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Adenovirus-Mediated Transfer of the CFTR Gene to Lung of Nonhuman Primates: Biological Efficacy Study

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ABSTRACT

We have evaluated the biological efficacy of E1-deleted adenoviruses in baboons for lung-directed gene therapy of cystic fibrosis (CF). The experimental design attempted to simulate a phase I clinical trial with animals receiving a single dose of virus to an isolated pulmonary segment. A total of 14 animals divided into four groups, each of which received escalating doses of virus, were used. Individual animals were necropsied 4 and 21 days after gene transfer and tissues were carefully surveyed for gene expression. Expression of the transgene was localized primarily to the area into which it was infused; the efficiency of recombinant gene expression and the abundance of transgene sequences were proportional to dose and both diminished with time. Transgene expression was found predominantly in alveolar cells with patches of expression in the proximal and distal airway. Analysis of adenoviral protein expression within transgene-expressing cells revealed infrequent expression of the E2a gene and no detectable expression of late genes (*i.e.*, fiber protein). These results suggest that recombinant adenovirus can be used to transfer genes efficiently to the lung of nonhuman primates and that therapeutic strategies of cystic fibrosis may require repetitive administration with current vectors.

OVERVIEW SUMMARY

Recombinant adenoviruses hold tremendous promise for gene therapies of lung disease in cystic fibrosis (CF). An evaluation of the feasibility and safety of this technology in nonhuman primates is critical in the design of clinical protocols. In a series of two papers, Wilson and colleagues describe an extensive study in baboons designed to evaluate the feasibility and safety of direct instillation of CF transmembrane conductance regulator (CFTR)-expressing adenoviruses into the airway. This paper by Engelhardt *et al.*, addresses the biological efficacy of E1-deleted adenoviruses for gene therapy of CF lung disease.

INTRODUCTION

ADVANCES IN GENE TRANSFER TECHNOLOGY together with an enhanced knowledge of the pathophysiology of cystic fibrosis (CF) have paved the way for the development of gene therapies for this lethal inherited disorder (Collins, 1992). The first step in the development of these therapies was the isolation of the gene defective in CF that encodes the CF transmembrane conductance regulator (CFTR) (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). Viral-mediated transduction of the normal version of this gene into isolated cells from patients with CF was sufficient to correct the primary cellular defect in CF, abnormal cAMP-mediated regulation of chloride conductance (Drumm *et al.*, 1990; Gregory *et al.*,, 1990). Localization of CFTR in the human lung has identified a variety of cells throughout the proximal (Engelhardt *et al.*, 1992) and distal lung (Engelhardt *et al.*, in press) that express this gene and, therefore, are potential targets for CFTR gene transfer.

A variety of viral and nonviral approaches have been evaluated for lung-directed gene therapy of CF (Collins, 1992). The most promising technology is based on recombinant adenoviruses that are rendered defective in replication by virtue of deletions in the E1a and E1b genes (Rosenfeld *et al.*, 1991a, 1992; Engelhardt *et al.*, 1993). Adenoviruses can be isolated in large quantities at concentrations in excess of 10^{13} particles/ml, and are capable of transducing genes into nondividing cells (Kozarsky and Wilson,

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1993). In addition, many serotypes of human adenoviruses are naturally tropic to the airway epithelium (Chanock, 1974).

The potential usefulness of recombinant adenoviruses for the treatment of CF was initially studied in the cotton rat (Rosenfeld *et al.*, 1991a,b, 1992). Direct instillation of *lacZ*-expressing adenoviruses into the airway of these animals led to high-level recombinant gene expression in the majority of surface epithelial cells of the conducting airway (Rosenfeld *et al.*, 1991b). Similar results were obtained when adenovirus expressing human CFTR was infused into the lungs of cotton rats; a low level of human CFTR expression was detected for up to 3 weeks (Rosenfeld *et al.*, 1992). Similar studies have been performed using a model of human lung that is based on the growth of a human bronchial xenograft in athymic mice (Engelhardt *et al.*, 1993). Instillation of CFTR-expressing adenovirus into the lumen of CF and non-CF xenografts was associated with high levels of recombinant-derived CFTR protein that: (i) was localized primarily to the apical plasma membrane in up to 30% of the cells, (ii) was found in all cell types except basal cells and, (iii) was stable for the lifetime of the grafts, which was 8 weeks. Additional analyses of the adenovirus-infected xenografts indicated that a significant number of transduced cells expressed at least one adenoviral early gene and that recombinant virus could be recovered from the lumen of the xenografts for up to 28 days.

We describe in this report an evaluation in nonhuman primates of the safety and biological efficacy of recombinant adenoviruses for lung-directed gene therapy. The study was constructed to simulate a possible phase I clinical trial. Several issues were addressed in this preclinical study, which involved a dose escalation of recombinant CFTR and *lacZ* adenoviruses into segments of the lung: (i) characterization of the inflammatory response in the lung, (ii) clinical evaluation of lung function, (iii) dissemination of virus into bodily fluids, (iv) efficiency of gene transfer, (v) stability of transgene expression, (vi) evaluation of cellular targets, and (vii) expression of viral genes from recombinant adenoviral vectors. This manuscript describes analyses that address biological efficacy while the companion manuscript describes clinical and pathological consequences of adenovirus-mediated gene transfer (Simon *et al.*, 1993).

METHODS

Preparation of recombinant adenovirus

The recombinant CFTR adenovirus (Ad.CB*CFTR*) contains the cytomegalovirus (CMV) enhancer, β-actin promoter, human CFTR cDNA, and SV40 poly(A) minigene cassette inserted into the E1-deleted region (spanning 1.0–9.2 map units)

TABLE 1. SUMMARY OF EXPERIMENTS

Animal				*Virus*			
I.D.	*Species*[c]	*Age*	*Wt. (kg)*	*Conc. (pfu/ml)*	*Vol (ml)*	*Total (pfu × 10⁹/kg)*	*Analysis*
Study I							
B1[a]	PC	12 years	32.5	10^{10}	20	6.1	Necropsy day 3
Study II							
B2[a]	PC	12 years	32.5	10^{10}	20	6.1	Long term
Study III							
III-A							
B4[b]	PP	Adolescent	7.7	10^{10}	7	10	Necropsy day 4
B6[b]	PP	Adolescent	13.6	10^{10}	7	5.1	Necropsy day 21
B8[b]	PP	Adolescent	10.4	10^{10}	7	6.7	Long term
						Ave = 7.3	
III-B							
B11[a]	PP	2.2 years	6.7	10^{9}	7	1.0	Necropsy day 4
B13[b]	PP	Adolescent	8.8	10^{9}	7	0.8	Necropsy day 21
B15[b]	PP	Adolescent	9.3	10^{9}	7	0.75	Long term
						Ave = 0.97	
III-C							
B10[b]	PP	Adolescent	6.1	10^{8}	7	0.1	Necropsy day 4
B14[a]	PP	2.4 years	8.7	10^{8}	7	0.08	Necropsy day 21
B16[b]	PP	Adolescent	9.2	10^{8}	7	0.08	Long term
						Ave = 0.09	
III-D							
B3[a]	PP	4.3 years	10.4	10^{7}	7	0.007	Necropsy day 4
B5[a]	PP	4.3 years	12.0	10^{7}	7	0.006	Necropsy day 21
B7[a]	PP	5.0 years	11.4	10^{7}	7	0.006	Long term
						Ave = 0.007	

[a] Bred in captivity.
[b] Caught in the wild.
[c] PC, *Papia cynocephalus*. PP, *Papio papio*.

