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Transduction by Adeno-Associated Virus Vectors in the Rabbit Airway: Efficiency, Persistence, and Readministration

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The ability of recombinant adeno-associated virus (AAV) vectors to integrate into the host genome and to transduce nondividing cells makes them attractive as vehicles for gene delivery. In this study, we assessed the ability of several AAV vectors to transduce airway cells in rabbits by measuring marker gene expression. AAV vectors that transferred either a β-galactosidase (β-gal) or a human placental alkaline phosphatase (AP) gene were delivered to one lobe of the rabbit lung by use of a balloon catheter placed under fluoroscopic guidance. We observed vector-encoded β-gal or AP staining almost exclusively in the epithelial and smooth muscle cells in the bronchus at the region of balloon placement. The overall efficiency of transduction in the balloon-treated bronchial epithelium was low but reached 20% in some areas. The majority of the staining was in ciliated cells but was also observed in basal cells and airway smooth muscle cells. We observed an 80-fold decrease in marker-positive epithelial cells during the 60-day period after vector infusion, whereas the number of marker-positive smooth muscle cells stayed constant. Although treatment with the topoisomerase inhibitor etoposide dramatically enhanced AAV transduction in primary airway epithelial cells in culture, treatment of rabbits did not improve transduction rates in the airway. Vector readministration failed to produce additional transduction events, which correlated with the appearance of neutralizing antibodies. These results indicate that both readministration and immune modulation will be required in the use of AAV vectors for gene therapy to the airway epithelium.

The lungs are important targets for gene therapy because lung pathology is the primary cause of morbidity in cystic fibrosis (CF), which affects ~1 in 3,000 Caucasian births. Vectors based on viruses (7, 13, 34) as well as plasmid DNA (3, 22) have been investigated for CF gene therapy. Of the viral vectors, retroviral vectors were used in initial studies to successfully transfer a normal CF transmembrane regulator (CFTR) cDNA to correct the CF defect in cultured cells (7). These vectors are devoid of viral regulatory and structural genes that can elicit an immune response, and their ability to integrate into the host chromosome can result in persistence of gene expression. However, the requirement of commonly used retroviral vectors for cell replication to achieve efficient transduction (30) predicts a poor performance in the lungs, where the cellular proliferation rate is low. Indeed, although retroviruses can infect progenitor airway epithelial cells efficiently in vitro, infection was inefficient in the mature airway epithelium in a xenograft model (8) and in the adult rabbit airway (20). In contrast to retrovirus vectors, adenovirus vectors (34, 42) can infect nondividing cells relatively efficiently, but transduction is lost rapidly due to the clearance of transduced cells by immunity generated to viral capsid proteins produced by these vectors (41). Additionally, adenovirus vectors do not efficiently infect the columnar and more differentiated epithelial cells of the airway (18).

Vectors based on the adeno-associated virus type 2 (AAV) have also been evaluated for CF gene therapy. AAV is a nonpathogenic parvovirus having a single-stranded DNA genome of 4.7 kb (32). AAV requires helper functions provided by adenovirus for efficient replication and transcription. Like adenovirus, AAV is naturally tropic for the airway epithelium. Vectors based on AAV can be made that integrate (26, 27, 29, 38) and are capable of transducing a variety of cell types (14, 16, 19, 21, 23, 25). Furthermore, the low transduction rate in primary and nondividing cells by AAV vectors can be increased by a variety of agents such as DNA-damaging agents (1, 2, 25), topoisomerase inhibitors (36), and the adenovirus genes encoded in early regions 1 and 4 (9, 11).

The normal adult rabbit lung has been used as a model for CF gene therapy with AAV vectors. Bronchoscopic administration of an AAV vector containing a human CFTR cDNA resulted in efficient gene transfer to certain areas of the normal adult rabbit lung (13). The expression of human CFTR (by antibody staining) and the persistence of expression (by reverse transcriptase PCR) was detected for up to 7 months. In addition, AAV transduction in the developing neonatal rabbit lung has been observed in a variety of airway and alveolar cell types, but expression was analyzed for only up to 10 days after vector administration (44). These studies indicate that AAV vectors may have utility in CF gene therapy, but there is a need for more quantitative data regarding rates and persistence of gene expression in the airway epithelium.

We initially attempted to quantitate transduction efficiency and persistence of gene expression using the AAV-CFTR vector SA306 used previously (13) but were unable to do so due to the low level of CFTR expression from this vector and problems with antibody specificity for human CFTR in a rabbit background. Therefore, it was necessary to assess transduction by a more direct approach. AAV vectors that transferred ei-

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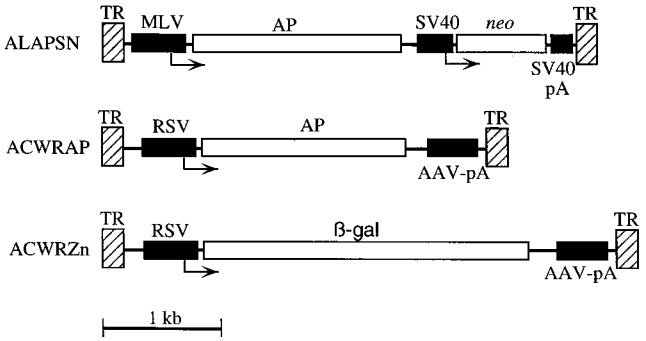

FIG. 1. AAV vectors. Arrows indicate promoters, open boxes indicate coding regions, and filled or hatched boxes indicate noncoding regions. Abbreviations: TR, AAV terminal repeat; MLV, Moloney murine leukemia virus long terminal repeat; RSV, Rous sarcoma virus long terminal repeat; pA, polyadenylation signal; *neo*, neomycin phosphotransferase gene.

ther the β-galactosidase (β-gal) or the human placental alkaline phosphatase (AP) gene were delivered to rabbit lungs by using a balloon catheter. Histochemical staining for marker protein activity was used in order to circumvent problems of nonspecificity and variability in antibody staining. We found that AAV vector transduction rates could be quite high in some localized areas of the airway epithelium but was low overall. Efforts to increase transduction rates by treatment with etoposide was successful in cultured epithelial cells but did not improve transduction in vivo. The smooth muscle population in the airway exhibited persistent gene expression, whereas the epithelial population did not. Readministration of vector failed to generate further transduction events, and this result was correlated with the appearance of neutralizing antibodies directed against AAV proteins.

MATERIALS AND METHODS

Cell culture. The 293 and IB3 cells (17, 43) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS)–100 U of penicillin per ml–100 μg of amphotericin B per ml at 37°C in a 10% CO_2-air atmosphere. Primary cells derived from the nasal polyps of CF individuals were isolated as previously described (19). Cells were grown in keratinocyte growth medium (KGM) supplemented with growth factors provided by the manufacturer (Clonetics, San Diego, Calif.).

AAV vector construction and production. The recombinant AAV vectors are shown in Fig. 1. ALAPSN (previously called AAV-LAPSN) has been described elsewhere (35) and contains a human placental AP cDNA (4) that is expressed from a Moloney murine leukemia virus promoter and a neomycin phosphotransferase cDNA that is expressed from a simian virus 40 promoter. ACWRAP (previously called CWRAP [12]) containing the AP cDNA driven from a Rous sarcoma virus promoter and enhancer sequence was obtained from S. Chatterjee (City of Hope National Medical Center, Duarte, Calif.). The vector ACWRZn was constructed by digestion of pD16.43 (10) with *Stu*I and *Sma*I restriction enzymes. The released fragment encoding the nuclear-localizing β-gal gene was ligated to *Xho*I linkers. After digestion with *Xho*I restriction enzyme, the fragment was inserted into ACWRAP previously digested with *Sal*I to release the AP cDNA.

