and electroporation may affect the results. The application of electric pulses at later times such as 5 or 10 min after injection did not increase the expression in the liver (data not shown). Furthermore, an increase in the amount of Gal-PLL or Gal-PEI in each complex

reduced the enhancement ratio produced by electroporation (Figure 3). These results, together with those obtained using naked pDNA, suggest that the polymers disturb to a certain degree the electroporation-mediated enhancement in transgene expression, although galactosylated vector-mediated gene transfer could be improved by *in vivo* electroporation. Different parameters involving the number, duration and electric field of electric pulses hardly improved the electroporation-mediated transgene expression by pDNA/vector complex (data not shown). The results of transgene expression in HepG2 cells strongly support the hypothesis that the free form of pDNA is more effective than its complexed form for transgene expression once it enters into the cytoplasm of cells.

In order to achieve liver-directed gene transfer, naked pDNA has been administered by various methods, including local tissue injection,<sup>16</sup> electroporation after local injection,<sup>12,13</sup> intraportal injection in a hypertonic solution,<sup>26,27</sup> gene gun,<sup>28</sup> and large-volume injection at a high velocity.<sup>7,8</sup> In some cases, transfection efficiency by naked pDNA exceeds that by the pDNA/vector complex. Electroporation generally increases the level of transgene expression up to 1000-fold, but the distribution of cells expressing the transgene product is still limited to around the injection site. It is expected that, when pDNA is injected into the vasculature instead of into the tissue parenchyma, it will be delivered to the vicinity of a large number of cells. Rapid injection of naked pDNA solution into the vasculature has resulted in high transgene expression in a large number of myotubes.<sup>29</sup> Furthermore, intraportal injection of pDNA in a hypertonic solution achieved widespread distribution of transfected cells throughout the liver.<sup>26,27</sup> These approaches, however, need to be studied to investigate the tissue damage induced by the procedures. Although electroporation might also induce tissue damage after its application onto the liver surface, we observed very little GPT leakage and little change in appearance, indicating that the conditions for electroporation used in this study do not result in severe tissue damage. This is marked contrast to the large volume injection of pDNA solution at high-velocity, which induced the leakage of enormous amounts of liver transaminases over a short period after injection.<sup>30</sup>

When intravenously injected, naked pDNA undergoes rapid degradation by nuclease and clearance by Kupffer cells, splenic macrophages, and sinusoidal endothelial cells in the liver.<sup>18,19</sup> No detectable transgene expression was observed in the liver. It is suggested that when electric pulses are applied to the surface of the liver, intravenously injected naked pDNA will pass through the discontinuous endothelium of the liver vasculature, reach the surface of hepatocytes, and then enter the cytoplasm through the pores created by electroporation. The application of electric pulses at different intervals clearly demonstrated that the electroporation-induced gene transfer is mediated by pDNA present in the circulation (Figure 6). There was a good correlation between the plasma concentration of undegraded pDNA and transgene expression (correlation coefficient:  $r^2 = 0.97$ ). These findings suggest that any approach to increase the plasma retention, not to increase delivery to the liver, of pDNA could further improve electroporation-mediated gene transfer to the organ.

pDNA is significantly taken up by liver NPC due to its strong negative charge.<sup>18,19</sup> Electroporation did alter the distribution of <sup>32</sup>P-pDNA between hepatocytes (PC) and NPC: the distribution ratio of naked <sup>32</sup>P-pDNA to PC was greater in the electroporated lobe compared with that in the non-electroporated lobe (Figure 9a). These results suggest that electroporation selectively increases the delivery of pDNA to PC. Anatomical features do not explain the selectivity because PC are localized underneath the lining of the sinusoidal endothelial cells that should have an initial contact with vascularly injected pDNA. Canatella and Prausnitz<sup>31</sup> experimentally and theoretically derived an equation to predict the effects of electroporation on the uptake of solute by cells and their viability. According to this equation, the cell volume is a major factor determining the number of solute (eg pDNA) molecules delivered to the cell; liver PC, or hepatocytes, are much larger in size than liver NPC. Such a difference in size would explain the selective increase in the uptake of pDNA by hepatocytes. Transgene expression correlated well with the amount of pDNA delivered to each type of cells, and hepatocytes had a greater amount of expression than NPC. The efficiency of expression would depend on the cell type, and macrophages such as Kupffer cells undergo transfection with difficulty, which would broaden the difference between hepatocytes and other cells in terms of transgene expression.

In the present study, we successfully demonstrated that the total number of transfected cells was much greater in the liver of mice given an intravenous injection of naked pDNA followed by electroporation than in animals given a local injection of pDNA into the liver (Figure 10). A further increase in this number could be achieved by modification of the size and shape of the electrodes, because the cells between or around them were the major cells transfected.

In conclusion, a more than 200 000-fold increase in transgene expression in the liver was achieved by *in vivo* electroporation after intravenous injection of naked pDNA. pDNA in blood circulation is delivered to the inside of the cells, leading to transgene expression. Although the galactosylated vector is effective in delivering pDNA to hepatocytes through the receptor-mediated process, the vector itself can be an obstacle in the electroporation-mediated increase in transgene expression. These results indicate that the application of electroporation to vascularly administered naked pDNA is a useful gene transfer method for reaching a large number of hepatocytes, which is a key factor in determining the therapeutic efficacy of the *in vivo* gene therapy approach to treating hepatic diseases lacking any intracellular protein, liver cancer, viral hepatitis and allograft rejection. Clinical application of this approach may be achieved in combination with abdominal operation, or by the development of endoscope-type electrodes.

## Materials and methods

### Chemicals

[ $\alpha$ -<sup>32</sup>P]dCTP was obtained from Amersham (Tokyo, Japan). Poly(L-lysine) (PLL: average molecular weight 29 000) and branched polyethyleneimine (PEI: 70 000)

were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Biowhittaker (Walkersville, MD, USA), respectively. All other chemicals were obtained commercially as reagent-grade products. Galactosylation of PLL and PEI was performed as reported previously,<sup>3,18</sup> by covalently binding 2-imino-2-methoxyethyl 1-thiogalactoside to each polymer. The numbers of galactose molecules per polymer were: 44 for Gal-PLL and 60 for Gal-PEI.

#### Animals

Male ddY mice (5 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained on a standard food and water diet and housed under conventional conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Kyoto University.

#### Preparation of pDNA

pDNA encoding firefly luciferase cDNA under the control of CMV-IE promoter was prepared as previously reported.<sup>2</sup> pCMV.SPORT- $\beta$ gal was purchased from GibcoBRL (GibcoBRL, Carlsbad, CA, USA). pDNA was amplified in DH5 $\alpha$ , isolated and purified using a QIAGEN Plasmid Giga Kit (QIAGEN, Hilden, Germany). The purity of the pDNA was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining. The pDNA concentration was measured by UV absorption at 260 nm. pDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by nick translation for biodistribution experiments.<sup>19</sup>

#### Formation of pDNA complex

pDNA complex was prepared in 5% dextrose solution. Various amounts of Gal-PLL or Gal-PEI were added to pDNA, and the mixture was tapped and then left for over 30 min at room temperature. The N/P ratio, the ratio of the concentration of total nitrogen atoms in the polymer to the phosphate group (P) in pDNA, was used as an index of formation. Based on the complex formation and previous studies, N/P ratios were set at 1.2 and 2.4 for the Gal-PLL complex, and 3 and 7 for the Gal-PEI complex.

#### Electrodes and electric pulse delivery

Electric pulses were delivered to the liver by a pair of 1-cm<sup>2</sup> forceps-type electrodes connected to a rectangular direct current generator (CUY21, Nepagene, Chiba, Japan).

#### Electric gene delivery after systemic administration of pDNA complex

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). A midline incision was made on the abdomen and the liver was exposed. Then 5% dextrose solution of naked pDNA or pDNA complex was injected into the tail vein at a fixed dose of 25  $\mu$ g pDNA/mouse. Unless otherwise indicated, electric pulses were delivered to the left lateral lobe of the liver through the electrodes at 30 s after injection. The electric pulse parameters were: 5 ms/pulse, 12 pulses, 4 Hz, and variable electric field from 50 to 500 V/cm. The incision was closed with metal clips or sutured. At 6 h after pDNA injection, the mice were killed and the liver was

excised and divided into two parts: the electroporated lobe (the left lateral lobe) and the other lobes. The liver lobes were homogenized with a five-fold excess of lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). Then the homogenates were subjected to three cycles of freezing and thawing, and centrifuged at 10 000 g for 8 min at 4°C. The supernatant was used for the measurement of luciferase assay. The protein content of each supernatant was also determined using a Protein Quantification Kit (Dojindo Molecular Technologies, Kumamoto, Japan). The GPT level in plasma was measured using a GPT-UV test kit (Wako, Osaka, Japan).

#### In vivo distribution experiment

<sup>32</sup>P-pDNA was added to unlabeled pDNA to give an injection dose of 25  $\mu$ g pDNA/mouse. Each mouse was injected with <sup>32</sup>P-pDNA or its complex in 5% dextrose solution. Then, electric pulses of 250 V/cm, 5 ms/pulse, 12 pulses, 4 Hz were applied to the left lateral lobe of the liver at 30 s after injection. At 1, 5, 10 and 30 min after injection, groups of three mice each were anesthetized with ether and blood was collected from the vena cava and plasma samples were obtained by centrifugation. The liver, kidney, spleen, lung and heart were excised, rinsed with saline and weighed. These organs were homogenized with 0.05% Triton X-100 solution. Each sample was dissolved in Soluene-350 (Packard, Netherlands), then scintillation medium (Clear-sol I, Nacalai Tesque, Kyoto, Japan) was added and the <sup>32</sup>P-radioactivity was counted in an LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan). Radioactivity derived from the plasma in each tissue was corrected for as previously reported.<sup>2</sup>

#### Isolation of liver PC and NPC

pDNA or <sup>32</sup>P-pDNA was injected and electric pulses were applied as above. At 10 min (for radioactivity measurement) or 6 h (for luciferase assay) after injection, liver cells were separated into hepatocytes (PC) and NPC as reported previously.<sup>18</sup> In brief, the liver was perfused with a buffer containing collagenase. The dispersed cells were separated into PC and NPC fractions by differential centrifugation. These cell fractions were subjected to <sup>32</sup>P-radioactivity measurement or luciferase assay.

#### Stability of pDNA in plasma after intravenous injection

Mice received 25  $\mu$ g pDNA into the tail vein. At indicated time points, 500  $\mu$ l blood was collected from the vena cava. pDNA was extracted and electrophoresed as reported previously.<sup>19</sup> The amount of pDNA was evaluated by computerized densitometry.

#### Assessment of transgene-positive cells by X-gal staining of whole liver

Naked pCMV.SPORT- $\beta$ gal (25  $\mu$ g/mouse) was administered as described above. The liver was removed at 24 h after injection, then placed in a fixing solution (4% paraformaldehyde, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 7.3, 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, and 0.02% IGEPAL C-630 (Sigma)) for 60 min at 4°C, and rinsed three times with a wash buffer (0.1 M NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 7.3, 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, and 0.02% IGEPAL C-630).  $\beta$ -Galactosidase activity was detected by immersing the liver in 5-bromo-

4-chloro-3-indolyl- $\beta$ -galactopyranoside (X-gal, Sigma) staining solution (1 mg/ml X-gal, 5 mM ferricyanide and 5 mM ferrocyanide at pH 7.3–7.6 in the wash buffer) for 16–24 h at 37°C. For comparison, the same amount of pDNA in 20  $\mu$ l 5% dextrose solution was injected directly into the lobe and the X-gal staining was performed in a similar manner.