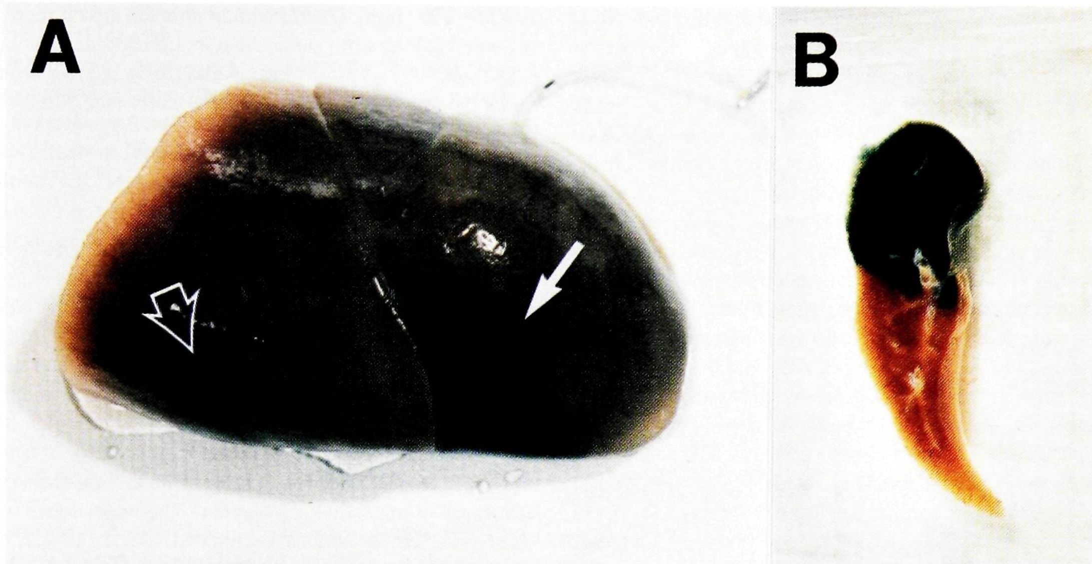

FIG. 1. Anatomical localization of *lacZ* transgene activity by *in situ* X-gal staining. Lungs from B1 infected with 10^{10} pfu/ml were harvested 3 days post-infection and histochemically stained for β-gal with X-gal by intrabronchial instillation. A. Left lung following 30 min staining in X-Gal. B. Wedge of the upper left lobe in which X-gal staining demarcates the posterior segment. Closed arrow designates area of infusion of virus in the posterior segment of the left upper lobe. Open arrow designates area of spillover of virus to the left lower lobe.

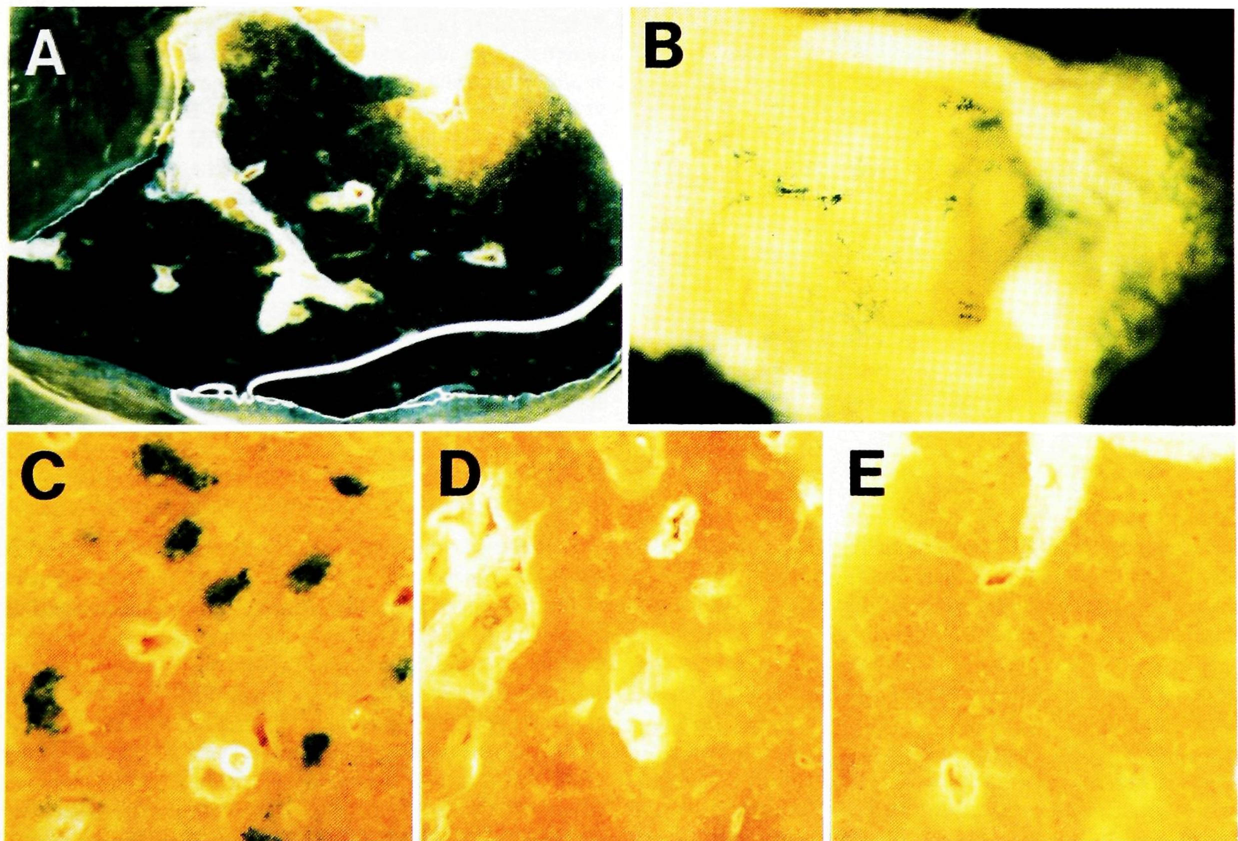

FIG. 2. Effect of viral dose on *lacZ* transgene expression. Tissue from the posterior segment of the left upper lobe of lungs infected with 10^{10} pfu/ml (A, demonstrating alveolar staining; B, demonstrating staining in a major bronchus), 10^9 pfu/ml (C), and 10^8 pfu/ml (D), at 4 days postinfection with Ad.CMV*lacZ* and histochemically stained in X-gal for 30 min. Absence of staining in the same region of the left upper lobe of an animal infected with 10^{10} pfu/ml at 21 days (E).

