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Aerosol gene delivery *in vivo*

(gene therapy/transfection/cationic liposomes)

ROSCOE STRIBLING*, ELISA BRUNETTE*, DENNY LIGGITT†, KARIN GAENSLER‡, AND ROBERT DEBS*§

*Cancer Research Institute, ‡Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0128; and †Department of Comparative Anatomy, University of Washington School of Medicine, Seattle, WA 98195

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ABSTRACT **The ability to express transgenes selectively within the lung will greatly facilitate the development of gene therapy for a variety of human diseases. We have demonstrated that aerosol administration of a chloramphenicol acetyltransferase (CAT) expression plasmid complexed to cationic liposomes produces high-level, lung-specific CAT gene expression in mice *in vivo*. Significant levels of CAT activity are seen in the lungs for at least 21 days following aerosolization. *In situ* immunostaining for intracellular CAT protein reveals that the majority of airway epithelial and alveolar lining cells are transfected *in vivo*. Histological analyses show no apparent treatment-related damage. These results have important implications for the development of human gene therapy.**

With the advent of molecular cloning techniques, an expanding array of genes with mutations responsible for important human diseases have been identified and isolated. To date, attempts to replace absent or mutated genes in human patients have relied on *ex vivo* techniques (1), since methods for safe and effective *in vivo* gene delivery are not currently available. Retroviruses (2), adenoviruses (3, 4), and liposomes (5) have been used in animal model studies in attempts to increase the efficiency of gene transfer. DNA has been introduced into animals by intratracheal (3, 4, 6, 7), intravenous (7, 8), intraperitoneal (9, 10), intramuscular (11, 12), and intraarterial (13) injections. The lung is a particularly attractive organ for *in vivo* gene therapy because it is directly accessible via the airway. The recent correction of defective chloride channel regulation in cystic fibrosis airway epithelial cells following *ex vivo* introduction of the cystic fibrosis transmembrane conductance regulator gene (14) suggests an obvious target for this type of therapy. Expression of introduced genes, either complexed to cationic liposomes (6, 7) or packaged in adenoviral vectors (3, 4), has been demonstrated in the lungs of rodents after intratracheal instillation. However, intratracheal injection is invasive and produces a nonuniform distribution of the instilled material (15). In contrast, aerosol delivery is noninvasive, results in deeper penetration of material into the lungs, and can deposit aerosolized material throughout the airways and alveoli of healthy individuals (16). Furthermore, aerosol administration has delivered biologically active macromolecules to the lungs of rodents (17, 18) and humans (19, 20). We have found that aerosol delivery of a chloramphenicol acetyltransferase (CAT) reporter gene complexed to a cationic liposome carrier can produce generalized CAT gene expression in mouse lungs *in vivo*, without any apparent toxicity.

MATERIALS AND METHODS

Animals. Two-month-old female ICR mice were used in all experiments.

Preparation of Plasmid DNA. We have used the bacterial CAT gene as a reporter to measure transgene expression levels (21). The plasmid vector used contains the CAT gene fused to the human cytomegalovirus (CMV) immediate early promoter/enhancer element (pCIS-CAT, generously provided by C. Gorman, Genetech). The plasmid was purified by alkaline lysis and ammonium acetate precipitation (22), and the nucleic acid concentration was measured by UV absorption at 260 nm. The CAT gene is not present in eukaryotic cells. Its product is an enzyme that catalyzes the transfer of acetyl groups from acetyl-CoA to the substrate chloramphenicol.

Preparation of Cationic Liposomes. Liposomes were prepared as small unilamellar vesicles (≈100 nm in diameter) containing the cationic lipid DOTMA as DOTMA/DOPE (1:1 mole ratio). DOTMA is *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-triethylammonium (a gift from Syntex, Palo Alto, CA), and DOPE is the neutral lipid dioleoyl phosphatidylethanolamine (Avanti Polar Lipids). Stock solutions of the lipids were dissolved in chloroform and stored under argon at −20°C. Lipids were mixed in a round-bottomed flask and evaporated to dryness on a rotary evaporator under reduced pressure. Double-distilled water was added to produce final lipid concentrations of 10 mM each, and the resulting mixture was sonicated for ≈20 min in a bath sonicator (Laboratory Supplies, Hicksville, NY). The liposomes were stored under argon at 4°C until use.