Recombinant AAV plasmids were propagated in the bacterial strain JC8111 (5). Vector stocks were generated as follows. On day 1, 293 cells were plated at 5×10^6 cells per 10-cm-diameter dish in 10 ml of DMEM containing 10% heat-inactivated FBS and antibiotics. The following day, the cells were infected with adenovirus type 5 at a multiplicity of infection of five. Two to 4 h later, vector plasmid (4 μg) and an AAV helper plasmid, pAAV/Ad (12 μg) (38), were cotransfected by the calcium phosphate transfection method. Under these conditions, the cells exhibited maximal lysis at 72 h and the lysed cells and supernatant were collected and subjected to three rounds of freezing and thawing in an ethanol dry-ice bath to completely disrupt the cells. The crude cell lysates were clarified by centrifugation at 6,000 × *g* for 30 min, followed by treatment with micrococcal nuclease (Pharmacia) (30 U/ml) at 37°C for 30 min. Routinely, cell lysates from 100 plates were pooled and concentrated by using a hollow fiber concentrator (CH2PR concentrator and S1Y100 cartridge; Amicon, Beverly, Mass.). One liter of cell lysate was reduced to 200 ml. After concentration, 20 ml of crude virus stock was placed over a 15-ml cushion of 40% sucrose in phosphate-buffered saline (PBS) and centrifuged at 27,000 rpm in a Beckman SW28 rotor at 5°C for 17 h. Virus pellets were resuspended in a total of 20 ml of CsCl (1.37 g/ml) and banded by centrifugation at 35,000 rpm for 24 h in a Beckman SW55 rotor. The AAV vector fraction was found below the adenovirus band, about two-thirds of the way from the top of the tube. Vector fractions were dialyzed in Ringer's solution using a 50,000 molecular weight cutoff membrane (Spectrum). Residual adenovirus in the stock was heat inactivated at 56°C for 1 h. Approximately 10 ml of vector stock was obtained from 100 10-cm dishes. Vector stocks were stored at −80°C prior to use in animals.

Characterization of vector stocks. The titers of cesium chloride-purified AAV vector stocks were determined with IB3 cells as targets for infection. IB3 cells were plated onto 12-well dishes at 5×10^4 per well. The next day the cells were refed with 1 ml of medium containing various dilutions of virus. The cells were fixed and stained for AP or β-gal 3 days later. The titer was expressed as AP-positive or β-gal-positive focus-forming units (FFU) per milliter. ACWRAP, ALAPSN, and ACWRZn routinely gave titers of approximately 5×10^7, 2×10^7, and 2×10^5 FFU/ml, respectively. For animal experiments, stocks of ACWRZn were further concentrated by using Centricon 100 filters (Amicon, Beverly, Mass.).

Southern analysis was also done on virus stocks to determine the number of genome-containing particles. Approximately 10^6 AP-positive FFU of ALAPSN or ACWRAP or 10^4 β-gal-positive FFU of ACWRZn was incubated with 100 U of DNase (Gibco, Gaithersburg, Md.) in a total volume of 150 μl for 15 min at 37°C. The DNase was inactivated at 68°C for 10 min after the addition of 1.5 μl of 0.5 M EDTA and 10 μg of tRNA. The solution was incubated at 37°C for 3 h after the addition of 50 μl of TE (10 mM Tris [pH 8], 1 mM EDTA), 2 μl of 10% sodium dodecyl sulfate, and 2 μl of proteinase K (20 mg/ml). Virion DNA was obtained by extraction with phenol-chloroform and precipitated using 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate. The virion DNA pellet obtained after centrifugation was resuspended in 50 μl of TE for 15 to 30 min at 45°C, and 10 to 20 μl was electrophoresed on a 1% neutral agarose gel along with plasmid DNA controls. DNA was transferred onto Hybond N+ membrane (Amersham International, Amersham, United Kingdom) according to the manufacturer's protocol for alkaline transfer; a probe containing AP cDNA was used for ALAPSN and ACWRAP, and one containing the β-gal gene was used for ACWRZn. Probes were labeled with ^{32}P by using the random priming labeling kit (Boehringer Mannheim, GmbH, Mannheim, Germany). Phosphorimager analysis was done on the hybridized single-stranded virion DNA band and compared to those of control DNA. A typical AAV vector stock contains approximately 10^{10} particles per ml of vector. The particle-to-infectivity ratio (as determined by FFU in IB3) of cells was approximately 500 for ALAPSN and ACWRAP and 100,000 for ACWRZn.

Generation of AAV vector by cotransfection of AAV vector plasmids and the AAV helper plasmid pAAV/Ad (38) produced *rep*/*cap*-positive AAV due to recombination of the plasmids used in transfection. This occurred with all combinations of AAV vector plasmids and pAAV/Ad so far. Therefore, parallel gels of virion DNA were run with control plasmid DNA that contained the AAV *rep* and *cap* genes. These blots were probed with a ^{32}P-labeled DNA fragment containing the AAV *rep* and *cap* genes. Results showed that the number of *rep*/*cap*-positive genome-containing particles was about 1% of the AAV vector particle number for ALAPSN and 5% for ACWRAP and ACWRZn. For example, a stock of ALAPSN had a titer of 2×10^7 AP-positive FFU/ml and contained 10^{10} genome-containing particles per ml of the AAV vector and 10^8 genome-containing particles per ml of the *rep*/*cap*-positive AAV.

The *rep*/*cap*-positive AAV was tested for its ability to replicate in the presence of adenovirus. 293 cells were plated in 96-well dishes at 3×10^4 cells per well. The following day, cells were infected with dilutions of the vector stock in the presence or absence of adenovirus (multiplicity of infection, 30). Controls included uninfected cells and cells infected with adenovirus only. The medium was gently removed 26 to 30 h later, and 100 μl of PBS containing 12.5 mM EDTA was added. The cells dislodged within 5 min at room temperature, and then the dish was placed on ice. Cells were gently pipetted into 5 ml of PBS and aspirated onto Hybond N membrane (Amersham International). The membranes were processed using a standard colony lift protocol (28) and hybridized to ^{32}P-labeled DNA fragment containing the AAV2 *rep* and *cap* genes. The ratio of genome-containing particles to AAV replication-positive cells was approximately 100:1 for the replication-competent AAV generated in the stocks. Wild-type AAV2 (wt-AAV) generated from transfection of pAAV2 also gave a similar ratio of particles to positive cells (37).

Etoposide treatment of primary CF cells. Primary cells derived from the nasal polyps of CF individuals (at passage 1 through passage 3) were plated in 12-well dishes at 5×10^4 cells per well in KGM medium. The following day, the cells were refed with KGM containing 3,000 AP-positive FFU of ACWRAP, 3,000 AP-positive FFU of ALAPSN, or 200 β-gal-positive FFU of ACWRZn. Etoposide at 0, 20, or 40 μM (Sigma, St. Louis, Mo.) was also added to the medium. In the experiment including wt-AAV, it was added to the inoculum at a particle ratio of 10, 100, and 1,000 (particle of wt:particles of AAV vector; particles determined by Southern analysis). Triplicate wells per vector per etoposide concentration were used. After a 16- to 18-h exposure to vector in the presence or absence of etoposide (and in the presence or absence of added wt-AAV), the medium was removed and the cells were washed with 1 ml of PBS and refed with

TABLE 1. Marker gene expression in rabbit bronchus 7 days after AAV vector administration

Histochemical stain	Vector	wt-AAV	No. of animals	Smooth muscle transduction[a]	Total marker-positive epithelial cells (±SEM)	Transduction rate (±SEM) (%)[b]
AP	Saline control	None	6	−	≤3	≤0.0001
	Vector control[c]	High	2	−	≤3	≤0.0001
		Low	2	−	≤3	≤0.0001
	ALAPSN	High	4	+	≤3	≤0.0001
	(10^7 FFU)	Low	3	++	808 ± 55	0.02 ± 0.00
	ACWRAP	High	5	+	700 ± 490	0.02 ± 0.01
	(10^7 FFU)	Low	7	+++	5,000 ± 1,100	0.17 ± 0.04
β-gal	Vector control[d]	High	2	−	≤3	≤0.0001
		Low	2	−	≤3	≤0.0001
	ACWRZn	High	2	−	6	0.0002
	(10^6 FFU)	Low	6	++	3,700 ± 1,100	0.10 ± 0.07

[a] −, no AP- or β-gal-positive smooth muscle cells were detected; +, 1 to 10 cells were detected; ++, 11 to 100 cells were detected; +++, greater than 100 cells were detected.