#### Quantification of $\beta$ -galactosidase-expressing hepatocytes

At 48 h after administration of pCMV.SPORT- $\beta$ gal, hepatocytes were isolated as above from the left lateral lobe of the liver, the lobe receiving the electric pulses after intravenous injection of naked pDNA (i.v. injection-n+EP group) or an injection of naked pDNA (direct injection group). The hepatocytes isolated and supplemented with William medium E containing 10% fetal bovine serum were seeded into culture plates. After 12-h incubation, the hepatocytes were washed, fixed with 0.25% glutaraldehyde for 20 min at 4°C, then stained with X-gal solution (1 mg/ml in phosphate-buffered saline, pH 7.4) for 16–24 h at 37°C. More than 100 individual cells per sample were observed under a microscope, and the number of blue cells, that is,  $\beta$ -galactosidase-expressing cells, was counted. Results are expressed as the percentage of  $\beta$ -galactosidase-expressing hepatocytes in the left lateral lobe of the liver.

#### Transfection to HepG2 cells

HepG2 cells, a human hepatoma cell line, were seeded to  $2 \times 10^6$  cells in Dulbecco's minimum essential medium/10% fetal bovine serum in six-well plates for 24 h prior to transfection. Naked pDNA or pDNA/Gal-PLL was added to the cells and electric pulses (95 V, 5 ms/pulse, 12 pulses, 10 Hz) were delivered to the cells through an electrode for cultured cells. The luciferase activity was assayed at 24 h after transfection.

#### Statistical analysis

Data were statistically analyzed by Student's *t*-test.

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## Research Paper

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# Tissue-Specific Characteristics of *in Vivo* Electric Gene Transfer by Tissue and Intravenous Injection of Plasmid DNA

Oranuch Thanaketpaisarn,<sup>1</sup> Makiya Nishikawa,<sup>2,3</sup> Fumiyoshi Yamashita,<sup>1</sup> and Mitsuru Hashida<sup>1</sup>

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**Purpose.** To evaluate the tissue-specific characteristics of electric gene transfer after tissue and intravenous injection of naked plasmid DNA (pDNA).

**Methods.** pDNA encoding firefly luciferase was injected directly into the liver, kidney, spleen, skin and muscle, or into the tail vein of mice, and electric pulses were then applied to one of these organs. The distribution of transgene expressing cells was evaluated using pDNA encoding  $\beta$ -galactosidase.

**Results.** Tissue injection of pDNA produced a significant degree of transgene expression in any tissue with the greatest amount in the liver, followed by kidney and spleen. The expression in these organs decreased quickly with time, and muscle showed the greatest expression at 7 days. Electroporation significantly increased the expression, and the expression level was comparable among the organs. Intravenous injection of pDNA followed by electroporation resulted in a significant expression in the liver, spleen, and kidney but not in the skin or muscle.

**Conclusions.** Electric gene transfer to the liver, kidney, and spleen can be an effective approach to obtain significant amounts of transgene expression by either tissue or intravenous injection of pDNA, whereas it is only effective after tissue injection as far as skin- or muscle-targeted gene transfer is concerned.

**KEY WORDS:** electroporation; gene transfer; intravascular injection; plasmid DNA; tissue distribution.

## INTRODUCTION

Of the nonviral gene transfer methods developed thus far, an injection of plasmid DNA (pDNA) is the simplest. Significant amounts of transgene products are produced after the injection of pDNA into tissues such as skeletal muscle (1), skin (2), and liver (3). Intramuscular injection of pDNA encoding hepatocyte growth factor or vascular endothelial growth factor is reported to be therapeutically effective in treatments for peripheral arterial disease in animal models as well as in clinical trials (4,5). However, a simple injection of pDNA into tissues generally gives less amount of transgene expression than that required for therapeutic benefit. A reliable approach developed so far to increase the expression by tissue-injected pDNA is the application of controlled electric pulses to the injection site: *in vivo* electroporation. It is able to facilitate both interstitial and intracellular transport of pDNA by the formation of transient pores on cell membranes and by electrophoresis (6). There have been several reports on successful applications of electroporation

for pDNA-based gene transfer in tissues including tumor (7–10). Although the application of electric pulses appears to increase the area of transfected cells (11,12), the distribution of these cells is still limited due to the large molecular size of pDNA.

Another pDNA-based gene transfer has been attempted by its intravenous injection. Because of digestion by nucleases and extensive clearance, a simple injection of pDNA into blood circulation did not lead to significant expression (13). On the other hand, a large volume injection of pDNA with high velocity, first described by Liu *et al.* (14) and Zhang *et al.* (15), has become an important experimental tool to produce very high transgene expression in the liver. This pressure-based gene transfer has also been applied locally, and significant amounts of transgene expression were obtained in skeletal muscle (16,17). As demonstrated in a previous paper (18), the administration of pDNA into blood circulation is theoretically superior to its local injection as far as the number of transfected cells is concerned. However, the harshness of the administration procedure has halted its clinical application. Again, electroporation can be used as a driving force for pDNA in the circulation to get into the inside of target cells. We and others have shown that the application of electric pulses to the surface of liver can induce significant transgene expression after intravenous injection of pDNA (19,20). However, the applicability of this approach to tissues other than the liver has not been examined so far.

When a protein encoded in pDNA exhibits its activity after being secreted into the blood circulation, such as blood

<sup>1</sup> Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.

<sup>2</sup> Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan.

<sup>3</sup> To whom correspondence should be addressed. (e-mail: makiya@pharm.kyoto-u.ac.jp)

coagulation factors, the level and persistence of transgene expression are very important, although the type of cells as source of production would be much less significant (21). In the case of gene therapy approaches for hemophiliacs, not only hepatocytes (22) that produce the coagulation factors in healthy subjects, but also other cells, such as fibroblasts (23) and muscle cells (24), have been investigated as target cells producing these factors. The properties of cells and tissues, such as the ease of transfection, the ability to synthesize proteins, location, life span of the cells, and tissue blood flow, are important factors determining the efficacy of gene therapy. Because of these different properties of tissues, it is necessary to investigate the differences in transgene expression in a variety of tissues in order to choose the most appropriate target for a specific disease.

In the present study, therefore, we examined the level of transgene expression in five different tissues, that is, liver, kidney, spleen, dorsal skin, and skeletal muscle, after tissue or intravenous injection of pDNA. The effect of electroporation was examined following both modes of administration. Here, we demonstrate that the level of transgene expression was dependent on the tissue and route of pDNA administration. The application of electric pulses to the injection site increases the expression level to almost an identical value, irrespective of the tissue examined. In contrast, intravenous injection of pDNA followed by electroporation was found to be a promising approach only for gene transfer to the liver, kidney and spleen.

## MATERIALS AND METHODS

### Animals

ICR mice (female, 18–20 g) were purchased from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan) and were maintained on a standard diet and water under conventional housing conditions. All animal experiments were carried out in accordance with the guidelines for Animal Experiments of Kyoto University.

### Plasmid DNA

pDNA encoding firefly luciferase cDNA under the control of CMV-IE promoter was constructed by subcloning the *HindIII/XbaI* firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) as previously reported (11). pCMV.SPORT- $\beta$ -gal containing a CMV promoter upstream of the  $\beta$ -gal gene, followed by the SV40 t-intron and polyadenylation signal were purchased from GibcoBRL (GibcoBRL, Carlsbad, CA, USA). Both pDNAs were amplified in the *E. coli* strain DH5 $\alpha$ , then isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN, Hilden, Germany). The purity was checked by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260 nm. For biodistribution experiments, pDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham, Tokyo, Japan) by nick translation (25).

### In Vivo Gene Expression Experiments

Four-week-old ICR female mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Then, a pDNA solution in saline or dextrose water (20  $\mu$ g/20  $\mu$ l) was injected directly into an injection site (1 injection/mouse). For injection into the liver, spleen or left kidney, a midline incision was made on the abdomen. Then, pDNA was injected and the incision was closed with metal clips. pDNA was injected into the gastrocnemius muscle of right hind leg or shaved dorsal skin. Square-wave electric pulses were applied with forceps-type electrodes to the injection site of the pDNA. In the case of intravenous injection, pDNA solution in saline (25  $\mu$ g/200  $\mu$ l) was injected into the tail vein following electroporation in the same manner as the cases of tissue injection. At optimal time points after injection, mice were killed and the tissue or organ receiving electroporation was excised, homogenized in a lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8), and subjected to three cycles of freezing in liquid nitrogen ( $-190^{\circ}\text{C}$ ) and thawing ( $37^{\circ}\text{C}$ ). Then, the homogenates were centrifuged at  $10,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  and 10  $\mu$ l of the supernatant was mixed with 100  $\mu$ l of luciferase assay buffer and the chemiluminescence produced was measured in a luminometer. The luciferase activity was expressed as relative light units (RLU) per tissue (RLU/tissue). The average weight (g) and protein content (mg protein/g tissue) of the tissues sampled were listed in Table I.

### Electrodes and Electric Pulse Delivery

Electric pulses were delivered to tissue using a pair of 1-cm<sup>2</sup> forceps-type electrodes connected to a rectangular direct current generator (CUY-21, Nepagene, Chiba, Japan). The parameters of the electric pulses were: 5 ms/pulse, 12 pulses, 4 Hz, and a variable electric field from 50 to 1,000 V/cm. Electric pulses were delivered to target tissue using the electrodes 30 sec after tissue or intravascular injection of plasmid DNA.

### Biodistribution Experiments After Intravenous Administration of <sup>32</sup>P-pDNA

<sup>32</sup>P-pDNA was added with unlabeled pDNA to adjust the injection dose to 25  $\mu$ g pDNA/mouse. Each mouse was injected with <sup>32</sup>P-pDNA in saline. A set of electric pulses (500 V/cm for liver, kidney and muscle or 1,000 V/cm for

**Table I.** Average Weight and Protein Content of the Tissues Sampled

Tissue	Weight (g)	Protein Content (mg protein/g tissue)
Liver	0.99 $\pm$ 0.13 (100)	325.0 $\pm$ 43.9 (100)
Kidney	0.12 $\pm$ 0.01 (12)	93.0 $\pm$ 16.6 (29)
Spleen	0.09 $\pm$ 0.02 (9)	95.4 $\pm$ 12.1 (29)
Skin	0.15 $\pm$ 0.03 (15)	51.2 $\pm$ 8.4 (16)
Muscle	0.11 $\pm$ 0.02 (11)	58.4 $\pm$ 8.0 (18)

Values are expressed as the mean  $\pm$  SD of tissues collected in this study ( $n = 50$  to  $60$ ).

The values in parentheses represent relative values to the liver.

spleen and skin, 5 ms/pulse, 12 pulses, 4 Hz) was applied to target tissues 30 sec after systemic injection. At 1, 3, 5, 10, and 30 min after injection, groups of 3–4 mice each were anesthetized with ether, blood was collected from the vena cava to obtain plasma by centrifugation. We examined the pDNA disposition only up to 30 min after injection since the disposition of pDNA after longer periods would not represent that of intact pDNA due to its rapid degradation (26). The liver, kidney, spleen, skin and muscle were excised, rinsed with saline and weighed. These organs were homogenized with 0.05% Triton X-100 solution and each sample was dissolved in soluene-350 (Packard, Netherlands), then Scintillation medium (Clear-sol I, Nacalai Tesque, Kyoto, Japan) was added and then  $^{32}\text{P}$ -radioactivity was measured in an LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan). Radioactivity derived from plasma in each tissue was corrected for as previously reported (27).