of modified adenovirus type 5 (Ad5). The remaining sequences are derived from *dl*7001, in which the majority of the E3 region (map units 78.4–86) was deleted (Engelhardt *et al.*, 1993; Yang *et al.*, 1993). Recombinant *lacZ* adenovirus (Ad.CMV*lacZ*) contains the CMV promoter, cytoplasmic *lacZ* gene, and SV40 poly(A) minigene cassette inserted into the E1-deleted (spanning 1.0–9.2 map units) region of Ad5 strain sub360, in which 78.4–85 map units of E3 has been deleted (Engelhardt *et al.*, 1993; Yang *et al.*, 1993). Viral stocks were generated from confluent monolayers of 293 cells infected with purified recombinant adenoviral stocks at a multiplicity of infection (m.o.i.) of 10. Cells were harvested 36–40 hr post-infection, pelleted, resuspended in 10 m*M* Tris-Cl pH 8.1, and lysed by three cycles of freeze-thawing. Cell debris was removed by centrifugation and supernatants were subjected to two rounds of CsCl gradient purification. Concentrated virus was desalted by gel filtration in phosphate-buffered saline (PBS) containing 10% glycerol for cryopreservation at −80°C. Concentrated stocks were diluted to a final concentration of 2% PBS, 0.2% glycerol, in isotonic saline for all viral instillations just prior to use.

General study design

Fourteen male baboons ranging in weights from 7 to 33 kg were used in this study; 7 were caught in the wild and the remaining 7 were bred in captivity. Descriptions of animals and the recombinant viruses used are provided in Table 1. Careful observation and physical examination indicated that all animals were clinically within normal limits. Several animals demonstrated mildly abnormal laboratory values or abnormal findings at necropsy (summarized in Simon *et al.*, 1993). One larger animal, B1, was used to evaluate the anatomical localization of *lacZ* transgene activity following instillation of 20 ml (10^{10} pfu/ml) of Ad.CMV*lacZ* into the posterior segment of the left upper lobe *via* a balloon catheter placed through a bronchoscope that was held in place for 10 min. Following instillation, the bronchoscope was removed with no attempt to aspirate remaining virus. On day 3 post-instillation of virus, the animal was necropsied and the whole lung was removed for *in situ* X-gal detection of β-galactosidase (β-gal) activity. The second large animal (B2) had 20 ml of 10^{10} pfu/ml Ad.CMV*lacZ* and Ad.CB*CFTR* placed into the posterior segments of the left and right upper lobes, respectively, by the same technique. The remaining 12 baboons were used in a dose-escalation study that evaluated the efficiency of CFTR and *lacZ* gene transfer at doses ranging from 10^7 to 10^{10} pfu/ml. The animals in the dose-escalation study were approximately three-fold smaller than those used in Studies I and II, therefore the volume of instilled virus was decreased from 20 ml to 7 ml. In these smaller animals, a pediatric bronchoscope was wedged into the posterior segments of the right upper lobe and left upper lobe to infuse Ad.CB*CFTR* and AdCMV*lacZ*, respectively. The bronchoscope was held in place for 10 min, after which it was removed without aspiration of remaining virus. The viral doses of the four treatment groups were as follows (see also Table 1): Group A—B4, B6, B8, 10^{10} pfu/ml, 7.3×10^9 pfu/kg (ave); Group B—B11, B3, B15, 10^9 pfu/ml, 9.7×10^8 pfu/kg (ave); Group C—B10, B14, B16, 10^8 pfu/ml, 9×10^7 pfu/kg (ave); and Group D—B3, B5, B7, 10^7 pfu/ml, 7×10^6 pfu/kg (ave). For each viral dose, one animal was necropsied at 4 days and another at 21 days. The remaining animals are currently being evaluated in long-term studies that include repeat bronchoscopy 4 days and 21 days after virus instillation to recover bronchoalveolar lavage and surface epithelial cells (see Simon *et al.*, 1993).

Necropsy protocol

Following euthanasia by an intravenous injection with pentobarbital/phenytoin, the lungs were analyzed for gross pathology and the heart lung cassette was removed *en bloc* from approximately the 10th most proximal tracheal cartilaginous ring. The lungs were immediately placed on ice and dissected into quadrants for analysis of transgene expression and histopathology. With the exception of animal B1, in which the segmental bronchus to the left lung was canulated for *in situ* detection of β-gal, the dissection of lungs from all other baboons was performed in the following manner. The upper lobes were divided into four quadrants designated LUL1, LUL2, LUL3, LUL4 for left upper lobe, and RUL1, RUL2, RUL3, RUL4 for right upper lobe. Typically the first two quadrants harvested from each lobe LUL1, LUL2, LUR1, and LUR2 were within the anatomical region predicted to have been instilled with virus. The left middle (LML), right middle (RML), left lower (LLL), and right lower (RLL) lobes were divided into two sections for analysis of transgene expression and histopathology. These samples were designated LML1, LML2, RML1, RML2, LLL1, LLL2, RLL1, and RLL2. All tissue quadrants were further divided into four parts: (i) fresh frozen in OCT for *in situ* hybridization and immunocytochemistry, (ii) fixed in formalin for histopathology, (iii) snap-frozen in liquid nitrogen for nucleic acid analysis, or (iv) fixed in glutaraldehyde for histochemical detection of β-gal activity, *en bloc*. All lobes of the lung were processed in this manner except for the azygos, which was too small to divide into quadrants and was retained solely for histopathology.

Two quadrants were also harvested from other organs, including the heart, liver, spleen, kidney, urinary bladder, brain, testes, and lymph nodes. These quadrants were divided into four subquadrants and processed for the analyses described above.

Histochemical and immunohistochemical analysis of lungs for β-gal, CFTR, and adenoviral proteins

To investigate the anatomical distribution of *lacZ* expression following bronchoscopic instillation into the posterior segment of the left upper lobe, the left lung from one animal (B1) infected with Ad.CMV*lacZ* was histochemically stained by intratracheal instillation of X-gal. Briefly, the left mainstem bronchus was canulated and distended with PBS containing 0.5% glutaraldehyde for 2 hr. Following fixation, the lung was lavaged four times with PBS containing 1 m*M* $MgCl_2$ for 15 min each. The lung was then distended with X-gal solution and stained at 37°C for 30 min (Price *et al.*, 1986). After histochemical staining, the lungs were lavaged with four changes of PBS followed by instillation of buffered formalin. Lungs from the remaining animals were similarly evaluated for *lacZ* expression, except that specific subquadrants of the dissected lobes were stained *en bloc*. Brushings from long-term animals were

evaluated on day 4 and 21 for *lacZ* activity on cytospun slides stained in X-gal for 4 hr.

