**Table III.** Area Under the Blood Concentration-Time Curve (AUC) and Tissue Uptake Clearance of [$^3$C]probucol Dissolved in Serum, [$^{14}$C]probucol Incorporated Emulsions and Liposomes After Intravenous Injection into Mice[a]

| Formulations | AUC (% of dose × h/ml) | Clearance[a] (μl/h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $CL_{liver}$ | $CL_{kidney}$ | $CL_{spleen}$ | $CL_{lung}$ | $CL_{heart}$ | $CL_{urine}$ |
| Serum | 5.9 | 2300 | 60.5 | 1250 | 131 | 55.9 | 0 |
| Gal-emulsions | 2.3 | 28,700 | 540 | 1800 | 150 | 403 | 170 |
| Gal-liposomes | 3.2 | 17,596 | 680 | 1620 | 430 | 160 | 145 |

[a] AUC and clearance (CL) were calculated for the period until 10 min after injection. An average of three experiments is shown.

physicochemical considerations (11,15). We found that the required lipophilicity of drugs for stable incorporation into lipid carries was $10^6$ for liposomes and $10^9$ for emulsions based on values for the partitition coefficient between *n*-octanol and water ($PC_{oct}$). Also, we investigated the relationship between the movement of galactosylated liposomes and incorporated drug after intravenous injection in relation to the lipid composition of the liposomes. Thus, probucol, with a $PC_{oct}$ of $10^{10.8}$, was selected as a model lipophilic drug having a suitable lipophilicity for incorporation of both of emulsions and liposomes. In this study, we examined the applicability of probucol to examine the usefulness of Gal-emulsions. As shown in Fig. 6, [$^{14}$C]probucol incorporated Gal-emulsion exhibited blood concentration and liver accumulation profile similar to that of [$^3$H]CHE emulsions and different from free [$^{14}$C]probucol, suggesting that probucol was stably incorporated in Gal-emulsions. In contrast, a slight difference was observed between the blood concentration and liver accumulation profiles of [$^3$H]CHE and [$^{14}$C]probucol in Gal-liposomes, suggesting that probucol was released from liposomes to some extent. Pharmacokinetic analysis demonstrated that the liver uptake clearance of [$^{14}$C]probucol incorporated into [$^3$H]-labeled Gal-emulsions was 1.6-times higher than that into Gal-liposomes. Thus, these results suggest that Gal-emulsions are more efficient carriers of probucol, which is a model lipophilic drug, for hepatocyte-selective targeting than Gal-liposomes.

In the current study, we showed that introduction of galactosylated cholesterol derivatives into emulsions can be prepared Gal-emulsions. Our current results provide evidence that introduction of ligand-grafted lipids such as mannose (36,37), fucose (38), folate (39,40), and transferrin (41), for cell-selective targeting, to emulsions also allows cell-selective targeting.

In conclusion, we have demonstrated that Gal-emulsions, which incorporate Gal-C4-Chol in emulsions, are alternative hepatocyte-selective carriers for highly lipophilic drugs. We have also demonstrated that the recognition mechanism of Gal-emulsions is mediated by asialoglycoprotein receptors, and the recognition efficiency depends on the galactose density on the emulsion surface. These observations provide information to help in the design of Gal-emulsions for hepatocyte-selective carrier systems for lipophilic drugs.

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Review

# Development of Cell-Specific Targeting Systems for Drugs and Genes

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**Cell-specific targeting systems for drugs and genes have been developed by using glycosylated macromolecule as a vehicle that can be selectively recognized by carbohydrate receptors. Pharmacokinetic analyses of the tissue distribution of glycosylated proteins came to the conclusion that the surface density of the sugar moiety on the protein derivative largely determines the binding affinity for the receptors and plasma lectin. Many glycosylated delivery systems have been developed and their usefulness investigated in various settings. Galactosylated polymers, when properly designed, were found to be effective in delivering prostaglandin $E_1$ and other low-molecular-weight drugs selectively to hepatocytes. In addition, glycosylated superoxide dismutase and catalase were successfully developed with minimal loss of enzymatic activity. A simultaneous targeting of these two enzymes to liver nonparenchymal cells significantly prevented hepatic ischemia/reperfusion injury. On the other hand, galactosylated catalase, a derivative selectively delivered to hepatocytes, effectively inhibited hepatic metastasis of colon carcinoma cells in mice. Finally, hepatocyte-targeted *in vivo* gene transfer was achieved by synthesizing a multi-functional carrier molecule, which condenses plasmid DNA, delivering DNA to hepatocytes through recognition by asialoglycoprotein receptors, and releasing DNA from endosomes/lysosomes into cytoplasm.**

**Key words** receptor-mediated endocytosis; pharmacokinetics; tissue distribution; asialoglycoprotein receptor; reactive oxygen species; gene delivery

The completion of the Human Genome Project allows drug candidates to be screened based on their interactions with target protein. Analysis of aberrant profiles of protein expression in disease states has led to the development and marketing of drugs able to interact with specific molecular targets. These compounds can be highly specific and effective, because they have a degree of specificity as far as their pharmacological activity is concerned. However, they still induce significant side effects and a number of adverse responses have been reported. Not only low-molecular-weight chemical compounds but also proteins, which could exhibit beneficial effects in certain diseases, have become candidate therapeutic agents to treat inherited and acquired diseases. However, most proteins examined thus far have had serious problems associated with their pharmacokinetic properties.

Targeted delivery of these drug candidates to the site of action is one solution for increasing their therapeutic index, irrespective of their specific pharmacological activity. Since Ringsdorf first proposed a model for a water-soluble macromolecular prodrug,[1] targeted delivery of anticancer drugs, biologically active proteins, antisense oligonucleotides and genes has been examined in great detail in an attempt to achieve an improved therapeutic output. In addition, the development of monoclonal antibodies as well as the finding of numbers of receptors on the cellular surface have made it possible for researchers to use such molecules involved in specific recognition as natural glycoproteins for the target-specific delivery of pharmacologically active compounds. Of the many combinations investigated, the sugar-lectin interaction has several features that are appropriate for the cell-specific targeting of pharmaceuticals; (i) the expression of lectins is specific to some types of cells, (ii) the affinity of ligands can be high enough for *in vivo* targeting, and (iii) the use of the recognition system produces little interference with the interactions that are important for life. Asialoglycoprotein receptors on hepatocytes and mannose receptors on several macrophages like Kupffer cells and liver sinusoidal endothelial cells recognize the corresponding sugars on the non-reducing terminal of sugar chains.[2] The use of sugar moieties for receptor-mediated drug targeting started with the pioneering work by Rogers and Kornfeld in 1971,[3] and a number of applications have been reported with antiviral drugs, antitumor agents, diagnostic agents, toxins, enzymes, antisense oligonucleotides and genes. Although sugar-containing delivery systems offer the possibility of improved delivery of the agent to the target when evaluated *in vitro*, some or most of the systems may not be effective *in vivo*. This is largely due to undesirable pharmacokinetic properties; for example, a highly potent antibody can bind to its antigen only when it reaches the site where the antigen is located. Therefore, in the development of cell-specific targeting system, the tissue distribution characteristics should be evaluated *in vivo* in order to investigate the various obstacles to targeted delivery such as limited passage through the endothelium, extensive uptake by mononuclear phagocyte system and rapid loss by glomerular filtration. Here, I will discuss how to develop cell-specific drug and gene targeting systems for liver cells, such as hepatocytes and liver nonparenchymal cells (Fig. 1), based on the pharmacokinetic analysis of the tissue distribution of macromolecular compounds. Then, I shall review the targeted delivery of various compounds to cells, aiming at the prevention of ischemia/reperfusion injury, inhibition of tumor metastasis and gene therapy.

