Complexes of Adenovirus with Polycationic Polymers and Cationic Lipids Increase the Efficiency of Gene Transfer in Vitro and in Vivo*

(Received for publication, July 31, 1996, and in revised form, October 31, 1996)

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Improving the efficiency of gene transfer remains an important goal in developing new treatments for cystic fibrosis and other diseases. Adenovirus vectors and nonviral vectors each have specific advantages, but they also have limitations. Adenovirus vectors efficiently escape from the endosome and enter the nucleus, but the virus shows limited binding to airway epithelia. Nonviral cationic vectors bind efficiently to the negatively charged cell surface, but they do not catalyze subsequent steps in gene transfer. To take advantage of the unique features of the two different vector systems, we noncovalently complexed cationic molecules with recombinant adenovirus encoding a transgene. Complexes of cationic polymers and cationic lipids with adenovirus increased adenovirus uptake and transgene expression in cells that were inefficiently infected by adenovirus alone. Infection by both complexes was independent of adenovirus fiber and its receptor and occurred via a different cellular pathway than adenovirus alone. Complexes of cationic molecules and adenovirus also enhanced gene transfer to differentiated human airway epithelia in vitro and to the nasal epithelium of cystic fibrosis mice in vivo. These data show that complexes of adenovirus and cationic molecules increase the efficiency of gene transfer, which may enhance the development of gene therapy.

Transfer of the cystic fibrosis transmembrane conductance regulator (CFTR)1 cDNA to airway epithelia of patients with cystic fibrosis (CF) (1) could provide an important new treatment for this genetic disease. Although previous studies have demonstrated the feasibility of gene transfer to CF airway epithelia, such studies suggest that an increase in efficiency is desirable. Enhanced efficiency would allow a vector to correct the electrolyte transport abnormalities that characterize the disease and minimize toxicity by reducing the amount of vector administered. Efficiency and safety will in turn determine the therapeutic index and thus the ultimate utility of gene transfer as a potential treatment.

Several vectors have been explored for gene transfer to CF airway epithelia, but so far adenovirus vectors (2–5) and nonviral vectors, including cationic lipids (6), have received the most attention. While both vector systems are capable of expressing CFTR in airway epithelia, an improvement in the efficiency of gene transfer to mature ciliated human airway epithelia remains an important goal (2–10). The two systems have different limitations and advantages.

Adenovirus-mediated gene transfer to airway epithelia is suboptimal because binding to the apical surface of the epithelium is limited, perhaps because the apical surface does not express the fiber receptor that mediates viral attachment (7, 8). Limited infection in vitro and in vivo could be partially overcome when the contact time between the virus and the apical surface was increased, even with a relatively low multiplicity of infection (m.o.i.) (8). These findings suggest that if adenovirus binding or entry into the cell could be increased, then adenovirus-dependent processes subsequent to binding and entry would remain intact and would facilitate gene transfer. These functions, which include entry of virus into the cell, release of DNA from the vector, entry into the nucleus, and transcription, are influenced by specific adenovirus proteins (11).

Nonviral vector-mediated gene transfer to mature human airway epithelia (6) could also be improved. Cationic molecules, including cationic lipids complexed with DNA, bind to the cell surface, and they are often taken up into the cell. However, release of DNA from the endosome, entry into the nucleus, release of DNA from the cationic molecules, and transcription of the DNA are not specifically enhanced by the vectors and thus remain as partial barriers that limit the efficiency of transgene expression (9).

Previous reports have described gene transfer systems that combine viral and nonviral components (12–18). In most cases, adenovirus has been incorporated into gene delivery systems to take advantage of its endosomolytic properties. These studies
have involved either covalent attachment of the adenovirus to a gene delivery complex or co-internalization of unbound adenovirus with cationic lipid-DNA complexes. In these formulations, the transfected gene is contained in plasmid DNA that is exogenous to the adenovirus. However, such formulations do not take advantage of adenovirus-dependent functions other than endosome disruption, and as a result, large amounts of adenovirus are required, and the increase in gene transfer has often been modest.

