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Development of Novel Formulations That Enhance Adenoviral-Mediated Gene Expression in the Lung *in Vitro* and *in Vivo*

Maria A. Croyle,[1*] Xuan Cheng,[1] Arbans Sandhu,[2] and James M. Wilson[2]

[1]*The University of Texas at Austin College of Pharmacy, Division of Pharmaceutics, Austin, Texas 78712, USA*
[2]*Institute for Human Gene Therapy and Department of Molecular and Cellular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA*

**To whom correspondence and reprint requests should be addressed. Fax: (512) 471-7474. E-mail: macroyle@mail.utexas.edu.*

Despite remarkable progress in the development of both viral and non-viral gene delivery vectors for cystic fibrosis therapy, low efficiency of gene transfer to the airway epithelium is a major obstacle to clinical application. Here we develop formulations that enhance cellular absorption of adenoviral vectors. We selected excipients from a panel of pharmaceutically acceptable compounds known to enhance drug absorption. Transduction efficiency of the virus in the presence of each ingredient was assessed *in vitro* and *in vivo*. Mannitol and chitosan substantially enhanced transduction efficiency *in vitro* and augmented expression *in vivo* by 4 and 8 log units, respectively. The most successful formulation (a blend of sucrose, mannitol, and Pluronic F68) transduced 100% of an A549 cell population *in vitro* and produced areas of intense gene expression in both large and small airways *in vivo* with minimal toxicity. Dose response studies also indicate that when placed in this formulation, the viral dose can be lowered by 1/2 log while maintaining superior levels of transgene expression. This formulation also enhanced the physical stability of the virus. No significant loss in titer was detected from a lyophilized formulation after storage at 25°C for 30 days.

KEY WORDS: **adenovirus, formulation, lung, gene therapy, stability**

INTRODUCTION

Although successful adenovirus-mediated gene transfer to cultured epithelial cells and to various *in vivo* airway models has been reported [1–4], differentiated epithelial cells are relatively resistant to adenovirus transduction. This may be explained by lack of adenovirus receptors on the apical surface of the airway epithelium [5,6]. As a result, high doses of virus and long periods of physical contact between the vector and target cells are required for efficient gene transfer. Both of these requirements present unique challenges in the development of gene therapy strategies. High doses of virus can precipitate local inflammation that may present a substantial risk to the patient [7,8]. Extensive vector contact with the airway epithelium is prevented by natural clearance mechanisms as well as mucous and soluble factors in airway surface fluid [9,10]. It would be useful to overcome these limitations and improve the efficiency of adenovirus-mediated gene transfer to allow a reduction of vector dose and exposure time to the airway epithelium.

Use of positively charged non-viral vectors to successfully deliver genes to the respiratory epithelium prompted the development of adenoviral formulations consisting of poly-L-lysine, protamine, or cationic lipids to enhance cellular absorption of the vector [11–15]. The rationale was that these positively charged molecules would preferentially associate with negatively charged adenoviral particles and facilitate attachment to the negatively charged cell surface membrane. Others have attempted to enhance viral uptake by compromising the integrity of the airway epithelia by formulating the vector with lung surfactant or chelating agents in hypotonic buffer [16–18]. Although these approaches have enhanced adenovirus transduction efficiency, these reagents may independently stimulate an immune response [19–23].

Our interest in the development of formulations to enhance adenoviral-mediated gene transduction in the respiratory epithelium stems from our goal of reducing adenoviral vector dosages *in vivo*. This approach is supported by data from gene therapy clinical trials that indicate that higher viral vector doses are associated with adverse reactions [24–27]. Here we develop novel formulations that combine pharmaceutically acceptable ingredients in a manner that promotes interaction of vector

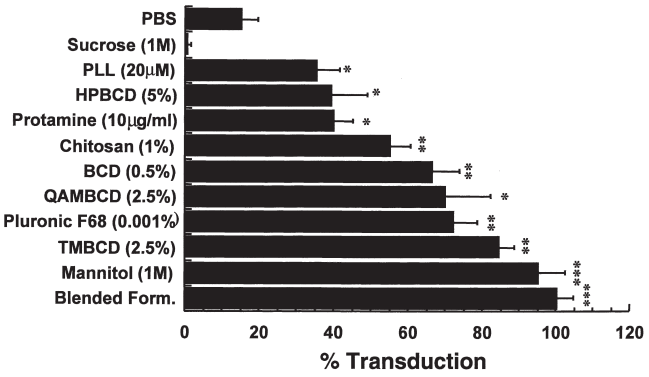

FIG. 1. *In vitro* transduction efficiency of formulated adenovirus preparations. Confluent A549 cells were infected with formulations containing AdlacZ at an MOI of 20. Poly-L-lysine (PLL) and protamine were included as standards for comparison to adequately assess transduction efficiency of the new formulations. Percent transduction is the ratio of lac^+ cells to the total number of cells in each culture. HPBCD, hydroxypropyl β-cyclodextrin; BCD, β-cyclodextrin; QAMBCD, quaternary amine β-cyclodextrin; TMBCD, tertiary amine β-cyclodextrin. Data are the results from 2 separate experiments. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, Student's *t* test.