Aerosol Delivery of Plasmid/Liposome Complexes to Mice. We aerosolized 12 mg of plasmid complexed to 24 μmol of DOTMA/DOPE liposomes over two different aerosol periods on the same day. To prevent aggregation and precipitation of the oppositely charged components, we diluted each separately in sterile water prior to mixing. Six milligrams of plasmid DNA and 12 μmol of DOTMA/DOPE liposomes were each diluted to 8 ml with water and mixed. Equal volumes were then placed into two Acorn I nebulizers (Marquest, Englewood, CO), the animals were loaded into an Intox small-animal exposure chamber (Albuquerque), and an air flow rate of 4 liters/min was used to generate the aerosol. Approximately 90 min were required to aerosolize this volume. The animals were removed from the chamber for 1–2 hr and then the above procedure was repeated.

Radiometric Assay of CAT Activity. Organs were dissected from animals sacrificed at 1–21 days after aerosolization, washed in cold phosphate-buffered saline (PBS), and homogenized in a hand-held tissue homogenizer containing 250 mM Tris·HCl, pH 7.5/5 mM EDTA, for lungs and spleen, or 250 mM Tris·HCl, pH 7.5/5 mM EDTA plus the protease inhibitors aprotinin, E-64, and leupeptin (Boehringer Mannheim), for liver, heart, and kidneys. These inhibitors prevent degradation of acetylated chloramphenicol species generated during the assay, thereby allowing optimal detection of CAT

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Abbreviations: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; DOPE, dioleoyl phosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-triethylammonium.

§To whom reprint requests should be addressed.

expression (data not shown). After homogenization, cells were lysed by three freeze/thaw cycles, and the lysate was heated (65°C for 10 min) and centrifuged (16,000 × g, 2 min). The protein concentrations of the extracts were measured with a Coomassie blue G250-based assay (Bio-Rad). Protein concentrations were normalized and a volume of extract was added to 10 μl of 100 mM acetyl-CoA (Sigma), 0.3 μCi of [^{14}C]chloramphenicol (Amersham), and distilled water to a final volume of 180 μl and allowed to react at 37°C for 8–10 hr (21). Following the reaction, the acetylated and unacetylated chloramphenicol species were extracted with cold ethyl acetate, spotted on silica TLC plates, and developed with a chloroform/methanol (95:5, vol/vol) solvent. The TLC plates were exposed to photographic film (Kodak X-Omat) for 1–3 days.

Preparation of Genomic DNA and Southern Hybridization. Immediately after aerosolization, mice were sacrificed and their lungs were removed. Genomic DNA was isolated and analyzed by Southern hybridization (22) using Hybond N$^+$ membrane (Amersham). CAT probe was prepared from a 1.6-kilobase (kb) fragment of the CAT gene, labeled with [α-^{32}P]dATP by random priming to yield a probe with an approximate specific activity of 2 × 10^9 dpm/μg. After hybridization, the membrane was washed three times in 2× standard saline citrate (SSC)/0.1% SDS at 65°C for 20 min and exposed to film for 24 hr. To determine the approximate copy number of the transfected CAT gene, blots were also hybridized with BSU 36-1, a 1.1-kb single-copy probe from a mouse factor VIII-A genomic clone, generously provided by Jane Gitschier (23). Relative amounts of the CAT plasmid deposited in individual mouse lungs were quantitated by Phosphorimager analysis using a Molecular Dynamics model 400A (24). The amount of retained probe in each lane following hybridization with the CAT probe was normalized to the amount of DNA loaded per lane, using the counts measured after hybridization with the single-copy factor VIII-A probe.