We reasoned that the efficiency of gene transfer would be improved if we could combine a nonviral vector system, which could mediate cell attachment, with an adenovirus vector system, which could mediate processes in gene transfer subsequent to binding. We thought that such a hybrid system might take advantage of the unique features associated with the two individual vector systems. We initially attempted to covalently link recombinant adenovirus to various ligands in order to increase vector binding and gene transfer. However, such attempts often reduced gene expression from the adenovirus vector, probably because chemical modification of viral proteins interfered with their functions in effecting gene transfer and expression (19). In the work described here, we tested the hypothesis that efficiency could be improved by using a noncovalent complex consisting of a cationic component and a recombinant adenovirus that contained the cDNA to be expressed. We postulated that a cationic component would charge-associate with adenovirus particles, which carry a net negative surface charge, and would facilitate attachment to the negatively charged cell membrane. We also postulated that if we could increase binding to the cell membrane, more adenovirus would be internalized, enhancing gene transfer and expression. To test this hypothesis and to examine the process, we used several cell systems in which the efficiency of adenovirus-mediated gene transfer varies. We then tested the gene transfer method on normal and CF airway epithelia in vitro and in vivo.

MATERIALS AND METHODS

Cell Culture—COS-1, HeLa, NIH-3T3, and 9L gliosarcoma (20) cells were cultured on 24-well plates (Corning 25820) in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. HeLa cells were cultured on 24-well plates in Eagle's MEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 10 mM nonessential amino acids (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were seeded at 3 × 10⁴/cm², except for 9L gliosarcoma cells, which were seeded at 7.5 × 10⁵/cm². Primary cultures of human umbilical vein endothelial cells were cultured in M199 medium (Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1% l-glutamine, Eagle's basal medium vitamin solution (Life Technologies, Inc.), and Eagle's basal medium amino acids (Life Technologies, Inc.) as described (21). Human umbilical vein endothelial cells were seeded at 1 × 10⁵/cm² 18–24 h prior to infection. Primary cultures of rat hepatocytes were isolated as described by Berry and Friend (22). The isolated cells were placed in culture medium consisting of 75% Eagle's MEM and 25% Waymouth medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 4 μg/ml dexamethasone (Sigma), 10 ng/ml triiodothyronine (Sigma), 50 ng/ml epidermal growth factor (Sigma), and ITS universal culture supplement (Collaborative Biomedical Products) and seeded onto collagen-coated 24-well plates at 4 × 10⁵ cells/cm² or onto collagen-coated 96-well plates at 7500 cells/well. Primary cultures of normal and CF human airway epithelia were grown on permeable filter supports at the air-liquid interface as described previously (8, 23).

Vectors and Vector-related Reagents—The recombinant adenovirus vectors expressing β-galactosidase, Ad2/βGal-2 (8) or Ad5RSVLacZ (24), and Ad2CFTR, Ad2ΔCFTR-8 (8), were propagated and titered as previously reported. Wild-type Ad2 was obtained from Dr. Sam Wadsworth (Genzyme Corp., Framingham, MA). For some studies, adenovirus was labeled by production in 293 cells in methionine-free medium containing 1 μCi/100 μl [35S]methionine (Amersham Life Science, Inc.). Fiber protein was a gift of Dr. Paul Freimuth (Brookhaven National Laboratory, Upton, NY).

Cationic Reagents—Various size poly-l-lysine (PLL) hydrobromide polymers were purchased from Sigma. Poly-l-lysine with an average molecular mass of 55.8 kDa (corresponding to ~250 lysine residues) was used in all experiments unless otherwise noted. Polyethyleneimine (average Mr = 25,000) was purchased from Aldrich. Histone (fraction V-N) and spermine were purchased from Sigma. Lipofectin, Lipofectace, and Lipofectamine were purchased from Life Technologies, Inc. DEAE-dextran, Tfx-50, and dioctadecylamidoglycercylerospermine were purchased from Promega (Madison, WI). The lipids GL-67, GL-53, and [N,N' N'-dimethylaminoethane]carbamoylchloester (DC-Chol) were gifts from Drs. Seng Cheng and David Harris (Genzyme Corp.). In some cases, these were formulated in a 1:2 molar ratio of GL-67 to dioleylophosphatidylethanolamine (DOPE), a 1:1 molar ratio of GL-53 to DOPE, or a 1:2 molar ratio of DC-Chol to DOPE. DOPE was purchased from Avanti Polar Lipids (Alabaster, AL). DMRIE/DOPE at a 1:1 molar ratio and βAE-DMRIE were gifts from Dr. Phil Felgner (Vical Inc., San Diego, CA).

