Beta Cyclodextrins Enhance Adenoviral-Mediated Gene Delivery to the Intestine

Maria A. Croyle,[1,2,5] Blake J. Roessler,[1,3] Cheng-Pang Hsu,[1] Rong Sun,[3,4] and Gordon L. Amidon[1]

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Purpose. In general, the intestinal epithelium is quite refractory to viral and non-viral methods of gene transfer. In this report, various cyclodextrin formulations were tested for their ability to enhance adenoviral transduction efficiency in two models of the intestinal epithelium: differentiated Caco-2 cells and rat jejunum.

Methods. Transduction efficiency of replication-deficient adenovirus type 5 vectors encoded with either the *E. coli* beta-galactosidase or the jellyfish green fluorescent protein gene was assessed by X-gal staining or visualization of fluorescence 48 hours after infection. *In vivo* experiments were performed using an intestinal loop ligation technique.

Results. Several formulations of neutral and positively charged beta cyclodextrins significantly enhanced adenoviral-mediated gene transfer in the selected models. The cyclodextrin formulations studied increased adenoviral transduction in the intestine by enhancing both viral binding and internalization. Viral binding was significantly increased on cell membranes treated with positively charged cyclodextrins, as seen with confocal microscopy and rhodamine-labeled virus. Permeability studies and TEER readings revealed that the most successful formulations gently disrupt cell membranes. This enhances internalization of viral particles and results in increased levels of gene expression.

Conclusions. These formulations can be of value in gene transfer to cells and tissues in which adenoviral infection is limited due to a lack of fiber and α_v integrin receptors. They are simple to prepare and do not affect the ability of the virus to transduce target cells.

KEY WORDS: adenovirus; intestine; gene therapy; cyclodextrins; jejunum.

INTRODUCTION

To date, the intestinal epithelium has been found to be rather refractory to nonviral methods of gene transfer. While some success has been reported in transferring DNA into Caco-2 cells by calcium phosphate coprecipitation with DNA, transfection efficiency is very low and idiosyncratic, an inherent difficulty with this method of gene transfer (1, 2).

Adenoviruses, however, have been shown to infect target cells with exceptionally high frequency in comparison to plasmid-based techniques (3). Not only can these vectors effectively transfer foreign genes to dividing cells, but they also transduce cells that have stopped dividing (such as cardiac and vascular smooth muscle (4) and cells of the central nervous system (5)), and cells that divide slowly (such as synoviocytes (6)). In addition, adenoviruses have been shown to produce large amounts of stable gene products in these cells (7).

From the information stated above, one could conclude that adenoviral vectors would deliver genes to the intestinal epithelium with extreme efficiency as all cell types described above are represented from rapidly dividing cells located in the crypts to the non-dividing enterocytes at the villous tips. However, while these viruses have demonstrated the ability to be potent vectors with promising futures in many tissue and cell types, data concerning their efficiency in transferring genetic material to the intestinal epithelium is quite limited and somewhat speculative (8–11).

We have found that replication-defective adenovirus type 5 vectors can transduce 80% of an undifferentiated enterocyte population with the *E. coli* β-galactosidase (lacZ) gene (12). This dramatically decreases to 0.5% as the cells begin to differentiate. One reason for this phenomenon is the inability of the vector to effectively bind to and enter enterocytes due to a reduction in α_v integrin expression (responsible for internalization) and possibly viral fiber receptors (responsible for viral attachment) on the apical surface of the epithelium (13). These findings suggest that, if adenovirus binding or entry into the cell could be increased, adenovirus-dependent processes subsequent to binding and internalization would continue and facilitate efficient gene transfer. These functions, which include release of DNA from the vector, entry into the nucleus, and transcription, are influenced by specific adenovirus proteins (14). Previous reports have described gene transfer systems that combine viral and non-viral components (8,15,16). In most cases, the transferred 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.

Cyclodextrins have a wide variety of uses in the food, cosmetic, and biotechnology industries. Recently, Hovgaard et al. have shown that cyclodextrin formulations enhance the transport of macromolecules across the intestinal epithelium (17). In this report, the hypothesis that transduction efficiency in enterocytes could be improved by using formulations consisting of either charged or uncharged cyclodextrins (which may facilitate viral binding and entry into enterocytes) and a recombinant adenovirus (which would mediate processes in gene transfer subsequent to viral binding and entry into enterocytes) that contained the cDNA to be expressed was tested. Formulations were assessed *in vitro* and *in vivo* in two models of the intestinal epithelium that have been found to be difficult to transduce with adenoviral vectors alone: differentiated Caco-2 cells and segments of rat jejunum.