## PHARMACOKINETIC CONSIDERATIONS INVOLVING CELL-SPECIFIC TARGETING SYSTEMS USING GLYCOSYLATED PROTEINS

The rational design of carbohydrate receptor-mediated cell-specific targeting systems can be achieved through an understanding of the molecular mechanism governing the interaction between the receptors and ligands. The interaction of galactosylated ligands with asialoglycoprotein receptors

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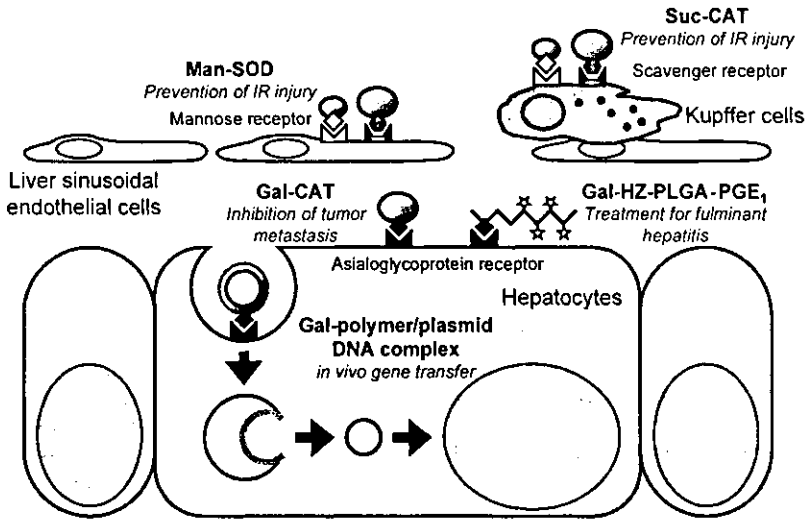


Fig. 1. Receptor-Mediated Cell-Specific Targeting of Drugs and Genes to Liver Cells

has been extensively investigated using isolated hepatocytes. Connolly *et al.* reported that highly clustered (branched) galactosides were more potent inhibitors than less clustered ones in their studies of the binding of $^{125}$I-asialoorosomucoid to hepatocytes.[4] The importance of a precise geometry of the sugar chains was suggested using multi-antennary oligosaccharides.[5,6] In order to modify proteins as well as other polymers with sugar moieties, monomeric sugar derivatives are suitable because they can be easily synthesized. Experiments using mannosylated bovine serum albumin (Man-BSA)[7] and galactosylated BSA (Gal-BSA)[8] suggest that the number of sugar residues play an important role in recognition by macrophage mannose receptors or asialoglycoprotein receptors, respectively. However, the information obtained in these studies is not sufficient to design efficient targeting systems.

In addition to the affinity for the corresponding receptors, which is the major factor determining ligand-receptor interactions *in vitro*, additional factors such as blood flow rate, capillary structure and interaction with blood components[9] will affect the overall interaction with receptors *in vivo*. In the theoretical design of hepatocyte-targeted delivery systems, we have developed galactosylated proteins having different molecular weights and different numbers of galactose units, and investigated their tissue distribution in mice and rats.[10—13] During the modification procedures, attention was paid not to alter the electric charge of the proteins, because reduction in the charge sometimes increases the affinity of the protein for scavenger receptors.[14] In addition, a residualizing radiolabel using $^{111}$In was used for tracing glycosylated proteins after administration, and this resulted in only minor efflux of radioactivity from tissues taking up the labeled compounds.[15—17]

After intravenous injection into mice, $^{111}$In-galactosylated proteins rapidly disappeared from the plasma. They were recovered in the liver in amounts that were highly dependent on the degree of galactose modification and the administered dose. Then, the time-courses of the plasma concentration and liver accumulation of the $^{111}$In-labeled galactosylated proteins were analyzed based on a physiological pharmacokinetic model, in which the body is represented by three compartments: plasma pool, the sinusoidal and Disse spaces in the liver, and the intracellular space in the liver.[10,12] The uptake of galactosylated protein in the liver was expressed as a saturable process with Michaelis–Menten kinetics having a maximum rate of uptake, $V_{max,I}$ (nmol/h), and a Michaelis constant, $K_{m,I}$ (nM). To estimate the pharmacokinetic parameters, differential equations derived from the model were simultaneously fitted to the experimental data of the plasma concentrations and liver accumulation of $^{111}$In-labeled galactosylated proteins using the non-linear least-squares method MULTI associated with the Runge–Kutta–Gill method. The Michaelis constant for the hepatic uptake of $^{111}$In-galactosylated recombinant human superoxide dismutase (Gal-SOD) was observed to be inversely correlated with the number of galactose residues, without a significant change in the maximum rate of uptake, $V_{max,I}$. This relationship could be successfully applied to other galactosylated proteins by using the surface density of the galactose residues as the degree of galactosylation (Fig. 2A), suggesting that this parameter controls the affinity of galactosylated proteins for asialoglycoprotein receptors. These analyses clearly demonstrated that an efficient delivery of proteins to hepatocytes by galactosylation can be achieved by adjusting the degree of galactosylation to a value for the distance between two vicinal galactose residues as short as 20—30 Å, which is of the same order as the naturally occurring sugar clusters arranged at the vertices of a triangle with sides of dimensions 15, 22, and 25 Å.[6]

A similar pharmacokinetic analysis was applied to the tissue distribution of mannosylated proteins.[18,19] We found that mannosylated proteins bind to serum-type mannan binding protein (MBP) in a structure-dependent manner. The binding to MBP was obvious at low concentrations of mannosylated proteins, and the disappearance from plasma was greatly retarded at doses less than 1 mg/kg when the molecular weight of the mannosylated proteins was 67000 or greater. Because $^{111}$In-Man-BSA showed capacity-limited plasma protein binding, this binding with a maximum binding concentration ($B_{max}$; nM) and a dissociation constant ($K_d$; nM) was included in the physiological model for the analysis of the tissue distribution of $^{111}$In-Man-BSA.[19] As shown in Figs. 2B, C, the $K_{m,I}$ values were fairly similar (34—68 nM) except for $^{111}$In-$Man_{12}$-BSA (300 nM), whereas the $K_d$ decreased dramatically

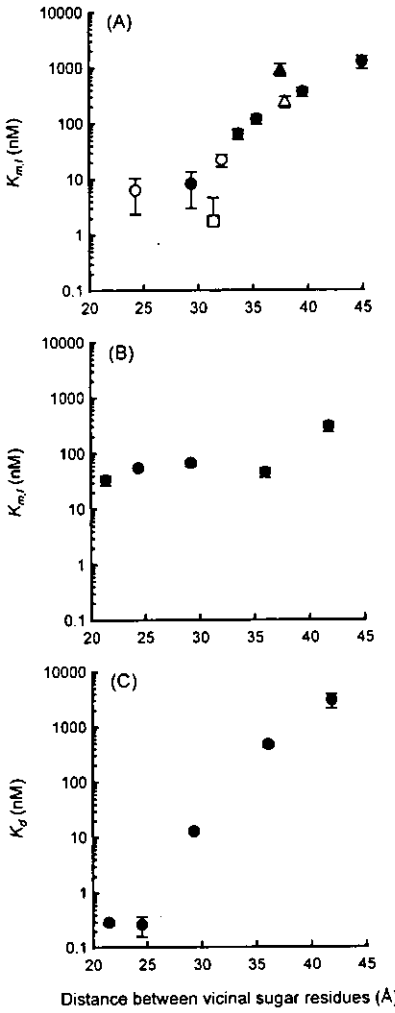


Fig. 2. Relationship between Pharmacokinetic Parameters and the Degree of Modification of $^{111}$In-Glycosylated Proteins

(A) $K_{m,I}$ of $^{111}$In-Gal-immunoglobulin G (□), Gal-BSA (○), Gal-SOD (●), Gal-soybean trypsin inhibitor (△), and Gal-chicken egg white lysozyme (▲). (B) $K_{m,I}$ and (C) $K_d$ of $^{111}$In-Man-BSA. Each parameter was plotted as the mean±S.D. against the average distance of two vicinal sugar residues.

on increasing the number or density of the mannose residues from 3000 nM for $^{111}$In-$Man_{12}$-BSA to 0.27—0.3 nM for $^{111}$In-$Man_{35}$-BSA and $^{111}$In-$Man_{46}$-BSA. This suggests that the *in vivo* recognition of MBP has a stronger cluster effect than that of mannose receptors. In addition, the relationship between the density of mannose and the $K_d$ (Fig. 2C) was comparable to that between the density of galactose on galactosylated proteins and the $K_{m,I}$ (Fig. 2A). The differences in the recognition of mannosylated ligands by hepatic mannose receptors and serum MBP could be explained by the fact that the mannose receptors contain different multiple carbohydrate recognition domains (CRDs) in the single polypeptide, whereas MBP is composed of six or more monomers with only a single CRD.[20] These findings will prove useful not only for designing cell-specific targeting systems for liver nonparenchymal cells but also for understanding the physiological roles of these lectins in the host defense system.