[b] Transduction rates were determined as described in Materials and Methods.

[c] 10^9 particles of a non-AP-expressing AAV vector containing high levels of wt-AAV and 10^6 FFU of ACWRZn containing low levels of wt-AAV were used in two animals each and stained for AP 7 days postinfection.

[d] 10^7 FFU of ALAPSN containing high levels of wt-AAV and 10^7 FFU of ACWRAP containing low levels of wt-AAV were used in two animals each and stained for β-gal 7 days postinfection.

1 ml of KGM medium. Cells were stained for AP or β-gal approximately 48 h later.

AP and β-gal staining of cultured cells. Cells were fixed in PBS containing 3.7% formaldehyde for 30 min followed by washing three times (15 min each) in PBS, and endogenous AP activity was heat inactivated at 68 to 70°C for 1 h. Afterwards the cells were stained for AP by incubation overnight at room temperature in AP reaction buffer (100 mM Tris [pH 8.5], 100 mM NaCl, 50 mM $MgCl_2$, 1 mg of nitroblue tetrazolium per ml, and 0.1 mg of X-phosphate per ml [Sigma]). For β-gal staining, cells were fixed in 3.7% formaldehyde for 15 min, rinsed three times with PBS, and stained in β-gal reaction buffer [25 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 25 mM $K_3Fe(CN)_6$, 25 mM $MgCl_2$, and 1 mg/ml X-Gal (Boehringer Mannheim)] for 4 h at 37°C and then overnight at room temperature.

AAV vector delivery to rabbit airways. The experimental procedures in animals were approved by the University of Washington Animal Care Committee. Adult New Zealand White rabbits (3 kg) were sedated by intramuscular injection of 35 mg of ketamine per kg–35 mg of xylazine per kg and intubated with a 3.5-mm-inner-diameter silicone endotracheal tube (Bivona Inc., Gary, Ind.). A 5 French (2-mm-diameter) balloon-tipped monitoring catheter (Baxter Healthcare Corp., Irvine, Calif.) was inserted and wedged in the left lower lobe bronchus under fluoroscopic guidance. With the animal breathing room air, the balloon was inflated with 0.3 ml of air to isolate the distal lung segment and secure the catheter placement; 2 ml of saline or AAV vector (ACWRAP, ALAPSN, or ACWRZn) was instilled. The catheter was left in place, and the animal was placed on its left side under a radiant heat source, the body temperature was monitored, and two half doses of sedation were given 30 min apart. The catheter and endotracheal tube were removed 60 to 90 min after instillation. The animals were returned to their cages when awake. There was no sign of tachypnea, stridor, or respiratory distress after recovery from anesthesia.

If the animal received an additional vector exposure on the contralateral side, the same protocol was followed, except that the animal was placed on its right side after instillation. If the animal received etoposide, it was administered immediately after the vector instillation while the animal was still sedated. Etoposide (15 mg of Vepesid/kg; Bristol Laboratories, Princeton, N.J.) was diluted in a total of 50 ml of 0.9% NaCl. The etoposide solution was infused into the ear vein at a rate of 100 ml/h.

AP and β-gal staining of rabbit lungs. Animals were sedated with an intravenous injection of sodium pentobarbital (50 mg/ml) and blood samples were taken for virus neutralization assays. After an intravenous injection of 300 U of heparin/kg, the animals were given an overdose of pentobarbital and the chest was opened and the pulmonary artery was catheterized. The lungs were perfused in situ with 100 ml of heparinized saline at 25 cm of H_2O pressure, followed by 100 ml of fixative (PBS containing 3.7% formaldehyde for AP staining or PBS containing 2% glutaraldehyde and 2% formaldehyde for β-gal staining). The lung was then dissected from the chest cavity, an endotracheal tube was inserted into the trachea, and fixative at 25 cm of H_2O pressure was instilled into and drained from the lungs three times. After the final filling, the trachea was ligated and the lungs were immersed in fixative overnight for the AP staining procedure or 1 h for the β-gal staining procedure. After fixation, the lungs were drained and refilled with PBS three times, cut into 3-mm-thick slices, and rinsed three additional times with PBS for 1.5 h each on a rocker. The tissue slices were stained in β-gal staining buffer overnight at room temperature. For AP staining, the tissue slices were placed in 50-ml conical tubes containing 25 ml of PBS and heated in a water bath at 70°C for 2 h. The tissue slices were then stained in AP staining buffer overnight at room temperature.

Quantitation of the overall transduction efficiency in the bronchial epithelium. The transduction efficiency was derived by dividing the total number of marker-positive cells by the estimated number of epithelial cells in the exposed bronchus. This is the value shown in Table 1 under the transduction rate. The estimated number of cells was derived by multiplying the calculated surface area in the bronchus (e.g., visible for analysis by the dissecting scope) by the mean density of cells per square millimeter (33). The surface area was derived by multiplying (the average circumference of the bronchus) by (the length of bronchus that was visible for quantitation). This value was approximately 115 mm^2. The average number of epithelial cells at this airway generation number is 180 per mm or 3.2×10^4 per mm^2. Therefore, the denominator is 3.7×10^6 cells (115 $mm^2 \times 3.2 \times 10^4$ cells per mm^2).

Maximum transduction rates were obtained from focal areas of intense staining in the bronchial epithelium. Serial sections were examined from two tissue samples from two animals for ACWRAP. A total of 28 sections was analyzed, totaling 10^4 cells. Serial sections from one tissue sample from one animal were examined for ACWRZn. A total of 10 sections was analyzed, totaling 2×10^3 cells. Rates were determined by dividing the number of positive epithelial cells by the total number of cells in the epithelium, expressed as a percentage.

Virus neutralization assay. Serum samples from rabbits were incubated at 56°C for 30 min to inactivate complement. ACWRZn was diluted in DMEM (containing 1% FBS) to obtain 1.5×10^3 β-gal-positive FFU (as determined on IB3 cells and equivalent to approximately 1.5×10^8 genome-containing particles of ACWRZn) per 100 μl of medium. Heat-treated serum (10 or 2 μl) was added to 200 μl of diluted virus to achieve a 1:20 or 1:100 dilution of serum, respectively. The virus and serum mixtures were incubated for 1 h at 37°C. Then 1 ml of DMEM containing 5% FBS was added to each sample, and each sample was split between two wells containing IB3 cells plated at 5×10^4 cells per well (12-well plates) the previous day. After a 60-min incubation at 37°C in a 5% CO_2 incubator, another 1 ml of DMEM containing 5% FBS was added to each well. Two days after infection, cells were fixed for 15 min in 3.7% formaldehyde in PBS, washed three times in PBS, and stained for β-gal-positive cells by incubation in β-gal staining buffer for 4 h at 37°C followed by overnight incubation at room temperature.

