

Cationic Liposome Conjugation to Recombinant Adenoviral Vector Reduces Viral Antigenicity

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Adenoviral (Ad) vectors are commonly used in gene therapy trials because of their efficiency in gene transfer. However, their use is limited by immune responses that reduce transgene expression and decrease the efficacy of repeated vector administration. In this study, we demonstrated that conjugation of Ad vector with our novel cationic liposomes could reduce viral antigenicity *in vivo*. Mice subcutaneously injected with liposome-conjugated Ad vector showed a 6.5-fold reduction of anti-Ad antibodies with neutralizing activity, compared to those with unconjugated Ad vector. Interestingly, we also found that the conjugated vector is less susceptible to inactivation by neutralizing antibodies *in vitro* and *in vivo*. Our results suggest that liposome conjugation reduces viral antigenicity, shields vectors from neutralizing antibody, and may allow repeated Ad vector administration.

Key words: Adenoviral vector — Repeated administration — Antigenicity — Liposome

Recent advances in the understanding of the molecular basis of disease have highlighted the potential of gene therapy. Vectors for gene transfer currently under investigation include retrovirus, herpes simplex virus (HSV), adenovirus, and adeno-associated virus.¹⁾ In considering strategies for effective gene transfer into the central nervous system (CNS), replication-deficient adenoviral (Ad) vectors offer a number of advantages for the transfer and expression of therapeutic genes into the brain. These include a broad host range of infection in humans, the capacity to transfer genes into nonproliferating or terminally differentiated cells such as neurons, and preparation of high titers. Several studies have succeeded in gene transfer into brain cells, including neurons, glia, and ependymal cells, using adenoviral vectors.^{2–5)} More recently, Ad vectors have been used for targeting solid brain tumors.⁶⁾ However, strong immune responses to Ad antigens may limit the success of gene transfer and expression and limit redosing with these vectors.^{7–10)}

Cationic liposomes are artificially generated lipid vesicles that can entrap DNA as well as drugs within their aqueous compartment or in the lipid bilayer, and have been widely used in the field of basic molecular biology and for gene therapy. We have developed novel cationic liposomes composed of small unilamellar vesicles (SUV), multilamellar vesicles (MLV) or large unilamellar vesicles (LUV) as gene transfer vehicles.¹¹⁾

We recently demonstrated that SUV liposome-conjugated Ad vector (Ad/SUV) resulted in a 10-fold higher transduction efficiency in human glioma cell lines *in vitro* than Ad vector alone, and no additional cytotoxicity was induced by SUV conjugation.¹²⁾ In this paper, we investigated whether Ad/SUV elicits a diminished host immune response *in vivo* and is less susceptible to inactivation by neutralizing antibodies.

We first examined if the ability to redose is correlated to anti-Ad antibody levels using a recombinant Ad vector expressing β -galactosidase (AxCALacZ, gift from Dr. I. Saito, University of Tokyo) and a vector expressing the herpes simplex virus-thymidine kinase gene (AxCAHS-tk). Immunocompetent C57BL/6 (B6) mice were immunized with 10^7 plaque forming units (pfu) of AxCAHS-tk via subcutaneous (s.c.) injection. Fourteen days later, mice were intracranially (i.c.) injected with 4×10^5 pfu of AxCALacZ. Animals killed 4 days later showed that a prior s.c. dose of AxCAHS-tk resulted in 1331 ± 52 ng/ml anti-Ad IgG levels and an 8-fold reduction in β -galactosidase activity compared with naive animals (Fig. 1). We speculate that the presence of neutralizing antibodies contributed significantly to the reduction in gene transduction efficiency.

SUV liposomes were prepared using N-(α -trimethylammonioacetyl)didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidylethanolamine (DOPE) in a molar ratio of 1:2:2 (total amount, 1 μ mol) in 200 μ l of chloroform. After the solvent was evaporated, the lipid film was wetted with 500

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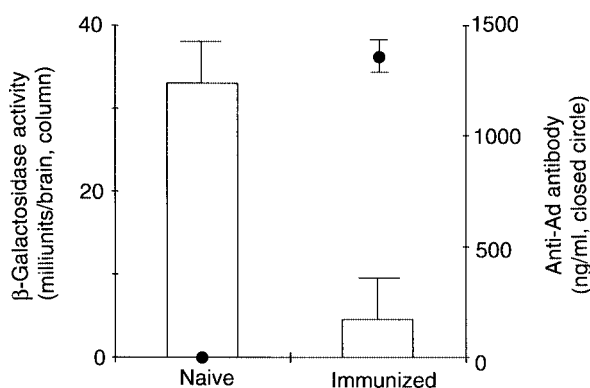