#### Characterization of LacZ pDNA Expression After Tissue or Intravenous Injection Followed by Electroporation

LacZ pDNA was administered by either tissue or intravenous injection as described above. After pDNA injection, at 24 h for liver, kidney, spleen and skin and at 7 days for muscle, these whole organs were collected and then stained using an X-gal histochemistry method to evaluate  $\beta$ -galactosidase activity.

#### X-gal Histochemistry

X-gal 5-bromo-4 chloro-3-indolyl- $\beta$ -D-galactoside is a chromogenic substrate for the  $\beta$ -galactosidase gene product expressed in the transgenic hepatocytes used. X-gal turns blue on exposure to  $\beta$ -galactosidase present in those cells. X-gal histochemistry was performed on the whole liver: fresh samples were placed in organ fixing solution (4% paraformaldehyde), 0.1 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  (pH 7.3), 2 mM  $\text{MgCl}_2$ , 0.01% sodium deoxycholate, and 0.02% IGEPAL C-630 (Sigma) for 60 min at 4°C, rinsed three times for 30 min with wash buffer [0.1 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  (pH 7.3), 2 mM  $\text{MgCl}_2$ , 0.01% sodium deoxycholate, and 0.02% IGEPAL C-630], then incubated in X-gal staining solution [1 mg/ml X-gal (Sigma), 5 mM ferricyanide, and 5 mM ferrocyanide at pH 7.3–7.6 in wash buffer] for 16–24 h at 37°C, and fixed for 24–48 h in 10% formalin (28).

#### Statistical Analysis

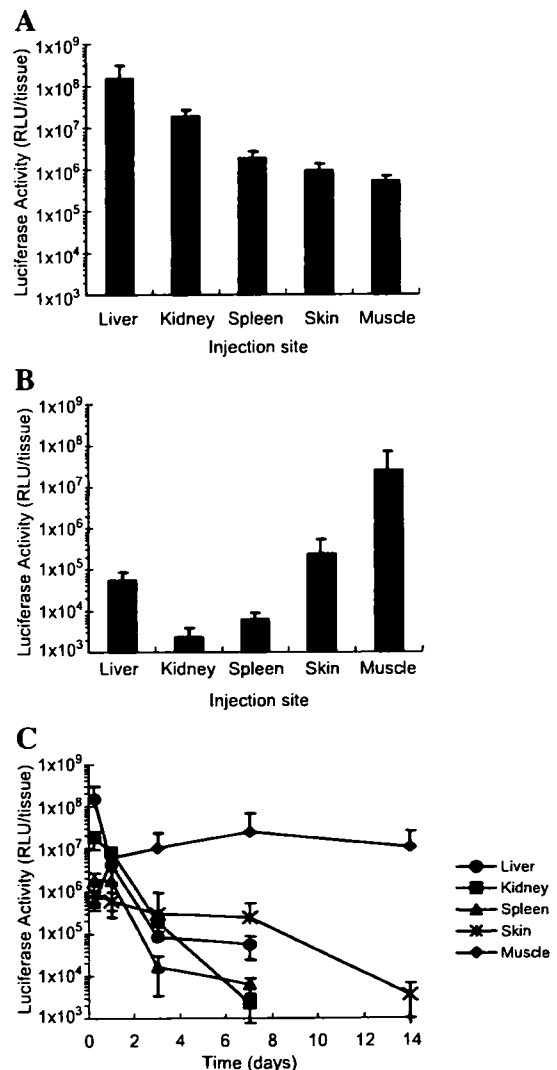
Experimental data were analyzed by Student's *t* test (see Fig. 3) or one-way ANOVA followed by the LSD multiple comparison test (see Fig. 2) using SPSS software. *p* values for significance were set at 0.05.

## RESULTS

#### Increased Transgene Expression by Electroporation After Tissue Injection of pDNA

Naked pDNA was injected into one of the mouse tissues under the same condition: 20  $\mu\text{g}$  pDNA in 20  $\mu\text{l}$  saline

solution was injected using an insulin syringe with a 29G-needle. Figure 1A shows the transgene expression in the tissues at 6 h after injection of pDNA. The liver gave the greatest expression at this earliest time point, followed by the kidney and spleen. The expression in skin and muscle was about 160- and 270-fold less than that in the liver. At 7 days, however, muscle showed the greatest transgene expression among the tissues examined, which was followed by skin, the liver, spleen and kidney (Fig. 1B). Figure 1C shows the time-courses of transgene expression in these organs. The expression in the liver, spleen and kidney reached a peak at 6 h, then decreased with time at a similar rate with an apparent half-life of 16 h. On the other hand, the expression in skeletal muscle was relatively low at 6 h but continuously increased with time and leveled off after 3 days. The expression in the



**Fig. 1.** Transgene expression after tissue injection of pDNA. ICR mice were injected directly with naked pDNA (20  $\mu\text{g}/20 \mu\text{l}$ ) into the liver, kidney, spleen, dorsal skin, or gastrocnemius muscle (1 injection/mouse). The total luciferase activity per tissue receiving an injection of pDNA is expressed in RLU (relative light units)/tissue as mean  $\pm$  SD of four mice: (A) 6 h, (B) 7 days, and (C) time course after injection.



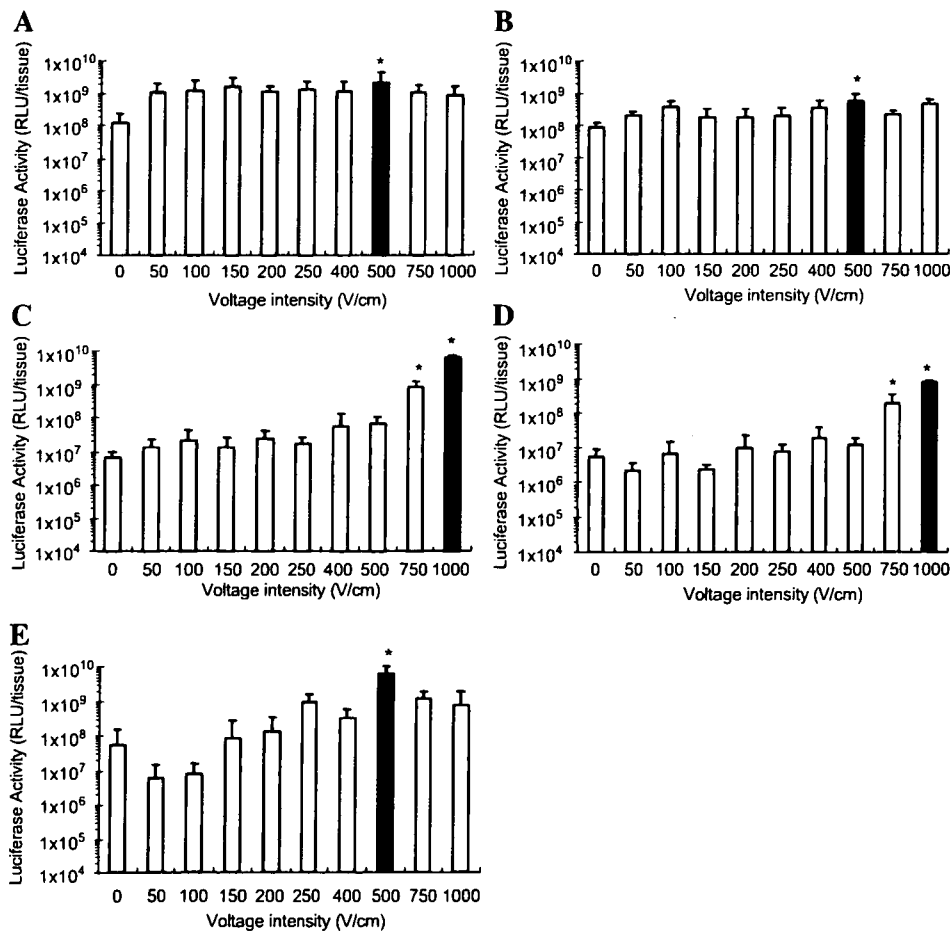
skin declined with time at a slower rate than that of the internal organs with an apparent half life of 44 h.

Twelve electric pulses of 5 ms-length, 4 Hz, with varying electric fields were applied to the injection site of pDNA 30 sec after injection using forceps-type electrodes. Figure 2A–E shows the transgene expression in the tissues examined. The electric field was changed from 50 to 1,000 V/cm. The expression was only evaluated at the time points when the expression in each tissue was at its peak: 6 h for the liver, kidney, spleen and skin, and 7 days for muscle. The application of electric pulses increased the expression in most cases, but the response to the pulses depended on the type of tissue. The expression in the liver was increased by the electric pulses of any given electric field by about 17-fold, with the greatest value of  $2.15 \times 10^9$  RLU/liver being obtained at 500 V/cm (Fig. 2A). A similar tendency was observed in the case of the kidney (Fig. 2B), but the greatest enhancement at an electric field of 500 V/cm was only seven-fold. The expression in muscle was also the greatest at 500 V/cm, and up to a 119-fold increase with a value of  $6.06 \times 10^9$  was obtained (Fig. 2E).

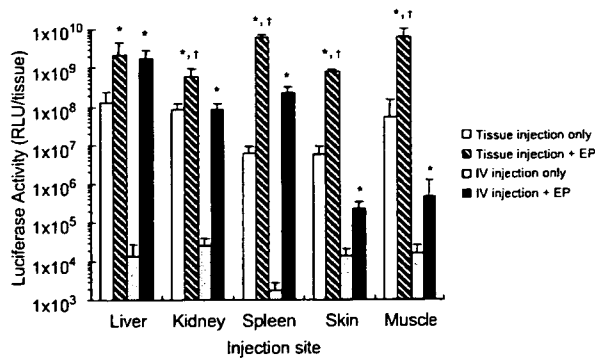
On the other hand, the expression in the spleen and skin simply increased as the electric field increased and the greatest expression was obtained at 1,000 V/cm (Fig. 2C and D). In skin, the increase in the expression was about 144-fold. Among the tissues examined, the spleen exhibited the greatest enhancement (997-fold) in the expression with the greatest value of  $6.16 \times 10^9$  RLU. These results show that the transgene expression in any tissue can be significantly improved by the application of electric pulses with parameters that need to be optimized depending on the target tissue.

#### Increased Transgene Expression by Electroporation After Intravenous Injection of pDNA

A normal intravenous injection of pDNA resulted in no significant transgene expression in any tissue examined (Fig. 3). To obtain detectable transgene expression, electric pulses that were optimized as above were applied to each of the tissues at 30 s after intravenous injection of pDNA. Electroporation significantly increased the expression in all



**Fig. 2.** Effect of the electric field strength on transgene expression after tissue injection of pDNA followed by electroporation the tissue: (A) liver, (B) kidney, (C) spleen, (D) dorsal skin, and (E) gastrocnemius muscle. ICR mice were injected directly with pDNA (20  $\mu$ g/20  $\mu$ l) into each tissue and received electric pulses: 12 pulse, 4 Hz, 5 ms/pulse, and variable electric field from 50 to 1,000 V/cm. After administration, mice were killed and the luciferase activity was measured at 7 days for gastrocnemius muscle and at 6 h for other organs. The results are expressed in RLU/tissue as mean  $\pm$  SD of four mice. \*A statistically significant difference against the control group ( $p < 0.05$ ).