***In Situ* Immunochemical Staining for CAT Enzyme.** At selected time points following aerosolization, mice were sacrificed and their lungs were immediately removed. The lungs were slowly inflated with PBS containing 33% (vol/vol) OCT (optimal-cutting-temperature compound, Miles), placed in a tissue cassette filled with OCT, and frozen in 2-methylbutane chilled in a dry ice/ethanol bath. Cryosections were cut at 5 μm and collected onto silanized slides. CAT was detected after fixation of cryosections for 10 min in either acetone at 4°C or 2% paraformaldehyde in PBS/0.1% Tween 20 at 4°C. (All subsequent dilutions and washes were done in PBS/0.1% Tween 20.) After fixation, sections were washed three times (5 min each), then covered with 10% normal rabbit serum for 10 min at 20°C. The serum was replaced with diluted (1:500) rabbit polyclonal antibody against CAT (a gift from Parker Antin and David Standring, University of California, San Francisco). The antibody-covered section was gently overlaid with a siliconized coverslip and incubated in a humid chamber at 4°C for 24 hr. Slides were then warmed to 20°C and washed three times. Bound rabbit antibody against CAT was detected by covering the sections with a 1:300 dilution of biotinylated affinity-purified goat anti-rabbit antibody (Vector Laboratories) for 1 hr, followed by three washes (10 min each) and replacement with streptavidin labeled with alkaline phosphatase (Zymed Laboratories) for 20 min. Immobilized alkaline phosphatase was detected using AP-red (Zymed Laboratories) as the chromagen; endogenous alkaline phosphatase was inhibited with levamisole (Zymed Laboratories). To control for potential spurious adherence of the streptavidin conjugate to bronchiolar epithelium, some sections were treated with free avidin and biotin prior to application of the primary antibody. Other controls, run concurrently, included the use of normal rabbit serum in place of primary antibody and the use of lung tissue from untreated mice. Photomicroscopy was performed using Kodak Ektachrome 64T film at ×50 (Fig. 4 *A* and *D*) and ×250 (Fig. 4 *B*, *C*, *E*, and *F*).

RESULTS AND DISCUSSION

Initially, mice were exposed either to an aerosol generated from a solution containing 12 mg of a CMV–CAT expression plasmid alone or to an aerosol generated from a solution containing 12 mg of CMV–CAT complexed to 24 μmol of DOTMA/DOPE (1:1) liposomes. Aerosols were administered to animals after they were placed individually in nose-out cones and inserted into an Intox small-animal exposure chamber. Mice showed no apparent ill effects or respiratory distress either during or after aerosol exposure. Fig. 1 shows the results of CAT assays from extracts of the lungs of mice sacrificed 72 hr after aerosol administration. Significant CAT gene expression was seen only in mice exposed to aerosolized DNA/liposome complexes. There was no CAT activity above background present in mice exposed to aerosolized DNA alone, demonstrating that the cationic liposome carrier was required for transfection. In addition, mice placed unrestrained in a 2-liter enclosed plastic box and exposed to DNA/liposome aerosols showed no CAT activity (data not shown).

We then investigated how long CAT protein was present in the lungs of mice and whether expression of the reporter gene was limited to the lungs. Despite inter-animal variation, high levels of CAT activity were present for at least 21 days following a single aerosol dose of DNA/liposome complexes (Fig. 2*A*). No CAT activity was detectable in extracts from the heart, spleen, kidneys, or liver of animals that showed high-level expression in the lungs (Fig. 2*B*), suggesting that transgene expression following aerosol delivery is restricted to the lungs. This is consistent with prior observations showing that penetration of very high molecular weight substances through the respiratory epithelium of normal animals is very limited (25). Plasmid DNA/liposome complexes have molecular weights > 10^6.

Although the small-animal exposure chamber we used was designed to efficiently deliver a uniform aerosol dose to multiple animals, we have observed significant variations in the level of CAT activity in the lungs of mice within a single experiment. One possible explanation for this variability is that the amount of DNA/liposome complex deposited in the lungs of mice is not uniform. To test this hypothesis, we

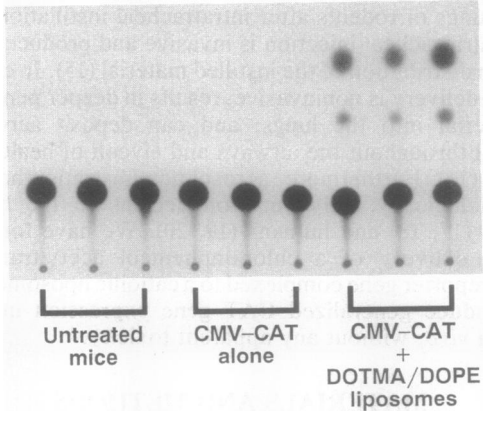

FIG. 1. TLC analysis of CAT activity in lung extracts from mice sacrificed 72 hr after receiving an aerosol containing either 12 mg of CMV–CAT plasmid alone or 12 mg of CMV–CAT plasmid complexed to 24 μmol of DOTMA/DOPE liposomes. Untreated mice were also assayed. Each lane represents a different mouse. Upper spots on the autoradiograph represent acetylated chloramphenicol.