Fig. 1. Immunization with adenoviral vectors elicits production of anti-adenoviral antibodies and prevents transgene expression of redosed adenoviral vectors. C57BL/6 mice were subcutaneously immunized with AxCAHS-tk (10^7 pfu). Fourteen days later, mice were intracranially injected with AxCALacZ (4×10^5 pfu). A further 4 days later, β -galactosidase activity (column) in the brain and anti-adenoviral antibody levels in serum (closed circle) were measured. All values are the mean and SD of 5 animals.

μ l of phosphate-buffered saline (PBS) and sonicated in a probe-type sonicator. To prepare Ad/SUV, 1×10^7 pfu of Ad vector was mixed with 1 μ mol of SUV, and incubated at 37°C for 30 min prior to use. The virus-SUV ratio represented the optimal conditions as previously determined in our lab (data not shown). No toxicity of Ad/SUV was observed under these conditions.¹²⁾

To test if Ad/SUV was less antigenic *in vivo*, AxCALacZ/SUV and an equivalent dose of AxCALacZ (10^7 pfu) were injected s.c. into B6 mice twice during a 14-day period. Serum anti-Ad levels were measured 7

Table I. Anti-adenoviral Antibody Level and Neutralizing Activity in Serum from Mice Immunized with Liposome-conjugated Adenoviral Vector

Vector	Anti-Ad IgG (ng/ml)	Neutralizing activity (%)
AxCALacZ	3990 \pm 150	52.5 \pm 3.0
AxCALacZ/SUV	611 \pm 14	17.8 \pm 2.5

All values are the mean and SD of 4–6 animals.