MATERIALS AND METHODS

Materials. Tissue culture reagents were obtained from Gibco BRL (Grand Island, NY) and Becton Dickinson (Lincoln

[1] College of Pharmacy, The University of Michigan, Ann Arbor, Michigan 48109-1065.
[2] Current address: Institute of Human Gene Therapy, Department of Molecular and Cellular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
[3] Department of Internal Medicine, Division of Rheumatology, University of Michigan Medical Center, Ann Arbor, Michigan 48109.
[4] Department of Surgery, University of Michigan Medical Center, Ann Arbor, Michigan 48109.
[5] To whom correspondence should be addressed. (e-mail: macroyle@mail.med.upenn.edu)

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Park, NJ). Alpha, beta, gamma, 2-hydroxypropyl beta (mean degree of substitution: 4-10), methyl beta (mean degree of substitution: 10.5–14.7), and Heptakis (2,6-di-O-methyl)- beta cyclodextrins were purchased from Sigma (St. Louis, MO). Sulfated, tertiary amine, and quaternary amine beta cyclodextrins were kindly supplied by Cerestar USA, Inc. (Hammond, IN). Tetramethylrhodamine-5 (and 6) isothiocyanate (TRITC), phosphate buffered saline (PBS), and D-mannitol were purchased from Sigma (St. Louis, MO). 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid (HEPES) was purchased from Boehringer Mannheim (Indianapolis, IN).

Preparation of Adenovirus. Replicative deficient strains of human adenovirus serotype 5 expressing either nuclearly-targeted *E. coli* β-galactosidase or the jellyfish green fluorescent protein (GFP) marker genes under the control of a rous sarcoma virus promoter were amplified in the 293 cell line using a modification of established methods (18). Virus was purified from cell lysates by banding twice on CsCl gradients followed by desalting on a Sephadex G-50 column (Sigma Biochemicals, St. Louis, MO) in PBS (pH 7.4). 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 previously (18).

Adenovirus Infection Studies. Caco-2 cells (ATCC HTB37) of passage 41–45 were seeded at a density of 1×10^5 cells/well in six well culture dishes for all infection studies. When the monolayers became fully differentiated (15 days after seeding), medium was removed and 0.25 ml of cyclodextrin formulation with adenovirus containing the lacZ gene at a multiplicity of infection (moi) of 50 was added. After incubation at 37°C for 2 hours, 2.5 ml maintenance medium was placed on the monolayers and infection allowed to continue for 48 hours, the time at which adenoviral transduction peaks in this cell line. Transduction efficiency of the virus in these formulations was assessed by X-gal staining as described previously (13). Uninfected enterocytes were also assayed for lacZ expression and endogenous levels subtracted from all samples.

TRITC Labeling of Adenovirus Virions. Adenovirus capsid proteins were labeled using modifications of established methods (19). Concentrated fractions of virus in 0.1 M sodium bicarbonate buffer (pH 8.7) were pooled and 20 μl TRITC (2 mg/ml in methanol) were added/milliliter of viral stock. The labeling reaction occurred in the dark at room temperature for four hours. Unconjugated dye was separated from viral particles on a Sephadex G-50 column in PBS (pH 7.4). Fractions were collected and analyzed by spectrophotometry at 580 and 260 nm. Samples containing rhodaminated virus consistently had A_{580}/A_{260} ratios of 0.2–0.3.

Adenoviral Binding Studies. Caco-2 cells were seeded at a density of 4.4×10^4 cells/well in 2 chamber glass cell culture slides (Super Cell, Fisher Scientific, Pittsburgh, PA) and maintained for 15 days. Preparations containing 1×10^3 TRITC-labeled viral particles/cell were placed on the monolayers for 2 hours at 4°C. Monolayers were washed twice with PBS/1 mM sodium azide and fixed with 3% paraformaldehyde (EM Sciences, Fort Washington, PA) in PBS for 30 minutes at 25°C. Samples were assessed with a Bio-Rad MRC 600 confocal microscope at 586/610 nm.

Transepithelial Electrical Resistance (TEER) Measurements. Caco-2 cells were seeded at a density of 100,000 cells/well on porous polycarbonate cell culture inserts with a pore size of 0.4 μm(Costar Transwell, Cambridge, MA) and maintained for 15 days. At this time, 1.5 ml of each cyclodextrin formulation was added to the apical sides of the monolayers and 3 ml DMEM 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 elsewhere (13).

Preparation of Intestinal Loops for Adenoviral Infection. A 3/4 inch midline incision was made in the anterior abdominal wall of anesthetized Sprague-Dawley rats. The jejunum was located and carefully brought to the surface of the cavity. A loop of approximately 7 mm in length was ligated loosely with fine surgical silk. Cyclodextrin formulations containing GFP-adenovirus at a concentration of 2×10^{12} particles/ml were introduced into the proximal end of the loop using a 30 gauge needle affixed to a 1 cc syringe. Care was taken during the surgical procedure to maintain viability of the blood supply and normal peristalsis. Each loop remained outside the peritoneum for 2 hours. The ligation was released and the intact intestine returned to the abdominal cavity of the animal. The abdominal incision was stitched closed and the animal allowed to recover. Twenty-four hours after dosing, animals were euthanized and treated intestinal segments processed for cryosectioning. GFP expression was detected with a Nikon Diaphot-TMD inverted microscope with a fluorescent attachment.