Preparation of Cationic Molecule-Adenovirus Complexes—Recombinant adenovirus was prepared by the University of Iowa Gene Transfer Vector Core at titers of ~1 × 10⁹ IU/ml. Complexes between cationic molecules and adenovirus particles were formed by prediluting the cationic component and the adenovirus components in Eagle's MEM in 12 × 75-mm polystyrene tubes (Fisher). Ratios of cationic molecules to adenovirus particles and volumes of the dilutions are described in the figure legends. The cationic component dilution was added to the viral particle dilution, mixed by inversion or gentle pipette tip aspiration, and allowed to incubate for 15–30 min at room temperature before application to cells or tissue. We describe cationic molecule-adenovirus complexes based on the calculated average number of cationic molecules/adenovirus particle. The ratio of adenovirus particles to infectious units varied from 50 to 150.

Infection of Cells in Culture—Cultured cells were infected 18–24 h after seeding when the cells were ~70% confluent unless otherwise noted. Airway epithelial cells were allowed to mature in culture for at least 10–14 days before use so that they developed a ciliated apical surface that resembles the in vivo airway surface (8, 23). The medium was replaced with 500 μl of Eagle's MEM containing 1.1 × 10⁹ particles of adenovirus complexed with cationic component at the indicated ratios of cationic molecules to particles. The cells were incubated for 15 min to 6 h (times indicated in the figure legends) in a 5% CO₂ humidified environment at 37°C, the infection solution was removed, and
fresh serum-containing medium was added. Cells were then incubated for an additional 24 h unless otherwise specified. In experiments where uptake of 35S-labeled adenovirus was assessed, cells were harvested for the measurement of cell-associated radiolabeled virus at the end of the infection period, while paired groups of cells were incubated for an additional 20–24 h (unless otherwise noted) and harvested for assessment of gene expression. Human airway epithelial cells were infected as described in the figure legends.

**Gene Transfer to the Nasal Epithelium**—Ad2/CFTR-8 was applied to the nasal epithelium of unanesthetized DDDF/DDSDF mice (25) as a 5-μl drop containing 5 × 10^7 IU/nostril adenovirus alone or PLLzAd. The transepithelial electric potential difference (Vt) across the nasal epithelium was measured using techniques similar to those we previously described (25). During measurement of Vt, the nasal mucosa was perfused at a rate of 50 μl/min with a Ringer’s solution containing 135 mM NaCl, 2.4 mM KH2PO4, 0.6 mM K2HPO4, 1.2 mM CaCl2, 1.2 mM MgCl2, and 10 mM HEPES titrated to pH 7.4 with NaOH. Three solutions were used: (a) Ringer’s solution alone; (b) Ringer’s solution containing 100 μM amiloride (Merck); or (c) Ringer’s solution containing 135 mM sodium gluconate (substituted for NaCl), 10 μM terbutaline, and 100 μM amiloride. Measurements were made after perfusion for 5 min.

**Assays**—β-Galactosidase activity was assayed using a Galacto-Lite kit (Tropix Inc., Bedford, MA) and a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA). Cells were removed from dishes or millicell filters by incubation with 120 μl of lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol) for 15 min, followed by scraping. A 4-μl aliquot from each 24-well plate or millicell was used for each Galacto-Lite assay. Protein was measured using Bio-Rad protein assay reagent. Data for β-galactosidase activity represent total values from all cells in one well or from one millicell. All conditions were performed in triplicate on at least two different occasions. For X-gal staining, cells were washed 24 h after infection, fixed with 1.8% formaldehyde and 2% glutaraldehyde, and then incubated for 16 h in X-gal solution as described previously. Blue staining of nuclei was evaluated by light microscopy. For anti-hexon staining, airway epithelia cultured on permeable filter supports were studied 30 h after infection. They were fixed with acetone/methanol and stained with a polyclonal fluorescein isothiocyanate-labeled anti-hexon antibody (Chemicon International, Inc., Temecula, CA). Hexon-positive cells were counted using low magnification fluorescence.
Cationic Molecule-Adenovirus Complexes Enhance Gene Transfer