**Fig. 3.** Transgenic expression after intravascular (IV) injection of pDNA with or without subsequent electroporation (EP). ICR mice were injected with pDNA solution into the tail vein (25  $\mu$ g/200  $\mu$ l). Electric pulses were applied at 30 s after pDNA injection under the optimal conditions: 500 V/cm for liver, kidney and gastrocnemius muscle and 1,000 V/cm for spleen and dorsal skin, 12 pulse, 4 Hz, 5 ms/pulse. After administration, the luciferase activity was determined at 7 days for gastrocnemius muscle and at 6 h for other organs. The results are expressed in RLU/tissue as mean  $\pm$  SD of four mice. \*A statistically significant difference ( $p < 0.05$ ) against the control (without electroporation) group; †a statistically significant difference ( $p < 0.05$ ) against the tissue injection group. The expression data after tissue injection (Figs. 1 and 2) are also shown for comparison.

tissues receiving electric pulses. However, no significant expression was observed in tissues that did not receive electric pulses. The enhancement ratios of transgene expression by electroporation were 130,000, 125,000 and 3,300 for the liver, spleen and kidney, respectively. The liver showed the greatest level of expression among the tissues, and this was almost identical to that achieved by the tissue injection of pDNA followed by electroporation. The expression in the spleen and kidney was also efficient, but that in the skin and muscle was significantly lower than that obtained by the tissue injection followed by electroporation. These findings indicate that intravenous injection of pDNA followed by electroporation is a suitable approach for tissue-selective gene transfer to the liver, kidney and spleen, but it is much less effective in achieving skin- and muscle-targeted gene transfer.

#### Effect of Electroporation on the Tissue Distribution of pDNA After Intravascular Injection of pDNA

Figure 4 shows the time courses of the concentration in the plasma (upper panel) and the amounts of radioactivity in tissues (lower panel) in the electroporated tissues, that is, the liver (A), kidney (B), spleen (C), dorsal skin (D), and muscle (E). A set of electric pulses (500 V/cm for liver, kidney and muscle or 1,000 V/cm for spleen and skin, 5 ms/pulse, 12 pulses, 4 Hz) was applied to each tissue 30 s after intravascular injection of <sup>32</sup>P-pDNA. As reported previously (22,23), <sup>32</sup>P-radioactivity derived from <sup>32</sup>P-pDNA quickly disappeared from the blood circulation and about 40% of the injected dose accumulated in the liver (Fig. 4A). The delivery of <sup>32</sup>P-pDNA to the tissues receiving electric pulses was increased by electroporation. However, the differences in the distribution were not so significant and did not explain the great increase in transgene expression in these organs receiving electroporation.

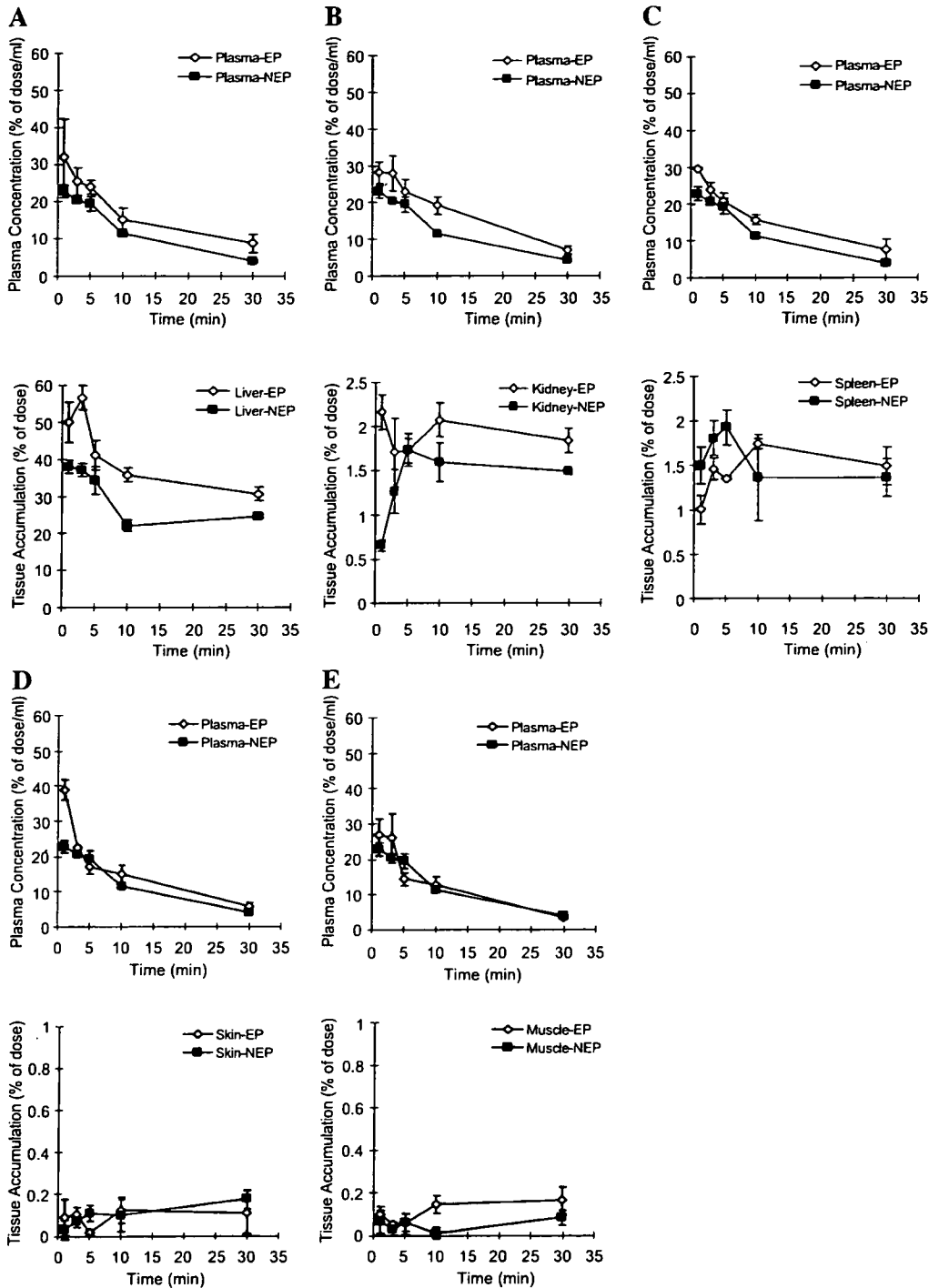
#### Distribution of Transgene Expressing Cells

X-gal histochemistry staining was used to assess the gross three-dimensional pattern of LacZ expression in whole organs at 24 h for the liver, kidney, spleen and skin and at 7 days for muscle after tissue or intravenous injection of pDNA encoding LacZ (Fig. 5). The patterns of  $\beta$ -galactosidase activity corresponded to the quantitative results of the firefly luciferase activity described above. After direct tissue injection, transgene products were limited to the area around the injection site. Electroporation expanded the area, but the transgene expression was still limited. In addition, electric gene transfer clearly showed its potential as a driving force for pDNA in the blood circulation to get into the inside of the target cells, not only in the liver but also in the spleen and kidney. The patterns of  $\beta$ -galactosidase activity in the liver indicate the possibility of obtaining an increased number of transfected cells when pDNA was injected into the blood circulation compared with that obtained after its tissue injection. However, this approach was less effective for skin and muscle-targeted gene transfer.

#### DISCUSSION

*In vivo* gene transfer appears to be a promising technology to treat various diseases including monogenic diseases such as Duchenne muscular dystrophy, hemophilia, and OTC deficiency, as well as cancers and viral infection. Except for lysosomal enzymes, intracellular proteins such as dystrophin can hardly be supplemented in the protein form, but they can be easily delivered to the site of action by using the gene-based approach. The supplementation of intracellular proteins by means of gene delivery, therefore, requires tissue-specificity as far as the delivery and expression of the target gene is concerned. In a marked contrast, extracellular or plasma proteins, including blood coagulation factors, cytokines, and growth factors, do not need to be delivered to the target tissue to exhibit their pharmacological activity. The target tissue for gene transfer should be optimized to obtain the best result as far as therapeutic efficacy is concerned. The parameters that need to be considered are: the efficacy of gene delivery, transfection efficiency, and duration of transgene expression. In the present study, therefore, we examined the tissue-specific characteristics of gene transfer after tissue and intravenous injection of pDNA.

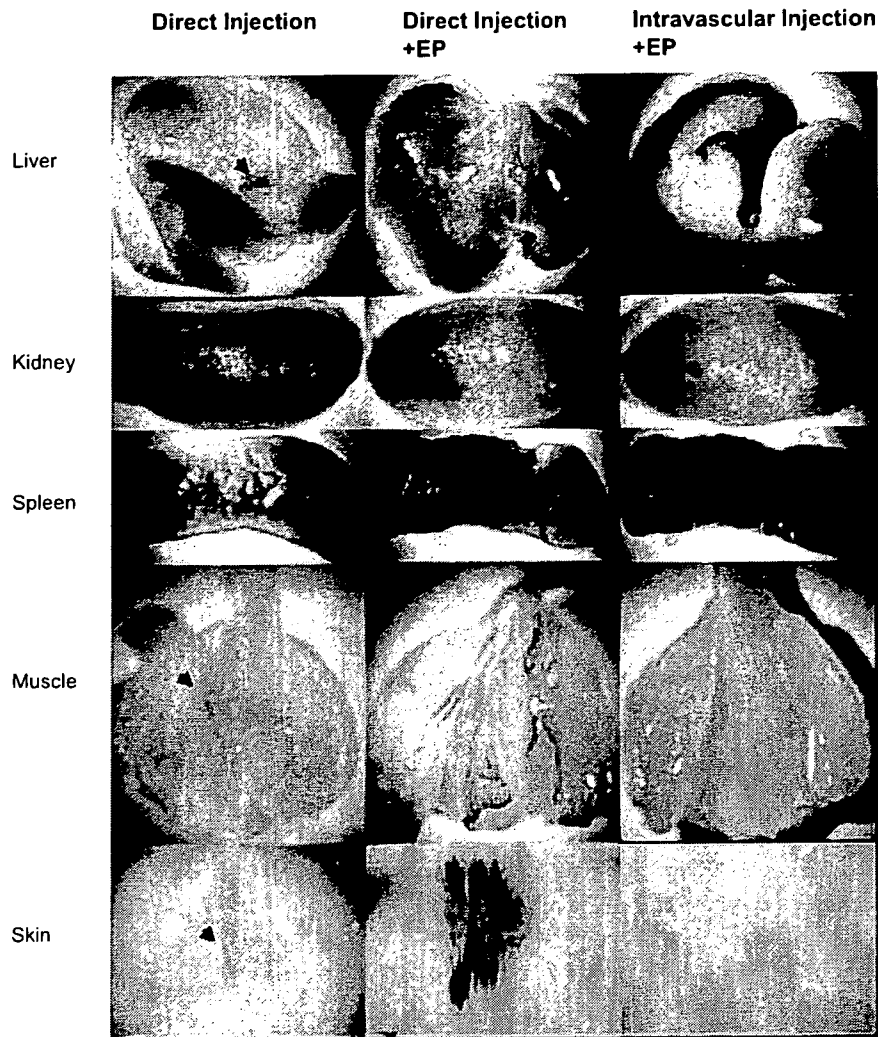
After tissue injection, pDNA can be taken up by cells adjacent to the injection site, which results in a substantial level of transgene expression. Among the tissues examined, we clearly demonstrated that the liver, kidney and spleen are good sites for the protein production when immediate expression is required. On the other hand, skeletal muscle is an attractive tissue for prolonged expression of therapeutic proteins. One explanation of this long-term stability and transcriptional activity in muscle is the terminally differentiated and long-living cells of myofibers including their postmitotic nature. However, this gene transfer approach involving simple pDNA injection has a relatively low efficiency of expression due to the limited distribution of plasmid DNA within the interstitial space of tissues only around the injection site (21). As first reported by Wolff *et al.* (29), *in vivo* electroporation can be used to improve the transfection efficiency of pDNA delivered



**Fig. 4.** Plasma concentration (upper panel) and tissue accumulation (lower panel) of  $^{32}\text{P}$ -radioactivity after injection of naked  $^{32}\text{P}$ -pDNA with electroporation (EP) or without electroporation (NEP). Naked  $^{32}\text{P}$ -pDNA was injected into the tail vein at a dose of 25  $\mu\text{g}/\text{mouse}$ , and electric pulses (500 V/cm for liver (A), kidney (B) and gastrocnemius muscle (E); 1,000 V/cm for spleen (C) and dorsal skin (D); 5 ms/pulse; 12 pulses; 4 Hz) were applied to the injection tissue at 30 s after injection. The results are expressed in % of dose/ml for plasma concentration or % of dose for tissue accumulation as mean  $\pm$  SD of four mice.

by tissue injection. The application of an electric field can induce a transient structural reorganization and produce reversible permeability of the cell membrane, allowing direct entry of pDNA into cytoplasm via pores (6,21).