with the airway epithelium and cellular uptake of adenoviral particles. Certain excipients were chosen based on their ability to enhance long-term physical stability of the virus [28,29]. Many of these ingredients have been routinely incorporated in drug formulations as absorption enhancers or bioadhesive agents to promote bioavailability of poorly absorbed compounds [30]. Excipient concentrations were derived from the pharmaceutical literature and were screened for toxicity and effect on adenoviral transduction efficiency on A549 cells before intratrachial administration to C57BL/6 mice.

RESULTS

Effect of Formulation on Adenovirus Transduction Efficiency *in Vitro*

We screened multiple excipients for their ability to enhance adenoviral transduction in A549 cells. Forty-eight hours after the addition of AdlacZ at a multiplicity of infection (MOI) of 20 in 10 mM phosphate buffered saline (PBS; pH 7.4), 15.2 ± 4.4% of the monolayer expressed the transgene (Figs. 1 and 2B). Formulations consisting of poly-L-lysine (36% transduction) and protamine (40% transduction) were included as standards by which to compare the transduction efficiencies of our novel formulations. Addition of 1% chitosan, a bioadhesive polymer, to the formulation increased transduction to 55.2 ± 5.3% (Figs. 1 and 2D). Addition of β-cyclodextrin, an excipient commonly used in formulations of water-insoluble compounds, produced a transduction efficiency of 66.6 ± 7.3%. The positively charged β-cyclodextrins, quaternary amine

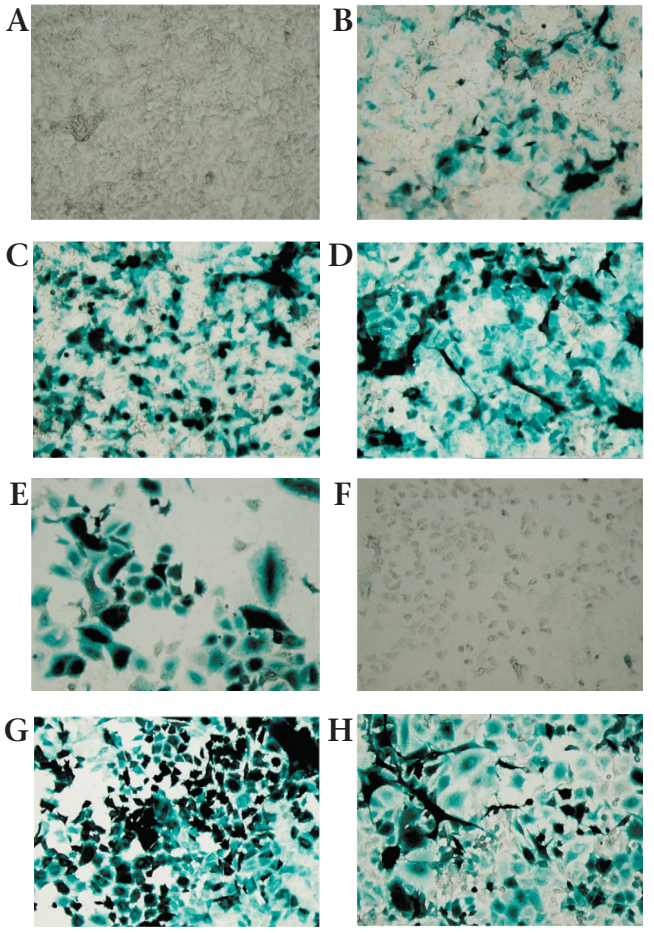

FIG. 2. Micrographs of A549 cells treated with formulations of first-generation adenovirus. Confluent monolayers were infected with virus (MOI 20) in various formulations. Forty-eight hours after infection, cells were fixed and stained for β-galactosidase expression. (A) Cell monolayer treated with PBS. There was no endogenous expression of β-galactosidase in this cell line. (B) Monolayer treated with virus in PBS. (C) Cells treated with virus in a solution of β-cyclodextrin. (D) Chitosan formulation. (E) Mannitol formulation. (F) Sucrose formulation. (G) Cell monolayer treated with a formulation consisting of a 1:4 ratio of sucrose and mannitol with 0.001% Pluronic F68. (H) Pluronic F68 formulation.