Fig. 4. Effect of complexes of adenovirus with various cationic polymers and cationic lipids on expression in 9L gliosarcoma cells. Data are β-galactosidase expression relative to expression with adenovirus alone, which was assigned a value of 1.0. Cells were incubated with 5000 particles/cell (m.o.i. = 50) for 2 h; the virus solution was then replaced with fresh medium; and expression was assessed 24 h later. Each cationic molecule was complexed with Ad3/5RSVLacZ over a range of cationic molecule/adenovirus particle ratios. The optimal ratio varied with different cationic molecules, and only data from the optimal ratio are plotted. The top panel shows nonlipid molecules; the middle panel shows cholesterol-based cationic lipids; and the bottom panel shows noncholesterol-type cationic lipids. For each cationic substance, the number of cationic molecules/adenovirus particle and nanograms/9 × 10⁶ particles, respectively, were as follows: histone (fraction V-S), 5000 molecules and 1620 ng; polyethyleneimine (PEI), 500 molecules and 412 ng; DEAE-dextran, 25,000 molecules and 9370 ng; spermine, no optimal ratio was found; DC-Chol/DOPE, 2.6 × 10⁵ molecules and 19,200 ng; DC-Chol, 1.7 × 10⁶ molecules and 128,000 ng; GL-53/DOPE, 2 × 10⁸ molecules and 1760 ng; GL-53, 9.2 × 10⁴ molecules and 8000 ng; GL-67/DOPE, 1 × 10⁸ molecules and 9400 ng; GL-67, 8.7 × 10⁴ molecules and 8000 ng; DMRIE/DOPE, 1.6 × 10⁵ molecules and 3800 ng; βAE/βAE-DMRIE, 1.7 × 10⁴ molecules and 128,000 ng; Tfx-50, 5 × 10⁸ molecules and 4700 ng; dioctadecylamidoglycylpermine (DOGS), 1.7 × 10⁴ molecules and 2000 ng; and Lipofectamine, 6.9 × 10⁷ molecules and 1500 ng (n = at least three for each molecule).
Projection of the number of PLL molecules to adenovirus particles on β-galactosidase expression in COS-1, 9L gliosarcoma, and NIH-3T3 cells. Cells were incubated for 2 h in 500 μl of Eagle’s MEM containing 5000 Ad2/βGal-2 particles/cell (m.o.i. ~ 50) complexed with PLL at the indicated ratio (n = at least three at each ratio). L.U., light units.

Because both the cell and the adenovirus have a net negative surface charge, we postulated that a cationic molecule might facilitate association of the vector with the cell. To learn whether a cationic molecule would bind to the cell surface, we applied Cy3-labeled PLL to different cell types. Fig. 2 shows Cy3-labeled PLL bound to the surface of the COS-1, HeLa, NIH-3T3, and 9L gliosarcoma cell lines. The surface of nearly every cell was fluorescently labeled in all cell types.

Therefore, we prepared PLL:Ad complexes and measured vector binding and transgene expression in the different cell lines. When we applied the PLL:Ad complex to NIH-3T3 and 9L gliosarcoma cells, both adenovirus binding (Fig. 1B) and transgene expression (Fig. 1A) increased to the same range as we had observed with adenovirus alone in COS-1 and HeLa cells. In contrast, complexing PLL with adenovirus had only small effects on binding and expression in COS-1 and HeLa cells. Fig. 3 shows photomicrographs of cells stained with X-gal after application of Ad2/βGal-2. PLL:Ad not only increased total β-galactosidase activity in NIH-3T3 and 9L gliosarcoma cells, but also increased the number of cells expressing the transgene.

Complexes of PLL:Ad also increased expression in primary cell cultures. In human umbilical vein endothelial cells, which are poorly infected by adenovirus alone, PLL:Ad increased expression 63 ± 3-fold (n = 3). In contrast, in primary cultures of rat hepatocytes, which are readily infected by adenovirus, PLL:Ad produced little enhancement (PLL:Ad was 1.1 ± 0.1 the level of expression with adenovirus alone (n = 3)).

Complexes Containing Adenovirus and Other Polycations Augment Gene Transfer—Encouraged by the results with PLL, we asked whether other cationic polypeptides would enhance gene transfer. We tested polyethyleneimine, DEAE-dextran, and histone (fraction V-S) and found that all facilitated gene transfer to poorly infected cell types (Fig. 4). As expected, with each polypeptide, the optimal cationic molecule/adenovirus particle ratio varied, producing data similar to those shown below in Fig. 5. However, not all cationic polypeptides were effective; spermine (a low molecular mass polypeptide with a potential +4 net charge) failed to enhance expression in 9L gliosarcoma cells despite testing of a range of molecule/adenovirus particle ratios (Fig. 4).

Cationic lipids complexed with adenovirus also augmented gene transfer to 9L gliosarcoma cells (Fig. 4). We obtained similar results in human umbilical vein endothelial cell cul-

The effect of PLL on adenovirus-mediated gene transfer was then replaced with fresh medium, and the cells were assayed for β-galactosidase activity 24 h later (n = at least three for each). Asterisks indicate p < 0.05 compared with control. L.U., light units.
crease β-galactosidase expression by COS-1 cells; in fact, at a PLL molecule/particle ratio of 25, we frequently noted a decrease in expression (Fig. 5). These results suggest the possibility that PLL may have interfered with the normal mechanism of adenovirus attachment and infection at intermediate ratios of PLL molecules to adenovirus particles, but then as the amount of PLL increased, PLL may have mediated adenovirus binding to the cells.