As listed in Table I, the tissues examined are largely diverse in the weight and protein content. Therefore, if the expression were expressed as the amount per tissue weight (RLU/g tissue) or protein content (RLU/mg protein), the



**Fig. 5.** The gross three-dimensional patterns of lacZ expression in whole organs after tissue or intravascular injection of lacZ DNA with electroporation (EP) or without electroporation. ICR mice were injected locally with pDNA solution (20  $\mu$ g/20  $\mu$ l) into target tissue or intravenously into the tail vein (25  $\mu$ g/200  $\mu$ l). Electric pulses were applied 30 s after pDNA injection at an optimum voltage (500 V/cm for liver, kidney and gastrocnemius muscle; 1,000 V/cm for spleen and dorsal skin) under the following conditions: 12 pulse, 4 Hz, 5 ms/pulse. After administration, X gal histochemistry staining was performed at 7 days for gastrocnemius muscle and at 24 h for other organs.

rank order of the tissues is different from that shown in Fig. 1; the kidney showed the greatest expression, followed by the liver. Although these parameters could be also important in considering the differences in transgene expression among tissues, the total amount of the expression should be used for the comparison, because it would directly correlate with the concentration of the transgene product in blood when secreted.

In the current study, the application of electric pulses to the injection sites greatly increased the amounts of transgene product in all tissues examined. However, at any electric field examined, the response to the pulses was dependent on the type of tissue, which is due to differences in the characteristics of cells and the relative conductivity of the extracellular matrix and interstitial fluid within each tissue (6,30). The greatest transgene expression was obtained at 500 V/cm for

the liver, kidney and muscle, whereas the expression in the spleen and skin simply increased as the electric field increased and exhibited the greatest values at 1,000 V/cm. When compared using the peak levels of expression, electroporation gave the greatest expression in the spleen, followed by muscle, liver, skin and kidney in this order. In addition, the spleen showed the greatest enhancement by electroporation up to 998-fold with a value of  $6.16 \times 10^9$  RLU. A recent study of electric gene transfer to the spleen indicated that transgene-expressing cells were found mostly in the white pulp of the spleen where the lymphocytes are located (31). Therefore, electric gene transfer can be an effective approach for spleen-targeted DNA vaccination to cancers and infectious diseases.

The patterns of  $\beta$ -galactosidase activity in the tissues confirmed that the application of electric pulses can signifi-

cantly increase the area of transfected cells after the injection of pDNA into the interstitial space of tissues. Therefore, electro-gene transfer can improve not only pDNA delivery to nucleus but also the distribution of transgene expressing cells.

As mentioned above, direct tissue injection of pDNA has the drawback of limited distribution of transfected cells (32). To overcome this limitation, intravascular administration of pDNA has been used to deliver pDNA to a greater number of target cells through the capillaries. Hydrodynamic pressure or electroporation was used as a driving force to deliver pDNA into cells, because pDNA itself lacks any ability to get into the cytoplasm in an active form for transgene expression. In an actual fact, no significant transgene expression was detected in any tissue examined after intravenous injection of naked pDNA. The application of electric pulses to the tissues significantly increased the amount of the transgene product in any case examined after systemic injection of pDNA. The assumption that electroporation creates pores on biological membranes makes it reasonable to speculate that it can increase the amount of pDNA delivered to the cytoplasm prior to degradation by nucleases within tissues and blood circulation. The longer time interval between the intravenous injection of pDNA and electroporation resulted in less transgene expression (20), indicating that the circulating intact pDNA is responsible for transgene expression.

Intravenous injection of pDNA followed by electroporation was found to be a promising approach for gene transfer to the liver, spleen and kidney, but it was much less effective in achieving muscle- and skin-targeted gene transfer. *In vivo* gene expression will take place only at restricted cells that are reached by intact pDNA after systemic administration. After intravenous administration, pDNA distributes within the body in a manner that depends on its interaction with biological components. Due to the limitation in huge size of pDNA, only capillaries in the liver, spleen and bone marrow possess pores and intercellular gaps that allow pDNA to reach parenchymal cells (33). Therefore, gene transfer to parenchymal cells in tissues having continuous-type endothelial cells, such as skeletal muscle and skin, after intravascular injection of pDNA, may require the delivery of pDNA across the endothelium. Although electroporation was expected to open the intercellular gaps in the endothelium, the very low degree of distribution of <sup>32</sup>P-pDNA in muscle and skin even after electroporation would explain the inability to achieve enhanced extravasation of pDNA from the capillaries to the interstitial space in these tissues.  $\beta$ -galactosidase expression data also supported the poor transfection results of skin and muscle electric gene transfer via the systemic circulation. Although the location of endothelial cells makes them the most liable cells to be transduced by intravascular pDNA with electric pulses, the endothelial cells are generally difficult to transfect as clearly demonstrated after hydrodynamics-based or electric pDNA delivery (20,34).

Among the tissues examined, the liver gave the greatest expression after systemic injection combined with electroporation, and this was almost identical to that achieved by the tissue injection of pDNA followed by electroporation. The discontinuous-type capillaries in the liver may help

pDNA distribute to the parenchymal cells. We found that electroporation selectively increases the delivery of pDNA to liver parenchymal cells and transgene expression in these cells, probably due to the difference in size of cells (20). This may also be applied to the spleen, which showed a high level of transgene expression after electroporation. The  $\beta$ -galactosidase activity in these tissues indicated that the cells expressing the transgene are still limited to the area around the injection site. This limited distribution of transfected cells could be simply explained by the limited area of the effects of the electric pulses applied. Therefore, improvements in electrode design should make it possible to increase the area of transgene-expressing cells. Although directly-injected pDNA in the liver might be present within the tissue in a greater amount than pDNA after intravenous injection, the levels of transgene expression suggest that directly-injected pDNA in the liver rapidly leaks out from the tissue through the well-developed vasculature. In addition, it is also suggested that only a tiny fraction of the pDNA seems to contribute to the final output of transgene expression.

Although electroporation could induce tissue damage, we found little leakage of transaminases such as glutamyl oxaloacetic transaminase and glutamyl pyruvic transaminase from liver parenchymal cells after electric gene transfer to the liver (20). In addition, we observed little changes in the appearance of any tissue after electroporation in the present study. These results suggest that the conditions of the electroporation used in this study do not induce severe tissue damage. In previous studies, no histological changes were observed in glomeruli and tubular epithelial cells of the kidney after electroporation at 100 V (35), and no obvious damages were found in skin tissue at 24 and 48 h after electroporation at 1,750 V/cm (12). An electroporation with a high voltage of 900 V resulted in the generation of some necrotic cells in skeletal muscle (36); however, the damaged tissue was replaced with muscle fibres with central nuclei by 2 weeks, indicating that the tissue damage can be regenerated in a short period of time.

In summary, *in vivo* electroporation increases the transgene expression in all the cases of both tissue and intravascular injection of pDNA. Intravenous injection of pDNA followed by electroporation has been shown to be a promising approach for tissue-selective gene transfer to the liver, kidney and spleen, but it is much less effective in achieving skin- and muscle-targeted gene transfer. The level of transgene expression was clearly demonstrated to be dependent on the tissue and the route of pDNA administration. Liver and spleen are the most interesting tissues for prompt supply of proteins because of the highest transgene expression in both modes of administration in electric gene transfer. To prolong the supply of therapeutic proteins, intramuscular administration followed by electroporation should be concerned especially its advantage in the easiness of application of the electric field. Initially, the clinical applicability of these approaches for internal organs was achieved in combination with abdominal surgery, or by the modification of endoscope-type electrodes. However, the use of chest electrodes for the introduction of pDNA into the lungs after inhalation has been reported in an animal model (37). This type of electrode can be used to apply electric pulses to internal organs without surgery after intravenous injection.

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# Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways

Kei Yasuda, Yoshiyuki Ogawa, Ikuko Yamane, Makiya Nishikawa, and Yoshinobu Takakura<sup>1</sup>

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Japan

**Abstract:** Previously, we showed that bacterial DNA and vertebrate DNA/cationic liposome complexes stimulate potent inflammatory responses in cultured mouse macrophages. In the present study, we examined whether endocytosis and subsequent acidification are associated with these responses. The endocytosis inhibitor, cytochalasin B, reduced tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) production by a plasmid DNA (pDNA)/cationic liposome complex. The endosomal acidification inhibitor, monensin, inhibited cytokine production by pDNA or a calf thymus DNA/liposome complex. These results suggest, similarly to CpG motif-dependent responses, that endocytosis and subsequent endosomal acidification are also required for these inflammatory responses. It is intriguing that another inhibitor of endosomal acidification, bafilomycin A, stimulated the production of TNF- $\alpha$  mRNA and its protein after removal of the pDNA/liposome complex and inhibitors, although it inhibited the release of interleukin-6. Similar phenomena were observed in the activation of macrophages by CpG oligodeoxynucleotide, calf thymus DNA, and *Escherichia coli* DNA complexed with liposomes. Moreover, bafilomycin A also induced a high degree of TNF- $\alpha$  release after stimulation with naked pDNA. These results suggest that bafilomycin A increases TNF- $\alpha$  production induced by DNA at the transcriptional level via an as-yet unknown mechanism. Furthermore, we investigated the contribution of Toll-like receptor 9 (TLR9), the receptor of CpG motifs, to the cell activation by the DNA/cationic liposome complex using the macrophages from TLR9<sup>-/-</sup> mice. We observed a reduced inflammatory cytokine release from macrophages of TLR9<sup>-/-</sup> mice compared with wild-type mice. However, the cytokine production was not completely abolished, suggesting that the DNA/cationic liposome complex can induce macrophage activation via TLR9-dependent and -independent pathways. *J. Leukoc. Biol.* 77: 71–79; 2005.

**Key Words:** macrophages · CpG motifs · tumor necrosis factor (TNF)- $\alpha$  · gene therapy

## INTRODUCTION

Unmethylated CpG sequences (CpG motifs) in bacterial DNA, but not in vertebrate DNA, are recognized by the immune system as a danger signal [1, 2]. When macrophages or dendritic cells (DC) take up CpG DNA, it is recognized by Toll-like receptor 9 (TLR9), which is one of the pattern recognition receptors [3]. TLR9 is present in the intracellular compartment [4], and inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and IL-12 are secreted. These cytokines significantly influence DNA-based therapies in different ways. In gene therapy, cytokine production generally seems inappropriate, as these inflammatory cytokines significantly reduce transgene expression in target cells through their direct cytotoxicity and promoter attenuation [5, 6]. Conversely, they are essential for the efficacy of DNA vaccination, as these cytokines can enhance the immune responses, and the balance of these cytokines profoundly affects the nature of the immune responses [7, 8].