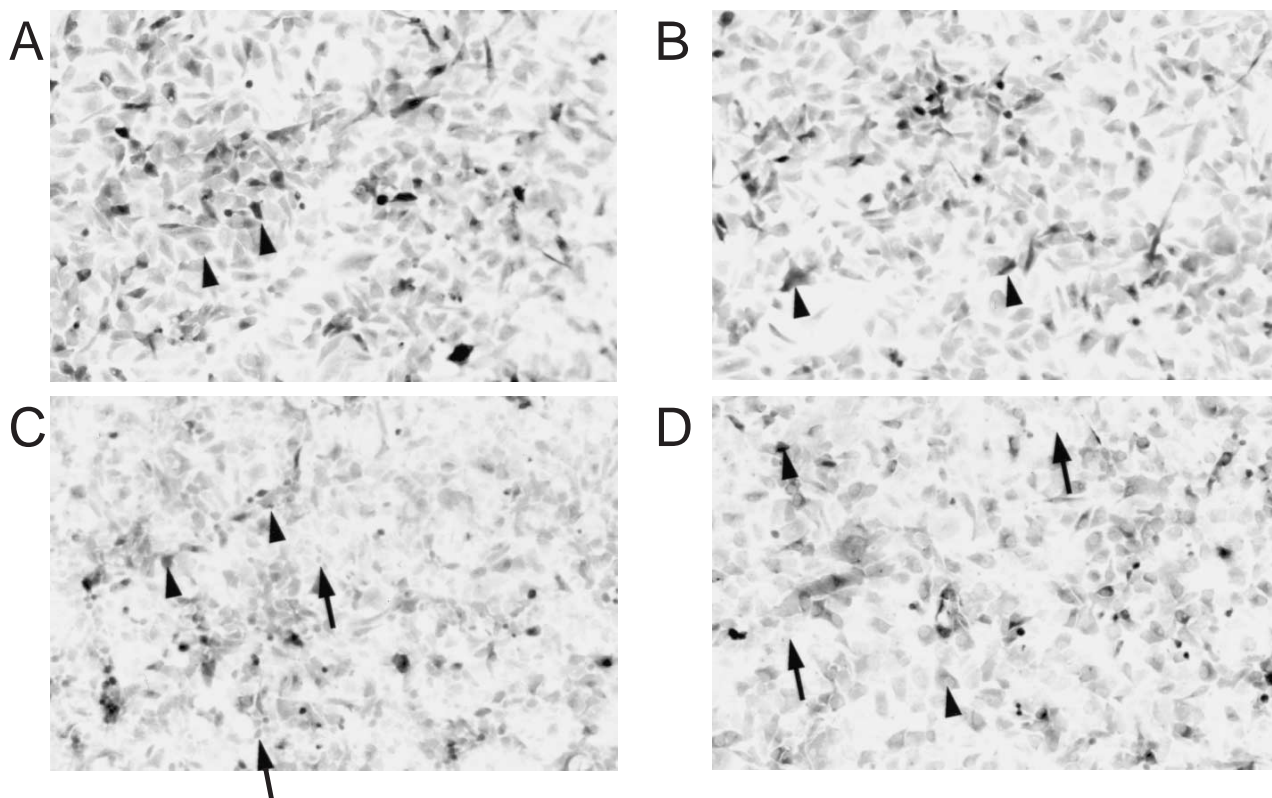


Fig. 2. Liposome-conjugated adenoviral vectors are less susceptible to inactivation by neutralizing antibodies *in vitro*. AxCALacZ or AxCALacZ/SUV (5×10^4 pfu) was incubated with serum containing neutralizing antibodies (NAb) at 37°C for 30 min and the mixture was added to HeLa cells (5×10^5 cells), which were assayed by X-gal staining 24 h later. A, AxCALacZ alone; B, AxCALacZ/SUV alone; C, AxCALacZ + NAb; D, AxCALacZ/SUV + NAb. The percentages of X-gal-positive cells were approximately 95, 97, 30 and 60% in A, B, C, and D, respectively. Arrowheads, examples of X-gal-positive and counter-stained cells showing the whole cell bodies in relatively dark gray; arrows, examples of X-gal-negative but counter-stained cells showing nuclei alone in light gray.

days after the second dosing.¹³⁾ Anti-Ad IgG levels from AxCALacZ/SUV-injected mice were 6.5-fold lower than those from AxCALacZ-injected mice (Table I). The neutralizing activity in blood from mice treated with AxCALacZ/SUV or AxCALacZ was assayed using HeLa cells. Briefly, 5×10^5 cells were seeded in 6-well plates. The following day, serum samples from vector-treated mice and normal mouse serum were diluted 10-50 fold with Dulbecco's modified Eagle's medium (DMEM). AxCALacZ (5×10^4 pfu) was incubated with each dilution at 37°C for 30 min, and was added to HeLa cells in the plates. After incubation for 24 h at 37°C, cells were stained with X-gal and counter-stained with hematoxylin. Neutralizing activity in the sample was calculated by use of the formula:

$$\text{Neutralizing activity (\%)} = \frac{[1 - (\text{the number of X-gal-positive cells in sample serum}) / (\text{the number of X-gal-positive cells in normal control serum})] \times 100}{1}$$

Neutralizing activity in blood from AxCALacZ-injected mice was significantly elevated, being 3-fold higher than in AxCALacZ/SUV-injected mice (Table I, 52.5 ± 3.0 and 17.8 ± 2.5 %, respectively).

Our recent studies and several reports by others showed that cationic molecules and/or lipids could bind to adenovirus and increase the efficiency of gene transfer *in vitro* and *in vivo* to a variety of cells and tissues, including human glioma cells, human smooth muscle cells and vascular tissue.^{12, 14-16)} The greatest benefit occurred in cells and tissues that are relatively resistant to Ad infection. Fasbender *et al.* suggested that the binding was dependent on an electrostatic interaction with the cell surface, that entry did not require an interaction of Ad fiber protein with cell surface, and that complexes entered cells via a pathway different from that utilized by adenovirus alone.¹⁶⁾

The goal of this study was to test if SUV liposome-conjugated Ad vector is less susceptible to inactivation by neutralizing antibodies *in vitro* and *in vivo*. AxCALacZ or AxCALacZ/SUV was incubated with serum containing neutralizing antibodies at 37°C for 30 min. The mixture was added to HeLa cells, which were assayed by X-gal staining 24 h later. We used HeLa cells because they are readily infected with adenovirus and therefore AxCALacZ/SUV produced no significant improvements in gene transfer to HeLa cells compared with AxCALacZ (Fig. 2, A and B). Prior incubation of AxCALacZ with 1/10 diluted neutralizing serum blocked gene transfer by more than 70%, while AxCALacZ/SUV retained approximately 60% of the control activity (Fig. 2, C and D). After we had confirmed that AxCALacZ/SUV injected into mouse brain parenchyma was able to express similar amounts of β -galactosidase to AxCALacZ alone (left portion of Fig. 3), we examined if SUV liposome conjugation had the ability to mask Ad vector in CNS from anti-Ad

