Adenovirus-Mediated Persistent Cystic Fibrosis Transmembrane Conductance Regulator Expression in Mouse Airway Epithelium

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Replication-defective adeno vectors have been used for gene transfer to the respiratory epithelium of experimental animals and individuals with cystic fibrosis. Studies from several laboratories have suggested that administration of first-generation Ad vectors results only in transient gene expression in the lung, due at least in part to destruction of vector-transduced cells by host cellular immune responses directed against viral proteins and/or immunogenic transgene products. We have constructed new Ad2-based, E1-deleted vectors encoding a weakly immunogenic transgene, the human cystic fibrosis transmembrane conductance regulator (hCFTR) under the control of the cytomegalovirus enhancer-promoter. These vectors contain wild-type E2 and E4 regions. These new Ad/CFT vectors were instilled into the lungs of immunocompetent C57BL/6, BALB/c, and C5H mice. In vitro cytotoxic T lymphocyte (CTL) analysis indicated the presence of Ad-specific CTLs in treated mice. However, we were not able to demonstrate a CTL response specific for hCFTR. Reverse transcriptase PCR analysis demonstrated that hCFTR mRNA expression continued in all three strains of mice for at least 70 days, the last time point analyzed. The E3 region did not play a significant role in persistence of the Ad/CFT vectors in the mouse lung. Functional hCFTR expression was also observed in the nasal epithelia of CF mutant mice. These results suggest that long-term expression of hCFTR is possible in the airway epithelia of immunocompetent mice without radical modification of Ad vector and in spite of the presence of CTLs.

MATERIALS AND METHODS

Ad vectors. Ad2/CFT-2 is an Ad2-based vector with most of the E1 region (nucleotides 357 to 3328) deleted and replaced with the CFTR expression cassette (9). Ad2/CFT-2 contains a PGK promoter driving hCFTR as the transgene, followed by a bovine growth hormone poly(A) signal and retains wild-type (wt) E2 and E3 regions. The E4 transcription unit has been replaced with open reading frame 6 (ORF6) of E4.

Ad2/CFT-5 is identical to Ad2/CFT-2 except in the CFTR expression cassette, where Ad2/CFT-5 contains a cytomegalovirus (CMV) enhancer-promoter-driven hCFTR followed by a bovine growth hormone poly(A) signal.

Ad2/CFT-16 has the same CFTR expression cassette as Ad2/CFT-5. It contains wt E2 and E4 regions. The E5 region of Ad2/CFT-16 has a 1,549-bp deletion in the E3B region corresponding to Ad2 nucleotides 23929 to 30840.

Ad2/CFT/E3 has the same CFTR expression cassette as Ad2/CFT-5 and Ad2/CFT-16. It contains wt E2 and E4 regions. The E3 region corresponding to Ad2 nucleotides 27971 to 30937 is completely deleted.

Ad2/CMVβgal-1 is a vector that has the CMV enhancer-promoter driving β-galactosidase and luciferase, which were used in these experiments.

More recent studies have demonstrated persistent expression in several strains of mice following intramuscular injection of an Ad vector encoding mouse erythropoietin (19). Other studies have shown that Ad vectors expressing human alpha 1-antitrypsin or human factor IX as the transgene can give rise to long-term expression when the vectors are delivered intravenously to the lungs of C57BL mice but not with other strains (2, 11–13, 20). The prolonged expression in all these studies appears to correlate with the absence of antibodies to the secreted transgene product (11, 12). To date, there have been no reports of an Ad vector capable of persistent transgene expression in the airways of adult immunocompetent animals. Here we describe the construction and in vivo characterization of Ad vectors which encode a therapeutic gene, the human CF transmembrane conductance regulator (hCFTR), and give persistent transgene expression in the lungs of normal immunocompetent mice and functional CFTR expression in the nasal epithelia of CF mutant mice.