and tertiary amine β-cyclodextrin, raised the transduction efficiency of the virus to 70.1% and 84.6%, respectively. Low concentration (0.001%) of Pluronic F68, a nonionic block copolymer surfactant, transduced 72.3 ± 6.5% of the cell population. Mannitol (1 M) produced a transduction efficiency of 95.3 ± 7.2% (Figs. 1 and 2E). A solution of sucrose at the same concentration, however, was toxic to the monolayer and failed to produce gene expression (Fig. 2F). As a result, this formulation was not tested *in vivo*. The most successful formulation consisted of a 1:4 ratio of sucrose to mannitol with 0.001% Pluronic F68 in PBS. When this formulation was applied to monolayers, every cell expressed the transgene (Figs. 1 and 2G).

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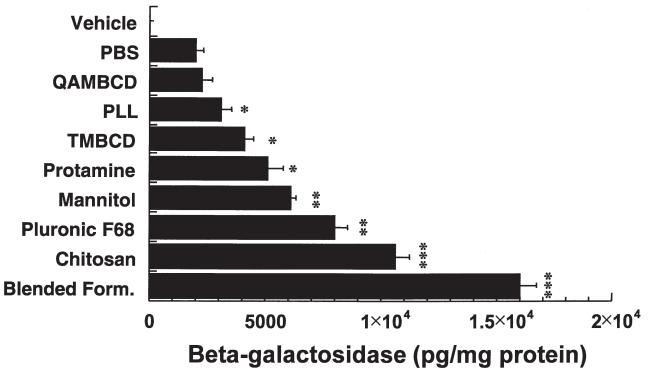

FIG. 3. Effect of formulation on adenoviral-mediated gene expression in the lung. First generation adenoviral vectors were administered intratracheally (5×10^{10} particles/ml), alone (PBS), or with formulation to C57BL/6 mice. Four days after infection, animals were sacrificed and lung tissue processed for analysis of β-galactosidase by ELISA. Data is the result of three separate experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, Student's *t* test.

In Vivo Transduction Efficiency of Formulated Adenovirus in the Lung of C57BL/6 Mice

To determine if *in vitro* transduction efficiency is an accurate predictor of formulation performance *in vivo*, 5×10^{10} particles of adenovirus in each respective formulation were delivered by intratrachial injection to C57BL/6 mice. There was no difference in the amount of β-galactosidase produced in the lungs of mice treated with virus in saline without any additional excipients (PBS) and the quaternary amine β-cyclodextrin formulation (2000 pg β-gal/mg protein; Fig. 3). Addition of poly-L-lysine to the formulation increased gene expression by 50% to 3100 pg β-gal/mg protein. Addition of either tertiary amine β-cyclodextrin or protamine to the viral preparation increased β-galactosidase to 4087 and 5221 pg β-gal/mg protein, respectively. Mannitol increased transduction efficiency threefold to 6125 pg β-gal/mg protein; however, the lung epithelium appeared extremely compromised in sections from animals treated with this formulation (Fig. 4F). The Pluronic formulation produced β-galactosidase at a level of 8265 pg/mg protein. Animals treated with this formulation displayed concentrated areas of gene expression mainly in large airways (Fig. 4C). The chitosan formulation produced an eightfold increase in transduction efficiency. Although sections from all animals treated with this formulation showed intense areas of gene expression, some local inflammation was also detected (Fig. 4E). The most successful formulation *in vitro*, the blended formulation, was also the most effective *in vivo*, increasing β-galactosidase expression in the lung by 1 log. Almost every cell in both the large and small airways expressed the transgene (Figs. 4G and 4H) and there was minimal inflammation. This formulation also enhanced the physical stability of the virus. After storage for 30 days at 4°C, titer dropped by 10% (Fig. 5). When this virus was stored under the same conditions in PBS, titer fell below detectable levels within five days (data not shown). Lyophilization of this formulation also allowed the vector to be stored at either 4°C or room temperature without significant loss of viral titer for 30 days.