## GLYCOSYLATED POLYMERS AS NOVEL DRUG/GENE CARRIERS

Polymers with multiple sites for conjugation with sugars as well as drugs can be more versatile carriers for drugs and genes than glycosylated proteins. Therefore, we have developed glycosylated polymers and examined their tissue distribution characteristics in mice.[21—24] In these studies, carboxymethyl-dextran, poly-L-glutamic acid (PLGA) and poly-L-lysine (PLL) were modified with galactose or mannose to obtain cell-specific targeting systems. A good correlation between the apparent hepatic uptake clearance of galactosylated PLGA (Gal-PLGA) and the number of galactose residues per PLGA was observed,[23] suggesting that the rate and extent of the delivery can be controlled by the number of galactose units as observed with galactosylated proteins. However, when the hepatic targeting of Gal-PLGA was compared with galactosylated proteins using the clearance values and the estimated surface density of galactose, it was found that Gal-PLGA requires more galactose residues to be recognized by asialoglycoprotein receptors than galactosylated proteins.[25] Cationic PLL derivatives accumulated in the liver irrespective of the modification with galactose or mannose, because of the charge-mediated interaction with both parenchymal and nonparenchymal cells in the liver.[24] However, pharmacokinetic analyses indicated that the hepatic uptake clearance of PLL increased when the polymer was modified with galactose or mannose.

Glycosylated polymers have been used for the targeted delivery of low-molecular-weight drugs and nucleotides.[25—28] Targeted delivery of vitamin $K_5$ by conjugating it with Gal-PLGA resulted in a rapid and continuous antihemorrhagic activity. Prostaglandin $E_1$ ($PGE_1$) was also successfully delivered to hepatocytes following its conjugation to Gal-PLGA hydrazide (Gal-HZ-PLGA) *via* a hydrazone bond. This targeting effectively inhibited the increase in plasma transaminase activity in the mouse model of fulminant hepatitis.[26]

## TARGETED DELIVERY OF ANTIOXIDANT ENZYMES BY DIRECT GLYCOSYLATION: PREVENTION OF ISCHEMIA/REPERFUSION INJURY

Biologically active proteins are candidates for a variety of diseases and disorders, but most proteins have failed to exhibit any therapeutic benefits due mainly to problems associated with their pharmacokinetic properties. For instance, SOD, an antioxidant enzyme degrading superoxide anion, was regarded as a potential therapeutic agent for reactive oxygen species (ROS)-mediated diseases such as rheumatoid arthritis; however, it is rapidly cleared by glomerular filtration in the kidney, leading to a plasma elimination half-life of only 5—10 min following intravenous injection in rodents. Catalase detoxifying hydrogen peroxide, the metabolite of the superoxide anion, is also rapidly cleared by hepatocytes after intravenous injection.[29] Effective applications of these antioxidant enzymes to ROS-mediated injuries, therefore, can be achieved by their targeted delivery to sites where ROS are generated.

Ischemia/reperfusion injury is widely recognized as a significant source of morbidity and mortality in a number of clinical disorders including myocardial infarction.[30] In addi-

tion, this injury is one of the main causes of the initial poor liver function after liver transplantation. Because many of the injury-induced pathophysiological events are mediated through the production of ROS, targeted delivery of antioxidant enzymes could be useful in the treatment of various ischemia/reperfusion injuries.

The local hepatic injury associated with ischemia/reperfusion is considered to involve two phases,[31] with the initial injury being mediated by activated Kupffer cells and the subsequent injury being mediated by neutrophils that are primed during the initial period. We have tried to apply glycosylation methods as well as succinylation[32] to the delivery of SOD and catalase to the liver in a cell-specific manner, aiming at preventing hepatic ischemia/reperfusion injury.[29,33–37] Galactosylated and mannosylated derivatives of SOD and catalase have been successfully developed with enzymatic activities of 90% or higher. In addition, succinylated catalase (Suc-CAT) was also synthesized as a catalase to target liver sinusoidal endothelial cells through a scavenger receptor-mediated process. Although the ischemia following reperfusion resulted in a striking increase in serum transaminase activity, the administration of SOD and catalase derivatives significantly inhibited this. Among various combinations, Suc-CAT and Man-SOD showed the greatest inhibitory effect against the injury evaluated by serum transaminase activity and by the integrity of the liver tissues.[36] This combination also significantly suppressed the expression of intercellular adhesion molecule-1 along the hepatic sinusoids and prevented neutrophil infiltration in the liver.[37] The numbers of mannose receptors and scavenger receptors and the affinity of these derivatives for the receptors suggest that the liver sinusoidal endothelial cells have a higher level of catalase activity than SOD activity, whereas Kupffer cells have mainly SOD activity. Therefore, a plausible mechanism of the protection by Suc-CAT and Man-SOD is the dismutation of superoxide anion that Kupffer cells generate by Man-SOD, followed by Suc-CAT-mediated elimination of hydrogen peroxide, which is a stable amphiphilic molecule that can diffuse through the cellular membrane. Thus, this 'double targeting' of SOD and catalase to liver nonparenchymal cells appears to be a promising approach to reducing the ROS produced by Kupffer cells and neutrophils infiltrating tissue.

## INHIBITION OF TUMOR METASTASIS BY TARGETED DELIVERY OF CATALASE

Although high levels of ROS are cytotoxic, as indicated in hepatic ischemia/reperfusion injuries, low levels of ROS act as second messengers in the activation of cellular responses. It has been reported that ROS are involved in the regulation of the expression levels of adhesion molecules and matrix metalloproteinases (MMPs).[38] Furthermore, a low level of hydrogen peroxide increases cell proliferation. Therefore, detoxification of ROS, especially hydrogen peroxide, would be beneficial for the prevention of tumor metastasis. Some studies have already shown significant, but limited, effects of antioxidant enzymes on experimental tumor metastasis in animal models,[39,40] but no attempts have been made to achieve their targeted delivery.

Therefore, we examined the effects on tumor metastasis of targeted delivery of catalase to the liver and lung, two major sites of metastatic events.[41–43] An intraportal injection of $1\times10^5$ mouse colon carcinoma colon 26 cells resulted in the formation of more than 50 metastatic colonies on the surface of the liver at 14 d after injection. An intravenous injection of catalase (CAT; 35000 units/kg of body weight) significantly reduced the number of colonies in the liver. Among the catalase derivatives examined, Gal-CAT showed the greatest inhibitory effect on hepatic metastasis, and the number of colonies was significantly smaller than that following treatment with catalase, Man-CAT or Suc-CAT.[42] High activity of MMPs, especially MMP-9, were detected in the liver of mice bearing metastatic tumor tissues, and this was significantly reduced by Gal-CAT. The *in situ* zymography suggested that the gelatinase activities in the tumor-bearing liver were close to the sinusoids of the liver. In addition, the gelatin zymographic analysis of liver homogenates clearly demonstrated that MMP-9 is the major contributor to gelatinolysis in tumor-bearing mouse liver after intraportal inoculation of colon 26 tumor cells. Further studies are needed to identify which cells in the liver contribute to the gelatinase activity in tumor-bearing liver, because MMPs are known to be produced from various cells including tumor cells, endothelial cells, macrophages and hepatocytes. Our preliminary results obtained using cultured hepatocytes and colon 26 tumor cells show that, under oxidative stress, MMP-9 is largely produced by hepatocytes, whereas MMP-2 is from colon 26 cells. These findings suggest that hepatocytes are the major source of the MMPs detected in the liver, which supports the experimental data showing that Gal-CAT, the hepatocyte-targeting type, has the greatest inhibitory effect on tumor metastasis in the liver.

