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Repeated Aerosolized AAV-CFTR for Treatment of Cystic Fibrosis: A Randomized Placebo-Controlled Phase 2B Trial

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ABSTRACT

Previous studies have demonstrated that delivery of a recombinant adeno-associated virus (AAV) vector encoding the complete human cystic fibrosis transmembrane regulator (CFTR) cDNA (tgAAVCF) to the nose, sinus, and lungs of subjects with cystic fibrosis (CF) was safe and well tolerated. In a small randomized, double-blind study of three doses of aerosolized tgAAVCF or placebo at 30-day intervals, encouraging but nonsignificant trends in pulmonary function and induced sputum interleukin 8 (IL-8) levels were seen at early time points. This larger study was conducted to verify these trends. One hundred and two subjects aged 12 years and older with mild-to-moderate cystic fibrosis (forced expiratory flow in 1 sec [FEV_1] : 60% predicted) were randomized to two aerosolized doses of 1×10^{13} DNase-resistant particles of tgAAVCF ($n = 51$) or matching placebo ($n = 51$) administered 30 days apart. Although tgAAVCF was well tolerated, the study did not meet its primary efficacy end point of statistically significant improvement in FEV_1 30 days after initial administration of tgAAVCF compared with placebo. There were no significant differences in spirometric lung function over time, induced sputum biologic markers, or days of antibiotic use in either treatment group. Thus repeated doses of aerosolized tgAAVCF were safe and well tolerated, but did not result in significant improvement in lung function over time. Because gene transfer is the simplest, most basic way to correct the underlying genetic defect that leads to disease in CF, further research is warranted to develop an effective gene transfer agent for the treatment of CF.

OVERVIEW SUMMARY

In this study, 102 subjects aged 12 years or older with mild-to-moderate cystic fibrosis (FEV_1 : 60%) received two doses of tgAAVCF or matching placebo administered 30 days apart via nebulizer. The study drug was safe and well tolerated. The pattern of adverse events was similar between subjects who received tgAAVCF and those who received placebo. In contrast to the smaller, phase 2 study that preceded this study, no difference in the change in FEV_1 or

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IL-8 levels was noted between treatment groups. The number of days of antibiotic use was comparable between treatment groups. This study, the largest gene transfer study conducted in CF patients to date, demonstrates the safety of adeno-associated virus vectors administered by aerosol delivery but also suggests the need for further improvement in gene transfer technology and/or more appropriate clinically relevant efficacy end points.

INTRODUCTION

CYSTIC FIBROSIS (CF) is an autosomal recessive disease with an incidence of approximately 1 in 4000 live births in large U.S. newborn screened populations (Therrell *et al*., 2005). CF is characterized by progressive lung disease due to chronic bacterial colonization, malabsorption due to exocrine pancreatic insufficiency, abnormal regulation of salt transport across the gastrointestinal and respiratory epithelium, and male infertility due to absence or stenosis of the vas deferens (Koch and Hoiby, 1993; Rosenfeld *et al*., 1993). In the United States, the predicted median survival of an individual with CF is 36.5 years (Cystic Fibrosis Foundation, 2006). More than 90% of mortality in CF is due to respiratory failure from progressive lung disease.

The cystic fibrosis transmembrane regulator (CFTR) gene, located on chromosome 7, encodes a 1480-amino acid residue protein that functions, at least in part, as a cAMP-regulatable chloride channel on the apical surface of the epithelium. The most common mutation of the CFTR gene is ΔF508, a deletion of the codon for the phenylalanine at location 508 (Koch and Hoiby, 1993; Rosenfeld *et al*., 1993). When normal copies of the CFTR gene are transferred to human respiratory epithelial cells in cell culture, CFTR DNA and mRNA are expressed with accompanying restoration of normal ion transport, suggesting potential correction of the defect and of the phenotype.

Delivery of the normal gene to the lungs of subjects with CF has presented several challenges, including the identification of an acceptable vector. Adeno-associated virus (AAV) is a capable vector for this purpose. AAV is not known to cause disease, nor is it a transforming or oncogenic virus. AAV exists mainly as an unintegrated episomal concatemer *in vivo* (Schnepp, 2005), and no significant alterations in cell growth or morphology have been documented when AAV is integrated into human cell lines *in vitro*. AAV is a replication-defective vector, capable of high-frequency stable gene transfer and expression in a variety of cells, including respiratory epithelial cells from subjects with cystic fibrosis (Carter, 1992; Egan *et al*., 1992; Flotte *et al*., 1992, 1993). tgAAVCF is a recombinant vector encoding the complete human CFTR cDNA developed for clinical use. When tgAAVCF was administered to rabbits and monkeys, physiologically active, nonmutated, human CFTR DNA and mRNA were recovered from lung cells (Conrad *et al*., 1996). Human respiratory epithelial cells that have been transfected with tgAAVCF *in vitro* also express human CFTR DNA and mRNA (Flotte *et al*., 1993).