CFTR protein expression was evaluated by indirect immunofluorescence using a purified polyclonal antibody generated against the carboxy terminus of human CFTR (α-1468 CFTR [a generous gift from Jonathan Cohn, Duke University]) as previously described (Marino *et al.*, 1991; Engelhardt *et al.*, 1992, 1993, and in press; Yang *et al.*, 1993a,b). Frozen sections (6 μm) or cytospun bronchial brushings were post-fixed in methanol for 10 min at −20°C followed by air drying. After blocking in PBS containing 20% donkey serum (DS), sections were incubated for 90 min in 1.5% DS/PBS containing 5 μg/ml α-1468 CFTR followed by washing in three changes of 1.5% DS/PBS for 8 min each. Sections were incubated in 1.5% DS/PBS containing 5 μg/ml donkey anti-rabbit FITC-conjugated antibody for 30 min. Finally, sections were washed, mounted in antifadent (Citifluor), and analyzed by fluorescent microscopy.

Studies evaluating the expression of adenoviral proteins from recombinant vectors were performed as previously described by immunocytochemical colocalization of β-gal, adenoviral fiber protein, and adenoviral DBP protein (Engelhardt *et al.*, 1993). Sections were incubated sequentially with 66 μg/ml rabbit anti-β-gal (5′→3′ Inc), a 1/10 dilution of hybridoma supernatant to Ad5 DBP, 5 μg/ml of both donkey anti-rabbit-AMCA and donkey anti-mouse-texas red, followed by a 1/10 dilution of mouse anti-Ad3 fiber-FITC (Ab5016, Chemicon Inc).

In situ detection of CFTR mRNA

In situ hybridization was performed as previously described using 6-μm frozen sections (Engelhardt *et al.*, 1992). A viral-specific probe (116 bp) to the 3′ untranslated region of the CFTR transcript was used to specifically detect CFTR transgene expression (Engelhardt *et al.*, 1993). This probe contains sequences specific to adenovirus-derived CFTR and hence will not hybridize to endogenous baboon CFTR transcripts. Sense and antisense ^{35}S RNA probes were generated using the Promega riboprobe system. Briefly, sections were fixed in 4% paraformaldehyde followed by dehydration through a graded series of ethanol. Sections were treated with 10 μg/ml proteinase K at 30°C for 30 min followed by acetylation in acetic anhydride. Following prehybridization for 4 hr at 54°C, sections were hybridized in 1×10^7 cpm/ml with sense and antisense probes for 16–18 hr. Sections were exposed to NBT-2 photoemulsion for 1 week.

Southern analysis for adenoviral DNA

Tissues frozen in liquid nitrogen were used to generate total cellular DNA. Samples were digested in TNE containing 0.5 mg/ml proteinase K, 0.1% SDS at 37°C for 16 hr. Samples were extracted twice with phenol, once with phenol/chloroform (1:1), and twice with chloroform, followed by precipitation in isopropanol. DNA was digested with *Eco* RI, resolved on a 1% agarose gel, transferred to nylon, and probed with a random-primed ^{32}P-labeled human CFTR cDNA fragment (2,226–3,714 bp). Controls for the quantitation of adenoviral copy number were generated by reconstitution of a known quantity of pAd.CB*CFTR* with baboon DNA from uninfected animals.

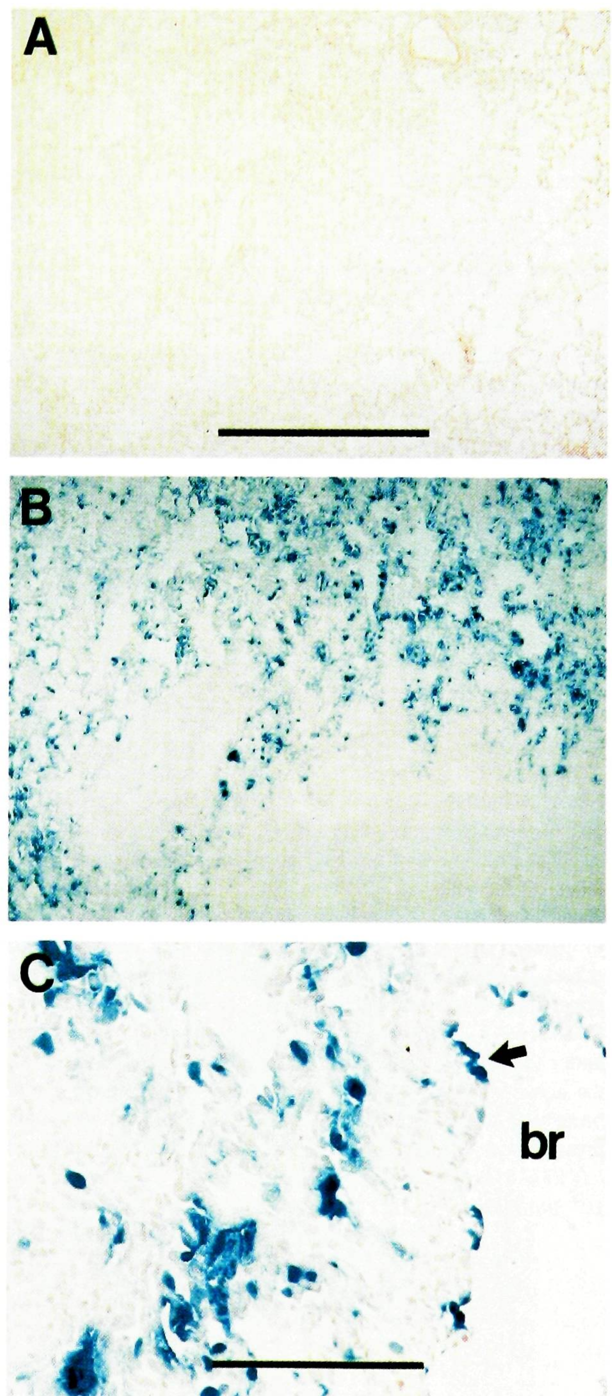

FIG. 3. Cellular distribution of *lacZ* expression in histochemically stained sections. Frozen (6-μm) sections from the posterior segment of 4-day animal B4 from the left upper and right upper lobes infected with 10^{10} pfu/ml of Ad.CB*CFTR* (A) and Ad.CMV*lacZ* (B and C), and histochemically stained in X-gal for 4 hr. br, bronchiole. Bar, 645 μm, A and B; 128 μm, C.

RESULTS

Distribution of transgene expression within the lung

The distribution of recombinant gene expression within the lung following instillation of Ad.CMV*lacZ* (20 ml of 10^{10} pfu/ml) into an isolated pulmonary segment was evaluated in animal B1. Three days after gene transfer, the lung was harvested *en bloc*, inflation fixed, and stained *in situ* with X-gal. Inspection of the lung indicated intense reaction product that was primarily confined to the posterior subsegment of the left upper lobe, the area into which the virus was instilled (Fig. 1A, see closed arrow). A smaller area of blue staining was seen within the posterior basal segment of the left lower lobe, probably due to spillover of virus at the time of bronchoscopy (Fig. 1A, see open arrow). The cellular distribution of *lacZ* expression in this sample analyzed by histologic sections of GMA-embedded tissues revealed a predominantly alveolar pattern of staining with infrequent patches of blue staining in bronchus and brochioles (data not shown).