RESULTS

AAV vectors can transduce epithelial and smooth muscle cells in the bronchus, but transduction is inhibited by replication-competent AAV. AAV vectors encoding human placental AP (ALAPSN and ACWRAP) or a bacterial β-gal protein

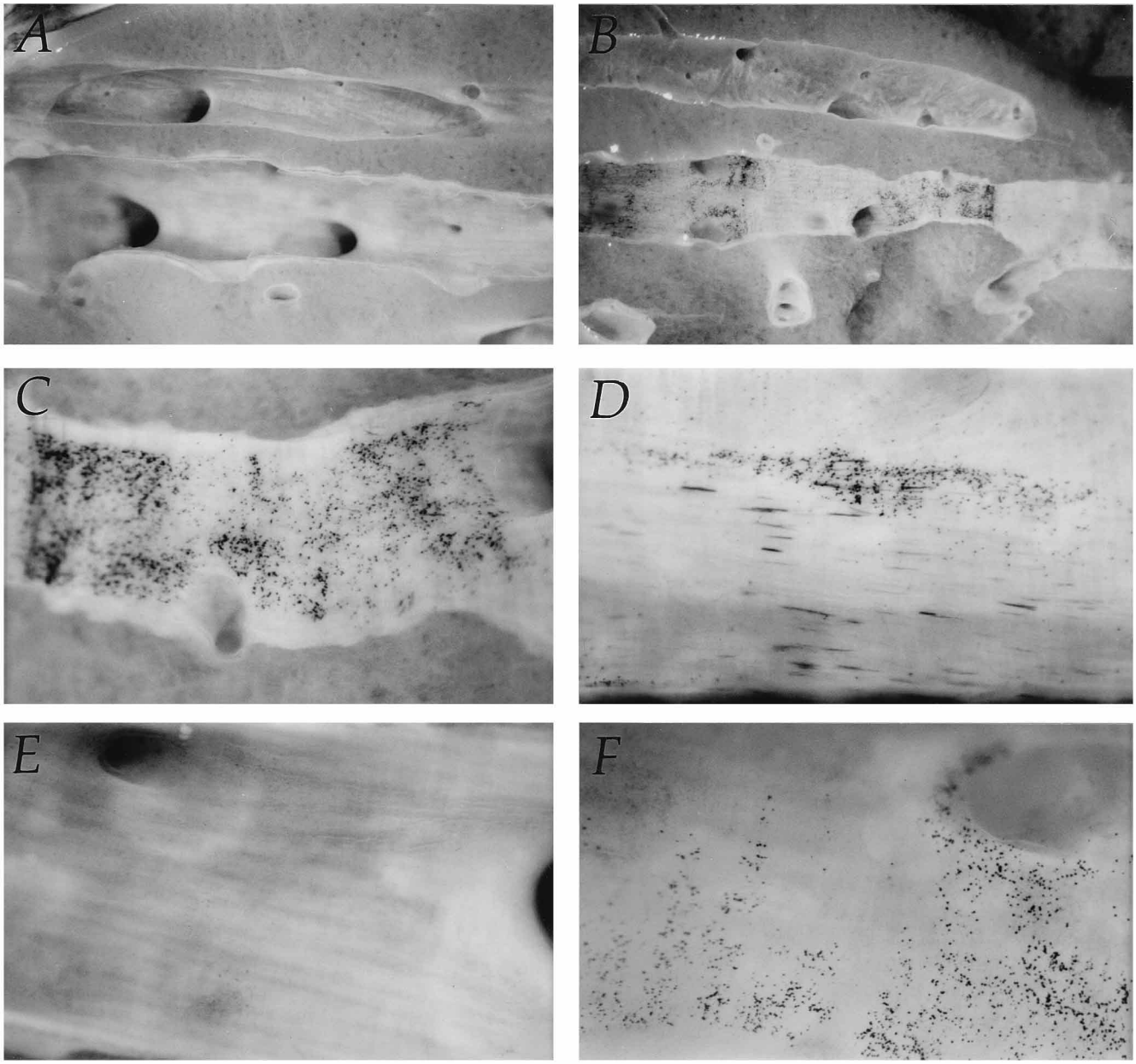

FIG. 2. Staining of marker gene products 7 days after vector exposure in longitudinal exposures of the bronchus in the left lower lobe of the lung. (A) Saline control. The animal received saline, and the lung was stained for AP expression (original magnification, ×8). (B, C, and D) The animal received 10^7 AP-positive FFU of ACWRAP, and the lung was stained for AP expression. Original magnification, ×8 for panel B and ×32 for panel C. (D) Epithelial and smooth muscle cell staining is shown. Magnification, ×32. (E) Vector control. The animal received ACWRAP, and the lung tissue was stained for β-gal expression. Magnification, ×32. (F) The animal received 10^6 β-gal-positive FFU of ACWRZn, and the lung tissue was stained for β-gal expression. Magnification, ×32.

modified to contain a nuclear localization signal (ACWRZn) was delivered to the left lower lobe of the rabbit lung by using a balloon catheter (see Materials and Methods). After 7 days, the animals were euthanized and their lungs were examined for gene expression by histochemical staining in order to evaluate transduction by the AAV vector. Figure 2 shows longitudinal exposures of the bronchi in the left lower lobe of the lung where the balloon catheter was placed. Round, punctate staining indicative of epithelial staining was observed in the epithelium of the bronchi with all three vectors (Fig. 2B, C, D, and F, and data for ALAPSN not shown). Animals that received saline (Fig. 2A) or control AAV vectors (ACWRAP, stained for β-gal, or ACWRZn, stained for AP) (not shown) did not exhibit staining.

Transduction of the smooth muscle cells beneath the bronchial epithelium was also observed for ACWRAP (Fig. 2D) as well as for ACWRZn and ALAPSN (not shown). The transduction of smooth muscle cells was more obvious with the AP vectors than with the β-gal vector because the Golgi and cell-surface localization of AP resulted in the AP stain defining the long extended body of the smooth muscle cells. The nuclear localization of β-gal in the ACWRZn vector precluded analysis of cell body morphology. However, the β-gal-stained nuclei of smooth muscle cells appeared more elliptical and elongated