E1-deleted replication-defective adeno vectors are attractive candidates for gene transfer because of their ability to transduce a wide variety of dividing and nondividing tissues in vivo (4, 14, 16, 17, 19, 30). We and others have used such Ad vectors for gene transfer to the respiratory epithelia of experimental animals and patients with cystic fibrosis (CF) (3, 9, 14, 24, 28–30). Early studies from several investigators have suggested that administration of high doses of E1-deleted Ad vector results in transient gene expression in vivo (4, 5, 23, 26, 27, 33). Results of experiments carried out with a variety of immunodeficient and immunocompetent strains of mice have suggested that the transience of gene expression is due, at least in part, to the destruction of vector-transduced cells by host cellular immune responses (predominantly CD8+ cytotoxic T cells) directed against viral proteins (4, 5, 23, 26, 27, 33). Reduction of this cellular immune response with second-genera- tion Ad vectors with modification or deletion of the E2 and E4 regions (5, 21, 24) has been reported. However, interpretation of these studies is complicated because of the immunogenic nature of the transgenes such as Escherichia coli β-galactosi-dase and luciferase, which were used in these experiments.

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effector/target cell ratios in triplicate. After 5 h of incubation at 37°C with 5% CO2, 20 μl of cell-free supernatant was collected from each well and counted in a Microbeta Trilux liquid scintillation counter (Wallace Inc., Gaithersburg, Md.). The percentage of lysis was calculated as follows: % lysis = [(sample counts per minute) – (spontaneous counts per minute)]/[total counts per minute] – (spontaneous counts per minute)] × 100.

**RNA extraction and analysis.** Total RNA was extracted from lung tissue with acid guanidinium thiocyanate–phenol-chloroform and treated with DNase (RNase-free DNase I; Promega Madison, Wis.). Levels of hCFTR mRNA were determined by quantitative reverse transcriptase (RT)-PCR essentially as described previously (10). RNA samples from animals in the same group were pooled. The RT reaction was performed with a cDNA kit (Invitrogen). Following reverse transcription, cDNA was amplified with primers differing in length (human sequence, 5'-CCAGACCAATTTTGAGGAAAG-3'; mouse sequence, 5'-CCACACCAATTTTGAGGAAAG-3') and was used in conjunction with the RT primer to amplify the identical regions of the endogenous CFTR mRNA and hCFTR mRNA derived from the vector. PCR conditions were as follows: 30 cycles of 95°C denaturation, 55°C annealing, and 72°C elongation. Control reactions with the PCR primers with either the human or murine sequence gave identical results with Ad2/CFTR-16-transfected mouse lung RNA, indicating that the single base difference near the 5' end of the primers did not affect PCR amplification. Within the 405-bp fragment that is amplified by this method, there are restriction endonuclease cleavage site differences that can be used to distinguish between the mouse and human products. For example, Mpl cleaves the hCFTR (i.e., vector-derived) RT-PCR product into two fragments of 268 and 137 bp; Mpl does not cleave the mouse-derived 405-bp fragment.

**β-Galactosidase assay.** Lungs from individual animals were homogenized, and β-galactosidase activity was measured by the Galactolight assay (Tropix). The protein concentration in lung homogenate was measured with the Bio-Rad DC protein assay. Protein concentration in lung homogenate was measured with the Bio-Rad DC protein assay.

**RESULTS**

**Construction of new Ad2/CFTR vectors.** A primary goal in designing new Ad2 vectors for CF gene therapy was to obtain vectors that are capable of directing persistent hCFTR gene expression in vivo. The CMV enhancer-promoter was chosen to direct expression of hCFTR cDNA because we have shown previously that this promoter can direct prolonged transgene expression in the lung, the target organ for CF gene therapy (1). Sustained CMV promoter-driven expression is dependent on E4 gene activity; thus, the wt E4 region was included in the vector design. The wt E4 region is known to encode several proteins that are involved in the immune response (22). In Ad2/CFTR-16, the gp19K coding sequences from E3A were retained on the basis of reports that expression of the gp19K protein may improve the longevity of gene expression from Ad vectors, presumably by inhibiting major histocompatibility complex (MHC) class I antigen presentation (13). The gp19K protein may improve the longevity of gene expression from Ad vectors, presumably by inhibiting major histocompatibility complex (MHC) class I antigen presentation (13).
hCFTR mRNA expression in the lung was measured over time by quantitative RT-PCR. Similar to our results in nude C57BL/6 mice, CFTR mRNA expression in normal C57BL/6 mice was essentially undiminished over time, until 70 days postexposure in this experiment (Fig. 2a). Expression of hCFTR mRNA was not measured beyond 70 days, since cells in the airway epithelium are not permanent but are replaced several times per year. A parallel group of C57BL/6 mice were instilled intranasally with $2 \times 10^9$ IU of Ad2/CFTR-5, a vector bearing the same gene expression cassette as Ad2/CFTR-16 but having an E4 region deleted for all sequences except ORF6 (7). Expression of hCFTR from this vector declined rapidly (Fig. 2a).