Effect of virus dose on efficiency of gene transfer

The efficiency and cellular localization of transgene expression was analyzed in 4 baboons at 4 days and in 4 baboons at 21 days after infection with Ad.CMV*lacZ* (left upper lobe) and Ad.CB*CFTR* (right upper lobe) administered at doses of 10^7 pfu/ml (Group D—B3, B5), 10^8 pfu/ml (Group C—B10, B14), 10^9 pfu/ml (Group B—B11, B13), and 10^{10} pfu/ml (Group A—B4, B6). Tissue blocks harvested from subquadrants of all lobes of the lung, except the azygos, were stained *en bloc* in X-gal. Analysis of tissue from animal B4 harvested 4 days after infusion of 10^{10} pfu/ml of virus revealed high levels of *lacZ* expression in all blocks from the posterior segment of the left upper lobe (Fig. 2A) and essentially no expression in blocks from all other lobes (data not shown). Examination of this tissue under the dissecting microscope localized *lacZ* expression to the alveolar region with patches of blue staining in the larger bronchi (Fig. 2B) and bronchioles (data not shown). *LacZ* expression was also detected in the posterior segment of the upper left lobe 4 days after gene transfer in the animal that received 10^9 pfu/ml of virus (B11); the abundance of *lacZ*-positive cells was reduced in comparison to tissues of the animal that received the higher dose of virus (compare Fig. 2A to 2C). *LacZ* expression was no longer detected in lung tissues of animals 21 days postinfection with a similar dose of virus [B6, 10^{10} pfu/ml (Fig. 2E) and B13, 10^9 pfu/ml (data not shown)]. Transgene expression was not detected in tissues harvested 4 and 21 days after gene transfer of animals that received 10^8 or 10^7 pfu/ml of virus (Fig. 2D and data not shown, respectively).

The cellular distribution of *lacZ* expression was precisely defined in X-gal-stained cryosections. As expected, sections taken from adjacent subquadrants of tissue that revealed positive X-gal staining *en bloc* showed significant levels of *lacZ* expression. Figure 3 presents X-gal histochemical analysis of tissues harvested 4 days after administration of 10^{10} pfu/ml virus. Within the posterior segment of left upper lobe, *lacZ* expression was confined predominantly to the alveolar regions with lower levels of infrequent patchy staining in bronchioles (Fig. 3B,C). No *lacZ* staining was observed in segments of the right upper lobes that received 10^{10} pfu/ml of CFTR virus (Fig. 3A). Infrequently, small regions of X-gal-stained tissue could be seen in areas outside the left upper lobe in the 4-day animal receiving 10^{10} pfu/ml virus. This supports the finding in animal B1 that recombinant gene expression is essentially localized to the area of the lung into which the virus was instilled. Cells in the alveoli that expressed *lacZ* appeared by morphologic criteria to be both alveolar type I (squamoid morphology) and alveolar type II cells (cuboidal morphology). The patchy bronchiolar staining appeared to be localized to both nonciliated and ciliated cell types.

Other organs including the heart, liver, spleen, kidney, urinary bladder, brain, and testes were analyzed for *lacZ* expression by *en bloc* histochemical staining of tissues from all necropsied animals; in addition, frozen sections from all tissues from the animals that received the highest dose of virus were analyzed for *lacZ* expression. The X-gal reaction product was absent in all nonpulmonary tissue of all animals with the exception of the spleen, which consistently demonstrated low levels of staining in all animals that was independent of virus dose. Similar levels of staining have been demonstrated in spleen from untreated mice, rats, and rabbits not treated with *lacZ* viruses, suggesting that this may be due to endogenous β-gal activity.

TABLE 2. TRANSGENE EXPRESSION IN CELLS DERIVED FROM BRONCHOALVEOLAR LAVAGE AND BRUSHINGS

			BAL[a]		*Brushing*	
Animal	*Day*	*Location*	*% X-gal*	*% CFTR*	*% X-gal*	*% CFTR*
B2	0	Right middle lobe	<0.1	<0.1	<0.1	<0.1
	3	*lacZ* segment	5.2	<0.1	<0.1	<0.1
	3	CFTR segment	0.3	3.4	<0.1	2.1
	21	*lacZ* segment	<0.1	ND	<0.1	ND
	21	CFTR segment	<0.1	ND	<0.1	ND
B8	0	Right middle lobe	<0.1	ND	<0.1	<0.1
	4	*lacZ* segment	1.5	ND	5.0	<0.1
	4	CFTR segment	<0.1	ND	<0.1	2.5
	21	*lacZ* segment	<0.1	ND	<0.1	<0.1
	21	CFTR segment	<0.1	ND	<0.1	<0.1

[a] Bronchoalveolar lavage.