Cationic liposomes are often used for easy and efficient transfection of plasmid DNA (pDNA) in vitro and in vivo. Several recent studies have shown that intravenous (i.v.) administration of a pDNA/cationic liposome complex leads to systemic gene expression especially in the lung. However, pDNA/cationic liposome complexes are well known to induce high amounts of inflammatory cytokines in vivo [9–12]. When delivered intranasally, pDNA/liposome complexes have a marked toxic effect on the lung [12]. Empty pDNA complexed with liposomes can produce a potent antitumor effect [13]. Even when inflammation is not critical, gene expression using a pDNA/liposome complex is only transient [14]. Qin et al. [5] have shown that interferon- $\gamma$  (IFN- $\gamma$ ) and TNF- $\alpha$  inhibit gene expression by promoter attenuation. In vitro gene expression of lung endothelial cells was reduced by TNF- $\alpha$  at low concentrations even when no obvious toxicity was observed [15]. We have demonstrated that tissue macrophages play an important role in cytokine induction following i.v. injection of pDNA cationic liposome formulations [16]. The important role of

<sup>1</sup>Correspondence: Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: takakura@pharm.kyoto-u.ac.jp

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immunostimulatory effects mediated by the CpG motif in gene therapy and DNA vaccination has been clearly defined. However, most of the in vitro studies focusing on the mechanisms of activation mediated by CpG DNA have been carried out using naked phosphorothioate CpG oligodeoxynucleotide (CpG S-ODN) or naked bacterial DNA in combination with macrophage cell lines.

We have studied the in vivo disposition characteristics of naked pDNA and found that the liver nonparenchymal cells, probably Kupffer cells (liver-resident macrophages), play an important role [17, 18]. Further in vitro studies using cultured mouse peritoneal macrophages have demonstrated that a specific receptor, such as the class A scavenger receptor, may be involved in the endocytotic uptake of naked pDNA by macrophages [19, 20]. Conversely, pDNA/cationic liposome complexes should be taken up by macrophages via a nonspecific mechanism based on electrostatic interaction. pDNA/cationic liposome complexes and naked CpG-ODN have been assumed to induce immune responses via similar mechanisms. However, we have demonstrated that not only bacterial DNA but also vertebrate calf thymus DNA complexed with cationic liposomes induce inflammatory cytokines from murine macrophages [21].

In the light of these findings, the present study was undertaken to characterize the inflammatory responses by a DNA/cationic liposome complex in mouse peritoneal macrophages in vitro. We examined whether endocytosis and endosomal acidification are also required for the inflammatory responses to pDNA or calf thymus DNA complexed with liposomes in macrophages. In addition, we examined whether this macrophage activation induced by the DNA/cationic liposome complex is dependent on TLR9.

## MATERIALS AND METHODS

### Chemicals

RPMI-1640 medium and Hanks' balanced salt solution were obtained from Nissui Pharmaceutical (Tokyo, Japan). Cytochalasin B, chloroquine, monensin, *Escherichia coli* DNA, and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). Bafilomycin A was purchased from Wako (Tokyo, Japan). LipofectAMINE plus™ (LApus or LA) reagent and Opti-modified Eagle's medium (MEM) were purchased from Invitrogen (Carlsbad, CA). Dextran sulfate (molecular weight, 500,000) and Triton X-114 were purchased from Nacal Tesque (Kyoto, Japan).

### Cell cultures

Male Institute for Cancer Research (ICR; 5 weeks) mice or C3H/HeJ mice [lipopolysaccharide (LPS) nonresponder; 5 weeks] were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Japan). C57BL/6 mice were obtained from Harlan Winkelmann (Borchen, Germany). TLR9<sup>-/-</sup> mice and littermate wild-type C57BL/6 mice were used at 8–12 weeks of age. Resident macrophages were collected from the peritoneal cavity of unstimulated mice with RPMI-1640 medium. Cells were washed, suspended in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (1.2 µg/ml), and then plated on 24-well culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at a density of  $5 \times 10^5$  cells/well for the activation experiments. For confocal microscopic observations, cells were plated on a cover glass in 12-well culture plates at a density of  $5 \times 10^5$  cells/well. After a 2-h incubation at 37°C in 5% CO<sub>2</sub>-95% air, adherent macrophages were washed three times with RPMI-1640 medium to remove nonadherent cells and

then cultured under the same conditions for 24 h. RAW264.7 cells were cultured with RPMI-1640 medium supplemented with 10% FBS, penicillin G (100 U/ml), and streptomycin (100 µg/ml). They were then plated on 24-well culture plates at a density of  $5 \times 10^5$  cells/ml and cultured for 24 h. Peritoneal macrophages from TLR9<sup>-/-</sup> mice or littermate wild-type C57BL/6 mice were plated on 96-well culture plates at a density of  $1 \times 10^6$  cells/well.

### pDNA

pcDNA3 vector was purchased from Invitrogen. The cytomegalovirus promoter-luciferase (pCMV-Luc)-encoding firefly Luc gene was constructed as described previously [22]. pcDNA3 has 26 5'-Pur-Pur-CpG-Pyr-Pyr-3' sequences including two CACCTT sequences reported to be the most potent CpG motifs for mice [23]. pDNA was purified using an Endo-free™ plasmid Giga kit (Qiagen, Valencia, CA). Methylated-CpG pDNA was synthesized by methylation of pDNA (pCMV-Luc) with 1 unit CpG methylase (New England Biolabs, Beverly, MA) per µg pDNA for 24 h at 37°C. The methylated-CpG pDNA was tested for digestion with *HpaII* (Takara, Kyoto, Japan) to confirm methylation. pDNA mobility was analyzed by 1% agarose gel electrophoresis.

### Purification of DNA

To minimize the activation by contaminated LPS, DNA samples were purified extensively with Triton X-114, a nonionic detergent. Extraction of endotoxin from pDNA, methylated-CpG pDNA, *E. coli* DNA, and calf thymus DNA samples was performed according to previously published methods [24, 25] with slight modifications. DNA samples were purified by extraction with phenol:chloroform isoamyl alcohol (25:24:1) and ethanol precipitation. DNA (10 mg) was diluted with 20 ml pyrogen-free water, and then 200 µl Triton X-114 was added followed by mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55°C. Subsequently, the solution was centrifuged for 20 min at 25°C, 600 g. The upper phase was transferred to a new tube, 200 µl Triton X-114 was added, and the previous steps were repeated three or more times. The activity of LPS was measured by Limulus amoebocyte lysate (LAL) assay using the Limulus F single test kit (Wako). After purification using the Endo-free™ plasmid Giga kit, 1 µg/ml pDNA was found to contain 0.01–0.05 EU/ml endotoxin. After Triton X-114 extraction, the endotoxin levels of DNA samples could no longer be determined by LAL assay; i.e., 1 µg/ml DNA contained less than 0.001 EU/ml. Without extraction of endotoxin by Triton X-114, 100 µg/ml naked pDNA, which contains 1–5 EU/ml endotoxin, could release  $521 \pm 73$  pg/ml TNF-α over 24 h.

### ODNs

Phosphorothioate ODNs were purchased from GENSET (Paris, France). The sequences of CpG S-ODN 1668 are 5'-TCC ATG ACC TTC CTC ATG CT-3', a proven activator of murine-immune cells as described previously [26, 27]. Phosphorothioate non-CpG-ODN 1720 (5'-TCC ATG ACC TTC CTC ATG CT-3') was used as a control.

### Cationic liposome formulation

LApus complexes were prepared according to the manufacturer's instructions. In brief, DNA or dextran sulfate was diluted with 75 µl Opti-MEM, and Plus reagent was added at a concentration of 1.2 µl per 1 µl DNA. LA was diluted in 75 µl Opti-MEM. After a 15-min incubation, the LA solution was added to the mixture containing DNA and Plus reagent. After a 15-min incubation, complex was added to the cells. In the case of the liposomal formulation for TLR9<sup>-/-</sup> mice, DNA was diluted with 100 µl serum-free RPMI. LA was diluted in 100 µl serum-free RPMI medium, and then the DNA solution and LA solution were mixed. After a 15-min incubation, 200 µl RPMI medium containing 10% fetal calf serum was added to the DNA/LA complex solution.

### Cytokine secretion

Mouse macrophages, resident peritoneal macrophages from ICR and CH3/HeJ mice, and RAW264.7 were washed three times with 0.5 ml RPMI 1640 before use. Cells were incubated for 2 h with 0.3 ml of the solutions containing the DNA/LApus complex. Then, the cells were washed with RPMI 1640 and incubated with RPMI 1640 containing 10% FBS for specified periods up to 48 h. In the case of the inhibition experiments, cells were incubated with the medium containing an inhibitor alone at various concentrations for 30 min and



were then incubated with the medium containing DNA/liposome formulations together with the inhibitor. After 2 h, the cells were washed and incubated with growth medium. At the indicated times, the supernatants were collected for enzyme-linked immunosorbent assay (ELISA) and kept at  $-80^{\circ}\text{C}$ . In the case of TLR9 $^{-/-}$  mice, cells were incubated in 100  $\mu\text{l}$  complete medium, and 100  $\mu\text{l}$  DNA/liposome complex was added to the cells. After 8 h, the supernatants were collected for ELISA. The levels of TNF- $\alpha$  and IL-6 in the supernatants were determined by the ANALYSA<sup>TM</sup> immunoassay system (Cenzyme, Minneapolis, MN).

## Confocal microscopy

pCMV-Luc was labeled using a Fasttag fluorescein-labeled (FL) labeling kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Cells were washed three times and incubated with the medium containing the FL-pDNA/LAplus complex. After a 1- or 3-h incubation at  $4^{\circ}\text{C}$  or  $37^{\circ}\text{C}$ , the cells were washed four times and fixed with 1% paraformaldehyde for 1 h. The cells were examined by confocal microscopy (MRC-1024, Bio-Rad, Hercules, CA). When the effect of inhibitors was examined, the cells were treated in the same manner as described above.

## RNase protection assay (RPA)

Total RNA was extracted from cells using TRIzol (Invitrogen) and subjected to RPA. Detection of mouse cytokines was performed with the RiboQuant multiprobe RPA system (PharMingen, San Diego, CA). The multiprobe template set involved mCK-3b: TNF- $\beta$ , lymphotoxin- $\beta$ , TNF- $\alpha$ , IL-6, IFN- $\gamma$ , transforming growth factor (TGF)- $\beta$ 1, TCF- $\beta$ 2, TCF- $\beta$ 3, migration inhibitory factor (MIF), L32, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for *in vitro* transcription using the T7 RNA polymerase to direct the synthesis of highly specific, [ $^{32}\text{P}$ ]-labeled antisense RNA mixtures. Each template set was transcribed using the Riboprobe system (Promega, Madison, WI) in the presence of [ $^{32}\text{P}$ ]-uridine triphosphate (3000 Ci/mmol, NEN, Boston, MA). Total RNA (20  $\mu\text{g}$ ) was hybridized with [ $^{32}\text{P}$ ]-labeled antisense RNAs at  $56^{\circ}\text{C}$  overnight and then subjected to RNase treatment. Protected fragments were precipitated and separated on a 5% acrylamide gel. The gel was dried and sensitized to an X-ray film.