RESULTS

Effect of Cyclodextrin Formulations on Adenoviral Transduction Efficiency in Differentiated Enterocytes

Multiple cyclodextrins were screened for their ability to enhance adenoviral transduction efficiency in differentiated Caco-2 cells. When adenovirus at a moi of 50 was mixed with preparations of 0.5, 2.5 and 5% (w/v) alpha cyclodextrin, 2.9, 4.2, and 9.7% transduction was seen in Caco-2 cells infected 15 days after seeding (Fig. 1a). These are 6, 8, and 19-fold increases from the 0.5% transduction seen in cells infected with the virus alone.

When the same dose of virus was mixed with formulations of 0.1, 0.5, and 1.8% (w/v) beta cyclodextrin, 3.4, 14.4, and 13.1% transduction was seen in differentiated Caco-2 cells (Fig. 1b). These results are greater than those of alpha cyclodextrin with 6.8, 28.8, and 26.2-fold increases in transduction detected.

When 50 moi of adenovirus was added to formulations of 5, 10 and 20% (w/v) gamma cyclodextrin, 3.6, 4.5 and 8.8% transduction was seen in differentiated Caco-2 cells (Fig. 1c). These are 7.2, 9, and 17.6-fold increases from that detected in cells infected with the virus alone.

Effect of Beta Cyclodextrin Derivatives on Transduction Efficiency in Differentiated Enterocytes

Several beta cyclodextrin derivatives were tested to determine if transduction efficiency of the adenovirus in differ-

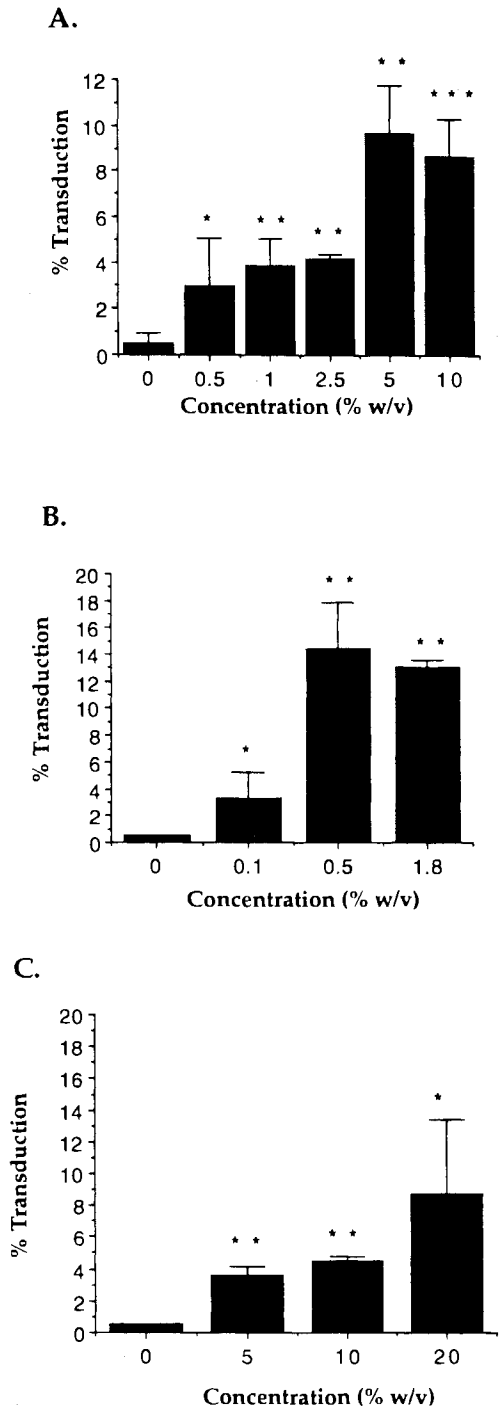


Fig. 1. Cyclodextrin formulations enhance transduction efficiency of adenovirus type 5 in differentiated enterocytes. Caco-2 cells were infected with A) alpha B) beta and C) gamma cyclodextrin formulations containing adenovirus encoding the lacZ gene at a moi of 50 15 days after seeding. Note differences in level of transduction between panels b and c and panel a. Data is the result of three separate experiments (n = 4). Error bars reflect the standard deviation of the data. *p < 0.01, **p < 0.001, ***p < 0.0001 (Student's t-test).

entiated Caco-2 cells could be enhanced further by these compounds. 2-hydroxypropyl beta cyclodextrin was somewhat effective at 2.5 and 5% (w/v) (Fig. 2a). 4.1 and 7.7% transduction was detected for each of the respective formulations resulting in 8.3 and 15.4-fold increases from control samples.