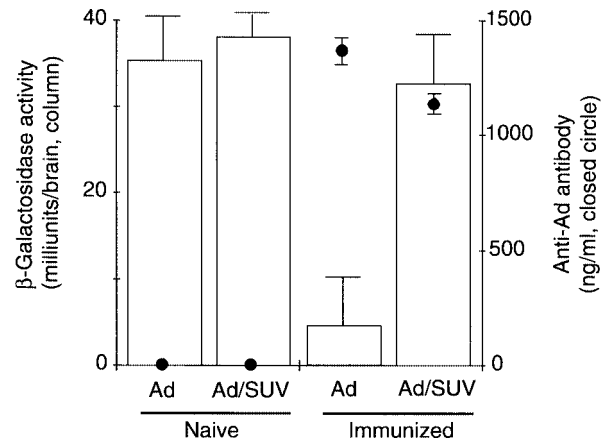


Fig. 3. Liposome-conjugated adenoviral vectors are less susceptible to inactivation by neutralizing antibodies *in vivo*. C57BL/6 mice were subcutaneously immunized with AxCAHS-tk (10^7 pfu). Fourteen days later, naive and the immunized mice were intracranially injected with AxCALacZ or AxCALacZ/SUV (4×10^5 pfu). A further 4 days later, β -galactosidase activity (column) in brain and anti-adenoviral antibody levels in serum (closed circle) were measured. All values are the mean and SD of 5 animals.

antibodies. Fourteen days after B6 mice were s.c. immunized with 10^7 pfu of AxCAHS-tk, the mice were i.c. injected with 4×10^5 pfu of AxCALacZ or AxCALacZ/SUV. Animals were killed 4 days later, and anti-Ad IgG levels in serum and β -galactosidase activity in brain tissue were measured. Although there was no significant difference in anti-Ad antibody levels between the two groups (1331 ± 52 , 1089 ± 34 ng/ml, respectively), β -galactosidase activity in AxCALacZ/SUV-injected mice was 7-fold higher than that in AxCALacZ-injected mice (right portion of Fig. 3).

Our results in the previous study and this are encouraging in three respects when considering repeated administration. First, delivery of cationic liposome-conjugated Ad vector may increase gene transduction efficiency to a variety of cell types that are especially resistant to Ad infection. Use of a more efficient delivery system could allow us to deliver smaller doses of Ad vector, thereby decreasing the humoral immune response. Second, mice injected with cationic liposome-conjugated Ad vector produced less anti-Ad antibodies compared with an equivalent dose of unconjugated Ad vector (Table I). The reduced antigenicity may prove beneficial for redosing. Finally, we also noted that cationic liposome-conjugated Ad vector was less susceptible to inactivation by neutralizing antibodies than Ad vector alone (Figs. 2 and 3).

The immune effectors against Ad antigens have been characterized.¹⁷⁾ Major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) are

directed toward viral antigens and the transgene product. Presentation of exogenous viral antigens by MHC class II molecules has been suggested to induce the Th1 subset of CD4⁺ T cells that strengthen the cytotoxic response, as well as the Th2 subset of CD4⁺ T cells involved in mounting an efficient humoral response by producing tumor necrosis factor alpha (TNF- α) and/or lymphotoxin α (LT α).¹⁸⁾ The B-cell response to an Ad infection consists essentially of IgG antibodies. Since some of these antibodies are neutralizing, efficient redosing is prevented. Although this study cannot discount a contribution from CTL, we hypothesize from the results shown in Fig. 1 that the presence of neutralizing antibodies plays a significant role in the reduction in transduction efficiency.

As well as the CTL response described above, several other issues remain unanswered. While liposomes have been reported to act as powerful adjuvants and delivery systems, their effect on the immune response varies with the nature of this linkage, surface-linked and encapsulated

antigens having different properties.¹⁹⁾ Although we conjugated 1×10^7 pfu of Ad with 1 μ mol of SUV, which represented optimal conditions from our previous study, it seems likely that not all of the liposomes would have been associated with virus. We have not yet attempted to purify discrete particles. However, studies addressing these issues might produce better performance.

This study suggests that it may be feasible to develop a system utilizing cationic liposomes which reduces antigenicity of Ad vector *in vivo* and shields the virus from neutralizing antibodies. Such an approach could circumvent the humoral response in people who receive multiple Ad administrations or people with pre-existing Ad immunity, allowing us to eliminate the requirement for systemic immunosuppression in the case of repeated administration. Our data suggest that additional investigation is warranted.

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