## RESULTS

### Cytokine production induced by cationic liposomes with DNA or dextran sulfate

Previously, we reported that pDNA and calf thymus DNA complexed with cationic liposomes induced cytokines [21]. However, it is not clear whether DNA itself is required for this activation of macrophages. Therefore, we used another polyanion, dextran sulfate, and made a complex with LAplus. Highly purified pDNA or calf thymus DNA without liposome could not activate peritoneal macrophages of ICR mice as shown previously (Fig. 1; ref. [28]). With liposomes, pDNA and calf thymus DNA could induce almost the same amount of TNF- $\alpha$  and IL-6. Conversely, the dextran sulfate/LAplus complex could not stimulate the induction of cytokines, although naked dextran sulfate could. Naked pDNA as well as LAplus complexes with pDNA and calf thymus DNA stimulated macrophage cell line RAW264.7 cells to produce TNF- $\alpha$  and IL-6 as shown before [28] (data not shown). Peritoneal macrophages seem less sensitive to CpG DNA than the RAW264.7 cell line. These cytokines were also induced from RAW264.7 cells upon stimulation with naked dextran sulfate. However, the dextran sulfate/LAplus complex did not activate them (data not shown). LAplus alone could not induce significant amounts of cytokines (Fig. 1). These results suggested that the cationic liposome complex required DNA to activate macrophages.

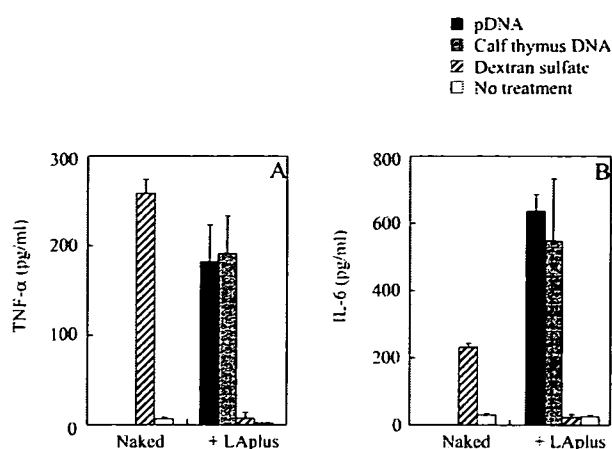


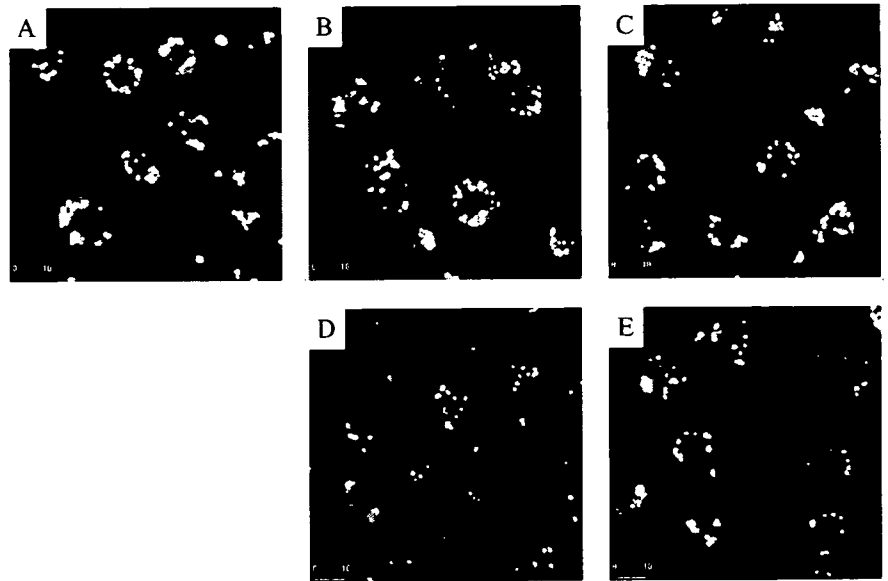
Fig. 1. Cytokine secretion induced by polyanions from peritoneal macrophages of ICR mice. The cells were incubated with naked DNA (100  $\mu\text{g}/\text{ml}$ ), naked dextran sulfate (100  $\mu\text{g}/\text{ml}$ ), or the DNA/LAplus complex (2.5:5  $\mu\text{g}/\text{well}$ ) or dextran sulfate/LAplus complex (2.5:5  $\mu\text{g}/\text{ml}$ ) for 8 h. After incubation, culture supernatant was collected, and the levels of TNF- $\alpha$  (A) or IL-6 (B) were determined by ELISA. Each result represents the mean  $\pm$  SD ( $n=3$ ).

### Effect of endocytosis and endosomal acidification inhibitors on the uptake or cellular localization of pDNA

The mechanism of naked CpG DNA immunostimulation appears to depend on the internalization, as CpG-ODN linked to a solid support has no stimulatory effect, and non-CpG-ODN inhibits the responses of CpG-ODN [27]. Endosomal acidification of DNA is also required for the CpG DNA-mediated activation. Inhibitors of endosomal acidification, such as bafilomycin A, chloroquine, and monensin, abolish the immune responses by CpG motifs [29, 30]. To examine whether these responses are also required for the cytokine production induced by pDNA complexed with cationic liposomes, we studied the effect of endosomal acidification inhibitors, such as bafilomycin A, chloroquine, and monensin, and an endocytosis inhibitor, cytochalasin B. These inhibitors may affect the cellular uptake and subsequent intracellular localization of pDNA. Therefore, confocal microscopic studies were carried out using the FL-pDNA/LAplus complex with inhibitors in macrophages from ICR mice. In the control cells, intense signals derived from FL-pDNA were observed mostly in vesicular structures, and some fluorescence diffused into the cytosol (Fig. 2). No apparent changes were observed for bafilomycin A and chloroquine (Fig. 2, B and C). In contrast, cytochalasin B significantly inhibited the uptake of the FL-pDNA/LAplus complex (Fig. 2D). Monensin inhibited the diffusion of the FL-pDNA/LAplus complex (Fig. 2E).

### Effect of endosomal acidification and endocytosis inhibitors on cytokine release

Bafilomycin A, cytochalasin B, chloroquine, and monensin significantly reduced the amount of TNF- $\alpha$  release induced by naked CpG-ODN 1668 from macrophages [21]. The effect of these inhibitors on TNF- $\alpha$  release induced by the pDNA/LAplus complex was examined in resident peritoneal macro-



**Fig. 2.** Effect of endocytosis and endosomal acidification inhibitors on uptake and cellular localization of the FL-pDNA/LAplus complex in macrophages of ICR mice. The cells were preincubated without inhibitor (A) with 250 nM bafilomycin A (B), 2.5 µg/ml chloroquine (C), 10 µg/ml cytochalasin B (D), or 10 µM monensin (E) for 30 min and were then incubated with the FL-pDNA/LAplus complex (2.5:5 µg/ml) in the absence or presence of the same inhibitor. After a 3-h incubation, the cells were washed and scanned by confocal microscopy.

phages from ICR and C3H/HeJ mice (LPS nonresponder) and a macrophage cell line RAW264.7. In these experiments, inhibitors were washed out after a 2-h incubation because of the cytotoxicity of the complexes, and cytokine determination was carried out after an additional 6 h incubation. Cytochalasin B slightly reduced the TNF-α release induced by the pDNA/LAplus complex from the macrophages of ICR mice (Fig. 3A). Monensin significantly reduced the TNF-α secretion by the pDNA/LAplus complex from the macrophages of ICR and C3H/HeJ mice and to a lesser extent, from RAW264.7 (Fig. 3, A–C). TNF-α release was slightly reduced by chloroquine in RAW264.7 (Fig. 3B). These endocytosis or endosomal acidification inhibitors alone showed no induction of TNF-α over the concentration range tested in these experiments (data not shown).

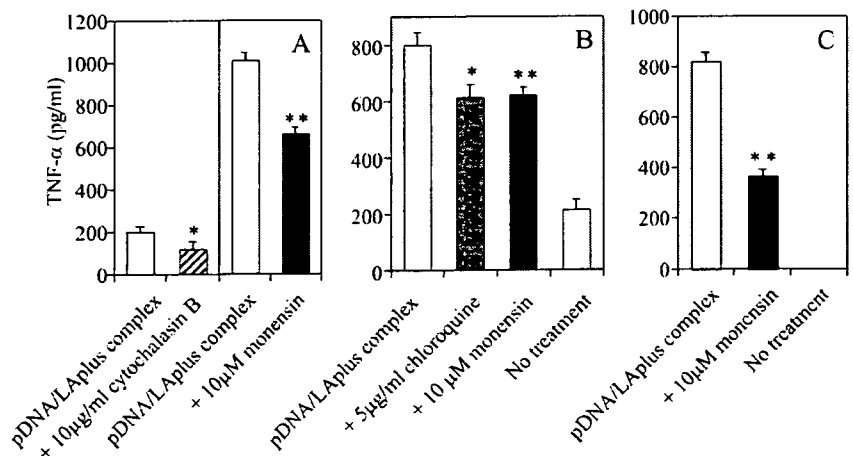
Further experiments were carried out to see whether only the pDNA/LAplus complex or other DNA/LAplus complexes were also affected by endosomal acidification. After an 8-h incubation, the methylated-CpG pDNA/LAplus complex stimulated peritoneal macrophages of C3H/HeJ mice to produce TNF-α,

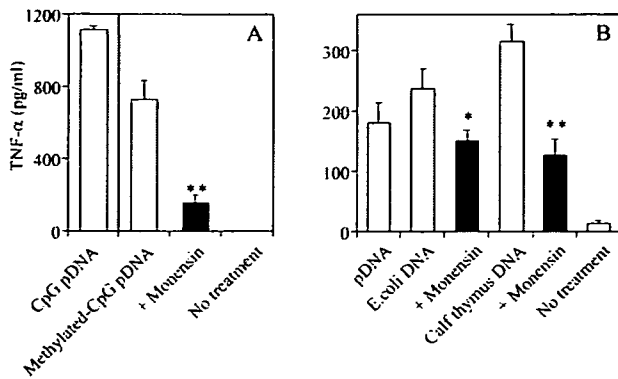
as shown previously [20]. Monensin reduced this TNF-α induction (Fig. 4A). Similar results were observed in the TNF-α release stimulated by *E. coli* DNA or the calf thymus DNA/LAplus complex (Fig. 4B). These results suggested that TNF-α production by the DNA/LAplus complex was inhibited by endosomal acidification inhibitors.

#### Effect of bafilomycin A on cytokine release induced by the DNA/LAplus complex

Next, we examined the effect of bafilomycin A, another inhibitor of endosomal acidification, on cytokine release by the DNA/LAplus complex. It was unexpected that bafilomycin A induced TNF-α production by the pDNA/LAplus complex from peritoneal macrophages and RAW264 cells, and this cytokine increased in a time-dependent manner (Fig. 5, A and C). Conversely, cytokine IL-6 production induced by the pDNA/LAplus complex was inhibited by bafilomycin A (Fig. 5, B and D). Bafilomycin A alone did not induce TNF-α production from peritoneal macrophages or RAW cells (data not shown). These results indicate that bafilomycin A stimulates TNF-α secretion

**Fig. 3.** Effect of inhibitors on TNF-α release by the pDNA/LAplus complex from peritoneal macrophages of ICR mice (A), mouse macrophage cell line RAW264.7 cells (B), or peritoneal macrophages of C3H/HeJ mice (LPS nonresponder; C). The cells were incubated with or without various inhibitors, cytochalasin B (hatched bar), chloroquine (shaded bar), or monensin (solid bars), for 30 min and were then incubated with the pDNA/LAplus complex (2.5:5 µg/well) in the presence or absence of inhibitors. After a 2-h incubation, liposomes were removed, and growth medium was added to the macrophages. The supernatants were collected 8 h after incubation with liposomes. TNF-α levels were determined by ELISA. Each result represents the mean ± SD (n=3). Differences in the cytokine levels in the samples treated with DNA only and DNA + inhibitors (cytochalasin B, chloroquine, and monensin) were statistically analyzed by the Welch *t*-test. \*, *P* < 0.05; \*\*, *P* < 0.01.