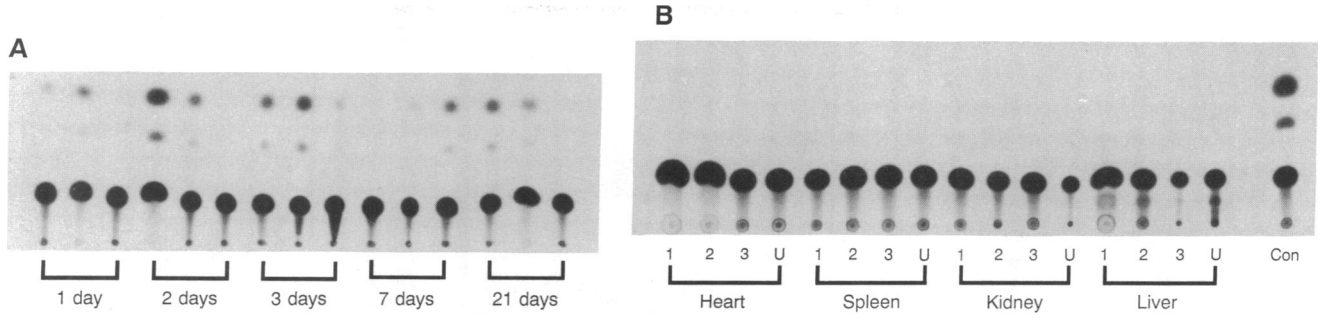

FIG. 2. (*A*) CAT activity in lung extracts from mice sacrificed from 1 to 21 days after receiving an aerosol containing 12 mg of CMV–CAT plasmid complexed to 24 μmol of DOTMA/DOPE liposomes. Each lane represents a different mouse. (*B*) CAT activity in several different tissue extracts from the mice sacrificed at the 3-day time point in *A*. Control extract (Con) contained CAT enzyme. Each lane represents a different mouse. U, untreated control.

measured initial lung deposition of liposomes by fluorescence analysis and of DNA by Southern blot analysis. We administered to mice either aerosolized cationic liposomes alone or DNA/liposome complexes containing a fluorescently labeled lipid, rhodamine-phosphatidylethanolamine, at 0.5 mol%. Immediately following aerosolization, the animals were sacrificed, their lungs were removed and homogenized, and rhodamine fluorescence was measured in a fluorimeter, as described (26). The recovered fluorescence per animal was 0.06 ± 0.02% (mean ± SD) of the total amount aerosolized. This suggests that <10 μg out of the 12 mg of DNA aerosolized per experiment was actually deposited in the lung. In addition, there was no significant difference in lipid deposition between animals receiving liposomes alone and those receiving the DNA/liposome complexes (data not shown). Since it was possible that a disruption of the complex could have occurred during nebulization, we also assessed the amount of CAT gene deposited during aerosolization (Fig. 3). Immediately after aerosol delivery of DNA/liposome complexes, mice were sacrificed and total lung DNA was prepared. Southern blots were probed with ^{32}P-labeled CAT probe. Labeled bands were scanned and demonstrated a <4-fold difference in plasmid deposition between animals in the same experiment (Fig. 3). These results suggest that the mouse-to-mouse variation in CAT gene levels following aerosol delivery (up to 10-fold) is not only a function of the amount of complex initially deposited in the lung but may also reflect differences in the site of uptake, rate of lung clearance, and/or variation in the ability of different lung cell types to express the transgene.