Clinical trials of tgAAVCF have been conducted. Phase 1 studies focused on safety, evaluating tgAAVCF-mediated gene transfer to the maxillary sinus cavity by direct instillation, to the right lower lobe delivered by bronchoscopic lavage, and to the whole lung by aerosol administration. The vector was well tolerated in these studies and dose-dependent gene transfer was observed (Wagner *et al*., 1998, 1999, 2002; Aitken *et al*., 2001; Flotte *et al*., 2003).

A phase 2 multicenter double-blind placebo-controlled study was conducted to evaluate the safety of multiple doses of aerosolized tgAAVCF in CF subjects. tgAAVCF was well tolerated and safe. Gene transfer was noted in a subset of subjects undergoing bronchoscopy, with a median copy number of 29–100 genomes per cell, depending on the site of brushing. Nonsignificant trends to improved pulmonary function and reduced sputum interleukin (IL)-8 levels were observed at early time points (Moss *et al*., 2004). A follow-up phase 2B study was designed to extend these observations and further investigate the safety, potential durability, and benefit of aerosolized multidose delivery of tgAAVCF.

MATERIALS AND METHODS

Study agent

The active study agent was tgAAVCF, a recombinant AAV2 vector genetically engineered to contain the complete coding region of the human CFTR cDNA. The vector was constructed by replacing the entire wild-type AAV viral coding sequence with the full-length human CFTR cDNA and a synthetic polyadenylation sequence based on murine β-globin. This construct was flanked by the AAV inverted terminal repeat sequences that are required for viral replication and packaging during the manufacturing process. tgAAVCF was formulated in a sterile isotonic buffered salt solution containing calcium and magnesium. Placebo consisted of the sterile isotonic buffered salt solution containing calcium and magnesium. tgAAVCF was produced under current Good Manufacturing Practice guidelines at Targeted Genetics (Seattle, WA). The tgAAVCF product used in the present study was produced by the same process, and as part of the same manufacturing campaign, as the tgAAVCF product that was used in the previous smaller phase 2 trial (Moss *et al*., 2004).

Both tgAAVCF and matching placebo were administered with the PARI LC Plus nebulizer (PARI, Midlothian, VA) using an AutoNeb controller that restricted the aerosol generation to the inspiratory phase of the respiratory cycle of the subject. The nebulizer was equipped with a one-way valve and an exhalation filter. It is estimated that about one-third of the nominal tgAAVCF dose reaches the lower respiratory tract with this system (Leung *et al*., 2007).

Study population

Subjects were recruited to this randomized, placebo-controlled, double-blind study from 12 CF centers in the United States. The study was approved by the institutional review boards and institutional biosafety committees of the participating institutions, and informed consent was obtained from all subjects. The protocol was reviewed and approved by the U.S. Food and Drug Administration (FDA, Rockville, MD) and the National Institutes of Health (Bethesda, MD) Recombinant DNA Advisory Committee.

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS[a]

	tgAAVCF (n = 51)	Placebo (n = 51)
Age, years	23.9 ± 10.9	21.3 ± 8.7
Gender		
Male	26 (51%)	28 (55%)
Female	25 (49%)	23 (45%)
CF genotype		
AF508 homozygous	27 (53%)	27 (53%)
AF508 heterozygous	18 (35%)	20 (39%)
FEV_1, % predicted	84.7 ± 13.7	87.9 ± 15.5

[a]Data are presented as number (%) or mean ± standard deviation.

Abbreviations: CF, cystic fibrosis; FEv1, forced expiratory volume in 1 sec; tgAAVCF, vector encoding the complete human cystic fibrosis transmembrane regulator.

Entry criteria included a confirmed diagnosis of CF, defined as clinical features of CF plus either a positive sweat chloride ≥60 mEq/liter or two identifiable mutations consistent with CF; forced expiratory volume in 1 sec (FEV_1) ≥60% predicted for age; and age ≥12 years. Exclusion criteria included receipt of intravenous antibiotics for a respiratory infection within 30 days of screening, severe hemoptysis, lung transplantation, cigarette smoking within 90 days of screening, and known substance abuse within 30 days of screening.