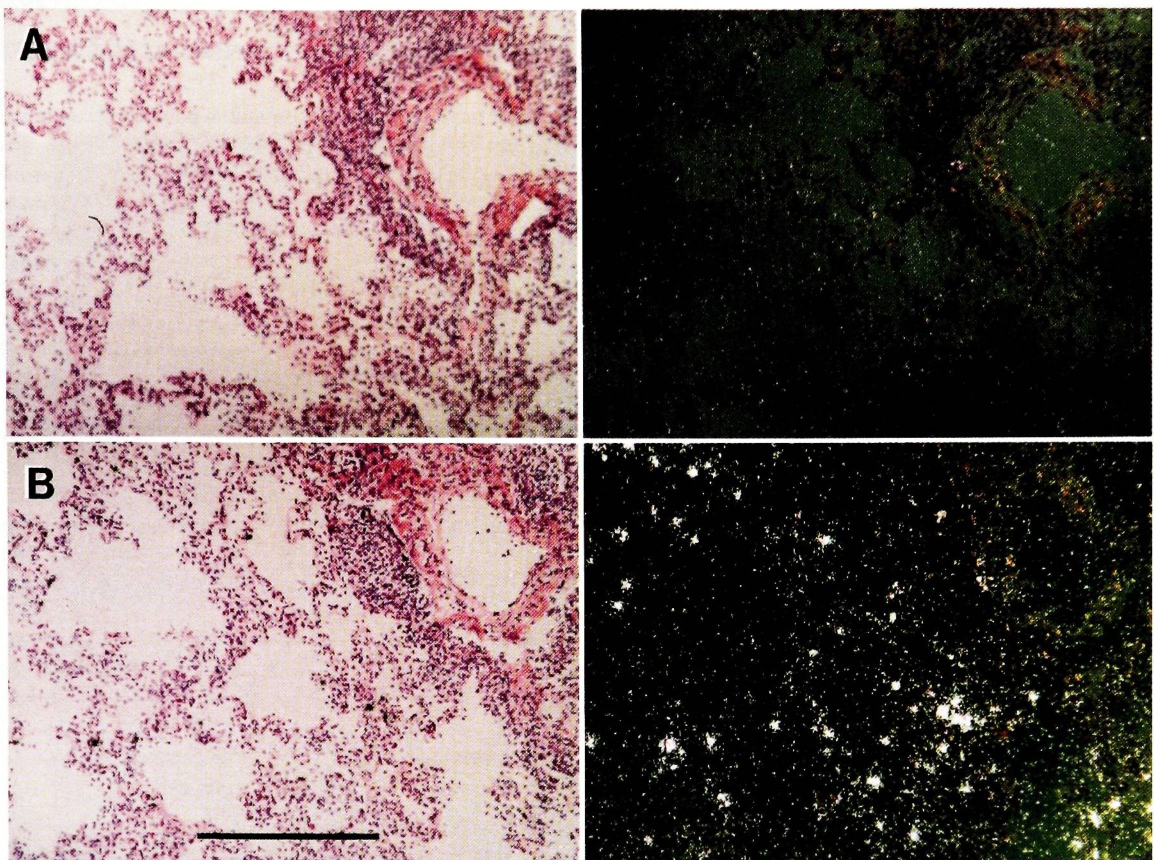

FIG. 4. Localization of CFTR mRNA by *in situ* hybridization. Serial frozen sections (6 μm) from the posterior segment of the right upper lobe from the animal infused with 10^{10} pfu/ml of Ad.CB*CFTR* and sacrificed at 4 days were analyzed by *in situ* hybridization to detect the recombinant derived CFTR mRNA. Sections were hybridized to a sense (A) and antisense (B) ^{35}S-labeled probes encoded in the 3′-untranslated sequence of the CFTR transgene. Bright field (left) and dark field (right) photomicrographs are shown. No signal above background was seen in sections from the posterior segment of the left upper lobe (infected with 10^{10} pfu/ml of Ad.CMV*lacZ*) hybridized to the antisense probes (data not shown) or in any sections harvested from all lobes of 21-day animals (data not shown). Bar, 320 μm.

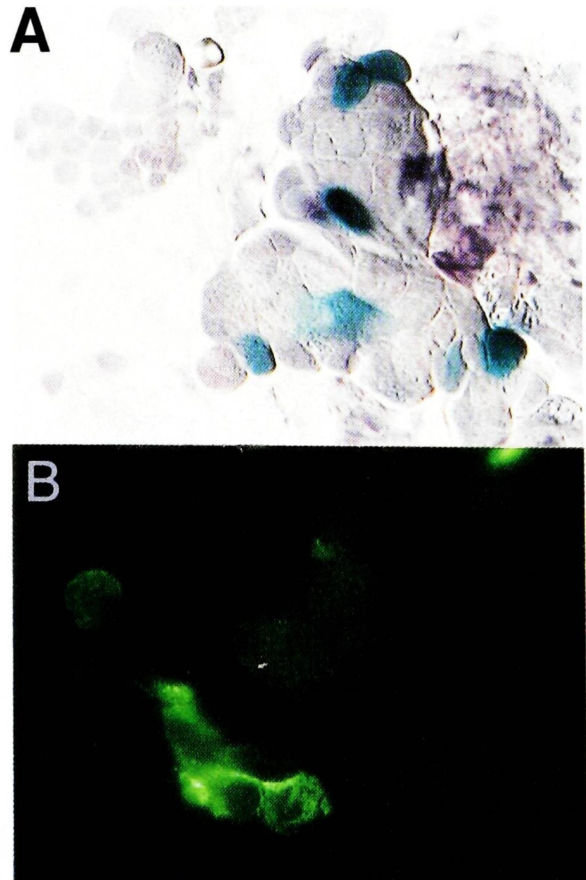

FIG. 5. Transgene localization in bronchial brushings. A. Histochemical X-gal staining of cytospun bronchial brushings from the Ad.CMV*lacZ*-infused left upper lobe of B4. B. Immunocytochemical CFTR staining of cytospun bronchial brushings from the Ad.CB*CFTR* infused right upper lobe of B4. No staining was observed in brushings taken from B4 before instillation of virus (data not shown).

Recombinant-derived CFTR mRNA in lung tissues

CFTR transgene expression was analyzed by *in situ* hybridization with a probe specific to the 3′-untranslated region of recombinant derived CFTR. Frozen sections from subquadrants of each lobe of all animals were hybridized to sense and antisense ^{35}S-labeled probes and exposed to photoemulsion for 7 days. Positive signal was seen at highest levels in lung tissue harvested 4 days after gene transfer in the animal that received 10^{10} pfu/ml of virus (Fig. 4B); the signal was localized to the segment of lung into which the Ad.CB*CFTR* virus was infused (*i.e.*, the posterior segment of the right upper lobe). Animal B11, which received 10^9 pfu/ml of virus, demonstrated high-level expression of recombinant CFTR in a smaller number of cells (data not shown). Serial sections hybridized to the sense probe gave no signal (Fig. 4A). No CFTR mRNA was detected in animals that received 10^8 pfu/ml (B10) and 10^7 pfu/ml (B3) of virus (data not shown). CFTR mRNA expression was undetectable in all lung samples analyzed 21 days after gene transfer. These localization studies substantiate the findings of anatomical confinement, cell distribution, and dose response of transgene expression that was demonstrated for Ad.CMV*lacZ* by histochemical analysis.

Animals were subjected to repeat bronchoscopy 4 and 21 days after instillation of virus in an attempt to recover cells expressing either *lacZ* or recombinant-derived CFTR (see Simon *et al.*, 1993 for methods). Samples were recovered from the right middle lobe before bronchoscopy and the posterior segments of the left upper lobe (*lacZ* segment) and right upper lobe (CFTR segment) 4 and 21 days after gene therapy. Surface epithelial cells recovered by brushing and cells in bronchoalveolar lavage fluid were sedimented onto slides. *LacZ* expression was detected by X-gal histochemistry while CFTR expression was detected by immunocytochemistry. Results from two animals (B2 and B8) that received 10^{10} pfu/ml of virus are summarized in Table 2 and an example of the cytochemical analysis is presented in Fig. 5.

Analysis of bronchoalveolar lavage (BAL) and brushing-derived cells by X-gal histochemistry revealed easily detectable *lacZ* expression in a significant number of cells at day 4 in the animals that receive 10^{10} pfu/ml of virus. Expression was restricted to cells from the *lacZ* segment and was no longer detected in cells recovered 21 days after gene transfer. Analysis of recovered cells for recombinant CFTR expression by immunocytochemistry revealed virtually identical results: recombinant protein was detected at day 4 and not at day 21 in cells of brushings and BAL from the segment into which the highest dose of virus was infused. Expression was not detected in cells from animals that received lower doses of virus (data not shown). The efficiency, distribution and stability of expression predicted from these analyses correlates with that demonstrated at necropsy.