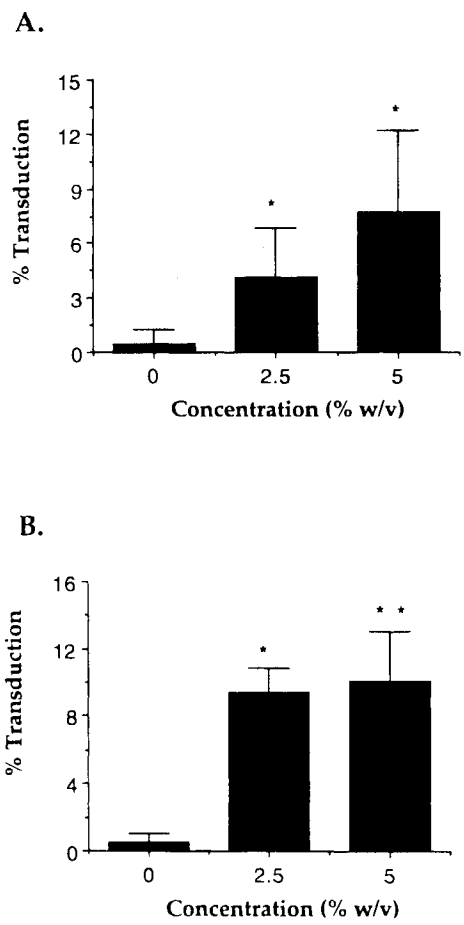


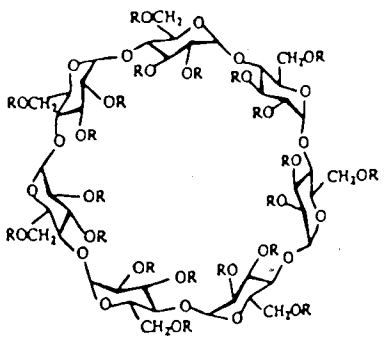
Fig. 2. Effect of beta cyclodextrin derivatives on transduction efficiency of adenovirus type 5 in differentiated enterocytes. Differentiated Caco-2 cells were infected with A) 2-hydroxypropyl and B) methyl beta cyclodextrin formulations containing adenovirus at a moi of 50. Data is the result of two separate experiments (n = 3). Error bars reflect the standard deviation of the data. *p < 0.01, **p < 0.001 (Student's t-test).

Methyl beta cyclodextrin was the most efficient of the derivatives screened at 2.5 and 5% (Fig. 2b). 9.4 and 10.1% transduction was detected for each respective formulation. While these formulations produced 18.7 and 20.2-fold increases in transduction, they did not surpass that of the 0.5 and 1.8% beta cyclodextrin formulations.

Effect of Charged Beta Cyclodextrins on Adenoviral Transduction Efficiency

Various derivatives of beta cyclodextrin were tested to determine if addition of charge to the molecule would influence adenoviral transduction efficiency (Fig. 3). The negatively charged cyclodextrin, sulfated beta cyclodextrin, was the least effective of all cyclodextrins tested (Fig. 3b,d). Formulations of 1, 2.5 and 5% (w/v) produced transduction efficiencies of 2.1, 0.65, and 0.39% respectively. The latter two results were not significantly different from control samples (Student's t-test, p = 0.7). Formulations of the positively charged quaternary amine beta cyclodextrin at 1, 2.5 and 5% (w/v) produced 2.0, 4.9, and 11.6% transduction in differentiated Caco-2 cells for 3.9, 9.8, and 23.2-fold increases in transduction. Tertiary amine

A.



B. R = -$CH_2CH_2SO_3Na$

C. R = -$NH(CH_3)_2$

D. R = -$NH(CH_3)_3Na$

E

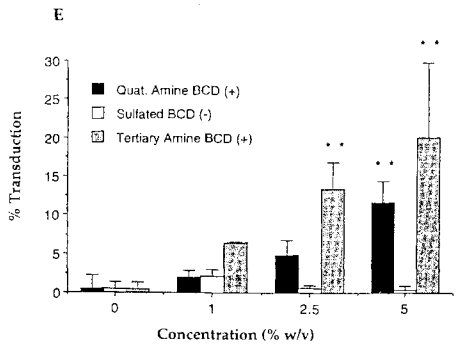


Fig. 3. Chemical structure of A) beta cyclodextrin B) sulfated beta cyclodextrin C) tertiary amine beta cyclodextrin D) quaternary amine beta cyclodextrin E) Effect of charge on the ability of beta cyclodextrin to enhance transduction efficiency of adenovirus type 5 in differentiated enterocytes. Adenovirus at a dose of 50 moi was added to each of the formulations. Data reflects results from triplicate experiments (n = 4). Error bars reflect standard deviations of the data. **p < 0.001 (Student's t-test) .

beta cyclodextrin was the most effective cyclodextrin tested with formulations of 1, 2.5, and 5% (w/v) cyclodextrin demonstrating 6.4, 13.3, and 20.04% transduction respectively. These results superseded those of the parent, beta cyclodextrin, with the 5% cyclodextrin formulation producing a 40-fold increase in transduction.