Study design

In this randomized, double-blind, placebo-controlled study, subjects (dosing goal, $n \geq 100$) were randomized at a 1:1 ratio to two aerosolized doses of either 10^{13} DNase-resistant particles (DRP) of tgAAVCF or matching placebo (buffered salt solution) administered 30 days apart with the PARI LC Plus nebulizer.

Sample size calculations were based on the effect sizes observed for the 30-day change in FEV_1 (liters) and 14-day change in sputum IL-8 concentration (log_{10} ng/ml) in the initial phase 2 multidose aerosol study, in which subjects received three doses of 1×10^{13} DRP of tgAAVCF or placebo at 30-day intervals (Moss *et al.*, 2004). Enrollment of 100 subjects, 50 in each treatment arm, would provide 93% power to detect a 0.14-liter difference in the 30-day change in FEV_1 between treatment groups, assuming a standard deviation of the change of 0.20 liter in each group. This sample size would also provide adequate power for detecting ≥0.3 log_{10}-ng/ml differences between treatment groups in IL-8. A planned interim futility anal-

ysis was also performed, which resulted in completion of the trial.

Study procedures

After completion of screening procedures, eligible subjects were randomized and received study drug by inhalation on study days 0 and 30. Pulmonary function testing was performed at screening, before dosing, and then every 14 to 15 days until 90 days after first study drug administration. Subjects underwent sputum induction for determination of sputum biologic markers (IL-8, DNA, neutrophil elastase, and protein concentrations) at screening, and then 14 and 45 days after first study drug administration. Sputum biomarkers were determined at a central laboratory as previously described (Moss *et al.*, 2005). Adverse events were collected at study visits for the first 90 days after first study drug administration, and then by telephone interview at study days 150 and 210.

The primary end point was 30-day change in absolute FEV_1. Secondary end points were changes in absolute FEV_1 values, FEV_1 % predicted, forced vital capacity (FVC), and mid-maximal expiratory flow rate (FEF_{25-75}) over serial time points from 14 to 90 days after first dosing, changes in induced sputum biologic markers over time, number of days of antibiotic use, and safety.

The study was overseen by a Data Safety Monitoring Committee (DSMC) of the Cystic Fibrosis Foundation. The DSMC was an independent, multi-disciplinary group consisting of physician subspecialists and a statistician who, collectively, have experience in treating patients with cystic fibrosis and in the conduct of randomized clinical trials. The DSMC convened on four occasions to review available, unblinded safety data. Each time the DSMC recommended study continuation.

RESULTS

A total of 122 subjects were screened for the study; 109 subjects were randomized and 102 received treatment. Of the 102 subjects who received treatment, 98 completed the study and 4 discontinued early. Of the four early discontinuations, one was in the tgAAVCF treatment group and three were in the placebo group. The reason the subject who received tgAAVCF discontinued early was loss to follow-up. Reasons for early discontinuation among the placebo recipients included experiencing an adverse event (unlikely to be related pulmonary exacerbation), death (unrelated motorcycle accident), and other (no response to day 210 phone call).

TABLE 2. ADVERSE EVENT OVERVIEW

	tgAAVCF (n = 51)		Placebo (n = 51)	
	Number of events	*Number (%) of subjects*	*Number of events*	*Number (%) of subjects*
Treatment-emergent AEs	515	50 (98%)	521	51 (100%)
Serious adverse events	22	13 (26%)	15	11 (22%)
Toxicity grade ≥3	30	13 (26%)	31	11 (22%)

Abbreviation: AE, adverse event.

TABLE 3. INCIDENCE OF MOST FREQUENT ADVERSE EVENTS[a]

	tgAAVCF (n = 51)	Placebo (n = 51)
Cough increased	34 (67%)	38 (75%)
Rhinitis	30 (59%)	35 (69%)
Sputum increased	19 (37%)	23 (45%)
Pharyngitis	22 (43%)	19 (37%)
Sinusitis	12 (24%)	15 (29%)
Headache	12 (24%)	15 (29%)
Lung function decreased	14 (28%)	10 (20%)
Asthenia	11 (22%)	13 (26%)
CF pulmonary exacerbation	13 (26%)	10 (20%)
Abdominal pain	8 (16%)	14 (28%)
Fever	14 (28%)	7 (14%)

[a]Data are presented as number (%) of subjects with AE. Most frequent adverse events are defined as adverse events occurring in ≥20% of subjects overall.