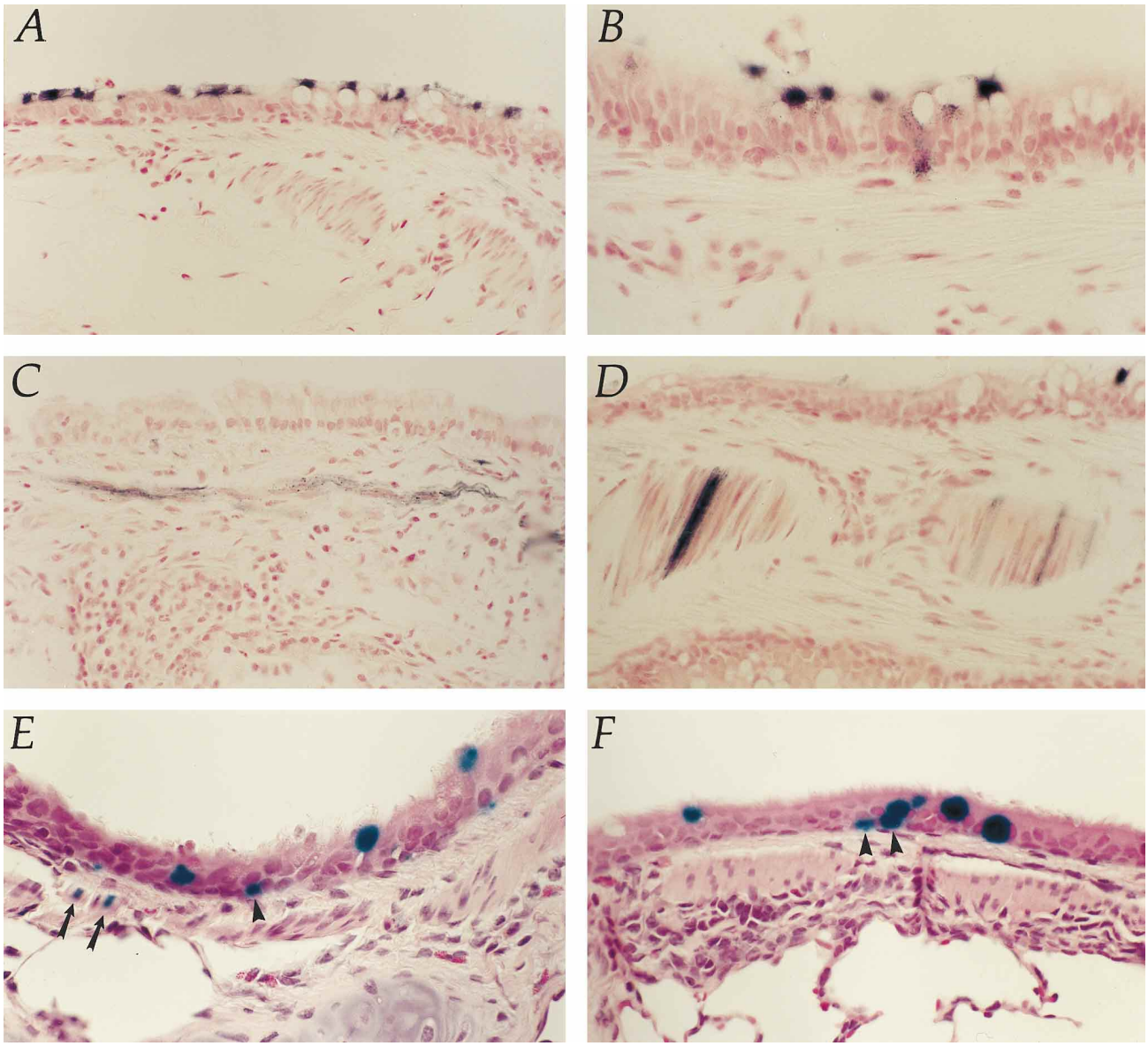

FIG. 3. Histologic sections of dense areas of marker protein staining in the bronchial epithelium. (A) AP staining is found primarily in ciliated cells and localized to the cilia of these cells (original magnification, ×400). (B) AP staining in basal and goblet cells (magnification, ×600). (C and D) AP staining in smooth muscle cells underlying the bronchial epithelium (magnification, ×400). (E and F) Transduction of epithelial and smooth muscle cells with the β-gal vector (magnification, ×600). Arrows indicate smooth muscle cell staining, and arrowheads indicate basal cell staining.

than the stained nuclei of epithelial cells in the bronchial epithelium. They also appeared in the plane of focus below that of the epithelial staining.

Quantitation of transduction efficiency for the β-gal vector was done only for the bronchus. The epithelial nature of the few β-gal-positive cells observed in the distal airways or in the alveolar regions of the lung could not be verified by histologic analysis (i.e., positive cells were not found in the sections examined). Quantitation of transduction efficiencies for the AP vectors was also done only in the bronchus because endogenous AP could not be totally inactivated in the distal airways. Although there may have been some AP staining due to vector, a significant increase in the total number of AP-positive cells in the distal airways was not consistently observed in vector-treated animals. The alveolar regions of the lung did not show any AP staining. Transduction was localized to the bronchus of the left lower lobe where the balloon catheter was placed and was not found in any other lobes of the rabbit lung. The photomicrographs in Fig. 2 also show that transduction occurred in patches and was not evenly spread throughout the epithelium. As a result, the mean transduction rate in the bronchial epithelium was low.

Table 1 compares the transduction efficiency of AAV vector stocks containing low and high levels of replication-competent AAV. Initial experiments were done with stocks containing high levels of wt-AAV coproduced with AAV vector because the adenovirus stock used for vector production was contaminated with wt-AAV. Delivery of vector preparations containing high levels of wt-AAV (at least a 100-fold excess of wt-AAV) resulted in little to no transduction of epithelial cells. Vector preparations made with reagents free of wt-AAV contamination contained low levels of replication-competent

AAV due to recombination of transfected DNA (see Materials and Methods). These vector stocks transduced rabbit airway epithelium at an efficiency of 0.02% for ALAPSN and 0.17% for ACWRAP, using 10^7 FFU of each vector. Transduction efficiency was 0.10% for ACWRZn using 10^6 FFU of the vector (Table 1). These rates represent a 10- to 1,000-fold increase over stocks of ACWRAP and ACWRZn containing high levels of wt-AAV, respectively. An increase in the number of transduced smooth muscle cells was also observed in stocks containing low levels of wt-AAV compared to those having high levels. These results show that wt-AAV interfered with AAV vector transduction.

Transduction can be efficient and is found in all three major epithelial cell populations. Histologic analysis was done in areas of dense staining to quantitate the maximum level of transduction in the airway epithelium as well as to verify the nature of the transduced cells. The efficiency of transduction ranged from 1 to 20%, with a mean of 6% for ACWRAP and 5% for ACWRZn (Fig. 3A, B, E, and F). The high transduction efficiency observed in these focal areas of staining in the region where the balloon catheter was expanded suggested that mechanical perturbation increased transduction by AAV vectors. Indeed, the transduction rate was about 100-fold lower in the epithelium of airway generations just adjacent to the bronchus containing the balloon catheter (an average of 0.001% compared to 0.11%, ACWRZn vector, $n = 4$ animals). Thus, an increased transduction rate was associated with the physical placement of the balloon catheter.

Figure 3 shows photomicrographs of β-gal and AP staining in airway epithelium. The majority of cells expressing AP were ciliated cells (Fig. 3A). However, a few AP-positive cells with the morphology of secretory and basal cells could be seen (Fig. 3B). In the ciliated cells, the cilia were stained intensely, while scattered granular AP staining was visible in the cell body at high magnification (data not shown). Transduced smooth muscle cells expressing AP are shown (Fig. 3C and D). The majority of cells expressing β-gal also appeared to be ciliated cells, although some stained cells appeared to be basal cells due to their size and location near the basement membrane (Fig. 3E and F). Results with both vectors are similar; the majority of transduced cells in the bronchial airway epithelium are ciliated cells.

Etoposide markedly increased AAV transduction of epithelial cells in culture but had little effect on transduction in rabbit airways. We have previously reported that DNA-damaging agents and topoisomerase inhibitors can increase AAV vector transduction in dividing and stationary cultures of primary human fibroblasts, with greater effects in stationary cultures (2, 36). We tested the effects of some of these agents in primary cultures of airway epithelial cells derived from the nasal polyps of CF patients. Initial analysis showed that gamma irradiation could increase AAV vector transduction; however, the treatment was toxic to cells (data not shown). Since irradiation of the lungs can result in lung fibrosis, the usefulness of this enhancement procedure may be limited for gene therapy to the lungs.

Another agent that can increase AAV vector transduction rates is etoposide, a topoisomerase inhibitor. The fact that it is currently used in cancer therapy for small cell lung cancer and that toxicity studies have already been done in rabbits and rodents (39) made this a reasonable candidate to test. Transduction by ALAPSN increased approximately 150-fold at concentrations of 20 and 40 μM etoposide in cultures of primary airway epithelial cells (Fig. 4A). Transduction by ACWRAP and ACWRZn was increased by 30- to 70-fold for the same concentrations of etoposide, respectively (Fig. 4A).