## APPLICATION OF CELL-SPECIFIC TARGETING SYSTEMS TO NONVIRAL GENE DELIVERY

The *in vivo* gene transfer profile required for effective gene therapy depends on the target disease.[44] Target cell-specific gene transfer is important for various aspects of *in vivo* gene therapy, because transgene expression in non-target cells could lead to the induction of side-effects. Generally speaking, plasmid DNA-based nonviral vectors offer the advantages of safety and versatility over viral vectors.[45] So far, several promising results of gene transfer using plasmid DNA-based approaches have been reported in preclinical and clinical settings.[46]

Gene transfer is expected to occur in cells reached by vectors directly or *via* the blood circulation. To achieve target cell-specific gene transfer, a variety of approaches has been examined, from the selection of the administration route of the vector to the use of tissue-specific promoters. Among those, targeted delivery of plasmid DNA using a receptor-mediated process would be an ideal approach. Plasmid DNA is a huge macromolecule with a strong negative charge and, therefore, its tissue distribution is highly restricted (Fig. 3).[45,47] The uptake by Kupffer cells and liver sinusoidal endothelial cells *via* a scavenger receptor-like mechanism largely determines its tissue distribution following intravascular administration of plasmid DNA in its naked form.[48,49]

Complex formation with positively charged molecules is an easy way to reduce the negative charge of plasmid DNA. We have synthesized a series of glycosylated polymers to

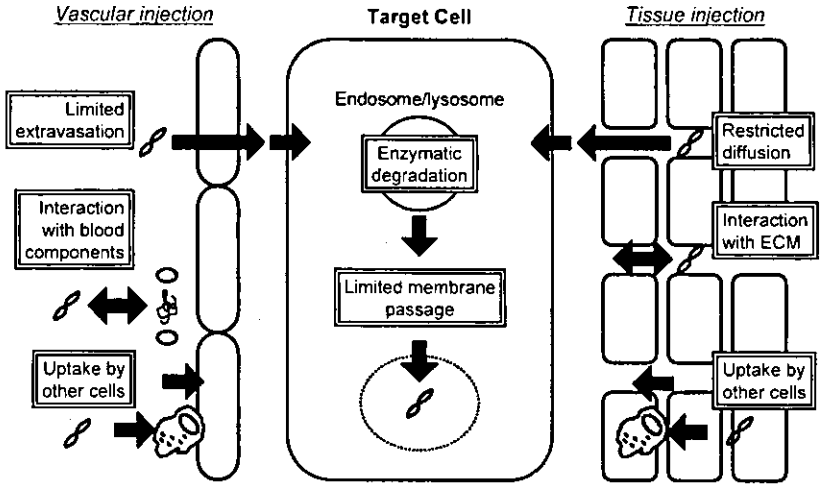


Fig. 3. Delivery Barriers for Nonviral Vector in Target Cell-Directed *in Vivo* Gene Transfer

achieve cell-specific gene transfer to carbohydrate receptor-positive cells.[50–53] Galactosylated PLL (Gal-PLL) synthesized using PLL with a molecular weight of 1800, 13000 or 29000 was mixed with plasmid DNA to form complexes.[50] A larger amount of $PLL_{1800}$ was required for complex formation than with $PLL_{13000}$ and $PLL_{29000}$, and increasing the number of galactose units on Gal-PLL resulted in reduced binding to plasmid DNA. The particle size and zeta-potential of the complexes varied depending on the mixing ratio and Gal-PLL used, and plasmid DNA/Gal-PLL complexes having diameters of 200 nm or less and a weak negative charge were prepared. After intravenous injection of $^{32}$P-plasmid DNA/$Gal_{13}$-$PLL_{13000}$ and $^{32}$P-plasmid DNA/$Gal_{26}$-$PLL_{29000}$, almost 80% of the radioactivity rapidly accumulated in the liver, preferentially in hepatocytes. Compared with these complexes, $^{32}$P-plasmid DNA/$Gal_5$-$PLL_{1800}$ and $^{32}$P-plasmid DNA/$Gal_5$-$PLL_{13000}$ had a smaller hepatic clearance, suggesting that both the molecular weight of PLL and the degree of galactose modification determine the hepatic targeting of plasmid DNA. *In vitro* and *in vivo* gene expression studies showed that plasmid DNA/$Gal_{13}$-$PLL_{13000}$ and plasmid DNA/$Gal_{26}$-$PLL_{29000}$ complexes were superior to plasmid DNA/$Gal_5$-$PLL_{1800}$ complex for introducing the DNA into cells. Thus, targeted delivery of plasmid DNA to hepatocytes *in vivo* was successfully carried out by controlling both the physicochemical properties of the carrier, Gal-PLL, and the particulate properties of the plasmid DNA/Gal-PLL complexes. The results obtained clearly indicate that the molecular weight and degree of galactose modification of Gal-PLL are major factors determining the stability of DNA/carrier complex formation; this in turn determines the cell-specific targeting and transgene efficiency.

However, the level of transgene expression by targeted gene delivery does not appear to be sufficient for treating diseases. To increase the expression efficiency by a nonviral approach, compounds that can enhance transgene expression, such as viruses or viral proteins, fusogenic lipids, and fusogenic, and membrane-disruptive peptides have been introduced into nonviral carrier systems. To preserve the advantages of nonviral systems, only synthetic compounds like fusogenic peptides are candidate helper molecules. In addition, from a pharmacokinetic point of view, they should be firmly attached to the delivery system. Based on these criteria, we have tried to improve the efficiency of transgene expression by synthesizing a multi-functional carrier molecule, galactosylated poly-L-ornithine (pOrn)-fusogenic peptide conjugate.[51] This molecule was designed so that it would (i) bind and condense DNA to optimize the systemic disposition profile, (ii) deliver DNA to hepatocytes through asialoglycoprotein receptor recognition, and (iii) release DNA from endosomes/lysosomes into cytoplasm after internalization. To this end, a cationic pOrn was modified first with galactose, then with a fusogenic peptide (mHA2) to obtain Gal-pOrn-mHA2. When applied with Gal-pOrn-mHA2 to HepG2 cells, an asialoglycoprotein receptor-positive cell line, fluorescein-labeled plasmid DNA showed a diffuse profile, suggesting the release of plasmid DNA from the endosome/lysosome compartment. A large amount of transgene product was obtained in the liver of mice injected with plasmid DNA/Gal-pOrn-mHA2 complex, which was much greater than that obtained with plasmid DNA/Gal-pOrn or plasmid DNA/cationic liposome complex. The luciferase activity in hepatocytes accounted for more than 95% of the total activity in all the tissues examined. Thus, hepatocyte-targeted *in vivo* gene expression was achieved by the intravenous injection of DNA complex with the multi-functional gene carrier, which can be an effective therapeutic option for hepatic diseases in which any important genes in hepatocytes are missing or mutated.[54]

## CONCLUSION

Cell-specific targeting of drugs and genes is a promising approach not only for increasing the therapeutic benefits but also for reducing the side-effects. Galactosylation and mannosylation of macromolecules have been proved to be effective in delivering a variety of pharmaceuticals from low-molecular-weight drugs such as $PGE_1$ to huge plasmid DNA. Careful examination of the tissue distribution of such delivery systems after *in vivo* administration is essential for the theoretical development of effective delivery systems, because there are many delivery barriers especially for nonviral vectors.[45] Therefore, analytical methods for the tissue distribution of macromolecular compounds are also important for the design of cell-specific targeting systems. We recently de-

veloped a novel radiolabeling method for plasmid DNA that is suitable for the analysis of the tissue distribution of plasmid DNA and its complexes.[55] Finally, it is fully expected that further basic studies on cell-specific targeting will lead to applications in routine medical practice in the near future.

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

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**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 α (TNF-α) 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-α 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-α release after stimulation with naked pDNA. These results suggest that bafilomycin A increases TNF-α 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$^{-/-}$ mice. We observed a reduced inflammatory cytokine release from macrophages of TLR9$^{-/-}$ 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)-α · 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 α (TNF-α), 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-γ (IFN-γ) and TNF-α inhibit gene expression by promoter attenuation. In vitro gene expression of lung endothelial cells was reduced by TNF-α 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

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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™ (LAplus 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 Nacalai 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$^{-/-}$ 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% $CO_2$–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$^{-/-}$ 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 GACGTT 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 *Hpa*II (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 amebocyte 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 ± 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 ACG TTC CTG ATG CT-3′, a proven activator of murine-immune cells as described previously [26, 27]. Phosphorothioate non-CpG-ODN 1720 (5′-TCC ATG AGC TTC CTG ATG CT-3′) was used as a control.

### Cationic liposome formulation

LAplus 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 liposome formulation for TLR9$^{-/-}$ 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/LAplus 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°C. In the case of $TLR9^{-/-}$ mice, cells were incubated in 100 μl complete medium, and 100 μl DNA/liposome complex was added to the cells. After 8 h, the supernatants were collected for ELISA. The levels of TNF-α and IL-6 in the supernatants were determined by the AN'ALYSA™ immunoassay system (Genzyme, Minneapolis, MN)

## Confocal microscopy

pCMV-Luc was labeled using a Fasttag fluorescein-labeled (FL) labeling kit according to the manufacturer's instructions (Vector Laboratories, Burlinghame, 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°C or 37°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-β, lymphotoxin-β, TNF-α, IL-6, IFN-γ, transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, 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, [$^{32}$P]-labeled antisense RNA mixtures. Each template set was transcribed using the Riboprobe system (Promega, Madison, WI) in the presence of [$^{32}$P]-uridine triphosphate (3000 Ci/mmol, NEN, Boston, MA). Total RNA (20 μg) was hybridized with [$^{32}$P]-labeled antisense RNAs at 56°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-α 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-α 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.