The demographics and clinical characteristics of the subjects dosed in the two treatment groups were similar (Table 1). In general, subjects had mild lung disease (mean FEV_1, 86.3% predicted overall), and were relatively young, with 23 subjects between 12 and 14 years of age, and 23 subjects between 15 and 17 years of age.

Safety results

A total of 1036 treatment-emergent adverse events were reported among 101 of 102 subjects. The proportion of subjects experiencing one or more adverse events, one or more serious adverse events, or one or more toxicity grade ≥3 adverse events was similar between the two treatment groups (Table 2).

A total of 37 serious adverse events were reported among 13 tgAAVCF subjects and 11 placebo subjects. None were considered definitely or probably related to study drug. Serious adverse events that occurred among tgAAVCF subjects included 16 CF pulmonary exacerbations and 1 episode each of hemoptysis, asthma, pneumonia, colitis, fractured elbow, and intestinal obstruction. Serious adverse events that occurred among placebo recipients included 10 CF pulmonary exacerbations, 2 cases of hemoptysis, and single instances of fatal motorcycle accident, nasal polyps, and dyspnea.

The proportion of subjects experiencing frequent adverse events (occurring in ≥20% of subjects overall) was similar between treatment groups. Although fever was more common in subjects receiving tgAAVCF than placebo, the difference was not statistically significant. As expected among subjects with CF, respiratory adverse events were common (Table 3).

Sera for neutralizing titers to AAV2 capsid were obtained at baseline and on days 14, 45, and 75. At baseline, 68% of subjects had anti-AAV2 neutralizing titers below the limit of detection (<1:4). Titers ranged from undetectable to 1:65,536, with a geometric mean of 1:15. As expected, all subjects exposed to tgAAVCF developed 4-fold or greater rises in anti-AAV2 capsid-neutralizing titers (Table 4). Among subjects exposed to tgAAVCF, geometric mean titers were 1:90, 1:1963, and 1:1370 on days 14, 45, and 75, respectively. In contrast, geometric mean titers for subjects who received placebo were 1:15, 1:15, and 1:14 on days 14, 45, and 75, respectively. No

adverse events associated with the development of AAV2 neutralizing titers were noted.

Efficacy results

Spirometric pulmonary function tests were performed every 14 or 15 days from screening until 90 days after the first dose of study medication. Pulmonary function was comparable between treatment groups at baseline. No improvement in FEV_1 was noted 30 days after initial dosing, or at any time point, when the tgAAVCF group was compared with the placebo group (mean difference in day 30 − baseline FEV_1 between tgAAVCF and placebo = 0.03 liter, $p = 0.42$; Fig. 1A). In contrast, there was improvement in FEV_1 30 days after initial dosing ($p = 0.04$) in the preceding, smaller phase II study when the tgAAVCF group was compared with the placebo group (Fig. 1B). No significant differences in other spirometric pulmonary function parameters, including FEV_1 % predicted, mean forced expiratory flow during the middle half of the forced vital capacity ($FEF_{25-75\%}$), and forced vital capacity (FVC), were noted between treatment groups over time (data not shown).

Adequate induced sputum samples at all time points were available for 48 actively treated and 49 placebo-treated subjects. IL-8 concentrations were measured in samples obtained at baseline, and on days 14 and 45. There were no differences in sputum IL-8 levels between treatment groups at any time point (Fig. 2A). In contrast, there was a significant change in

TABLE 4. SERUM AAV2 NEUTRALIZING ANTIBODY TITERS AND CHANGE OVER TIME BY TREATMENT GROUP[a]

	tgAAVCF (n = 51)	Placebo (n = 51)
Positive baseline titer	17 (33%)	16 (31%)
Fourfold or greater rise		
Day 14	29 (59%)	1 (2%)
Day 45	49 (100%)	3 (6%)
Day 75	50 (100%)	1 (2%)