To ensure that the persistence of hCFTR expression observed was not due to a limited immune responsiveness of the C57BL/6 mouse strain, Ad2/CFTR-16 was administered to mice from two other inbred strains. Groups of BALB/c and C3H mice were instilled intranasally with Ad2/CFTR-16, and hCFTR mRNA expression was measured at intervals up to 70 days. As shown in Fig. 2, Ad2/CFTR-16 gave rise to expression of hCFTR at day 70 that was not markedly different from that measured at day 3 in all three strains of mice examined. For comparison, a parallel group of BALB/c mice were administered Ad2/CFTR-2, a vector using the PGK promoter to direct hCFTR gene expression and having only ORF6 from the E4 region (9). Expression of hCFTR mRNA from this vector declined rapidly (Fig. 2b). Analysis of DNA levels in the lungs of mice treated with different Ad/CFTR vectors did not reveal a correlation between loss of gene expression and the loss of vector DNA; in all cases approximately 90% of the vector DNA was lost by day 21 (data not shown), suggesting that prolonged expression was directed by a small proportion of input vector. This result suggests that the rapid decrease in transgene expression that we observed by day 21 with the E4ORF6 containing vectors Ad2/CFTR-5 and Ad2/CFTR-2 is not a result of a relatively faster destruction of E4ORF6 containing vector-transduced cells.

Given that quantitation of mRNA levels by RT-PCR methods is challenging, and because the finding of prolonged hCFTR expression from Ad2/CFTR16 runs counter to the broad perception that Ad vectors direct transient expression in vivo, we felt that it was crucial to confirm our expression results by an alternative method. This alternate method of RNA detection involves a set of RT-PCR primers that coamplify a segment of the endogenous mouse CFTR mRNA and the cognate segment of hCFTR mRNA. The proportion of the RT-PCR product that is derived from the vector is revealed by
digested of the mixed RT-PCR product with a restriction endonuclease that cleaves the hCFTR product but does not cleave the mouse product. RT-PCR was performed on RNA isolated from lung tissue from experiments similar to those shown in Fig. 2a and b. The RT-PCR products were then digested with MspI, which cleaves only hCFTR, and analyzed by gel electrophoresis. As seen in Fig. 3, murine CFTR mRNA was present at steady levels throughout the experiment and served as an internal control for the RT-PCRs. The levels of Ad2/CFTR-16-encoded hCFTR mRNA did not vary markedly over the time course of the experiment. Expression of hCFTR mRNA from Ad2/CFTR5, however, declined to background levels by day 45. Although this method is not quantitative, the levels of hCFTR mRNA expressed from the Ad2/CFTR-16 vector in the mouse lung appear to be equal to or greater than the levels of endogenous mouse CFTR mRNA expression over the time course of this experiment (70 days).

Functional CFTR expression in nasal epithelia of CFTR mutant mice. It is clear from the above-mentioned experimental results that Ad2/CFTR-16 can direct long-term expression of hCFTR mRNA in the lungs of immunocompetent mice. Under the experimental conditions used, the majority of gene transfer is to the small airways within the lung, the desired location for CF gene therapy. However, the small airways of the mouse are inaccessible to current techniques for measurement of chloride secretion. Previously, we have measured the ability of Ad2/CFTR-5 to correct the CF chloride secretion defect in the nasal passage of a strain of CFTR knockout mouse (7). We found that Ad2/CFTR-5 can correct the nasal defect on day 2 postadministration but that this correction had largely vanished by 7 days postadministration (7). To measure the ability of Ad2/CFTR-16 to correct the CF chloride secretion defect, similar gene transfer experiments were carried out in the nasal cavities of mice defective for CFTR activity. The mice employed in these experiments bear a knock-out deletion within the endogenous CFTR gene but are transgenic for hCFTR cDNA controlled by an intestine-specific promoter (32). This genetic composition renders the animals functionally defective for CFTR within the respiratory tract, including the nasal epithelium.