Effect of Charge on Viral Binding to Differentiated Caco-2 Cells

In order to elucidate a mechanism by which charged cyclodextrin preparations enhanced adenoviral transduction in differentiated Caco-2 cells, preparations containing TRITC-labeled virus were added to monolayers. Viral binding was detected by confocal microscopy. 0.5% beta cyclodextrin significantly enhanced adenoviral binding to the monolayer (Fig. 4b) when compared to control samples where virus was added in the absence of cyclodextrin (Fig. 4a). 5% sulfated beta cyclodextrin prevented the virus from binding to the monolayer (Fig. 4c). Areas of fluorescence were scarce on monolayers treated with this formulation in each of three separate experiments. The 5% tertiary amine beta cyclodextrin formulation greatly enhanced adenoviral binding with 90% of the surface area of monolayers treated with this formulation covered with virus (Fig. 4d).

Pre-Treatment of Caco-2 Cells with Beta Cyclodextrin Formulations

In order to develop dosing schedules that would further enhance adenoviral transduction efficiencies, beta cyclodextrin formulations were added to enterocytes prior to and after the addition of the virus. Transduction in cells pre-treated with the 0.5% formulation was significantly reduced with 1.3% transduction detected while the adenoviral-cyclodextrin combination showed 14.4% (Student's t-test, $p < 0.0001$) (Fig. 5). A similar effect was seen in cells pre-treated with the 1.8% formulation as 1.4% transduction was detected with this schedule. The cyclodextrin-viral combination produced 13.2% transduction. When cyclodextrins were added 2 hours after adenoviral infection, transduction efficiencies were still lower than the original virus-cyclodextrin formulations. These results indicate that the presence of both the virus and the cyclodextrin is critical for the enhancement of adenoviral transduction in differentiated enterocytes and that treatment with cyclodextrin formulations alone will not add to this effect.

Effect of Osmolarity on Adenoviral Transduction in Differentiated Enterocytes

Because the charged cyclodextrins were produced as sodium salts with degrees of substitution of 2–5, high osmolarities associated with these formulations could be responsible for enhanced adenoviral transduction in differentiated monolayers. Osmolarities of cyclodextrin formulations were measured using a Wescor 5500 vapor pressure osmometer (Wescor, Logan, Utah). Mannitol solutions that had similar osmolarities were prepared. A slightly hypertonic solution of 1% mannitol tripled transduction efficiency (Table 1). However, a 2% mannitol preparation did not significantly enhance transduction efficiency further. Five percent manntiol (575 mOsm) produced a 5-fold increase in adenoviral transduction efficiency, but 10% offered no additional enhancement. Cyclodextrin formulations that had osmolarities similar to that of the manntiol preparations produced higher transduction efficiencies in all instances. Thus, the effect of cyclodextrins on transduction efficiencies in differentiated cells can be attributed to mechanisms other than solvent drag of the virus through tight junctions of the monolayer due to high osmolarity of the preparation.

Effect of Cyclodextrins on Transepithelial Electrical Resistance (TEER) Measurements of Caco-2 Monolayers

Drops in TEER measurements of differentiated monolayers were seen as early as one hour after exposure to 1.8% beta cyclodextrin as resistances fell from 1723.3 ohm × cm^2 to 1640.3 (Fig. 6a). After two hours of exposure, a significant drop to 1374 ohm × cm^2 was detected ($p < 0.05$, Student's t-test) to 1374 ohm × cm^2 and remained at this level for the remainder of the study. When the adenovirus was added to the