To learn whether it was necessary to form a complex between PLL and adenovirus, we first pretreated 9L gliosarcoma cells with PLL (5 ng/µl) for 5 min, removed excess PLL by washing, and then added adenovirus. Fig. 6 shows that separate application of PLL followed by adenovirus was not nearly as effective as adding the preformed PLL:Ad complex to the cells. Fig. 6 also shows that applying PLL to the cells before application of PLL:Ad or GL-67:Ad complexes largely prevented the augmentation of gene transfer observed with these complexes. These results suggest that PLL competed with sites that bind both PLL:Ad and GL-67:Ad, thereby attenuating gene transfer.

We did several additional studies to optimize PLL:Ad-mediated gene transfer. 1) We found that the size of PLL influenced gene transfer (Fig. 7). A size of 55.8 kDa (used throughout this study) produced the greatest augmentation of gene transfer when compared with PLL of other sizes, even though the calculated ratio of positive charges to adenovirus particles remained constant. 2) Many cationic lipid-DNA complexes have a tendency to aggregate and lose their effectiveness with prolonged incubation (27). We observed a similar property with PLL:Ad. With a 5-h delay (at room temperature) between preparation of the complex and application to cells, expression was 28 ± 10% (n = 6) of the value obtained when the delay interval was only 15 min. In contrast, adenovirus alone retained its infectivity; expression after a 5-h delay at room temperature was 104 ± 7% (n = 6) of the value after a 15-min delay (data not shown). 3) We found that it was necessary to prepare the PLL:Ad complex in the absence of serum. When serum was
present during complex formation, expression in 9L gliosarcoma cells was reduced to 2.8 ± 0.1% (n = 3) of the value obtained after preparation under serum-free conditions. 4) Serum inhibits gene transfer by some cationic lipid-DNA complexes and by some nonviral vector preparations that contain PLL. To examine the effect of serum, we used COS-1 cells so that we could compare the effect of PLL:Ad with adenovirus alone. Fig. 7B shows that PLL:Ad-mediated gene transfer to COS-1 cells was decreased in the presence of serum, but the decrease was no greater than that observed with adenovirus alone. We obtained similar results with 9L gliosarcoma cells. These data indicate that after the complex was formed, it could be used in either the presence or absence of serum. 5) We previously reported that the concentration of adenovirus was an important variable in effecting gene transfer (26). Likewise, we found that as the concentration of PLL increased (produced by decreasing the applied volume with a constant m.o.i.), expression increased in 9L gliosarcoma cells (Fig. 7C). 6) The enhancement produced by complexing adenovirus with PLL was not dependent on the time after seeding of 9L gliosarcoma cells (Fig. 7D). In confluent 9L gliosarcoma cells, expression with PLL:Ad was 32-fold greater than with adenovirus alone, and with subconfluent cells in log-phase growth, expression was 29-fold greater.

We also examined the appearance of the PLL:Ad complex by transmission electron microscopy. Fig. 8 shows photomicrographs of adenovirus alone and PLL:Ad complexes at a suboptimal infection ratio (4 PLL molecules/particle), an optimal ratio (250 PLL molecules/particle), and another suboptimal ratio (10,000 PLL molecules/particle). As the number of PLL molecules adenovirus particle increased, we saw that the adenovirus particles tended to clump together, although we found individual adenovirus particles even at the highest ratio. These observations suggest that PLL is linking the negative surface charge on the adenovirus particle to other particles and that excessive amounts of PLL produce aggregation, which could decrease the efficiency of gene transfer, as shown in Fig. 5.

**Gene Transfer by PLL:Ad Complexes Does Not Depend on the Fiber Receptor**—In some cell types, adenovirus infection is mediated by binding of the adenovirus fiber protein to an unidentified receptor on the cell surface (28). Consistent with this conclusion, we found that excess fiber inhibited gene transfer by adenovirus alone in primary hepatocytes, which are readily infected cells (Fig. 9A). However, fiber did not inhibit expression by PLL:Ad and only partially inhibited expression by GL-67:Ad.