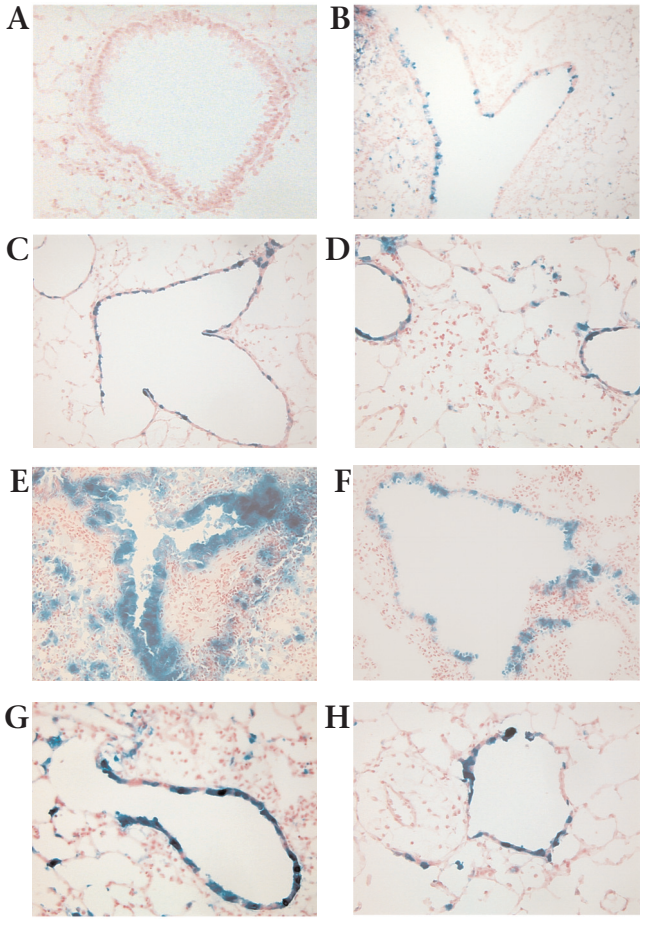

FIG. 4. Histological evaluation of adenoviral-mediated gene expression in the lung in the presence of formulation. C57BL/6 mice were given intratracheal injections of adenovirus at a dose 5×10^{10} particles/ml in various formulations. Four days after injection, animals were sacrificed and lung tissues processed and evaluated for *lacZ* expression by X-gal histochemistry. (A) Micrograph from an animal treated with a saline bolus. (B) Micrograph from an animal given adenovirus in saline. (C) Animal treated with virus in a 0.001% Pluronic formulation. (D) Animal that received virus in 2.5% tertiary amine β-cyclodextrin. (E) Airway of an animal treated with virus in 1% chitosan. (F) Animal treated with virus in 1 M mannitol. Every section from animals given the blended formulation (a 1:4 ratio of sucrose:mannitol with 0.001% Pluronic F68) demonstrated extremely high levels of gene expression in both the large (G) and small (H) airways.

Successful Adenoviral Formulations Produce Superior Levels of Gene Expression with Lower Viral Doses

One of our main objectives was to develop formulations that could enhance adenoviral transduction efficiency

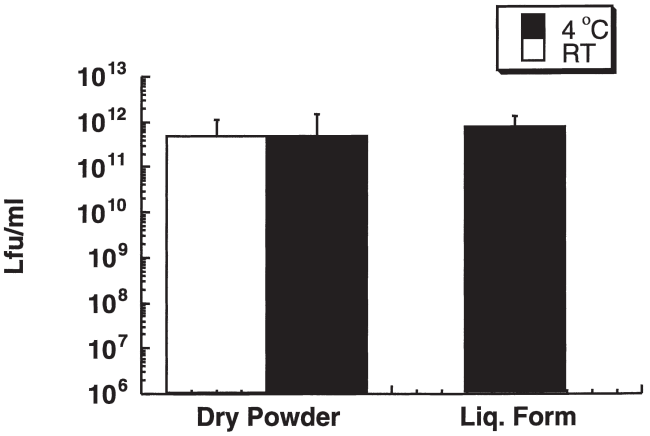

FIG. 5. Physical stability profile of the blended formulation 30 days after storage at 4°C and room temperature. After CsCl purification, adenovirus was desalted over a Sephadex column equilibrated with the blended formulation (a 1:4 ratio of sucrose:mannitol with 0.001% Pluronic F68). Half the samples were lyophilized and stored at either 25°C or 4°C. The remaining samples were stored in stoppered, sealed Type 1 borosilicate glass vials at 4°C in a liquid form. Titer of each preparation was 1×10^{12} lfu/ml at the initiation of the study. Data reflect the average titer from six samples stored under each condition. The slight decrease in titer of the dry powder formulation can be attributed to loss that occurred during the lyophilization process (data not shown). lfu/ml, lac forming units/ml.