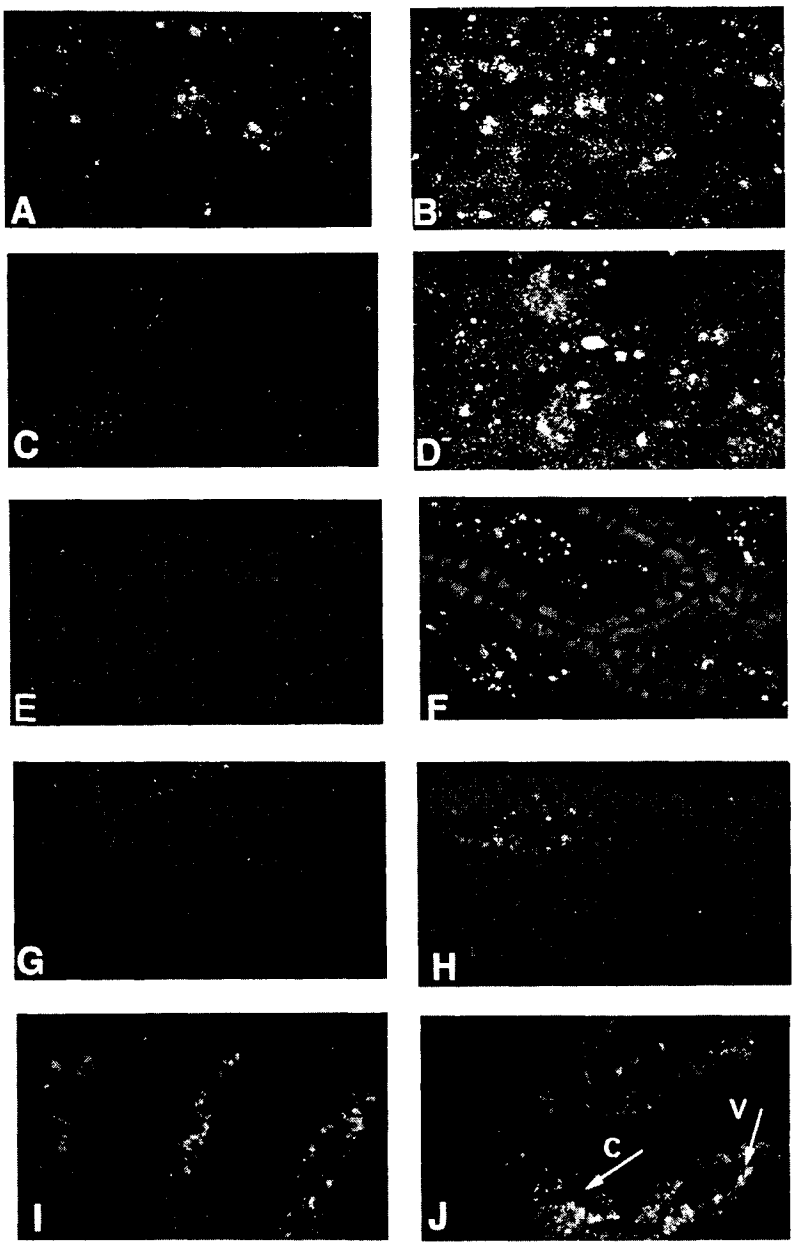


Fig. 4. Positively charged beta cyclodextrins enhance viral binding to the apical surface of differentiated enterocytes and adenoviral-mediated gene transfer in the rat jejunum. A) Confocal micrograph of the apical surface of differentiated Caco-2 cells treated with 1×10^3 TRTIC-conjugated adenoviral particles/ml in PBS (control sample). B) 0.5% beta cyclodextrin significantly enhanced adenoviral binding to cell monolayers. C) 5% sulfated beta cyclodextrin inhibited viral binding. Little fluorescence could be detected in samples from three separate experiments. D) The 5% tertiary amine beta cyclodextrin formulation greatly enhanced viral binding. Approximately 90% of the surface area of the monolayers were coated with virus. In all samples, areas of intense fluorescence represent concentrated areas of viral binding. E) Little autofluorescence was detected in ligated rat jejunum treated with placebo dose of PBS (control segment). F) Gene expression is sparse in sections 24 hours after treatment with 2×10^{12} GFP adenovirus particles/ml in absence of cyclodextrin. G) Slight levels of fluorescence were detected in segments treated with adenovirus containing the lacZ gene in saline. H) Segments treated with 5% sulfated beta cyclodextrin showed some expression of the GFP protein but to a lesser degree than control segments. I) Significant gene expression was seen in the villi of tissue treated with a 05.% beta cyclodextrin adenovirus preparation. J) The most effective formulation *in vitro*, 5% tertiary beta cyclodextrin also demonstrated the highest transduction levels *in vivo*. with concentrated areas of gene expression in the crypts (arrow C) and along the villus axis (arrow V). (magnification of a–d: 100 × e–j: 180 ×).

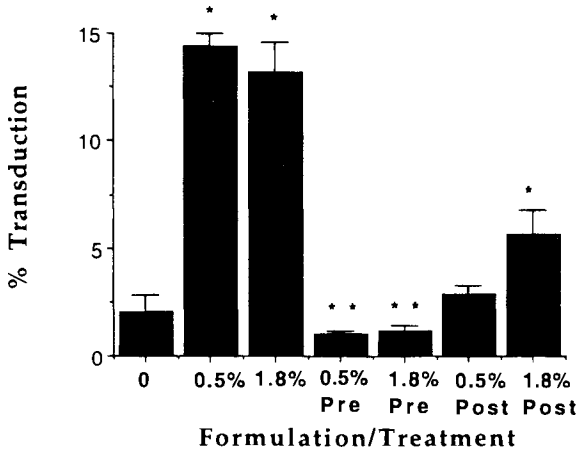


Fig. 5. Pre-treatment and post-treatment of differentiated enterocytes with beta cyclodextrin formulations significantly reduces transduction efficiency of adenovirus type 5. In pre-treatment studies, beta cyclodextrin formulations were added to differentiated Caco-2 cells for 2 hours prior to addition of adenovirus (moi of 100). Infection commenced for 48 hours. Post-treatment studies were performed in the reverse manner. Data reflects the results of two separate experiments (n = 3). Error bars reflect the standard deviation of the data. Pre = pretreated samples; Post = samples treated with cyclodextrin 2 hours after addition of adenovirus. *$p < 0.01$, **$p < 0.0001$ (Student's t-test).