We also examined the effect of neutralizing antibodies. Fig. 9B shows that neutralizing antibodies directed against fiber did not interfere with expression by PLL:Ad when we used optimal PLL molecule/particle ratios. However at a suboptimal ratio (25 PLL molecules/particle), anti-fiber antibody produced some inhibition of expression. These data, together with the adenovirus binding experiments, the effect of fiber, and the enhancement of gene transfer to poorly infected cells, suggest that PLL causes adenovirus to bind to and then infect cells through pathways other than the fiber receptor-mediated pathway.

In contrast to the effect of anti-fiber antibody, anti-hexon antibody inhibited infection by both adenovirus and PLL:Ad (Fig. 9B). Chloroquine, which inhibits adenovirus infection by raising the pH of the endosomes and by preventing release from the endosomes (29), also inhibited gene transfer by PLL:Ad. Treatment of 9L gliosarcoma cells with 100 μM chloroquine for 60 min decreased β-galactosidase expression following infection with PLL:Ad by 59 ± 10% compared with expression obtained in the absence of chloroquine (n = 6). These results suggest that adenovirus-dependent steps subsequent to binding and uptake into the cell are required for infection.

Because the functional data suggested that the mechanism for binding and uptake of PLL:Ad was different from that of adenovirus alone, we looked for a structural correlate using transmission electron microscopy. We used 9L gliosarcoma cells because they are poorly infected by adenovirus due to poor binding of virus. We also used a very large number of particles per cell in order to observe the vector-cell interaction. At high m.o.i. values, there were a substantial number of adenovirus particles in the cells, even in 9L gliosarcoma cells, which show limited binding of adenovirus (Fig. 10, A and B). The electron photomicrographs show that adenovirus was usually taken up into the cells as single particles or occasionally as a small (two or three) number of particles. In the cells treated with PLL:Ad (Fig. 10, C and D) or Lipofectamine-adenovirus (Fig. 10, E and F), there were a greatly increased number of adenovirus particles in the cells, consistent with the data shown in Fig. 1. It is also apparent that with the PLL:Ad and cationic lipid-adenovirus complexes, multiple adenovirus particles were present in the endosomes and that the endosomes were larger than those containing adenovirus alone. In addition, with cationic lipid-adenovirus complexes, we sometimes saw a lamellar appear-
ance of the lipid around and associated with the virus (Fig. 10, inset).

Gene Transfer by PLL-Ad Complexes to Airway Epithelia in Vitro and in Vivo—We also assessed the effect of complexes on airway epithelia. Primary cultures of human airway epithelia grown at the air-liquid interface differentiate into a respiratory epithelium that has a ciliated surface and many characteristics of the native epithelium (8, 23). To test infection of mature epithelia, we applied adenovirus alone or complexed with PLL to the apical surface for 30 min. Then the virus was removed, and the surface was washed twice to remove unattached virus. Fig. 11 shows that more cells were infected with PLL-Ad than with adenovirus alone; there were 94 ± 48 hexon-positive cells/low power field with adenovirus alone versus 797 ± 115 with PLL-Ad (p = 0.001; n = 4).

We also applied 20 m.o.i. of Ad2/βGal-2 alone, PLL-Ad, or GL-67-Ad to the mucosal surface of mature epithelial monolayers for 30 min. Fig. 12A shows that epithelia treated with PLL-Ad or GL-67-Ad generated more β-galactosidase activity than those treated with adenovirus alone. Fig. 12B shows that, as we previously reported (8), application of adenovirus expressing CFTR to the apical surface of CF airway epithelia for 30 min had little effect on cAMP-stimulated Cl− transport. However, treatment with PLL-Ad complexes restored a cAMP-stimulated Cl− current to CF epithelia. These data suggest that the complexes are more efficient than adenovirus alone at gene transfer to mature human airway epithelia.

Previous studies have administered adenovirus vectors encoding CFTR to the nasal epithelium of patients with CF and tested for correction of the electrophysiologic defect. We initially reported correction of the CF electrophysiologic abnormalities following application of Ad2/CFTR-1 to nasal epithelium that was injured during the application procedure (2). However, when adenovirus vector was applied to intact respiratory epithelium, we saw only limited evidence of gene transfer (5). Hay et al. (4) found evidence of partial electrophysiologic correction, and Knowles et al. (3) found no evidence of correction. These data suggest that more efficient gene transfer to human ciliated respiratory epithelia in vivo would be valuable.