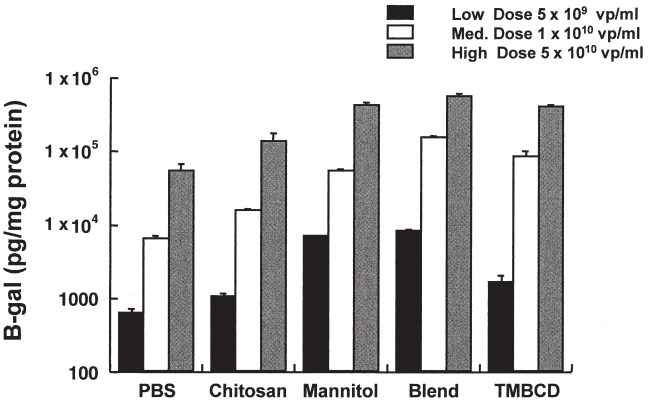

FIG. 6. Dose response of formulated adenoviral vectors in the lung. Low (5×10^9 viral particles), medium (1×10^{10} viral particles), and high (5×10^{10} viral particles) doses of adenovirus were formulated in either 10 mM potassium PBS (pH 7.4) or buffer with chitosan (1%), mannitol (1 M), tertiary amine β-cyclodextrin (TMBCD, 2.5%), or the blended formulation (a 1:4 ratio of sucrose:mannitol with 0.001% Pluronic F68) and administered by intratracheal injection to C57BL/6 mice. Animals were necropsied 4 d after administration of vector and gene expression assessed by an ELISA assay. Data reflect the average gene expression from five animals per dosing group. vp, viral particles.

in the airway epithelium in a manner that would reduce the amount of vector needed for efficient gene expression. To determine if this was possible with the formulations that we found to be highly effective *in vivo*, we formulated three doses of adenovirus (5×10^{10} (high dose, the standard dose in our laboratory), 1×10^{10} (medium dose), and 5×10^9 (low dose) viral particles) in either 10 mM potassium PBS (pH 7.4), buffer with chitosan (1%), mannitol (1 M), tertiary amine β-cyclodextrin (TMBCD, 2.5%), or the blended formulation and administered these by intratracheal injection to C57BL/6 mice. A distinct dose response was seen with each formulation and all produced significantly higher levels of gene expression than the PBS preparation at each of the doses studied ($P \leq 0.05$, Student's *t* test; Fig. 6). The blended formulation produced the highest level of gene expression of all the formulations studied (5.8×10^5 pg β-galactosidase/mg protein, high dose). When the dose was reduced to 1×10^{10} particles/ml, gene expression fell to 1.6×10^5 pg β-galactosidase/mg protein, a level that surpassed that seen with a high dose of vector (5×10^{10} particles/ml) formulated in PBS (5.4×10^4 pg β-galactosidase/mg protein). Levels of gene expression produced by the mannitol and tertiary amine β-cyclodextrin formulations at the medium dose were also comparable to that of the high dose of vector in PBS (5.5×10^4 and 8.6×10^4 pg β-galactosidase/mg protein). A similar effect was seen when the lowest dose of virus was formulated with the mannitol and blended formulations. Lungs from these animals contained 7,209 ± 23.1 and 8,223 ± 57.3 pg β-galactosidase/mg protein, which was comparable to those of animals that received the medium dose of virus (1×10^{10} particles/ml) in PBS (6,596 ± 51.7 pg β-galactosidase/mg protein). The chitosan formulation did show an increase in transgene expression when compared dose for dose with the PBS formulation, but this was not sufficient to justify a reduction in vector dose.

Effect of Formulation on Cell Membrane Integrity and Tight Junction Formation

To elucidate the mechanism by which formulations enhanced adenoviral transduction efficiency and to assess the immediate toxicity that may be associated with them, we initiated several *in vitro* tests. The first involved incubation of A549 cells with each formulation for a period of two hours. After this time, samples were taken from the cultures and assessed for lactate dehydrogenase (LDH), an intracellular enzyme. The percent lysis was determined as a ratio of LDH for each formulation to LDH from cells treated with a lysis buffer. The chitosan and sucrose formulations were the most toxic, as 48.4% and 39.8% lysis was observed from monolayers treated with these preparations (Fig. 7A). The mannitol and blended formulations were moderately toxic with 18% and 15.5% lysis reported. The remaining formulations were only mildly toxic, producing 9–10% lysis.