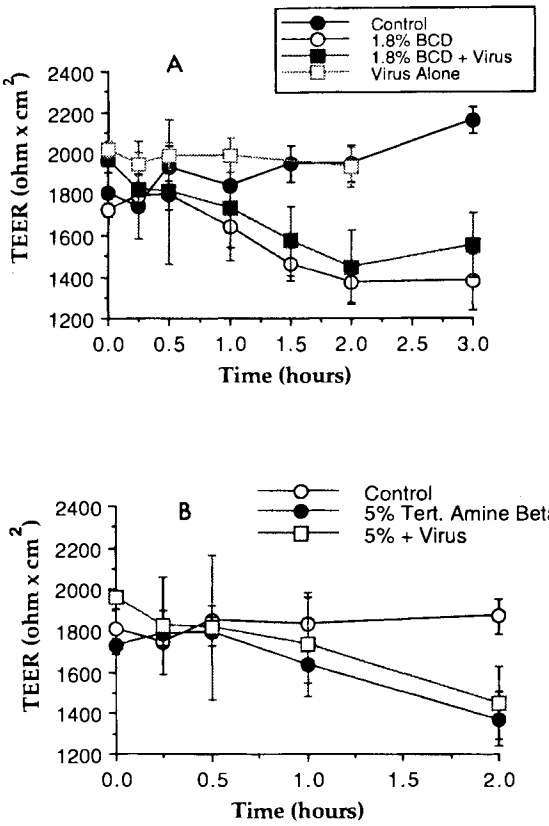


Fig. 6. Effect of beta cyclodextrin formulations on the transepithelial electrical resistance (TEER) of differentiated Caco-2 cells. When A) 1.8% beta cyclodextrin and B) 5% tertiary amine beta cyclodextrin formulations were added to monolayers, significant decreases in TEER were detected. Data is the result of two separate experiments (n = 3). Error bars reflect the standard deviation of the data.

remainder of the study. When the adenovirus was added to the preparation, similar TEER profiles were generated. The 5% tertiary amine beta cyclodextrin formulations showed similar results (Fig. 6b).

Effect of Adenovirus-Beta Cyclodextrin Formulations in Rat Jejunum

In order to assess the ability of cyclodextrin formulations to increase adenoviral-mediated gene delivery *in vivo*, rat jejunum was ligated and formulations containing adenovirus encoding GFP injected locally. Little autofluorescence was detected in control samples treated with a saline bolus (Fig. 4e). When adenovirus was administered in PBS, transduction levels were low with sparse patches of fluorescence located in only a few villi. Figure 4f represents the only positive section found after initial screening of 30 sections. In order to determine if intestinal cells are induced to autofluoresce as a result of adenoviral infection *in vivo*, animals were treated with adenovirus containing the lacZ gene in PBS. Slight levels of green fluorescence were detected in tissues treated with this virus (Fig. 4g) but were much lower than those seen in tissue treated with the GFP virus. 5% sulfated beta cyclodextrin produced some transduction of the GFP gene in the rat jejunum (Fig. 4h), but levels were similar to those seen with the PBS preparation. The 0.5% beta cyclodextrin formulation produced marked GFP expression *in vivo* with concentrated areas of expression in all villi of the jejunum (Fig. 4i). The most effective formulation *in vitro*, 5% tertiary amine beta cyclodextrin, also demonstrated the highest transduction levels *in vivo* (Fig. 4j). Concentrated areas of transgene expression were located near intestinal crypts (arrow C) and along the villus axis (arrow V).

Table 1. Effect of Osmolarity on Adenoviral Transduction Efficiency in Differentiated Caco-2 Cells

Formulation	Osmolarity (mOsm)	% Transduction (± S. D.)
DMEM	290	1.1 (± 0.2)%
0.5% Beta CD	296	14.4 (± 3.5)%
1.8% Beta CD	300	13.1 (± 0.5)%
1% Mannitol	345	2.9 (± 0.4)%
2% Mannitol	409	3.6 (± 0.6)%
5% Sulfated BCD	502	0.5 (± 0.4)%
5% Mannitol	575	5.0 (± 0.7)%
5% Quaternary BCD	595	11.6 (± 2.7)%
5% Tertiary Amine BCD	817	20.0 (± 9.2)%
10% Mannitol	862	5.2 (± 0.1)%

Note: Osmolarities of 290–300 mOsm are considered to be isosmotic.

DISCUSSION

Interaction of virus with Caco-2 cells could be enhanced by cyclodextrins at two stages in the adenoviral replication cycle: viral attachment and internalization. Even though most phases of adenovirus infection are well understood (20), little is known about the potential implications of charge interactions for cell binding and entry of adenoviruses. The adenovirus

carries a net negative charge density on its capsid originating from multiple runs of acidic residues on hexon and fiber proteins (21). Arcasoy et al. have found that removal of negatively charged sialic acid residues from cultured, polarized epithelial cells significantly improves the efficiency of adenovirus-mediated gene transfer to these cells (22). In the studies reported here, sulfated beta cyclodextrin did not significantly influence adenoviral transduction efficiency (Figs. 3 & 4). This could be due to static repulsion between the virus, the cyclodextrin, and residues on the cell membrane. This type of interaction has been reported to occur between human cytomegalovirus (CMV), human immunodeficiency virus (HIV), sulfated carbohydrates, and various cellular targets (23, 24).