[a]Data are presented as number (% tested). Seronegative, a titer value of <1:4; seropositive, a titer of 1:4 or greater.
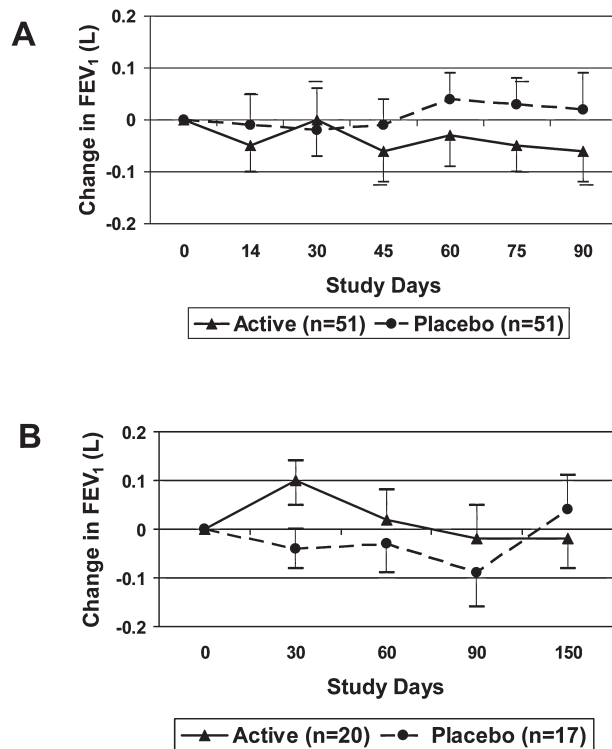
FIG. 1. (A) Mean change in FEV$_1$ over time in the current (study ID number 25E01) phase 2 study. (B) Mean change in FEV$_1$ over time in the preceding (study ID number 25B01) phase 2 study (Moss *et al*., 2004).

show that repeated administration of aerosolized tgAAVCF to the lungs is safe and well tolerated. The pattern and severity of adverse events were similar between the two treatment groups. CF genotype distributions were similar in active and placebo arms. However, the encouraging trends in pulmonary function and induced sputum IL-8 levels observed in an earlier, smaller phase 2 study were not confirmed in the current study. No improvement in lung function was observed 30 days after initial dosing when the tgAAVCF-treated group was compared with the placebo group. Moreover, there were no changes in lung function over time in either treatment group. No significant differences were found in induced sputum biologic markers, including IL-8, neutrophil elastase, DNA, or protein concentration, between treatment groups at any time point. Finally, the use of intravenous antibiotics for pulmonary infections was similar between treatment groups.

The failure of this trial to meet its primary end-point efficacy measure raises two key questions about transfer of CFTR to the respiratory epithelium via the tgAAVCF vector. First, there is the question of adequacy of transgene expression by this vector and route. Prior clinical studies have shown potent tgAAVCF transgene transfer by direct polymerase chain reaction (PCR) but low or undetectable transgene mRNA expression in respiratory epithelial cells as measured by reverse transcriptase (RT)-PCR in cells obtained by bronchial brushing (Aitken *et al*., 2001; Moss *et al*., 2004). However, because the level of endogenous CFTR expression, clearly sufficient for biological function, is only about one copy per cell (Trapnell *et*

induced sputum IL-8 levels on day 14 ($p = 0.03$) in the previous phase II study (Fig. 2B). No differences in other induced sputum biologic markers, including neutrophil elastase, DNA, and protein, were found between treatment groups over time (data not shown).
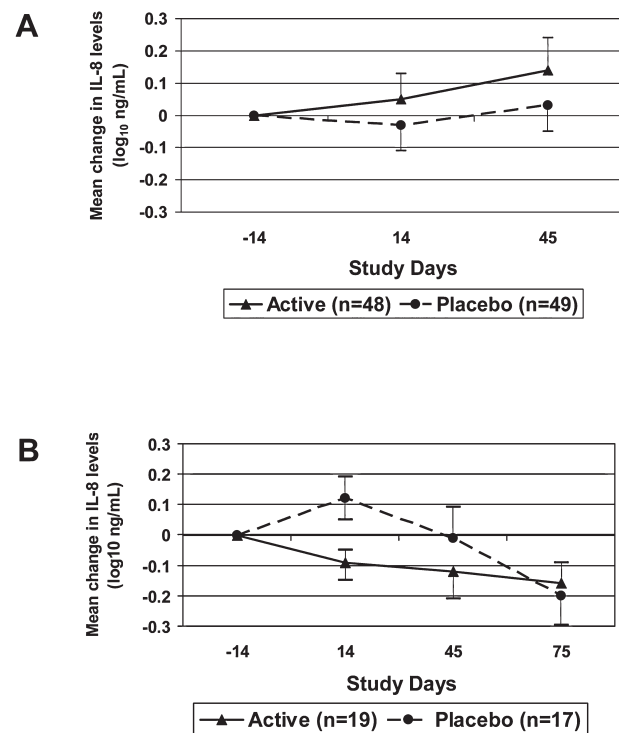
The use of intravenous antibiotics for the treatment of pulmonary infection was recorded throughout the study. The proportion of subjects who required intravenous antibiotics was similar between active and placebo recipients, with 14% of subjects who received tgAAVCF and 16% of subjects who received placebo requiring at least one course of intravenous antibiotics for pulmonary infection. The mean number of courses of antibiotics (0.16 ± 0.42 vs. 0.18 ± 0.43) and days of antibiotic treatment (2.3 ± 6.0 vs. 2.3 ± 6.9) was comparable between active and placebo recipients, respectively.