Analysis of tissues for adenoviral DNA

Tissues were evaluated for the presence and abundance of adenoviral DNA by Southern blot analysis of *Eco* RI-restricted total cellular DNA (Fig. 6). *Eco* RI releases an internal 1.5-kb fragment from the adenoviral genome that hybridizes to human CFTR cDNA probe. This probe also detects endogenous baboon CFTR bands that exhibit substantial polymorphism in the population of animals that were used in this study. DNA was isolated from various sites within the lung of each animal including the left upper, left lower, right upper, and right lower lobes. A representative autoradiograph is presented in Fig. 6. Samples from lungs harvested 4 days after gene transfer demonstrated viral DNA whose abundance varied with the dose of infused virus in the following manner: 10^{10} pfu/ml, 10 copies of viral DNA per cellular genome (Fig. 6, lane 1); 10^9 pfu/ml, 2 copies of viral DNA per cellular genome (Fig. 6, lane 3); 10^8 pfu/ml, 0.1 copies of viral DNA per cellular genome (Fig. 6, lane 5); and 10^7 pfu/ml, <0.05 copies of viral DNA per cellular genome (Fig. 6, lane 7). When detected, the viral DNA was restricted to the lobe into which the CFTR virus was infused. Southern analysis of lung samples harvested 21 days after gene transfer failed to detect viral DNA with the exception of one quadrant of the posterior segment of the right upper lobe of the animal infused with 10^9 pfu/ml virus (B16) which retained 0.1 copies of viral DNA per cellular genome (Fig. 6, lane 4). Recovery of viral DNA from tissue was greater than 95% as estimated by reconstitution experiments in which lung tissue homogenates were mixed with a known number of viral particles before isolation and analysis of DNA (data not shown).

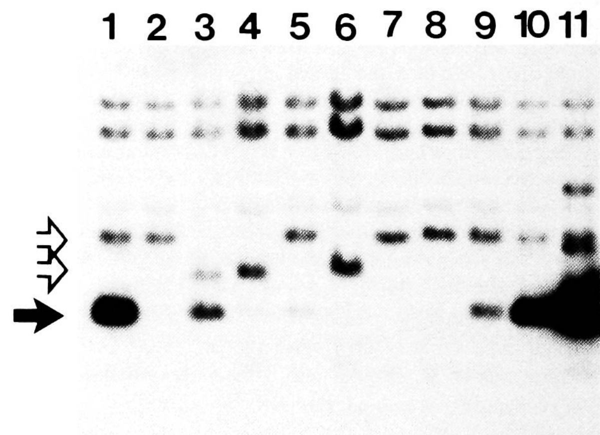

FIG. 6. Analysis of recombinant adenoviral DNA. Total cellular DNA (10 μg) from lungs infected with escalating doses of recombinant adenovirus were analyzed at 4 and 21 days. Southern blot was hybridized to a ^{32}P-labeled probe encompassing the internal *Eco* RI fragment of human CFTR. Lanes 1, 3, 5, and 7 show DNA samples from the posterior segment of the right upper lobe of 4-day animals. Lanes 2, 4, 6, and 8 show DNA samples from the posterior segment of the right upper lobe of 21-day animals. The various doses of Ad.CB*CFTR* include 10^{10} pfu/ml, lanes 1 and 2; 10^9 pfu/ml, lanes 3 and 4; 10^8 pfu/ml, lanes 5 and 6; 10^7 pfu/ml, lanes 7 and 8. DNA samples from the left middle, left lower, right middle, and right lower lobes showed no signal (data not shown). Reconstitution of genomic DNA with known quantities of human CFTR cDNA are shown in lanes 9, 1 copy; lane 10, 10 copies; and lane 11, 100 copies. The closed arrow marks the 1.5-kb diagnostic adenoviral DNA fragment. Open arrows mark bands that differ in mobility due to a restriction site polymorphism in the baboon population.

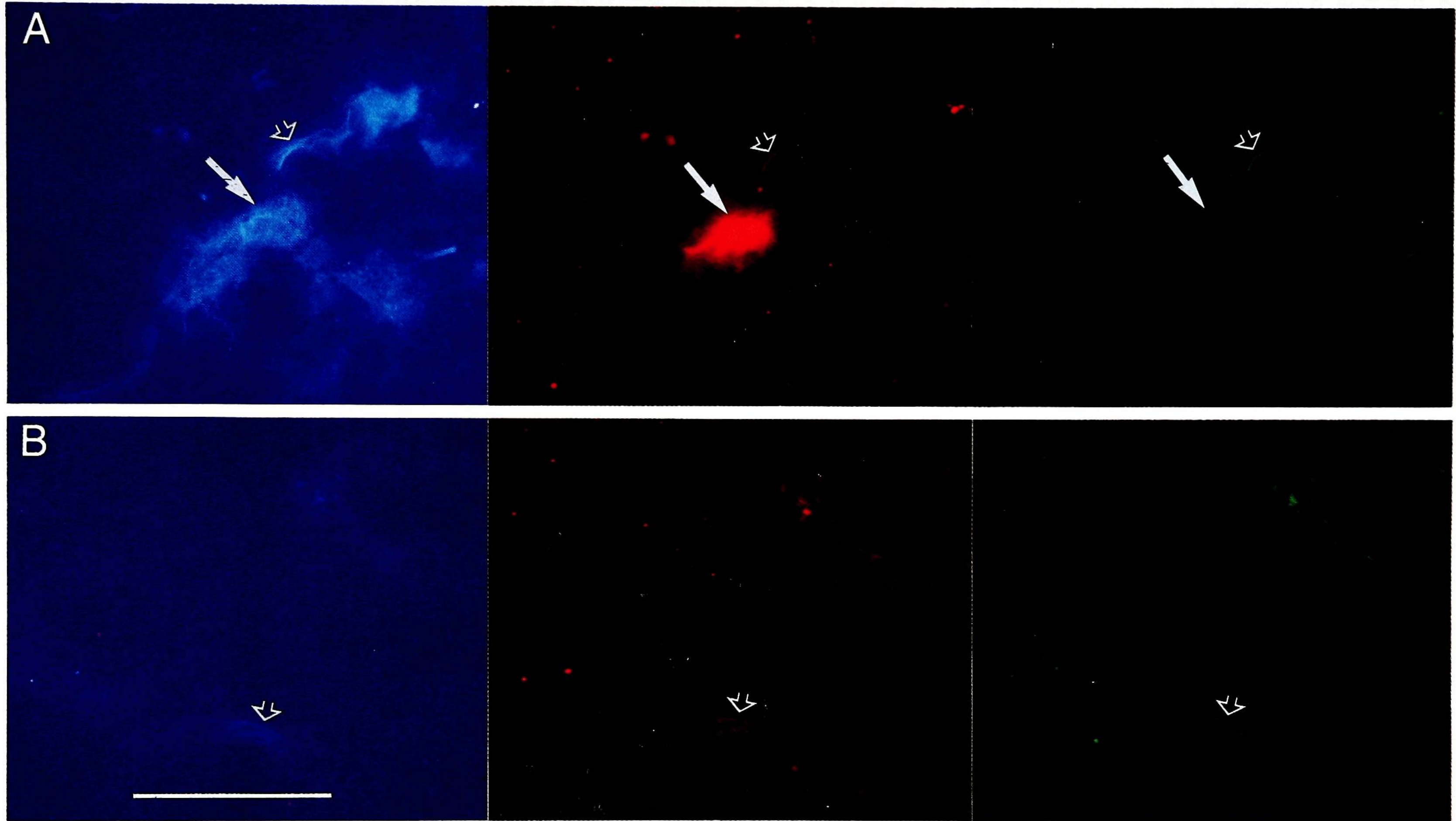

FIG. 7. Localization of adenoviral proteins DBP and fiber within *lacZ*-expressing cells. Frozen (6-μm) sections from posterior segment of the left upper lobe (A) of an animal infected with 10^{10} pfu/ml of Ad.CB*lacZ* were analyzed by triple immunofluorescence for β-gal (detected with AMCA in blue), left; DBP (detected with Texas Red), middle; fiber (detected with FITC in green), right. Tissue from the right lower lobe (B) of an animal receiving the lowest dose of virus (10^7 pfu/ml) that showed no evidence of CFTR or *lacZ* transgene activity as detected by *in situ* hybridization or histochemical staining in X-gal served as a negative control for antibody specificity (β-gal, left; DBP, middle; fiber, right). Closed arrows mark a cell that stained positively for β-gal and DPB but had no detectable staining for fiber. Open arrows mark areas of autofluorescence seen in all three channels and also detected in sections not treated with secondary antibodies (data not shown). Bar, 53 μm.