**Fig. 4.** Effect of an endosomal acidification inhibitor on TNF- $\alpha$  release by pDNA or methylated pDNA (A) or *E. coli* DNA or calf thymus DNA (B), complexed with LAplus from macrophages of C3H/HeJ mice. The cells were incubated for 30 min without inhibitor or with monensin (10  $\mu$ M, solid bars). Then the cells were incubated with the DNA/LAplus complex (2.5:5  $\mu$ g/well) in the presence or absence of the same inhibitor. After a 2-h incubation, liposomes were removed, and growth medium was added to the macrophages. The supernatants were collected 8 h after incubation with liposomes. TNF- $\alpha$  levels were determined by ELISA. Each result represents the mean  $\pm$  SD ( $n=3$ ). Differences in the cytokine levels in the samples treated with the DNA/LAplus complex alone and the DNA/LAplus complex + monensin were statistically analyzed by the Welch *t*-test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

induced by DNA/liposome formulation. To examine whether these results are also observed with other types of DNA, TNF- $\alpha$  release induced by pDNA, *E. coli* DNA, or calf thymus DNA complexed with LAplus after 8 h was determined. Bafilomycin A potently stimulated the TNF- $\alpha$  release induced by the DNA/LAplus complex from peritoneal macrophages of ICR mice (Fig. 6A). Similar results were observed in methylated-CpG pDNA or CpG-ODN complexed with LAplus from peritoneal macrophages of C3H/HeJ mice (data not shown). However, IL-6 release was reduced by bafilomycin A (Fig. 6B). Similar results were observed in RAW264.7 cells after an 8-h incubation (data not shown).

#### Bafilomycin A-increased TNF- $\alpha$ mRNA production

Among these inhibitors, only bafilomycin A increased TNF- $\alpha$  release by the DNA/LAplus complex. To examine how bafilomycin A affects the transcription or translation of TNF- $\alpha$ , we investigated the mRNA production of TNF- $\alpha$ . Figure 7 shows the expression of mRNA in peritoneal macrophages after stimulation with the pDNA/LAplus complex. The amount of TNF- $\alpha$  mRNA induced by the pDNA/LAplus complex from macrophages of ICR mice was increased by bafilomycin A, and this increase was time-dependent (Fig. 7B). No mRNA expression of IL-6 was detected, although IL-6 protein was released from the cells (Fig. 6). IFN- $\beta$  mRNA was detected at 2 h, and this was reduced by bafilomycin A treatment, and it was undetectable at 8 h.

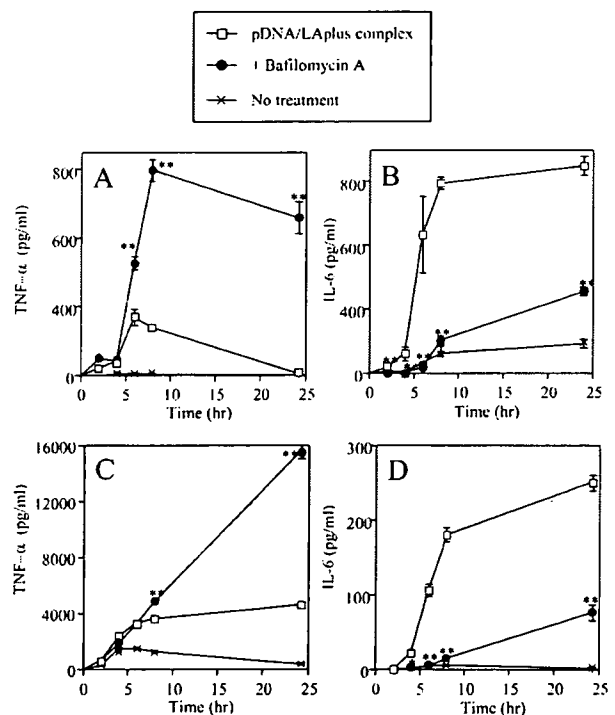
#### Effect of bafilomycin A on TNF- $\alpha$ production induced by naked pDNA

Bafilomycin A and other inhibitors of endosomal acidification inhibit TNF- $\alpha$  release induced by naked CpG-ODN 1668 [29].

However, in our study, after removal of the DNA/LAplus complex and bafilomycin A, TNF- $\alpha$  was increased, as shown in Figure 5. Therefore, the effect of these inhibitors on cytokine release induced by naked pDNA from RAW264.7 cells was examined. pDNA and inhibitors were incubated and then washed after a 2-h incubation. TNF- $\alpha$  production was monitored up to 24 h. TNF- $\alpha$  was induced by naked pDNA and was reduced after washout. Monensin inhibited the TNF- $\alpha$  production. Bafilomycin A also inhibited TNF- $\alpha$  release up to 2 h. However, after 2 h, it enhanced TNF- $\alpha$  production (Fig. 8).

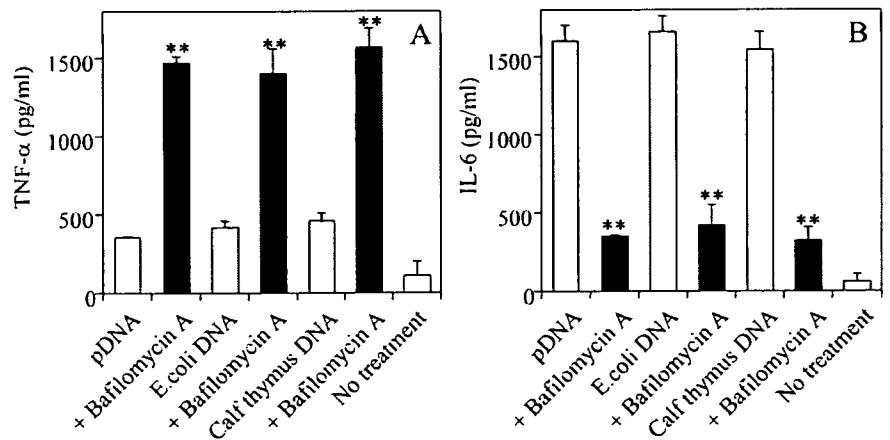
#### TLR9-dependent and -independent pathways are involved in macrophage activation by DNA/cationic liposomes

In general, the difference between bacterial DNA and vertebrate DNA is that the former has many unmethylated CpG motifs, and the latter does not. These CpG motifs are reported to be recognized by an intracellular receptor TLR9 [31]. To clarify whether the immunoactivation induced by the vertebrate calf thymus DNA/cationic liposome complex is TLR9-dependent, we used the peritoneal macrophages from TLR9<sup>-/-</sup> mice and control mice, and measured cytokine production.



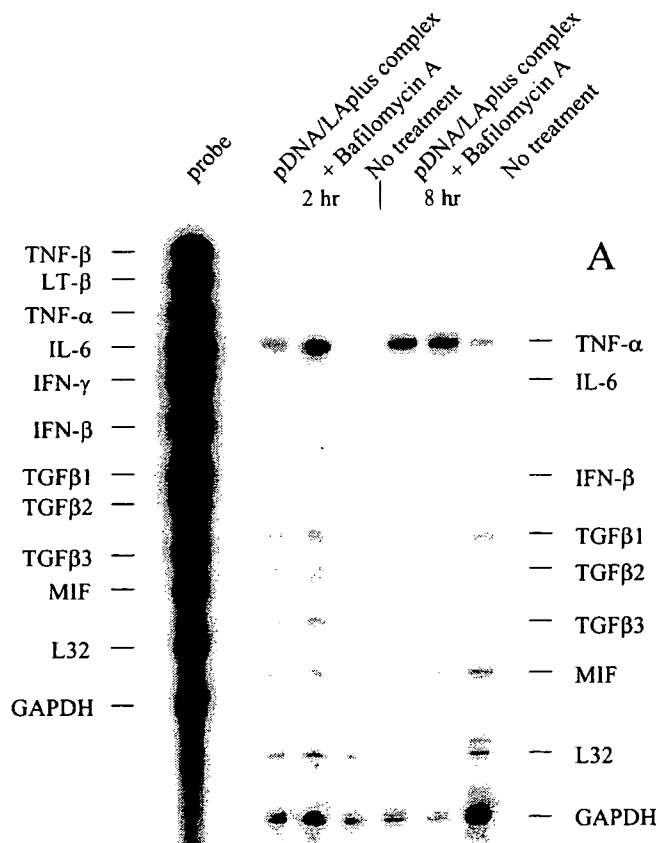
**Fig. 5.** Time-course of cytokine secretion by the pDNA/LAplus complex from resident macrophages of ICR mice (A, B) or RAW264.7 cells (C, D). The cells were incubated with or without various inhibitors, bafilomycin A, for 30 min and were then incubated with the pDNA/LAplus complex (2.5:5  $\mu$ g/well) in the presence or absence of inhibitors. After a 2-h incubation, liposomes were removed, and growth medium was added to the macrophages. The supernatants were collected at the indicated time after incubation with liposomes, and TNF- $\alpha$  levels were determined by ELISA. Each result represents the mean  $\pm$  SD ( $n=3$ ). Differences in the cytokine levels in the samples treated with the DNA/LAplus complex alone and the DNA/LAplus complex + bafilomycin were statistically analyzed by the Welch *t*-test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

**Fig. 6.** Effect of bafilomycin A on TNF- $\alpha$  or IL-6 release by the DNA/LAplus complex from resident macrophages of ICR mice. The cells were incubated for 30 min in the presence or absence of bafilomycin A (250 nM, solid bars). Then, the cells were incubated with pDNA, *E. coli* DNA, or calf thymus DNA (2.5  $\mu$ g/well) complexed with LAplus (5  $\mu$ g/well) with or without inhibitors. After a 2-h incubation, liposomes were removed, and growth medium was added to the macrophages. The supernatants were collected 8 h after incubation with liposomes. The cytokine concentrations were measured by ELISA. Each result represents the mean  $\pm$  SD (n=3). Differences in the cytokine levels in the samples treated with the DNA/LAplus complex alone and the DNA/LAplus complex + bafilomycin were statistically analyzed by the Welch *t*-test. \*\*, *P* < 0.01.



Naked pDNA or calf thymus DNA could not induce TNF- $\alpha$  production (Fig. 9), as shown previously [28]. When pDNA was complexed with LA, the peritoneal macrophages released TNF- $\alpha$ . This cytokine release was significantly reduced in the macrophages from TLR9<sup>-/-</sup> mice, indicating that the cytokine induction is dependent on TLR9. However, it is interesting that the cytokine production was not completely abolished. Moreover, calf thymus DNA, which should not be a ligand of TLR9,

also stimulated the macrophages from TLR9<sup>-/-</sup> mice, although the amount of TNF- $\alpha$  was less compared with that from control wild-type mice. Phosphorothioate CpG 1668, a typical TLR9 ligand, did not induce cytokine production from the cells of TLR9<sup>-/-</sup> mice. Both of the macrophages did not respond to LA alone. These results suggest that TLR9-dependent and -independent pathways are involved in the macrophage activation induced by the DNA/cationic liposome complex.



**B**

	2 hr	8 hr
pDNA/LAplus complex	1.089	4.069
+ Bafilomycin A	2.174	7.705
No treatment	0	0.133

**Fig. 7.** Cytokine gene expression measured by RPA. (A) Peritoneal macrophages of ICR mice were treated with or without bafilomycin A for 30 min. Then, the pDNA/LAplus complex was added to the cells in the presence or absence of bafilomycin A. After 2 h, liposomes were removed, and growth medium was added to the cells. At the indicated time, total RNA (20  $\mu$ g/lane) was extracted from the cells and subjected to RPA (A). The intensity of each protected band was normalized according to the intensity of the band of GAPDH (B).