Cells that line the airways form tight junctions that are a barrier to adenoviral transduction [5,31]. Many pharmaceutical excipients have been shown to disrupt the tight junctions of differentiated epithelial cells and enhance the bioavailability of drugs that are not easily absorbed. These absorption enhancers are routinely

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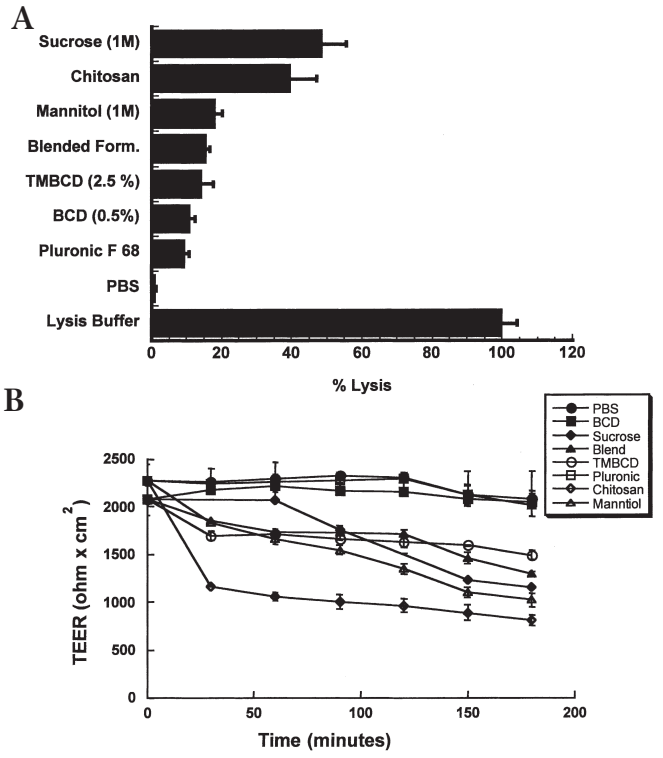

FIG. 7. Successful formulations enhance cell membrane permeability and weaken tight junctions of differentiated epithelial cells. (A) Effect of formulation on the integrity of A549 cells. We added each formulation (2 ml) to A549 cells in 12-well culture dishes. Samples (100 μl) were taken over a period of 2 h and assessed for the presence of lactate dehydrogenase (LDH), an intracellular enzyme. Percent lysis is the ratio of the LDH level from cells tested by each respective formulation to the LDH level of cells treated by a lysis buffer (0.1% Tween 20 in water). Monolayers with values of 10–20% lysis were viable for several days after removal of the formulation at end of the study period. Data are the average values from three monolayers for each formulation. (B) Effect of formulation on the transepithelial electrical resistance (TEER) of differentiated epithelial cells. Caco-2 cells were maintained on Transwell culture inserts for a period of 15 d. At this time, medium was removed from the apical side of the monolayers and formulations added. TEER, an indicator of tight junction integrity, was measured over a period of 3 h. Data are the average measurements from three Transwell cultures for each formulation.

tested on the Caco-2 cell line, differentiated intestinal epithelial cells known to form strong tight junctions. We added the formulations to the apical side of Caco-2 monolayers grown on Transwell culture inserts and measured the transepithelial electrical resistance (TEER) over a period of three hours. The chitosan formulation substantially compromised tight junction integrity, as TEER fell by 1100 ohms × cm^2 within 30 minutes after application of the formulation (Fig. 7B). After this initial decline, the TEER was constant for the remainder of the study period. The tertiary amine β-cyclodextrin, mannitol, and blended formulations were not as disrupting to the monolayer, as they reduced the TEER by approximately 400 ohms × cm^2 within 30 minutes after application. A second distinct drop in TEER of approximately 200 ohms × cm^2 was detected two hours after application in monolayers treated with these formulations. The PBS, β-cyclodextrin, and Pluoronic formulations had no significant effect on the TEER of the differentiated monolayers ($P \leq 0.05$, Student's t test).