Expression of functional CFTR protein was examined in the nasal epithelia of double-transgenic CF (−/−) mice than in wt mice. Perfusion of the nasal epithelia with Ringer's solution resulted in a decline in the basal PD of both wt (+/+ ) and CF (−/−) mice (data not shown). Subsequent perfusion with Ringer's solution containing amiloride (100 μM) resulted in further decreases in the PD for all groups. This reduction was significantly greater in CF (−/−) than in wt (+/+ ) mice (data not shown).

In the untreated double-transgenic CF (−/−) mice, in the presence of amiloride (100 μM), replacement of NaCl with sodium gluconate (low Cl) in the Ringer's solution caused a small depolarization (Fig. 4b) as observed previously in the CF null mice (6, 7, 31). These results suggest that the electrophysiological properties of the nasal epithelia of the bitransgenic CF mice are similar to those of CF null and ΔF508 mice (6, 7, 31). Administration of Ad2/CFTR-16 resulted in a decrease in basal PD (Fig. 4a) and restored the hyperpolarization in response to low Cl (Fig. 4b), indicating the presence of functional CFTR within the nasal epithelium. Furthermore, these electrophysiological changes were not significantly reduced between day 2 and day 15 after vector administration (analysis of variance, followed by Student-Newman-Keuls test; P > 0.05). These data demonstrate that the CFTR expression cassette in Ad2/CFTR-16 can give rise to expression of both mRNA and functional CFTR protein in vivo.

Anti-Ad vector CTLs are present in immunocompetent mice treated with Ad2/CFTR vectors. A CTL response against E1-deleted Ad vectors has been demonstrated by several investigators following vector delivery to the lungs or livers of immunocompetent mice, and it has been shown that viral proteins expressed from E1-deleted Ad vectors are targets for Ad-specific CTL in vitro (10, 11, 20, 23, 26, 27). We have shown previously that these vector-specific CTL do not necessarily limit vector expression from transfected cells in the liver (20). To test whether the longevity of expression from Ad2/CFTR vectors could be explained by an unforeseen failure to provoke a CTL response, leading to an escape from immune surveillance, we investigated the CTL response following intranasal delivery of Ad2/CFTR-16. Consistent with previous studies from our laboratory and others, we were able to demonstrate a vector-specific CTL response in C57BL/6 (data not shown) and BALB/c mice (Fig. 5). We do not find any significant differences in cytolysis with target cells infected with Ad2/CFTR-16, Ad2/CFTR-5, or Ad2/CFTRΔE3. In several studies conducted in our laboratory, we have not been able to demonstrate a
CTL response specific for hCFTR in Ad/CFTR vector-treated mice.

Repeat administration of Ad2/CFTR-16. Our results demonstrate that the CTL response against an Ad vector expressing a weakly immunogenic transgene is ineffective after a single administration of the vector to the lung, as has been demonstrated previously for administration to the liver (20). In terms of persistence of transgene expression, an important issue for effective use of gene therapy vectors for the treatment of CF is the CTL response following repeated administration. Effective repeated administration of Ad vectors can be achieved through the use of immunomodulatory drugs such as antibody to CD40 ligand (15, 25) or deoxyspergualin (8). The use of such agents depresses the neutralizing antibody response and allows for

FIG. 4. Functional CFTR expression in nasal epithelia of CF mutant mice. Ad2/CFTR-16 ($10^9$ IU) was perfused over the nasal epithelia of CF mutant mice over a 60-min period. Basal nasal PD (a) and change in PD in response to low Cl$^-$ substitution (b) were measured on days 2, 7, and 15 posttreatment. Data are expressed as mean ± standard errors of the mean ($n = 4$).
were instilled intranasally with 10^9 IU of Ad2/CMV transgene. Parallel groups of immunocompetent C57BL/6 mice persistence of these vectors expressing a weakly immunogenic endogenous E3 region does not play a significant role in the days, the last time point analyzed (Fig. 7a), indicating that the inclusion of the endogenous E3 region in Ad vectors does not improve persistence of an Ad vector expressing a highly immunogenic transgene.