We (8) and others (7) have also found that a single application of adenovirus to the nasal epithelium of CF mice is unable to correct the CF Cl− transport defect, although if the duration of contact with the epithelium is prolonged, some correction does occur (8). Therefore, we tested the ability of PLL-Ad to correct the electrophysiologic defect in the nasal epithelium of CF mice. Fig. 13 shows that CF mice bearing the ΔF508 mutation have a basal voltage that was more electrically negative than that of wild-type mice, and the voltage failed to hyperpolarize in response to perfusion with a solution containing a low Cl− concentration. As previously reported (8), administration of Ad2/CFTR-8 failed to correct either of these defects (Fig. 13). However, after addition of PLL-Ad, both electrophysiologic properties were corrected to the normal range. These data indicate that functional CFTR Cl− channels were restored in the nasal epithelium.

DISCUSSION

In this study, we combined adenovirus with cationic molecules to generate complexes that increased the efficiency of gene transfer. Expression of reporter genes was increased in a number of cultured cells, and gene transfer to primary cultures of mature human airway epithelia was enhanced. In vivo, the
complexes corrected the electrophysiologic abnormalities that characterize CF epithelia.

By including PLL or cationic lipids in the complex, we were able to increase binding and cellular uptake. These are important barriers to adenovirus-mediated gene transfer in cells that do not express a cell-surface receptor that binds adenovirus fiber (28, 30, 31). Binding and uptake of the complex did not appear to be dependent on fiber receptors because expression was not blocked by addition of excess fiber protein or a neutralizing antibody to fiber. Transmission electron microscopy also suggested that the complex enters the cell via a mechanism different from that of adenovirus alone.

By including adenovirus in the complex, we were able to take advantage of adenovirus-dependent processes that facilitate expression such as escape from the endosome and entry into the nucleus (11); these processes are significant barriers to nonviral vector-mediated gene transfer. Our data indicate that adenovirus retains its ability to facilitate DNA delivery, even when entry into the cell is via a pathway different from the usual receptor-mediated mechanism. But the finding that a neutralizing antibody against hexon and chloroquine pretreatment inhibited expression suggests that when viral processes after uptake are compromised, the efficiency of infection falls.

These processes may include escape from the endosome and transport to the nucleus.

We found that we could combine many different cationic molecules with adenovirus to facilitate gene transfer. However, there were important differences in the ability of various cationic molecules to enhance gene transfer, and not all cationic molecules were effective. The receptors for complex binding are most likely negatively charged molecules on the cell surface. Although we have not identified specific receptors, it seems likely that many different negatively charged molecules, e.g. sialic acids, may bind the complexes. This conclusion is consistent with our observation that pretreatment of cells with PLL blocked gene transfer by complexes containing either PLL or GL-67.

Although we did many of our studies with PLL to test the concept, PLL may not be the cationic component of choice for gene therapy applications. In this regard, cationic lipids might offer advantages because they have already been administered to humans (6) and function as well or better than PLL in airway epithelia. Cationic lipids might also offer other advantages by providing additional components that facilitate gene transfer. For example, the cholesterol in GL-67 could possibly facilitate cell binding. Other cationic molecules, such as dendrimers, might also prove to be effective. Future studies will be required to evaluate cell type-specific effects and to identify the relationship between the structure of the cationic molecule, its function in the complex, and its interaction with the cell.

Previous reports have described the incorporation of adenovirus into gene transfer complexes (12–14, 16, 17, 32–37). Such complexes took advantage of the endosome-disrupting properties of adenovirus to enhance gene transfer. Several investigators have chemically (12–14, 17, 32, 36) or immunologically (13, 36, 37) coupled adenovirus to PLL alone, to PLL conjugated to a receptor ligand (12–14, 32), or to PLL plus PLL conjugated to a receptor ligand (17, 32, 35, 36). This complex was then charge-associated with plasmid DNA, and expression of plasmid DNA was used to evaluate gene transfer. In addition, unbound adenovirus has been co-internalized with PLL-plasmid DNA complexes with and without an associated receptor ligand (16, 18, 38) and with cationic lipid-DNA complexes (9, 15). Many of these reports describe an increase in transgene expression. However, to our knowledge, previous reports have not used cationic molecule-adenovirus complexes in which the transgene is encoded in adenovirus DNA. This approach uses the cationic molecule to enhance adenovirus uptake into poorly infected cells and then utilizes adenovirus proteins to facilitate steps in gene transfer subsequent to uptake.