DISCUSSION

Our objective was to develop novel formulations to enhance adenoviral-mediated gene expression in the airway epithelium. One of the major findings that can be derived from this work is the lack of a correlation between transduction efficiency of the virus *in vitro* and gene expression levels *in vivo*. The A549 cell line, derived from human non-small cell lung carcinoma, is highly permissive to adenoviral infection [32]. Despite the fact that all formulations were tested with a low dose of adenovirus to detect differences in transduction efficiencies, some formulations that achieved high levels of gene expression *in vitro* did not produce similar results *in vivo*. It is important to note, however, that *in vitro* testing was useful in detecting the toxicity of the sucrose formulation and prevented it from being used in animals. A more appropriate model to assess transduction efficiency may be air-liquid interface cultures of human airway epithelial cells. This model more accurately represents the behavior of polarized, differentiated airway epithelial cells. But these cells are difficult to culture, expensive to maintain, and cannot be routinely maintained in large-scale quantities [33,34]. Additional efforts are currently underway to develop *in vitro* models of the airway epithelium, which can be maintained on a routine basis for rapid screening of novel gene delivery systems to the lung.

The discrepancy between our *in vitro* and *in vivo* data can also be attributed to the unique properties of each excipient. A good example of this is the chitosan formulation. Chitosan, a linear, positively charged polysaccharide, is a mucoadhesive capable of slowing down the rate of mucocilliary clearance [35–37]. Thus, addition of this excipient to adenoviral formulations promotes interaction with the airway epithelium and increases the contact time necessary for viral internalization. This effect was terminated prematurely in the cell culture system as formulation was removed and replaced with complete medium. Airways treated with this formulation demonstrated intense areas of gene expression as well as areas of acute inflammation. Several studies have demonstrated that chitosan is not toxic when applied to the respiratory tract [38,39]. Our toxicity data, however, indicate that this may not be the case, as this formulation substantially compromised cell membrane integrity and disrupted the tight junctions of differentiated monolayers (Fig. 7). This information, in conjunction with the areas of acute inflammation detected in lungs of mice dosed with this formulation, indicates that this formulation may not be suitable for use in gene therapy applications.

Addition of any substance to a drug product can substantially alter the bioavailability of the active compound. We chose excipients for these studies based on their demonstrated ability to enhance drug absorption. Some excipients, however, have been implicated as causing, contributing to, or playing some role in certain human reactions not related to the active compound [40]. Thus, it was imperative that we evaluate the toxicological profiles of our formulations with several *in vitro* assays. The assays that we chose, an enzyme release assay and TEER measurement across polarized epithelial monolayers, not only assessed the effect of each formulation on the overall health of the cell, but provided clues to the mechanisms by which the excipients enhance adenoviral transduction efficiency. Formulations that were the most successful at promoting adenoviral transduction efficiency (mannitol, tertiary amine β-cyclodextrin, and the blended formulation) increased tight junction permeability and weakened cell membranes. These data indicate that both transcellular and paracellular absorption of the virus is enhanced in the presence of the formulations.

So far, most efforts have focused on incorporation of a single, positively charged agent in the viral preparation to enhance binding and subsequent internalization at the target site. Our results indicate that a single entity will not adequately solve all formulation issues associated with the delivery of genetic material to the tissue of interest. Our most successful formulation consisted of a blend of sucrose, mannitol, and Pluronic F68. It is important to note that this formulation was not randomly chosen, but was derived from our screening different blends of sucrose and mannitol for their ability to retain moisture sufficient to preserve viral titer during lyophilization (M. A. C. and J. M. W., manuscript submitted). A hypertonic 1 M solution of sucrose (osmolarity 750 mOsm) effectively dessicated and shrunk cells in culture. Addition of mannitol to the preparation and reducing the amount of sucrose produced a moderately hypertonic solution (osmolarity 375 mOsm) that was well tolerated *in vitro* and *in vivo*. Pluronic F68 functions as a wetting agent in most formulations that promotes direct contact between an active ingredient and the lung epithelium and prevents aggregation of proteins in solution [41]. We saw this effect in our studies, as average particle size of a viral preparation fell from 163.2 ± 30.6 nm to 70.4 ± 6.2 nm (the size of a single viral particle) when Pluronic F68 was added to the formulation as determined by dynamic laser light scattering. We also have shown that this excipient also has slight membrane-disrupting capabilities that promote cellular absorption of viral particles, which was further accentuated in the presence of sucrose and mannitol to produce maximal transduction efficiency with minimal toxicity.

We have developed a formulation that substantially augments adenoviral transduction efficiency to large and small airways of the lung with minimal toxicity. As a result, the amount of virus necessary for sufficient gene expression and the potential for virus-associated toxicity can be reduced by 1/2 log. The lyophilized form of this formulation also enhanced physical stability of the virus at ambient temperature. Although this formulation may also enhance the transduction efficiency of other viral vectors in the lung and other target tissues, each vector and tissue type has different physical characteristics, which may present a unique challenge to the formulation scientist.