FIG. 5. CTL response to Ad2/CFTR vectors. BALB/c mice were instilled with 2 × 10^9 IU of Ad2/CFTR-16 on day 0. On day 21, spleen cells were collected, restimulated in vitro with Ad2/CFTR-16-infected syngeneic fibroblasts, and tested for cytolytic activity against target cells infected with different Ad/CFTR vectors. Results shown are the mean percentages of lysis from triplicate wells at various effector/target ratios. Targets: uninfected (C); Ad2/CFTR-5 (●); Ad2/CFTR-16 (○); Ad2/CFTRΔE3 (▲).

FIG. 6. Repeat dosing with Ad2/CFTR-16. BALB/c mice were instilled with 10^8 IU of Ad2/CFTR-16 or Ad2/CFTR-5 on day 0 and were then challenged with a dose of Ad2/CFTR-16 on day 14. During this time interval, a CTL response to vector antigens is stimulated, but neutralizing antibodies do not reach inhibitory levels (data not shown). On day 0, a parallel group of animals were treated with a dose of 10^9 IU of Ad2/CFTR-16, and the vector-derived hCFTR mRNA levels were measured until day 59 (Fig. 6). There was no indication that previous exposure of animals to either Ad2/CFTR-16 or Ad2/EV had any impact on the duration of expression from the challenge dose of Ad2/CFTR-16 compared to that in animals receiving only a single vector dose.

Role of E3 in persistence. Poller et al. (13) have reported that the E3A region plays an important role in persistence of a human factor IX expressing Ad vector in the livers of immunocompetent mice. To determine the role of E3 in the persistence of transgene expression with Ad2/CFTR-16 in the lungs of immunocompetent mice, normal BALB/c mice were instilled intranasally with 5 × 10^8 IU of Ad2/CFTR-16 or Ad2/CFTRΔE3, a vector that is completely deleted for the E3 region. hCFTR mRNA expression was measured over time in total lung RNA by quantitative RT-PCR. Expression of hCFTR mRNA was essentially undiminished with both vectors until 70 days, the last time point analyzed (Fig. 7a), indicating that the endogenous E3 region does not play a significant role in the persistence of these vectors expressing a weakly immunogenic transgene. Parallel groups of immunocompetent C57BL/6 mice were instilled intranasally with 10^8 IU of Ad2/CMVβgal-1 (wt for E3 and E4) or Ad2/CMVβgal-ΔE3 (E3 completely deleted and wt for E4). β-Galactosidase activity was measured over time by the Galactolight assay. Expression of β-galactosidase declined rapidly to background levels by day 21 with both vectors (Fig. 7b), indicating that the inclusion of the endogenous E3 region in Ad vectors does not improve persistence of an Ad vector expressing a highly immunogenic transgene.

DISCUSSION

Prominent among the potential limitations of Ad vectors for gene therapy is the belief that in vivo expression of Ad vector-encoded transgenes is inherently transient in nature. Early reports highlighted the transient nature of in vivo Ad vector-mediated gene transfer such that gene expression was seen to plummet within 2 to 3 weeks to levels representing only a small fraction of that measured just after administration (4, 24, 26, 33). In principle, any of a number of events can result in truncated gene expression: vector-induced cytotoxicity, vector-or transgene-specific CTL attack, complete loss of vector DNA, or promoter shutoff. Persistent gene expression can occur only in the absence of each of these potential limitations. The vast majority of studies on gene expression have relied on the use of foreign reporter genes, the protein products of which are highly immunogenic and capable of provoking strong cellular and/or humoral immune responses; this immune response appears to be responsible for extinguishing expression from the Ad vector under study. The realization that Ad vector-specific CTL may not necessarily be effective in eliminating transfected cells came from the demonstration that long-term gene expression could be achieved in immunocompetent animals under conditions in which there were no immune responses to the vector-encoded reporter gene product (2, 11–13, 19, 20). The conclusions of those studies were limited to liver and muscle and were mouse strain dependent. The current study employs human CFTR as the transgene, which, although potentially immunogenic in mouse, has failed to provoke a detectable immune response as expressed from our Ad vectors. Persistent gene ex-
pression in the lung has been achieved through the use of a specific vector genotype, the CMV enhancer-promoter for transgene expression in conjunction with a wt E4 region (1).