TABLE 2. AP- or β-gal-positive cells after readministration of recombinant AAV vectors[a]

Primary vector	Secondary vector	Marker-positive bronchial epithelial cells	Marker-positive smooth muscle cells
None	ACWRZn	1,145, 4,415	1,001, 1,622
	ACWRAP	8,782, 3,233	2,139, 2,148
ACWRAP	ACWRZn	0, 0, 0, 0	0, 0, 0, 0
	ACWRAP	0, 0, 0, 0	0, 0, 0, 0
ACWRZn	ACWRZn	ND	ND
	ACWRAP	0, 0, 0, 0	0, 0, 0, 0

[a] Animals were challenged with the primary vector or no vector in the left lower lobe, 14 days later they were rechallenged in the RLL with the same vector or one expressing a different marker gene (10^7 AP-positive FFU of ACWRAP or 10^6 β-gal-positive FFU of ACWRZn). Each value represents the total number of marker-positive cells in the bronchus of the RLL for an independent animal. ND, not done.

Since wt-AAV can affect transduction, we evaluated the effect of etoposide on AAV vector transduction in the presence and absence of added wt-AAV. In the presence of a 100-fold excess of wt-AAV, the relative increase in transduction by AAV vector was higher (compared to cultures with added wt-AAV and without etoposide): 400-fold for ALAPSN and 70- to 140-fold for ACWRAP and ACWRZn (Fig. 4A). However, the maximum number of transduced cells with etoposide treatment was the same with or without wt-AAV. The fold increase was greater because the presence of wt-AAV at 100-fold excess decreased the baseline transduction rates (Fig. 4B). The addition of a 10-fold excess (by particle number) of wt-AAV did not inhibit AAV vector transduction in vitro, but increasing wt levels to 100- and 1,000-fold excesses decreased transduction rates by approximately 2- and 10-fold, respectively (Fig. 4B). These results show that although the presence of wt-AAV inhibits transduction by AAV vector in cultured airway epithelial cells, its inhibitory effect is abrogated in the presence of etoposide.

The effect of etoposide on AAV vector transduction was evaluated in rabbits by infusing etoposide into the ear vein immediately after instillation of the vector and during the time when the balloon catheter was still in place. We used the highest dose of etoposide possible to maximize any potential effect while remaining consistent with animal welfare considerations (15 mg/kg; 50% lethal dose, 37 to 60 mg/kg). Treatment with etoposide did not increase transduction by ALAPSN or ACWRAP in vivo (Fig. 5). The addition of a 100-fold excess (by particle number) of wt-AAV to AAV vector stocks decreased transduction by ALAPSN and ACWRAP. Treatment with etoposide in the presence of wt-AAV did increase transduction by ALAPSN slightly but did not for ACWRAP. Thus, in contrast to its ability to dramatically enhance transduction in vitro, etoposide did not enhance transduction in vivo.

We observed pathologic changes in the lungs of animals treated with AAV vector stocks and etoposide. Granulomas were often seen in the vector-treated lobe of the lung, whereas granulomas were never seen when AAV vector was administered without etoposide (data not shown). Large and small infiltrating cells were seen in the alveolar spaces at and near these granulomatous sites (data not shown). Thus, intravenous injection of etoposide in conjunction with airway delivery of AAV vector by balloon catheter placement resulted in lung pathology, which may have affected transduction rates.

Readministration of AAV vector failed to produce transduction events, and persistence of expression is cell-type dependent. Histologic analysis showed that most of the epithelial

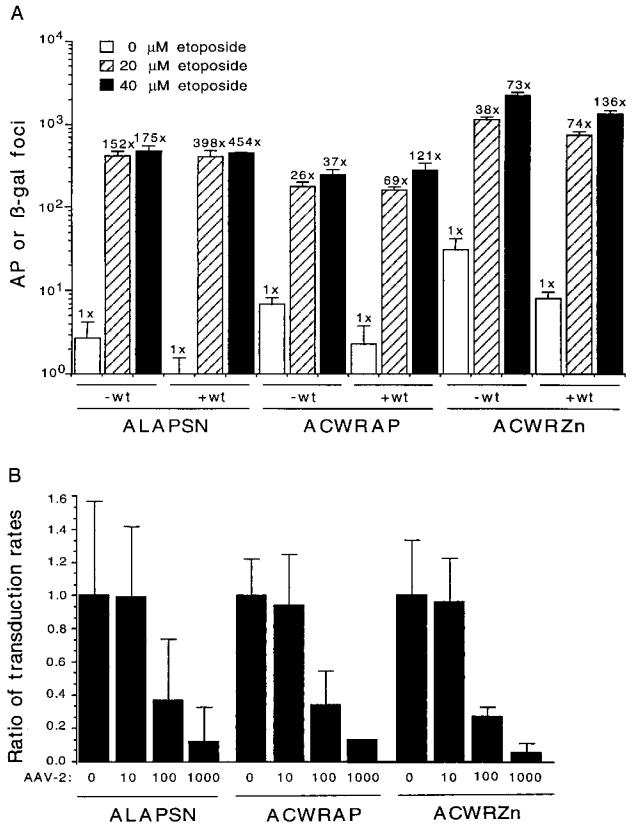

FIG. 4. Effect of etoposide and wt-AAV on transduction by AAV vectors. (A) Primary cells derived from the nasal polyps of CF individuals were transduced with ALAPSN, ACWRAP, or ACWRZn in the absence or presence of 20 or 40 μM of etoposide. The absence or presence of wt-AAV (added at 100-fold excess of genome-containing particles of wt-AAV2) to the AAV vector infection inoculum is indicated (−wt or +wt, respectively). Triplicate wells were scored, and standard deviations (SD) are given. The fold enhancement over transduction without the addition of etoposide is given on top of each bar. (B) Primary cells derived from the nasal polyps of CF individuals were transduced with ALAPSN, ACWRAP, or ACWRZn in the absence or presence of increasing amounts of AAV2 (wt). 0, 10, 100, and 1000 indicate the ratio of wt to vector as measured by genome-containing particles. Error bars represent SD.

cells that stained positive for marker-gene expression at 7 days postinfection were terminally differentiated cells. This result suggested that the persistence of transduced cells in the airway would be lost over time due to the turnover of cells in the epithelium. Thus, for long-term gene therapy there would be a requirement for readministration of the AAV vector. Therefore, we evaluated the possibility of vector readministration and the persistence of gene expression in animals. The right lower lobe (RLL) of the lung was used for readministration. Because primary inoculation of the left lower lobe did not result in marker-positive cells in the RLL, the detection of marker-positive cells in the RRL can be attributed to the second vector. Animals were given either the same vector as the initial one or a vector encoding a different marker gene in the RLL 14 days after the primary inoculation. Analysis of the RLL of the rabbit lung showed transduction in naive animals for both ACWRAP and ACWRZn (Table 2). Prior infection with ACWRAP prevented any transduction of epithelial cells or smooth muscle cells from a second administration of ACWRAP or ACWRZn (Table 2). Prior infection with ACWRZn also prevented a second challenge with ACWRAP (Table 2).

Persistence of AAV vector expression in animals that received only one inoculum of ACWRAP was examined by quantitating the number of marker-positive cells in the left lower bronchus at days 7 and 60. The four animals analyzed at day 60 showed an 80-fold reduction in the number of AP-positive epithelial cells compared to the value at day 7 (Fig. 6). It is evident that although transduction by the AAV vector can persist in the lung epithelium, it is dramatically reduced over time. In the same animals, the number of marker-positive smooth muscle cells did not decrease after administration of ACWRAP (Fig. 6).