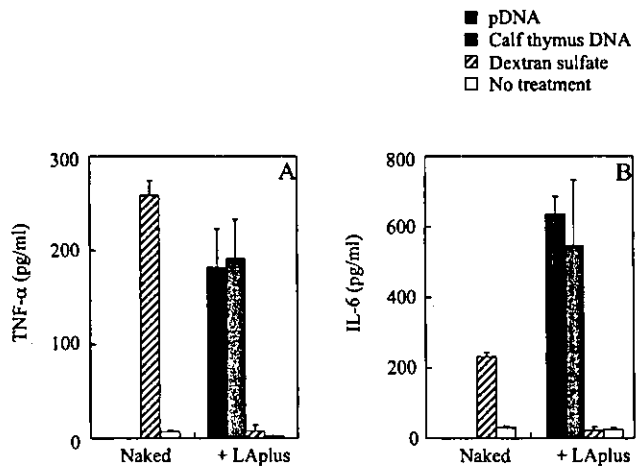



**Fig. 1.** Cytokine secretion induced by polyanions from peritoneal macrophages of ICR mice. The cells were incubated with naked DNA (100 μg/ml), naked dextran sulfate (100 μg/ml), or the DNA/LAplus complex (2.5:5 μg/well) or dextran sulfate/LAplus complex (2.5:5 μg/ml) for 8 h. After incubation, culture supernatant was collected, and the levels of TNF-α (A) or IL-6 (B) were determined by ELISA. Each result represents the mean ± 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-α release induced by naked CpG-ODN 1668 from macrophages [21]. The effect of these inhibitors on TNF-α release induced by the pDNA/LAplus complex was examined in resident peritoneal macro-

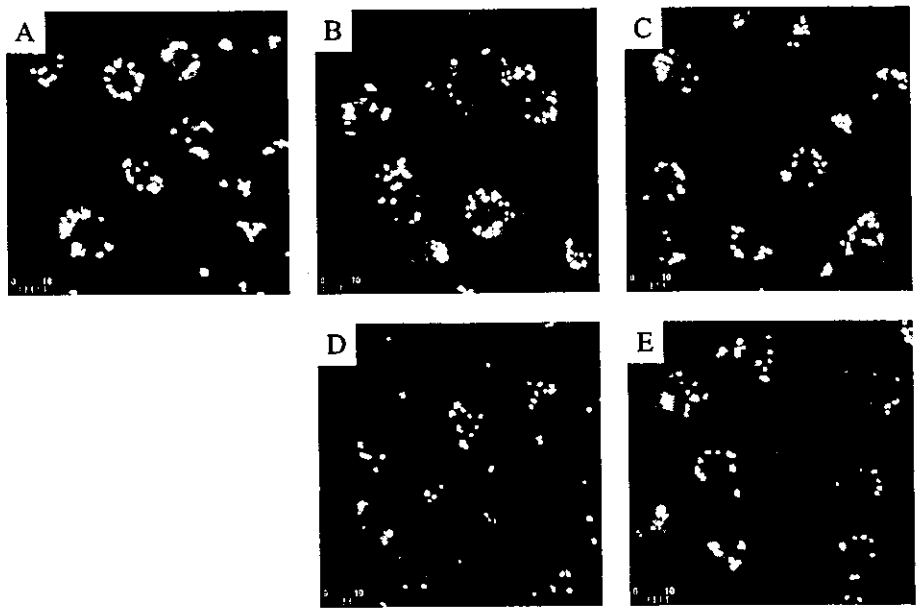



**Fig. 2.** Effect of endocytosis and endosomal acidification inhibitors on uptake and cellular localization of the FL-pDNA/LAplus complex in macrophages of ICR mice. The cells were preincubated without inhibitor (A) with 250 nM bafilomycin A (B), 2.5 μg/ml chloroquine (C), 10 μg/ml cytochalasin B (D), 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

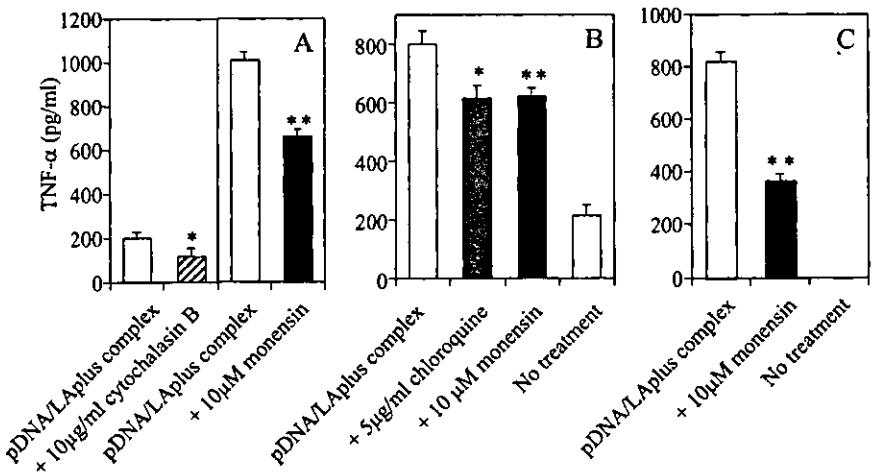



**Fig. 3.** Effect of inhibitors on TNF-α release by the pDNA/LAplus complex from peritoneal macrophages of ICR mice (A), mouse macrophage cell line RAW264.7 cells (B), or peritoneal macrophages of C3H/HeJ mice (LPS nonresponder; C). The cells were incubated with or without various inhibitors, cytochalasin 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. *, $P < 0.05$; **, $P < 0.01$.

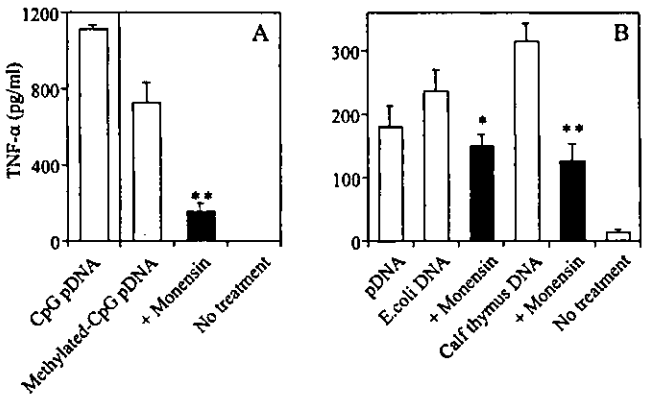



**Fig. 4.** Effect of an endosomal acidification inhibitor on TNF-α release by pDNA or methylated pDNA (A) or *E. coli* DNA or calf thymus DNA (B), complexed with LAplus from macrophages of C3H/HeJ mice. The cells were incubated for 30 min without inhibitor or with monensin (10 μM, solid bars). Then the cells were incubated with the DNA/LAplus complex (2.5:5 μg/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-α levels were determined by ELISA. Each result represents the mean ± 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-α 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-α 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-α mRNA production

Among these inhibitors, only bafilomycin A increased TNF-α release by the DNA/LAplus complex. To examine how bafilomycin A affects the transcription or translation of TNF-α, we investigated the mRNA production of TNF-α. **Figure 7** shows the expression of mRNA in peritoneal macrophages after stimulation with the pDNA/LAplus complex. The amount of TNF-α 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-β 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-α production induced by naked pDNA

Bafilomycin A and other inhibitors of endosomal acidification inhibit TNF-α release induced by naked CpG-ODN 1668 [29]. However, in our study, after removal of the DNA/LAplus complex and bafilomycin A, TNF-α 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-α production was monitored up to 24 h. TNF-α was induced by naked pDNA and was reduced after washout. Monensin inhibited the TNF-α production. Bafilomycin A also inhibited TNF-α release up to 2 h. However, after 2 h, it enhanced TNF-α 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$^{-/-}$ mice and control mice, and measured cytokine production.