The system we describe has a number of advantages. It may be of value in gene transfer for cells and tissues in which infection is limited because the cells lack fiber receptors. On the other hand, it probably offers no advantage for cells that are already easily infected by adenovirus, such as hepatocytes. Because of its nonspecific nature, it may be useful for tissues in which the vector can be applied selectively to the target cells, e.g. to the apical surface of airway epithelia. The system also has the advantage that many different cationic molecules can be evaluated readily; because covalent linkage is not required, less time may be needed to evaluate specific conditions, and there may be less danger of inactivating important viral functions. The system we describe also has some disadvantages. The complex needs to be used shortly after preparation to ensure optimal gene transfer. The system is more complex than either adenovirus or cationic lipid-DNA alone. Moreover, after in vivo delivery, there is the possibility of toxicity from each of the individual components. However, future studies may solve some of these problems, and their importance may be dimin-

**FIG. 11.** Infection of primary cultures of mature human airway epithelia by wild-type Ad2 alone (A) or complexed with PLL/Ad (B). Epithelia were studied 21 days after they were seeded when they were confluent and differentiated. Ad2 or PLL/Ad (m.o.i. = 50, particle/IU ratio = 12) was applied to the apical surface of epithelia (5 × 10⁶ cells/epithelium) for 30 min. Vector was then removed, and the epithelium was rinsed twice. Thirty h later, the epithelium was fixed and stained with anti-hexon antibody.
4–5 days later (for PLL D12 days after vector administration). Data for wild-type (n = 3). Asterisks indicate p < 0.01 compared with adenovirus alone. B, Ad2/CFTR-8 were prepared as described for A and applied to CF airway epithelia 10–14 days after they were seeded. The change in short-circuit current in response to cAMP agonists (ISCAMP) was measured 4–5 days later (n = at least eight in each group). Asterisks indicate p < 0.05 compared with adenovirus. L.U., light units.

FIG. 13. Effect of adenovirus and PLL/Ad complexes on basal voltage (Vt) (A) and change in voltage produced by perfusion with a solution containing a low Cl− concentration (∆Vtlow, cAMP) and cAMP agonists in the presence of amiloride (B). Either Ad2/CFTR-8 alone or complexed with PLL (250 PLL molecules/particle) was applied to the nasal epithelium of mice containing the ΔF508 mutation in the mouse CFTR gene. Vector (5 × 106 IU/nostril) was applied to the nasal mucosa in 5 μl. Electrophysiologic measurements were obtained 2 days after vector administration. Data for wild-type (+/+) and CFTR-ΔF508 (ΔF/ΔF) mice that were not exposed to adenovirus are from Zeiher et al. (25). Asterisks indicate p < 0.01 compared with ΔF/ΔF mice treated with adenovirus alone (p < 0.01; n = 12 for adenovirus and 13 for PLL/Ad).

We also speculate that the system we describe could be adapted to include additional functions. For example, specific ligands might be included, and the amount of cationic molecule available for binding to the cell might be decreased in an attempt to target the vector to specific cells. Using such a system, perhaps adenovirus infection of its normal targets, fiber receptor-bearing cells, could be minimized. Finally, it is conceivable that adenovirus could be coated with cationic molecules that replace the cell binding and internalization functions of the virus, yet shield it from neutralizing antibodies that can prevent repeat administration.

This work shows that complexes of adenovirus with cationic molecules enhance gene transfer in vitro and in vivo. Because they interact with the cell surface through charge association, these and related complexes may increase gene transfer to a number of different cell types. With improvement in the efficiency of gene transfer, it may be possible to deliver less vector and, as a result, to reduce toxicity and the immune response. A consequent increase in the therapeutic index would further enhance the development of successful gene therapy.

Acknowledgments.—We thank Pary Weber, Phil Karp, Christopher Welsh, Alaina Kehrli, Gina Hill, and Theresa Mayhew for excellent assistance. We thank Drs. Seng H. Cheng and David Harris for the gift of GL-67, GL-53, and DC-Chol; Dr. Paul Freimuth for the gift of fiber protein; Dr. Wisia Chroboczek for the gift of neutralizing antibodies directed against fiber and hexon; Drs. A. E. Smith and Sam Wadsworth for Ad2/CFTR-8, and wild-type Ad2; Phil Felgner for the gift of DMRIE/DOPE and βAE-DMRIE; and Dr. Alfredo Fabrega and Steve Struble for primary cultures of hepatocytes. We thank the University of Iowa Gene Transfer Vector Core for preparing the adenovirus, the University Central Microscopy Research Facility for help with microscopy, and the DERC DNA Core for assistance.

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