To determine the types and percentage of lung cells that were transfected *in vivo*, lungs of mice sacrificed 72 hr after exposure to an aerosol containing DNA/liposome complexes were cryosectioned, probed with a polyclonal anti-CAT antibody, and counterstained to detect intracellular CAT protein (Fig. 4). Lung sections taken from DNA/liposome-treated mice had a diffuse immunostaining pattern involving bronchiolar and alveolar components. The bronchiolar epithelial cytoplasm was stained with greatest intensity and uniformity. CAT antigen was detected (as demonstrated by red staining) in nearly all conducting airways, with only rare individual cells or two- to three-cell clusters not stained (Fig. 4 *A* and *B*). The diffuse alveolar pattern was due to moderately intense staining of the majority of alveolar lining cells (Fig. 4*C*). These areas occasionally faded into small, randomly scattered regions where lining-cell staining was faint. Focal, intense staining (arrows) occurred in the cytoplasm of scattered individual alveolar lining cells (Fig. 4*C*). Controls included replacement of the primary antibody with normal rabbit serum (Fig. 4*D*) and use of lung sections from untreated animals (Fig. 4 *E* and *F*). Immunostaining was not recognized in either of the control preparations. Examination of multiple sections of lung from treated and control mice demonstrated no significant lesions.

In summary, we have demonstrated that (*i*) a single aerosol dose of a CAT expression vector complexed to cationic liposomes transfects the majority of the cells lining both the conducting airways and the alveoli of the lung, (*ii*) the gene product is present in the lung for at least 3 weeks, (*iii*) the expression appears to be lung-specific, and (*iv*) there is no histological evidence of damage following exposure. There

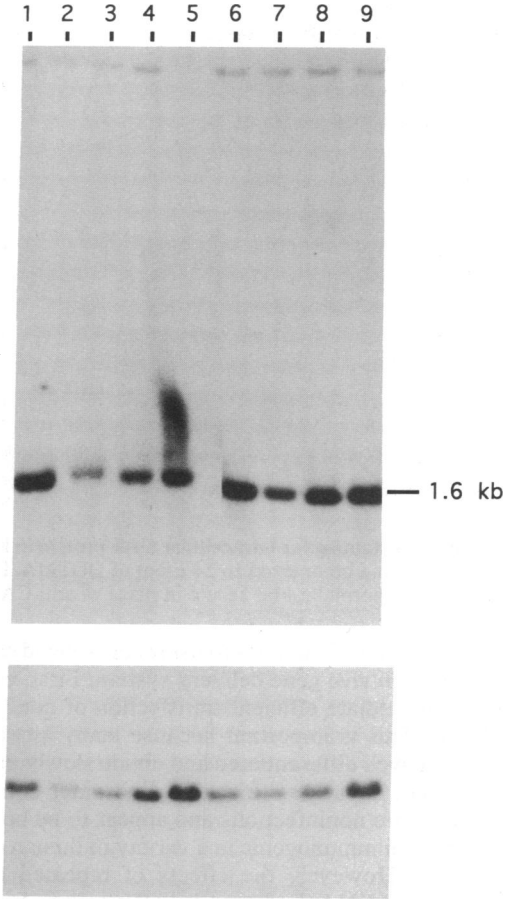

FIG. 3. Southern blot hybridization of genomic DNA from the lungs of mice sacrificed immediately after receiving an aerosol containing 12 mg of CMV–CAT plasmid complexed to 24 μmol of DOTMA/DOPE liposomes (lanes 1–4 and 6–9) and from an untreated control mouse (lane 5). Samples were digested with the restriction enzyme *Hin*dIII and probed with a 1.6-kb CAT fragment (*Upper*). The same membrane was hybridized with BSU 36-1, a 1.1-kb single-copy probe from a mouse factor VIII-A genomic clone (*Lower*). Each lane represents a different mouse.