We also examined persistence of gene expression due to the initial AAV vector exposure in animals that received two doses of AAV vectors. Persistence was examined by quantitating the number of marker-positive cells in the left lower bronchus at day 21 (after a secondary administration in the RLL at day 14), and a comparison was made to the value obtained at day 7. A 35-fold decrease in marker-positive epithelial cells was seen in animals that received ACWRAP or ACWRZn (Fig. 6). In the same animals, the number of marker-positive smooth muscle cells also did not decrease after readministration of ACWRAP or ACWRZn. The results show that readministration does not affect persistence of AAV vector gene expression in smooth muscle cells.

Lack of transduction by the second administration of AAV vector correlates with the appearance of neutralizing antibodies. The most likely explanation for the lack of transduction following administration of a second AAV vector was the generation of neutralizing antibodies. To test this possibility, serum samples from naive, saline-, and AAV vector-challenged rabbits were analyzed for neutralizing activities to AAV vectors. All naive and saline-treated animals showed minimal inhibition of AAV vector infection (Table 3). At day 7, all five sera from AAV vector-treated animals exhibited greater than 90% inhibition of ACWRZn at a 1:20 dilution and greater than 30% inhibition at a 1:100 dilution. Neutralizing activities for AAV vector increased for up to 60 days (compare the neutralizing activities for days 7, 14, and 60 at the 1:100 dilution). The neutralizing activity was directed at viral proteins because sera from ACWRAP-challenged animals inactivated ACWRZn vector in the neutralization assay. Also, the readministration experiments showed that transduction was inhibited even when vectors encoding a different marker gene was used in the sec-

TABLE 3. Neutralization of recombinant AAV vector by rabbit serum[a]

Vector	No. of animals	Days postinfection	Vector neutralization (%) for serum dilution indicated:	
			1:20	1:100
None	6	NA[b]	4.5 ± 4.0	2.7 ± 3.0
Saline	2	7	5.0 ± 3.3	3.2 ± 3.0
ACWRZn (10^6 FFU)	2	7	99.9 ± 0.6	35.0 ± 7.9
	4	14	99.9 ± 0.0	78.8 ± 24.7
ACWRAP (10^7 FFU)	3	7	91.0 ± 11.4	32.9 ± 6.8
	7	14	97.5 ± 3.4	53.1 ± 17.2
	4	60	100.0 ± 0.0	83.0 ± 3.1

[a] Values and standard deviations are expressed as percent inactivation of the total β-gal-positive FFU obtained in control samples (ACWRZn incubated with culture medium only).

[b] NA, not applicable.

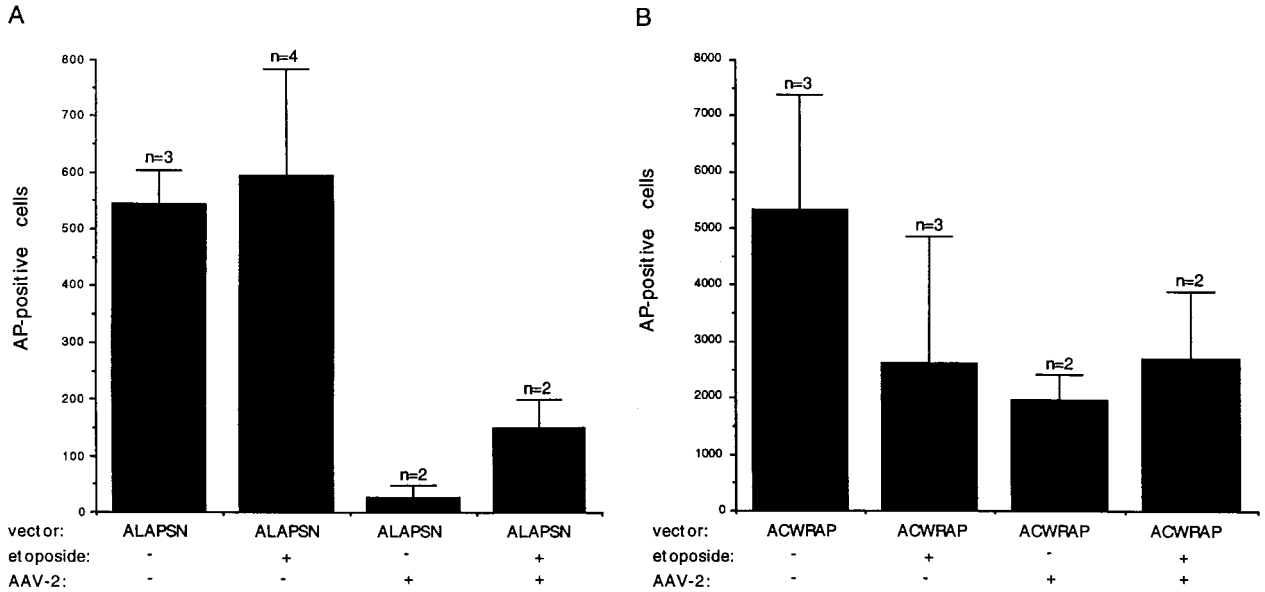

FIG. 5. The effect of etoposide and wt-AAV on transduction by AAV vectors in the rabbit airway epithelium. ALAPSN (10^7 AP-positive FFU) (A) and ACWRAP (10^7 AP-positive FFU) (B) were delivered to rabbit airways by using a balloon catheter. AAV2 was added to the vector stocks at 100-fold excess (particle number) in some animals. If the animal received etoposide (15 mg/kg), it was administered immediately after vector instillation by infusion into the ear vein. The lungs were processed for AP staining 7 days after vector instillation. The mean values and standard errors of the mean are shown, and *n* represents the number of animals in each group.

ond challenge. The results show that the failure of a second administration of AAV vector to transduce the airway was due to the generation of neutralizing antibodies to AAV, most likely to the viral capsid proteins.

DISCUSSION

The method used here for AAV vector administration involved delivery of the vector into one lobe of the rabbit lung using a balloon catheter. Initially, the purpose of using a balloon catheter was to localize the vector in the left lower lobe to promote viral uptake in the distal airways. In fact, we observed little to no transduction in the distal airways or in the alveolar regions. The primary site of transduction was in the bronchus where the balloon catheter was lodged, indicating that mechanical perturbation at the site of vector delivery enhanced transduction. The transduction rates in these areas were impressive. In some sections, almost every ciliated cell was positive. In contrast, areas of uninjured airway epithelium showed about a 100-fold reduction in the percentage of marker-positive cells. It will be important to understand why some areas of the epithelium were highly permissive for AAV transduction while other areas were refractory. Perhaps the balloon catheter affected the integrity of the mucin layer or the movement of the cilia. Alternatively, the epithelium could have been abraded and the injury increased the proliferation rate of the epithelial cells. This would be consistent with a higher transduction rate, since AAV vectors transduce dividing cells more efficiently than nondividing cells in cell culture. These results demonstrating enhanced transduction in injured epithelium are reminiscent of those obtained with adenovirus vectors (18). The basis for increased transduction in abraded and regenerating epithelium by adenovirus vectors is most likely due to more efficient vector entry into the exposed basal cells than into the columnar cells of the intact epithelium (15), and this possibility might also explain our results with AAV vectors. Experiments that evaluate the effects of injury, growth factors, mucolytic reagents, and agents that affect ciliary movement may help to increase AAV vector transduction in the airway epithelium.