Immunocytochemical detection of adenoviral proteins DPB and fiber

Genetically modified lung tissues were evaluated for expression of adenoviral proteins using techniques of immunocytochemistry in which the product of the *lacZ* gene was colocalized with protein products of the early adenoviral gene E2a [DNA binding protein (DBP)] and the fiber protein, which is a translation product of the late adenoviral transcriptional unit. The specificity and sensitivity of this analysis was previously studied in the context of human xenografts infected with wild-type adenovirus and has been described previously (Engelhardt *et al.*, 1993). Figure 7A presents the analysis of lung from the animal harvested 4 days after infusion of the highest dose of virus; on the same field β-gal was detected with AMCA in blue, DBP was detected with texas red, and fiber was detected with FITC in green. High levels of immunocytochemical staining for β-gal was seen within the posterior segment of the left upper lobe (Fig. 7A, left), the same area which demonstrated high-level *lacZ* expression by X-gal histochemistry. Within the group of *lacZ*-expressing cells were found infrequent cells (<0.1% of the total *lacZ*-expressing population) that expressed detectable quantities of DBP (Fig. 7A, middle). DBP always colocalized with *lacZ*, suggesting it was not derived from an unrelated virus. Fiber protein was not detected in any cells (Fig. 7A, right). Right lower lobes from animals that received the lowest dose of virus (10^7 pfu/ml) served as negative controls for antibody specificity and showed no immunoreactive staining for β-gal, fiber, or DBP (Fig. 7B).

Recovery of adenovirus from recipient animals

Each animal was evaluated for recovery of adenoviruses by infecting 293 cells with the biological sample and evaluating the cells for both *lacZ* expression and the development of cytopathic effects (CPE). Specimens from blood, urine, nasopharynx, stool, and bronchoalveolar lavage fluid were harvested 0, 4, 15, and 21 days after gene transfer and analyzed for virus. The only sample from which virus was recovered was the lavage fluid harvested 3 days after gene transfer from the *lacZ* segment of animal B2. All other samples were negative. The sensitivity of both the *lacZ* and CPE assays was consistently 10 particles of virus per plate.

DISCUSSION

Experiments in a variety of small animal models support the potential utility of recombinant adenoviruses for gene therapy of CF lung disease (Rosenfeld *et al.*, 1991a,b, 1992; Engelhardt *et al.*, 1993). Based, in part, on these studies, we proposed a clinical trial for evaluating the biological efficacy and safety of recombinant adenoviruses for CF gene therapy. The general construct of the proposed clinical experiment is to expose a segment of lung to a solution containing CFTR adenovirus and to evaluate the recipient for toxicity as well as efficiency and stability of recombinant gene expression. The experiments described in this and the following manuscript were performed in baboons in an attempt to simulate the proposed clinical trial with four treatment groups, each of which received a single dose of virus ranging from 10^7 pfu/ml to 10^{10} pfu/ml. Within each group of the baboon experiments, animals were necropsied 4 and 21 days after gene transfer to allow precise evaluations of the recipients for gene transfer and toxicity. Animals were also followed longitudinally to allow correlation between the clinical evaluation of gene therapy with findings at necropsy.

An important aspect of the study design was to expose a small and defined area of lung to the virus; the rationale was to minimize the portion of lung at risk if toxicity occurred. Analysis of lung tissue indicated that instillation of the virus through a bronchoscope resulted in gene transfer moderately well localized to the segment of lung into which the virus was infused. These studies also confirmed that recombinant gene expression is dose dependent over at least a 3-log concentration of virus. Histochemical evaluation of lung tissue revealed a consistent pattern of recombinant gene expression in which the majority of expression was found in alveolar cells with patchy areas of expression in bronchi and bronchioles. This is similar to the distribution of gene expression we have observed in mice, ferrets, and rhesus monkeys (Engelhardt and Wilson, unpublished results), however, it contrasts with experiments in cotton rats that demonstrated expression primarily in surface epithelial cells of the conducting airway (Rosenfeld *et al.*, 1991b; Engelhardt, unpublished results). The implications of the distribution of recombinant gene expression found in the baboons to the potential of gene therapy of CF is unclear. The regulation of CFTR expression in human lung is complex with the existence of subpopulations of surface epithelial cells throughout the conducting airway and the alveoli that express high levels of the gene (Engelhardt *et al.*, 1992 and in press). In light of this complexity, it will be difficult with any strategy of somatic gene therapy to reconstitute precisely CFTR expression in the lung. Whether the type of genetic reconstitution achieved with recombinant adenoviruses will be therapeutic will have to await efficacy trials in humans or a physiologically relevant animal model.

An effective gene therapy for CF will require that the recombinant CFTR is expressed for a prolonged period of time and/or that the treatment can be administered repeatedly. Experiments in baboons indicated that expression of recombinant-derived CFTR diminishes significantly within 3 weeks. This apparent instability of transgene expression may be explained by several mechanisms. In general, diminution of expression may be caused by (i) loss of viral DNA because of degradation of the episomal viral genome or turnover of the transduced cell, or (ii) inactivation of the transcriptional elements responsible for expression of the transgene. Southern analysis of lung DNA indicated that an important factor leading to loss of transgene expression was loss of viral DNA. The rate with which diminution of transgene expression occurred suggests that it is not likely caused by turnover of the cells even if that turnover were accelerated in the setting of injury. The most likely explanation is that the viral DNA does not integrate and is retained in an episomal form that is eventually degraded. This finding is in contrast to the results obtained in the human bronchial xenografts where adenovirus-expressed CFTR is retained at high levels for the life of the grafts (*i.e.*, 2 months, [Engelhardt *et al.*, 1993]). Reasons for these disparate results are not

known. However, they may illustrate the limitations of using nonhuman animal models to study the biology of human adenoviruses.

In conclusion, we have presented results from a comprehensive preclinical trial in nonhuman primates that address the efficiency of recombinant adenoviral vectors as a therapeutic vector for gene therapy of CF lung disease. We are able to achieve localized transgene expression in segments of the lung which persist for less than 21 days. The mechanism(s) of lack of persistence is unknown but suggests that current vectors used may require multiple administrations to be an effective therapeutic tool in the treatment of CF.

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