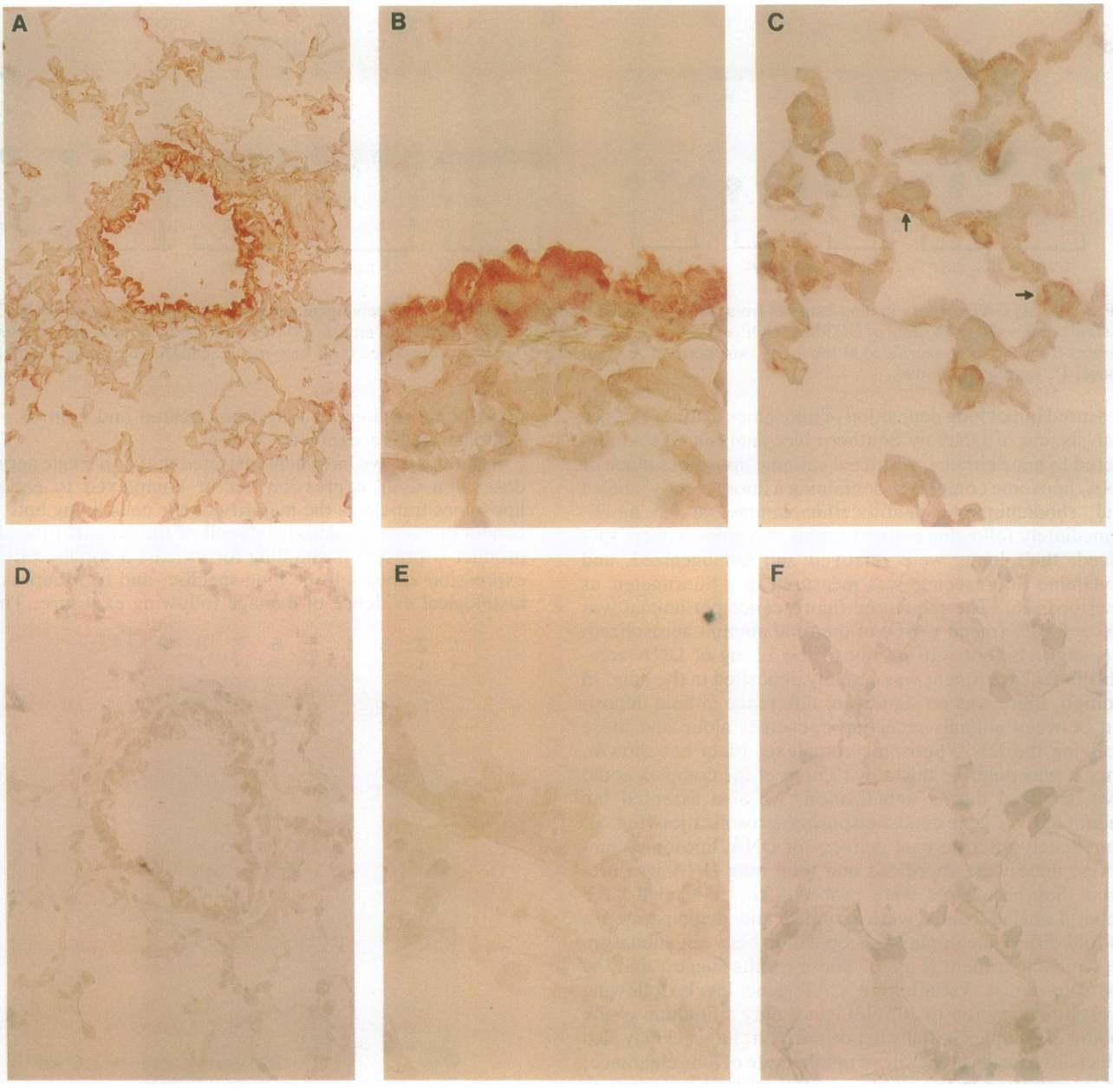

FIG. 4. Immunostaining for intracellular CAT protein in lung sections from mice sacrificed 72 hr after receiving an aerosol containing 12 mg of CMV–CAT plasmid complexed to 24 μmol of DOTMA/DOPE liposomes (*A–D*), or from untreated mice (*E* and *F*). The section shown in *D* was treated with normal rabbit serum in place of anti-CAT antibody. (×23 in *A* and *D*; ×113 in *B*, *C*, *E*, and *F*.)

are several potential advantages to using aerosolized cationic liposomes as an *in vivo* gene delivery system. First, cationic liposomes can mediate efficient transfection of nondividing cells (27, 28). This is important because many airway epithelial cells are well differentiated and divide slowly or not at all (29). Second, liposomes (including liposomes containing cationic lipids) are noninfectious and appear to be both well tolerated and nonimmunogenic in a variety of human clinical trials (30, 31). However, the effects of repeated aerosol administration of DNA/liposome complexes into both normal and diseased lungs will need to be tested. Finally, it may be possible to achieve more precise intrapulmonary targeting by altering aerosol particle size to preferentially direct the aerosol to alveoli or proximal versus distal airways (15) or by covalently coupling monoclonal antibodies to the liposome surface, thereby targeting lung cells expressing the corresponding cell surface antigen (32). Cationic liposome-mediated DNA delivery by aerosol appears promising as a method for achieving high-level, lung-specific transgene expression *in vivo*.

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