In this study, all stocks of AAV vectors contained some level of replication-competent AAV. Therefore, transduction rates obtained do not answer the question of whether AAV vectors can transduce in the absence of any replication-competent AAV. However, we have recently developed a system to pro-

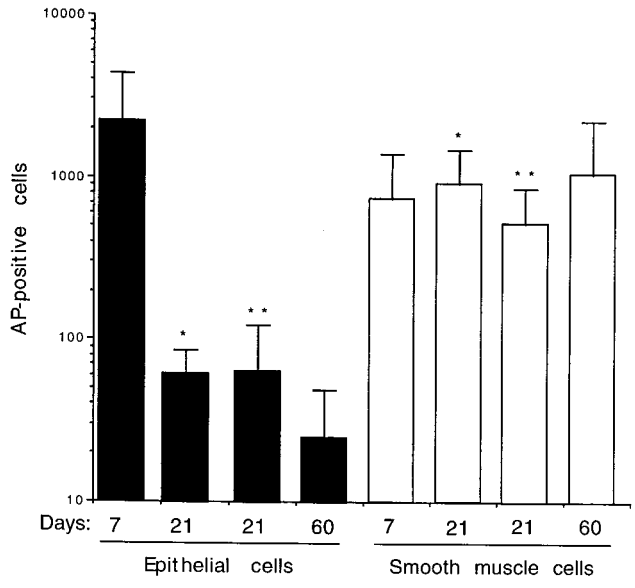

FIG. 6. Persistence of AAV vector expression in rabbit bronchus. Animals were given ACWRAP (10^7 AP-positive FFU) at day zero and were euthanized on day 7, 21, or 60. The numbers of AP-positive cells in the bronchial epithelium are shown. Some animals received a second AAV vector (ACWRZn [*] or ACWRAP [**]) on day 14. The means and standard deviations are shown, the number of animals was four in each group.

duce AAV vectors that are free of replication-competent AAV (<1 infectious unit per 1.5×10^6 FFU of the ACWRAP vector; 2a), and preliminary results show that these vector stocks yield transduction rates in the rabbit airway similar to those of stocks having low levels of replication-competent virus.

Experiments done in cultured primary airway cells and in the rabbit airway demonstrate that high levels of wt-AAV can interfere with transduction by the AAV vector. It is unlikely that wt-AAV inhibits by stimulating cytotoxic cells that clear the transduced cells by day 7, because cellular immunity would not likely be optimal this early in an immune response and it would not occur in vitro. In addition, other immune responses, such as the acute lung inflammation seen after administration of adenovirus vectors, were not observed after delivery of AAV vector stocks. If wt-AAV was in general toxic to cells, cell death would be seen in cultures of primary airway cells transduced with AAV vector stocks containing high levels of wt-AAV, but that did not occur. Although we did not detect expression of the AAV *rep* gene by Northern analysis of RNA from cultured cells exposed to AAV vector stocks containing high levels of wt AAV, it might have been expressed at a low level and may have affected vector transduction or transgene expression. Alternatively, wt-AAV could interfere by competing for receptors on the surfaces of the target cells, as has been observed in AAV-binding assays (31). However, the amount of wt-AAV required on a per cell basis to successfully compete in binding assays was several orders of magnitude higher than the amount used here. Additionally, the presence of etoposide abrogated the inhibitory effect of the wt-AAV in cultured epithelial cells. Etoposide presumably does not affect virus entry but acts at later steps in transduction. Thus, this result argues against a block at the level of virion entry. The results showing that etoposide can reverse the inhibition of transduction due to wt-AAV are consistent with the hypothesis that wt-AAV interferes with AAV vector transduction by competing for factors involved in second-strand DNA synthesis or integration. Etoposide may increase factors involved in DNA synthesis and repair and thereby facilitate expression from the AAV vector.

The dramatic increase in transduction of cultured epithelial cells after the addition of etoposide was not duplicated in the rabbit. Intravenous injection of etoposide in conjunction with airway delivery of AAV vector by balloon catheter placement resulted in pathologic changes in some areas of the lung, whereas other areas appeared normal. Etoposide seemed to predispose the animals to lung infections, which may have interfered with AAV transduction, and perhaps the dose and regimen of etoposide used may not have been optimal for enhancing AAV vector transduction in vivo. We treated rabbits with the highest dose of etoposide compatible with animal welfare. The 50% lethal dose in mice is higher than that in rabbits (39), thus, further evaluation of etoposide might be performed in the mouse where a larger number of variables such as dose and regimen can be tested and where genetic differences can be controlled.

The persistence of AAV vector transduction in smooth muscle cells and airway epithelial cells differed dramatically. The number of marker-positive epithelial cells dropped 80-fold, while the number of marker-positive smooth muscle cells remained constant over the 60-day time course of the experiment. It has been previously shown that transplanted smooth muscle cells expressing adenosine deaminase or human placental AP (after retroviral transduction ex vivo) continued to express these proteins for at least 1 year in vivo (6) and that skeletal muscle injected with an AAV vector encoding β-gal expressed this protein for more than 1.5 years (40). The long-term persistence of gene expression in muscle cells may be in part due to the fact that muscle cells are long-lived. In contrast to muscle tissue, the respiratory epithelium has a constant but slow rate of regeneration. In early studies of tracheobronchial epithelium in rodents, population turnover was found to vary from 8 to 257 days (24). This wide range in values is due to the great differences observed in labeling indices in the trachea, depending on the health, strain, and age of the animals. The turnover rate in the rabbit airway epithelium is not known. The 80-fold drop in 60 days shown by the four animals that received only one AAV vector challenge may be consistent with epithelial turnover. The persistence data also indicate that the numbers of transduced epithelial stem cells or progenitor cells were inadequate to replenish the population at day 60. Taken together, the issues of epithelial cell turnover and the apparent paucity of transduced epithelial stem cells indicate that the use of AAV vector in the airway would require vector readministration.

Transduction due to a second administration of AAV vector was completely inhibited, and this effect correlated with the production of neutralizing antibodies. The neutralizing activity was directed against the virus proteins and not the marker protein. Neutralizing activity elicited by one challenge with an AAV vector appeared within 1 week and was sufficient to prevent transduction after readministration of AAV vectors. There is the possibility that the low level of recombination-derived replication-competent AAV affected the production of neutralizing antibodies to AAV capsids. However, we did not detect a productive infection initiated by the replication-competent AAV in cultured cells infected at a high multiplicity of infection in the absence of adenovirus. Furthermore, preliminary results in rabbits showed that neutralizing activities of sera obtained from rabbits ($n = 3$) treated with AAV vector stocks that did not have detectable levels of replication-competent AAV (2a) were similar to those obtained here. It is possible that adenovirus and cellular proteins that contaminate AAV vector preparations could act as adjuvants to stimulate an immune response against AAV, and further purification of AAV vector stocks may prove beneficial in reducing the immunogenicity of vector stocks.

The results of this study indicate that the successful application of AAV vectors for gene therapy to the lungs must address several issues. First, the overall transduction rate in the airway epithelium is low. Further work to evaluate drug treatments or other procedures to enhance transduction rates will be necessary. Second, understanding the immune response to AAV vectors in vivo is important. While there seems to be no overt cellular immunity to transduced smooth muscle cells, the possibility of cellular immunity in the loss of persistent gene expression in the epithelial population needs further analysis. Third, the ability to readminister AAV vectors will most likely rely on prevention of the formation of neutralizing antibodies which might be accomplished by using inhibitors of immune responses. Despite these limitations to the use of AAV vectors in the lung, the high rates of gene transfer observed for AAV vectors in some areas of the airway indicate the potential utility of these vectors for gene therapy.

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