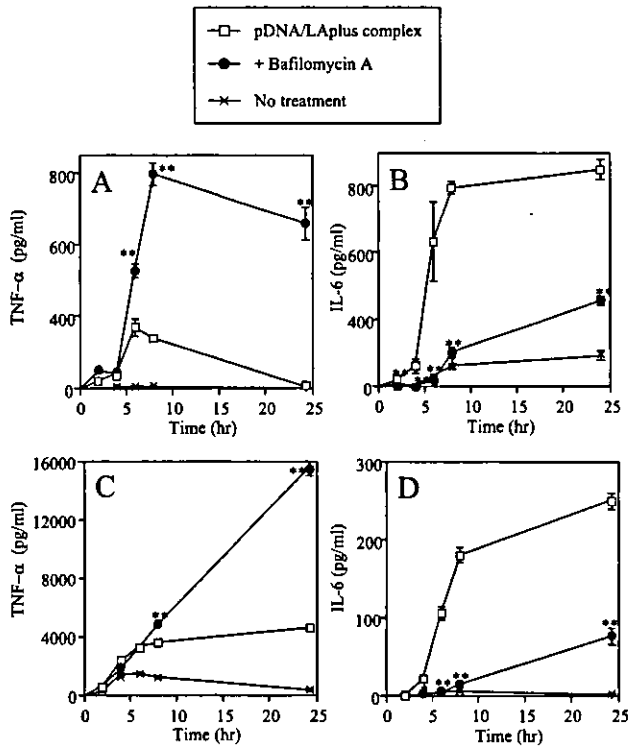



**Fig. 5.** Time-course of cytokine secretion by the pDNA/LAplus complex from resident macrophages of ICR mice (A, B) or RAW264.7 cells (C, D). The cells were incubated with or without various inhibitors, bafilomycin A, for 30 min and were then incubated with the pDNA/LAplus complex (2.5:5 μg/well) in 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 at the indicated time after incubation with liposomes, and 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 the DNA/LAplus complex alone and the DNA/LAplus complex + bafilomycin were statistically analyzed by the Welch *t*- test. *, $P < 0.05$; **, $P < 0.01$.

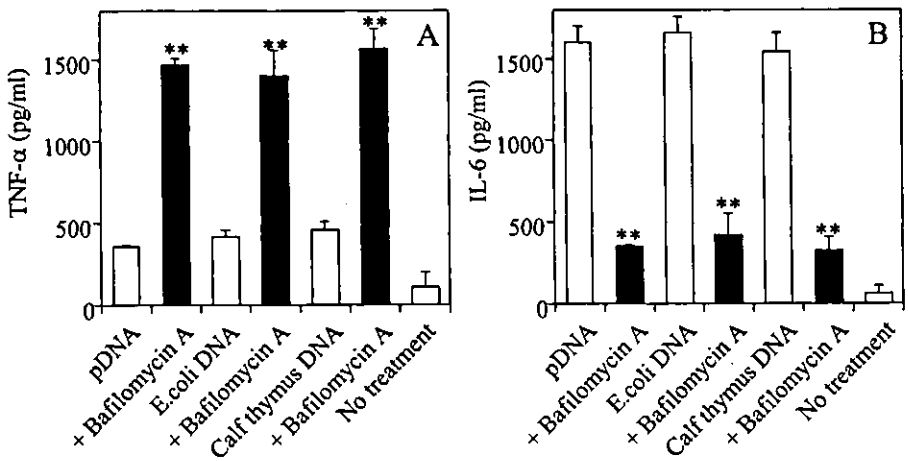
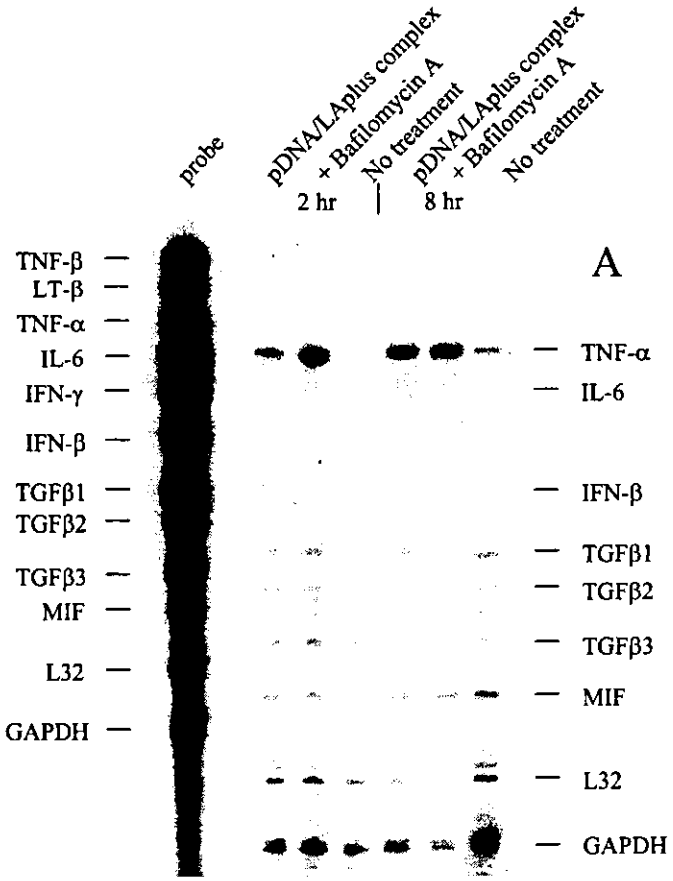



**Fig. 6.** Effect of bafilomycin A on TNF-α or IL-6 release by the DNA/LAplus complex from resident macrophages of ICR mice. The cells were incubated for 30 min in the presence or absence of bafilomycin A (250 nM, solid bars). Then, the cells were incubated with pDNA, *E. coli* DNA, or calf thymus DNA (2.5 μg/well) complexed with LAplus (5 μg/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 ± 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-α production (**Fig. 9**), as shown previously [28]. When pDNA was complexed with LA, the peritoneal macrophages released TNF-α. This cytokine release was significantly reduced in the macrophages from TLR9$^{-/-}$ 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$^{-/-}$ mice, although the amount of TNF-α 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$^{-/-}$ 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 |
| + Bafilomcyin 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 bafilomcyin A. After 2 h, liposomes were removed, and growth medium was added to the cells. At the indicated time, total RNA (20 μg/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).

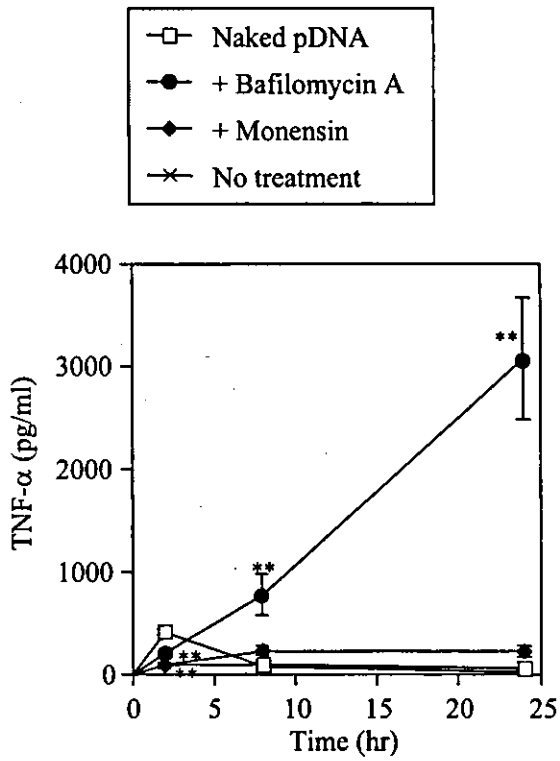



**Fig. 8.** Time-course of cytokine secretion induced by naked pDNA from RAW264.7 cells. Cells were incubated with or without inhibitors for 30 min. Then, naked pDNA (10 μg/ml) was added to the cells in the presence or absence of inhibitors. After 2 h, DNA was washed, and growth medium was added to the macrophages. Supernatants were collected at the time indicated. Cytokine concentrations were determined by ELISA. Each result represents the mean ± SD (n=3). Differences in the cytokine levels in the samples treated with the DNA/LAplus complex alone and the DNA/LAplus complex + bafilomycin or monensin were statistically analyzed by the Welch *t*- test. **, $P < 0.01$.

## DISCUSSION

Many in vivo studies in mice have shown that pDNA/cationic liposome complexes stimulate potent cytokine production [11, 15, 32–36]. However, the immune responses evoked by these complexes at a cellular level are poorly understood and seem to be similar to that of naked DNA with CpG motifs. Few in vitro studies using cultured cells have been reported [37–39].