Confocal microscopy studies with positively charged cyclodextrins provided additional evidence that viral attachment is enhanced by the presence of these compounds (Figs. 3 & 4). While the quaternary amine beta cyclodextrin formulations significantly enhanced gene transfer to differentiated enterocytes, they did so only to the level of that of the parent compound. Tertiary amine beta cyclodextrin, however, greatly enhanced transduction to a level that surpassed that of the parent compound. This cyclodextrin does not carry as strong of a positive charge as the quaternary amine derivative. Instead, it has a slightly positive dipole moment dispersed over the entire molecule, which may assist in effective removal the negative charge of the plasma membrane. The presence of three bulky ethyl groups associated with the amine nitrogen of the quaternary amine derivative may be responsible for lack of additional transduction efficiency because they physically prevent direct interaction with the cell membrane.

Toxicity studies have shown that cyclodextrin formulations which enhance adenoviral-mediated gene transfer permeabilize the cell membrane (data not shown). Additional studies have revealed that these formulations significantly reduce transepithelial electrical resistance measurements (Fig. 6). Thus, low pre and post treatment transduction levels could be attributed to i) inefficient delivery of the viral genome to the nucleus due to the inability of the virus to shed its protein coat in the cytoplasm in the absence of the change in pH experienced in endocytotic vesicles and ii) cells pre or post treated with these formulations were significantly damaged from extended contact with the concentrated cyclodextrin.

Cyclodextrins enhance bioavailability of poorly water soluble compounds because of their ability to entrap the hydrophobic molecules within their hydrophilic shells. Thus, the question arises as to whether cyclodextrins form complexes with the virus as a means of enhancing gene transfer. There was no correlation between cyclodextrin core diameter size and transduction efficiency. The cyclodextrin with the largest core diameter size, gamma cyclodextrin, was the least effective of all preparations tested at high concentrations (Fig. 1c). Direct interaction of a single adenovirus particle with the interior core of a single cyclodextrin molecule is physically impossible because the viral diameter is 70 nm (25). The diameters of beta and gamma cyclodextrins are 0.78 and 0.95 nm respectively. Adenovirus fiber proteins cannot even interact directly with the tiny interior core of these carbohydrates because the shaft diameter is 2.8 nm wide (26). Studies of strict particle:particle ratios of adenovirus:cyclodextrin showed no significant effect on adenoviral gene transfer (data not shown). Successful formulations were those in which cyclodextrin was in excess with respect to adenoviral particles by a factor of $1:1 \times 10^8$.

Particle size data indicates that beta cyclodextrin formulations at these concentrations interact with the virus regardless of charge (data not shown). Adenovirus suspensions had a mean particle diameter of 108 nm, a value slightly above that for single viral particles, indicating that aggregation occurs among virions. When cyclodextrin was added, mean particle sizes were similar in all cases to that of a single adenoviral particle. Thus, we can conclude that, beta cyclodextrins act as viral dispersants. They produce suspensions of single adenoviral particles which increase the number of virions that come in contact with enterocytes and, as a result, increase transduction efficiency.

Levels of gene expression in the rat correlated well with *in vitro* data (Fig. 4). It is important to note that in all cases described here, transgene expression is located in the lamina propria and not in the enterocytes that line the intestinal epithelium—the area originally thought to be the prime target for gene delivery to the intestine. These results present considerable questions about the mechanism of adenoviral gene transfer in the intestine as they are somewhat contradictory of those reported by others in the literature where expression of the lacZ gene was localized in epithelial cells at the brush border and in intestinal crypt areas (10,11). We believe that results obtained by these investigators are somewhat speculative due to the presence of high concentrations of microflora (and resultant high levels of endogenous lacZ gene expression) in the intestine—especially in the colon. For this reason, we selected the jellyfish green fluorescent protein (GFP), a marker not present in any amount in the intestine, to study gene transfer to the jejunum and are currently typing the cells in the lamina propria that express the transgene with antibodies against certain cell surface markers.

The formulations described here have a number of advantages. They can be of value in gene transfer to cells and tissues in which adenoviral infection is limited due to a lack of fiber and α_V integrin receptors. Positively charged formulations may also enhance gene transfer with plasmid cDNA and certain protocols for efficient gene transfer using this type of vector are currently under development in our laboratories. However, cyclodextrins may not offer any advantage to cells that are already easily infected by adenovirus, such as hepatocytes. Because of their non-specific nature, these formulations may be useful for tissues in which the vector can be selectively applied to target cells such as airway surface epithelia. These formulations are simple to prepare and do not affect the ability of the virus to infect and transduce target cells.

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