Materials and Methods

Materials. Sucrose USP, poly-L-lysine hydrobromide, protamine sulfate (Grade X), chitosan, and D-mannitol USP were purchased from Sigma (St. Louis, MO). β- and 2-hydroxypropyl β-cyclodextrins were also purchased from Sigma (St. Louis, MO). Tertiary amine and quaternary amine β-cyclodextrins were purchased from Cerestar USA, Inc. (Hammond, IN). Pluronic block copolymer F68 was provided by the BASF corporation (Mt. Olive, NJ).

Preparation of adenovirus. First-generation adenoviral vectors expressing *Escherichia coli* β-galactosidase (AdlacZ) under the control of a CMV promoter were amplified in the 293 cell line using a modification of established methods [42]. Virus was purified from cell lysates by banding twice on CsCl gradients followed by desalting on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA) equilibrated with each respective formulation. Concentration of the virus was determined by UV spectrophotometric analysis at 260 nm. All experiments were performed with freshly purified adenovirus stock. The number of plaque forming units (p.f.u.) per ml of stock was determined as described [42].

Adenovirus infection studies. A549 cells (ATCC CCL 185) were seeded at a density of 1×10^4 cells/well in 12-well culture dishes for all infection studies. When monolayers were confluent, medium was removed and 0.1 ml formulation with AdlacZ at an MOI of 20 was added. After incubation at 37°C for 2 h, formulation was removed and replaced with 2 ml culture medium. After 48 h, transduction efficiency was assessed by X-gal staining as described [29].

Cytotoxicity assays. A549 cells were seeded at a density of 10,000 cells/well in 12-well culture dishes for toxicity studies. Two days after seeding, culture medium was replaced with each of the formulations. Samples were taken from the cultures 2 h after addition of formulation. Samples were assessed for lactate dehydrogenase activity by a colorimetric assay (In Vitro Toxicology Assay kit, LDH based, Sigma, St. Louis, MO). Percent lysis was calculated as the ratio of LDH activity in the formulation samples to the LDH activity in samples from cells treated with 0.1% Tween 20 in water.

TEER measurements. Caco-2 cells (ATCC HTB37), a highly characterized differentiated cell line known to form tight junctions similar to those of lung epithelial cells, were seeded at a density of 300,000 cells/well on porous polycarbonate cell culture inserts with a pore size of 0.4 μm (Costar Transwell, Cambridge, MA) and maintained for 15 d. At this time, 1.5 ml of each formulation was added to the apical sides of the monolayers and 3 ml fresh cell culture medium added to basolateral chambers. TEER measurements were taken by an epithelial voltohmeter with a special "chopstick" electrode designed for this purpose (World Precision Instruments, Sarasota, FL) as described [31].

Administration of formulated vector to immunocompetent animals. C57BL/6 (H-2b) mice (6–8 weeks old) were anesthetized with ketamine and xylazine by means of intraperitoneal injection, and the trachea was exposed through a midline incision. Preparations were administered intratracheally at a dose of 5×10^{10} particles in 50 μl of each respective formulation. Four days after administration, animals were necropsied and one lung immersed in OTC compound for cryosectioning. The other lung was treated for direct assessment of β-galactosidase expression by an ELISA assay.

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X-gal histochemistry. Frozen sections (6 μm) were fixed in 0.5% glutaraldehyde and stained for β-galactosidase activity as described [43]. Sections were counterstained with hematoxylin.

β-*Gal assays*. Tissues were excised from euthanized animals and washed twice in cold PBS. When numerous samples were processed, excised tissues were stored for up to 2 h in cold DMEM. Tissues were rinsed in lysis buffer (provided with the β-gal ELISA kit, Boehringer-Mannheim) containing 4 mM Pefablock (Boehringer-Mannheim), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 μg/ml pepstatin, 5 μg/ml aprotinin (Sigma), 1 μg/ml leupeptin, 0.5 mM EDTA, and 0.5 mM dithiothreitol (DTT). Tissues were homogenized in 1 ml lysis buffer using a Brinkman polytron. Following homogenization, extracts were centrifuged at 14,000 rpm for 10 min. The protein concentration of the cleared supernatants was determined by a microplate assay (Bio-Rad DC Protein assay) using bovine serum albumin as a standard. Extracts were quick-frozen in a dry ice/ethanol bath and stored at –80°C until assayed. β-gal concentrations were determined by an enzyme-linked immunosorbent assay (ELISA; Boehringer-Mannheim) according to the manufacturer's instructions.

Statistical analysis. All data are reported as means ± standard deviations. The Student's *t* test was used for the comparison of two means.

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