Previously, we investigated the immune response induced by the DNA/cationic liposome complex and found that bacterial pDNA and vertebrate calf thymus DNA stimulate murine macrophages [21]. In this study, we showed that DNA is essential for this activation, as the dextran sulfate/cationic liposome complex could not induce any cytokine release from macrophages. Therefore, we investigated the activation mechanism of the DNA/cationic liposome complex and compared it with the activation mechanism of naked CpG-ODN or naked bacterial DNA. CpG-ODN is reported to require endocytosis to induce immunoactivation [28, 30, 40]. Intracellular TLR9 recognizes the CpG motifs [4]. Naked DNA, including pDNA and ODN, is taken up by macrophages via receptor-mediated mechanisms, which are still unknown. Conversely, the DNA/cationic liposome complex seems to be internalized into the cells via nonspecific mechanisms based on electrical interactions. Therefore, we investigated whether endocytosis was also required for the cytokine release induced by the DNA/cationic liposome complex. Cytochalasin B, an endocytosis inhibitor, causes depolymerization of actin filaments and blocks endocytosis and phagocytosis [41]. The effect of cytochalasin B was straightforward. The TNF-α release induced by the pDNA/LAplus complex, which is supposed to be taken up by adsorptive endocytosis, was inhibited (Fig. 3A). Reduced uptake in the presence of this inhibitor was confirmed by confocal microscopy (Fig. 2E). These results indicate that endocytosis is also essential for the immunoactivation induced by the DNA/cationic liposome complex.

Next, we investigated whether acidification of the endosomal compartment was also essential for the immune response by the DNA/LAplus complex, as it has been reported to be required for CpG-ODN [29, 30]. Three types of endosomal acidification inhibitors were used: Bafilomycin A is a specific inhibitor of vacuolar-type $H^+$-ATPase [42], monensin is a $Na^+/H^+$ ionophore, and chloroquine is a weak base [43]. Monensin exhibited an inhibitory effect on TNF-α and IL-6 release induced by the pDNA/LAplus complex. Restricted intracellular diffusion by monensin after internalization may be an indication of this (Fig. 1). Chloroquine slightly suppressed TNF-α production, and bafilomycin A inhibited IL-6 release. These results show that the pDNA/LAplus complex required endosomal acidification. Moreover, these immune responses are independent of the type of DNA, as monensin reduced the TNF-α and IL-6 production induced by *E. coli* DNA, methylated DNA, or calf thymus DNA complexed with the LAplus complex, and bafilomycin A reduced IL-6 production (Fig. 5).

CG sequences are suppressed in vertebrate DNA and are highly methylated compared with bacterial DNA. TLR9 recognizes these differences, namely unmethylated CpG motifs. Therefore, in principle, calf thymus DNA would not be recognized by TLR9. However, our results show that bacterial pDNA and vertebrate calf thymus DNA can induce TLR9-dependent and -independent activation of macrophages when these DNA are complexed with liposomes. The TLR9-dependent or -inde-

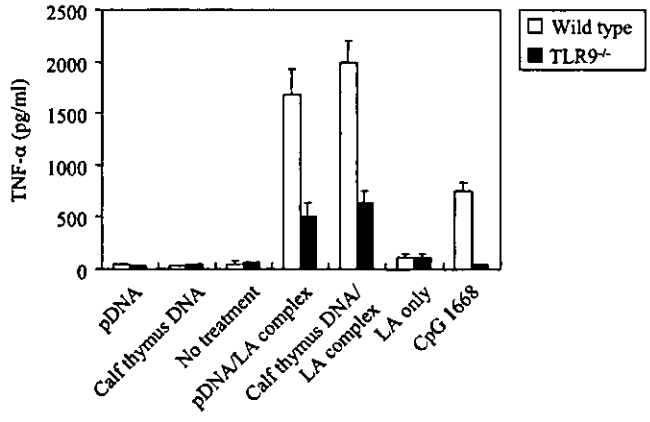



**Fig. 9.** Cytokine production induced by the DNA/cationic liposome complex from peritoneal macrophages from normal and $TLR9^{-/-}$ mice. Naked pDNA (10 μg/ml) or the DNA/LA complex (10:20 μg/ml) was added to the cells. After 8 h, supernatants were collected at the time indicated. Cytokine concentrations were determined by ELISA. Each result represents the mean ± SD (n=2). The results are the average of duplicate determinations and are typical of two experiments.

pendent mechanism is not fully understood. The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is reported to be another receptor for immunostimulatory CpG DNA [44]; however, the study using the DNA-PKcs-deficient mice shows that this protein does not recognize immunostimulatory CpG DNA [45]. One possible explanation of macrophage activation by calf thymus DNA/cationic liposome complexes is that calf thymus DNA has very few unmethylated CpG sequences [46]. The human genome has ~45,000 unmethylated CpG sequences (CpG islands), and the mouse genome has 37,000 CpG islands [47]. The limited uptake and subsequent degradation of naked calf thymus DNA may account for the inability to induce a significant macrophage activation by the naked form [46]. In fact, 40–50% of 0.1 μg/ml naked pDNA was associated with resident peritoneal macrophages or RAW264.7 cells, and ~30% of pDNA was degraded after a 3-h incubation. [25]. Complexation with liposome would increase DNA uptake and prevent DNA degradation, consequently enhancing the availability of CpG motifs in vertebrate DNA. Another possibility is that a non-CpG motif can induce activation when DNA is complexed with liposome. Tousignant et al. [48] have shown that i.v. injection of non-CpG-ODN/cationic liposome complexes can induce systemic IL-12 production. This ODN contains GATC sequences, and the inversion of AT to TA reduces the activity.

Other studies also support our observation. Double-stranded mouse genomic DNA can induce activation of the bone marrow-derived DC when it was transfected with FuGENE, another cationic lipid [49]. Zhao et al. [50] challenged the i.v. injection of pDNA/cationic liposome into TLR9$^{-/-}$ mice and showed that there was TLR9-independent toxicity at high amounts of the pDNA/cationic liposome complex, although a dramatic reduction in toxicity was observed. This finding also agrees with our results in the present study. Further investigation is required to identify the unknown mechanism of TLR9-dependent or -independent activation of macrophages.

Bafilomycin A showed unexpected effects on TNF-α release. If the DNA/LAplus complex requires endosomal acidification, bafilomycin A should inhibit cytokine production. However, bafilomycin A significantly enhanced the production of TNF-α, although it inhibited IL-6 release. This inhibitor did not affect the distribution of the pDNA/LAplus complex, as no apparent change was observed in the intracellular localization of FL-pDNA (Fig. 2). It also increased when macrophages were stimulated by the LAplus complex with *E. coli* DNA or calf thymus DNA (Fig. 6). Moreover, it increased TNF-α production by naked pDNA after removal of DNA (Fig. 8). These results indicate that bafilomycin A increases the degree of TNF-α release. The amount of TNF-α mRNA increased following bafilomycin A treatment, although the level of IFN-β expression was reduced (Fig. 7). Therefore, bafilomycin A affects the signal transduction before TNF-α mRNA production induced by the DNA/LAplus complex or the stability of mRNA. Bidani and Heming [51] reported that bafilomycin A increased TNF release from LPS-activated alvaeolar macrophages. Bafilomycin A is known to block vacuolar-type $H^+$-ATPase on endosomal and plasma membranes, which not only leads to inhibition of endosomal acidification but also to significant cytosolic acidification [42, 52, 53]. Moreover, the production of TNF-α is under post-transcriptional control. An adenine and uridine-rich element (ARE) in the 3′-untranslated region of TNF-α transcripts is an important determinant of post-transcriptional control [54]. Furthermore, unlike other cytokines, TNF-α is a membrane-binding protein and becomes soluble following proteolytic cleavage by TNF-α-converting enzyme (TACE) [55]. Therefore, there is the possibility that bafilomycin A changes the cytosolic pH and affects ARE or TACE. The detailed mechanisms underlying these phenomena await further investigation.

In conclusion, the present study has demonstrated that DNA complexed with cationic liposomes can induce CpG motif-independent activation to produce TNF-α and IL-6 in cultured resident peritoneal macrophages from mice or RAW264.7 cells. Endocytosis and endosomal acidification is also required for cytokine production by DNA complexed with cationic liposomes in cultured resident peritoneal macrophages from mice or RAW264.7 cells. Moreover, the DNA/cationic liposome complex stimulates mouse peritoneal macrophages in a TLR9-dependent and -independent manner.