Having demonstrated that human CFTR was weakly immunogenic in normal mice and that the CMV promoter combined with a wt E4 region could direct prolonged β-galactosidase expression in nude mice, new vectors incorporating these features were constructed. Although it was predicted that these new Ad/CFTR vectors would escape immune surveillance and that its gene expression cassette would remain active, the possibility remained that prolonged hCFTR expression in a normal mouse might elicit immune and/or inflammatory responses sufficient to extinguish gene expression. However, the results of the current study demonstrate clearly and consistently that Ad2/CFTR vectors can direct expression of hCFTR mRNA at undiminished levels for at least 70 days. Moreover, functional correction of the CF chloride secretion defect in the nasal epithelium was undiminished for up to 15 days, the longest interval tested.

Persistent gene expression was observed despite the presence of an immediate nonspecific response to vector administration characterized by inflammatory cells and cytokines and a vector-specific cellular immune response. These responses have been implicated broadly as representing intrinsic limitations to the effectiveness of Ad vectors for gene therapy. The results of this study indicate clearly that in the three immunocompetent inbred mouse strains tested, conventional Ad vectors of the appropriate genotype can direct long-term gene expression in the lung. However, if a highly immunogenic transgene like β-galactosidase is used, the inclusion of the E3 region does not improve persistence. This does not rule out the possibility that overexpression of E3 proteins may have benefits in the mouse or other experimental systems (6a). Ad vectors do provoke a CTL response in C57BL/6 and BALB/c mice (10), and the E3 gp19K protein does bind to the MHC I heavy chains encoded by these strains. However, the C3H mouse exhibits a weak CTL response to Ad vector (10), and the MHC I heavy chain from this strain binds only weakly to the E3 gp19K protein.

While the results of this study are encouraging in the sense that vector-directed gene expression was achieved for a period of several weeks, a key question that remains is the half-life of the respiratory epithelial cell. Since the results of numerous studies indicated that respiratory epithelial cells in a number of species are replaced over a period of 60 to 100 days, we arbitrarily terminated our gene expression studies at 70 days. The issue of longevity of respiratory epithelial cells in the CF patient’s lung clearly cannot be resolved in the rodent model and must be addressed in the context of a clinical study.

The treatment of certain genetic diseases such as CF will require repeated administrations throughout the lifetime of the patient. It is known that Ad vector-based gene delivery gives rise to a dose-dependent humoral immune response leading to the development of neutralizing antibodies to adenoviruses (2, 4, 9, 23, 28) which reduces the efficiency of repeated gene expression studies.

![Graphs](http://jvi.asm.org/) FIG. 7. Role of E3 region in persistence. (a) Mice were instilled with $5 \times 10^9$ IU of Ad2/CFTR-16 (○) or Ad2/CFTR/ΔE3 (★). RT-PCR analysis was performed as described in Materials and Methods. (b) C57BL/6 mice were instilled with $10^9$ IU of Ad2/CMVβgal-1 (●) or Ad2/CMV/βgal/ΔE3 (△). β-Galactosidase (βgal) expression was measured as described in Materials and Methods. RLU, relative light units.
gene transfer to a fraction of that achieved initially. Switching of vector serotypes may circumvent the neutralizing antibody issue, although practical considerations such as development, approval, and manufacturing of multiple vectors for a single disease indication may limit this approach. Recent studies have shown that transient immunosuppression can effectively block the humoral response and allow repeated Ad vector administration to the mouse lung (8, 15, 25). This strategy could have undesirable side effects and remains untested in humans.

We demonstrate of Ad vector-mediated long-term transgene expression in the murine lung taken together with the transient immunosuppression strategies to block the anti-Ad neutralizing antibody response provides hope that gene therapy for CF may be clinically feasible in the future. Further studies of this nature are clearly warranted in nonhuman primates.

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