## ACKNOWLEDGMENTS

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# Cationic charge-dependent hepatic delivery of amidated serum albumin

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**Abstract**

To obtain a quantitative correlation between the physicochemical properties of amidated bovine serum albumin (BSA) and their tissue distribution characteristics for the development of targeted delivery of proteins, BSA was amidated with hexamethylenediamine (HMD) or ethylenediamine (ED) to obtain cationized BSAs. Their structural changes were examined by spectroscopic and electrophoretic techniques then their tissue distribution was studied in mice. Circular dichroism (CD) and fluorescence measurements showed that spectroscopic changes occurred as the number of free $NH_2$ groups increased. Capillary electrophoresis revealed a linear relationship between the mobility and the increased number of free $NH_2$ groups. $^{111}$In-cationized BSAs were rapidly taken up by liver, but HMD-BSA showed a faster uptake than ED-BSA with a similar number of free $NH_2$ groups, suggesting that the diamine reagent with a longer carboxyl side chain results in more efficient hepatic targeting. The hepatic uptake clearance ($CL_{liver}$) of both derivatives increased significantly with a decrease in electrophoretic mobility ($\mu_{ep}$) towards the anode and reached a plateau at low electrophoretic mobility. The electrophoretic mobility is an appropriate indicator of the degree of amidation, which was closely correlated with the hepatic uptake clearance. The correlation between the mobility and the clearance shows that a low degree of amidation is sufficient for efficient hepatic targeting of proteins.
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*Keywords:* Bovine serum albumin; Amidation; Structural changes; Tissue distribution; Pharmacokinetics

*Abbreviations:* BSA, bovine serum albumin; HMD, hexamethylenediamine; ED, ethylenediamine; CD, circular dichroism; DTPA, diethylenetriaminepentaacetic acid; TNBS, trinitrobenzene sulfonic acid; bis-ANS, 4,4′ -dianilino-1,1′ -binaphthyl-5,5′ -disulfonic acid; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydorochloride; $CL_{liver}$, hepatic clearance; $\mu_{ep}$, electrophoretic mobility; Trp, tryptophan; Tyr, tyrosin.

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## 1. Introduction

For effective drug therapy, it is necessary to deliver the therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial and adverse effects. Among the various strategies for site-specific drug delivery, macromolecular carriers such as proteins and synthetic polymers are becoming widely used because of the diversity of their physicochemical and biological properties and functions [1–3]. As a method for site-specific drug delivery, chemical modification of protein carriers is an attractive strategy for the optimization of drug therapy as far as efficacy and safety are concerned.

In previous studies, we explored the tissue distribution characteristics of proteins and their derivatives in relation to their physicochemical and biological properties by examining the pharmacokinetic clearance and constructed a strategy for the rational design of targeting systems [4,5]. Based on the relationships obtained, targeted delivery systems for proteins have been developed using chemical modifications such as galactosylation, mannosylation or succinylation [6–9]. Biologically active proteins such as superoxide dismutase and catalase have been successfully targeted to liver nonparenchymal cells by succinylation or mannosylation [10,11]. During these investigations, it was found that the molecular weight and electric charge of the protein derivatives play critical roles in the tissue distribution, including the hepatic uptake, a very important distribution-determining process.

Cationization is another approach that can be used for targeted drug delivery. The cell surface membrane is negatively charged, so this charged surface provides sites of interaction for cationic macromolecules. Thus far, cationic delivery systems have been used for the delivery of drugs, proteins and genes to various tissues including the liver, brain and kidney [12]. The liver plays a significant role in the overall clearance of cationic macromolecules from the circulation because of its unique capillary architecture, and the hepatic targeting of a cationic macromolecular conjugate of mitomycin C [13] and superoxide dismutase [11] with dextran derivatives has been reported. Recently, this cationic charge-mediated delivery has been extensively studied in the field of nonviral gene delivery [14].

In spite of these promising characteristics, there are no systematic studies of the application of cationization techniques to the targeted delivery of proteins to the liver. In fact, the effects of cationization on protein conformation such as dimensional structure, fluorescent and electric properties have hardly been examined at all. In this study, therefore, the relationship between the physicochemical characteristics of macromolecules and their tissue distribution was systematically studied using bovine serum albumin (BSA, molecular weight 67 kDa) as a model protein. Two amidation reagents, ethylenediamine (ED) and hexamethylenediamine (HMD), were used for the cationization of BSA, and derivatives with different degrees of modification were synthesized. We examined the structural properties spectroscopically and electrophoretically as well as the tissue distribution characteristics of these cationized BSAs. Pharmacokinetic analyses were performed to obtain quantitative relationships between the various factors.

## 2. Materials and methods

### 2.1. Materials and animals

BSA, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydorochloride (EDAC), HMD and 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) were purchased from Sigma (St. Louis, MO). BSA was defatted according to the reported method [15] before use. Diethylenetriaminepentaacetic acid (DTPA) anhydride was obtained from Dojindo Laboratory (Kumamoto, Japan). $^{111}$Indium chloride ([$^{111}$In]$InCl_3$) was kindly supplied from Nihon Medi-Physics (Takarazuka, Japan). ED was obtained from Wako (Osaka, Japan). All other chemicals were of analytical grade.

Male ddY mice (24–26 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Animals were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experi-

mentation Committee of Graduate School of Pharmaceutical Sciences of Kyoto University.

### 2.2. Synthesis of cationized BSAs

Derivatives of BSA with HMD (HMD-BSA) were synthesized by covalent attachment of HMD to BSA [16]. Coupling reactions of BSA with ED (ED-BSA) were performed as described previously [17]. Native and cationized BSAs were subjected to SDS-PAGE analysis, and all were found to give one band (data not shown). The number of increased free amino groups in each derivative was determined by the trinitrobenzene sulfonic acid (TNBS) method [18].

### 2.3. Radiolabeling of cationized BSAs and tissue distribution experiments

BSA derivatives were radiolabeled with $^{111}$In using the bifunctional chelating regent DTPA anhydride according to the method of Hnatowich et al. [19], which has been described in a previous paper [8]. Mice received into the tail vein injections of $^{111}$In-labelled cationized BSA at a dose of 1 mg/kg in saline and housed in metabolic cages to allow the collection of urine. At appropriate intervals after injection, blood was collected from the vena cava under ether anesthesia and plasma was obtained by centrifugation. The liver, kidney, spleen, lung, heart and muscle were excised, rinsed with saline and weighed. The radioactivity of each sample was measured in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo). Radioactivity originating from the plasma in each tissue sample was corrected using the distribution data of $^{111}$In-BSA at 10 min after intravenous injection, assuming that $^{111}$In-BSA was not taken up by tissues during this 10-min period.

### 2.4. Pharmacokinetic analysis

The plasma $^{111}$In radioactivity concentrations were normalized with respect to the percentage of the dose per milliliter and analyzed using the nonlinear least-square program MULTI [20]. The tissue distribution was evaluated using the liver uptake clearance ($CL_{liver}$) as described previously [7,8].

### 2.5. Circular dichroism (CD) measurements

CD spectra were recorded using a JASCO-820 type spectropolarimeter (JASCO, Tokyo, Japan) at 25 °C. For calculation of the mean residue ellipticity [$\theta$], the molecular mass of the BSA derivatives was assumed to be 67 kDa. Far-UV (wavelength 200–250 nm) and near-UV (wavelength 250–350 nm) CD spectra were recorded at protein concentrations of 5 and 15 μM, respectively, in 67 mM sodium phosphate buffer (pH 7.4) [21].

### 2.6. Fluorescence measurements

Steady-state fluorescence measurements were performed using a Shimadzu RF-540 spectrofluorophotometer (Shimadzu, Kyoto, Japan) with 1-cm quartz cells, thermostatically controlled devices and 5-nm excitation and emission bandwidths at 25 °C. A fluorescence excitation wavelength of 295 or 280 nm was employed. The protein concentration was 2 μM in 67 mM sodium phosphate buffer (pH 7.4) [22].

### 2.7. Effective hydrophobicity

The effective hydrophobicity of cationized BSAs was estimated as reported previously [22] with slight modifications. In brief, each cationized BSA sample was dissolved in 67 mM sodium phosphate buffer (pH 7.4) to give a final concentration of 1 μM. To this solution was added bis-ANS (1 μM) at 25 °C and the fluorescence spectra excited at 394 nm were recorded on a Shimadzu RF-540 spectrofluorophotometer using a 1-cm quartz cell, thermostatic devices and 5-nm excitation and emission bandwidths.

### 2.8. Capillary electrophoresis

BSA derivatives (15 μM) were dissolved in 67 mM sodium phosphate buffer (pH 3.0), which was used as a background electrolyte. A CAPI-3000 (Otsuka Electronics, Osaka, Japan) capillary electrophoresis system was equipped with a bare fused silica capillary (total length 42 cm, effective length 30 cm, inner diameter 75 μm; GL Science, Tokyo, Japan). Hexadimethrin bromide solution (5%) was delivered through the capillary before use every day.