CONCLUSIONS

Cystic fibrosis has been considered a prime candidate for gene complementation because it is a monogenic, autosomal recessive defect; even small amounts of functional CFTR are sufficient to protect the lung against disease, and only a small proportion of the cells in an epithelium may need to express CFTR in order to correct epithelial chloride transport. Several gene transfer vectors, including AAV, have been used to deliver the CFTR in preclinical models and CF patients. In this paper, we

FIG. 2. (A) Mean change in induced sputum IL-8 levels over time in the current (25E01) phase 2 study. (B) Mean change in induced sputum IL-8 levels over time in the preceding (25B01) phase 2 study (Moss *et al*., 2004).

al., 1991), it is important to establish whether a similar level of transgene expression can result in physiological correction. By using more sensitive assays performed on primary nasal cells harvested from tgAAVCF recipients participating in one of the phase 1 trials (Flotte *et al.*, 2003), it was shown that physiological correction of cAMP-activated chloride channel function was in fact achieved, and that the presence of tgAAVCF vector genomes correlated with CFTR mRNA expression and physiological correction (Flotte *et al.*, 2005). Thus, the lack of transgene-specific CFTR mRNA in the prior phase 2 study does not mean that gene correction was not attained (Moss *et al.*, 2004), but rather that technical means of verifying correction in the lower respiratory tract remain insufficient to settle the question and need further refinement. To reduce invasive perturbation of the airways, bronchoscopy and bronchial brushings were not performed in the study reported here.

The second key question provoked by our negative primary efficacy end-point finding is that of appropriate selection of relevant and meaningful outcome measures for gene therapy of cystic fibrosis. The current study design and end point were based on a preliminary suggestion of short-term improvement in lung function following the first dose of tgAAVCF in the prior phase 2 trial (Moss *et al.*, 2004), which drove both sample size and choice of 30-day FEV_1 as the primary outcome measure. The previously observed improvement in FEV_1 30 days after treatment was not seen in this trial (Fig. 1). Because AAV2 is slow to express at the mRNA level, taking at least 30 days in airway epithelium, the primary time point may have been too early; however, later improvements were not observed either (Fig. 1A). In addition, inspection of 30-day FEV_1 changes in groups receiving placebo during numerous CF drug trials conducted by the Cystic Fibrosis Therapeutics Development Network reveals a wide variety in responses, from sharp drops to increases in this measure (N. Mayer-Hamblett, personal communication).

The treatment effect in placebo-controlled trials depends on the impact of both the active drug and placebo on the subjects in each group. Regarding the pulmonary effect of a gene therapy intervention, a more likely treatment effect would be slowing of disease progression (manifested in reduced loss of airflow over time or reduced rate of pulmonary exacerbations) rather than an early boost in airflow similar to what has been observed with inhaled mucolytic or antibiotic interventions (Fuchs *et al.*, 1994; Ramsey *et al.*, 1999). It thus remains entirely possible that the efficacy of tgAAVCF has been missed because of selection of the wrong primary end point. Longer term gene therapy trials using biomarkers and clinical end points most likely to reflect CFTR correction seem advisable if the field is to progress, a strategy adopted by the U.K. Cystic Fibrosis Gene Therapy Consortium (Griesenbach *et al.*, 2006). In our study early improvement in sputum biomarkers (secondary efficacy measures in this trial included IL-8, DNA, and elastase levels) did not occur, and may also require longer observation periods to be affected by a gene correction strategy.

Gene transfer remains the most basic way to correct the underlying genetic defect that leads to disease in CF. Although repeat administration of tgAAVCF did not result in early improvement in pulmonary function as measured by FEV_1, treatment with this AAV vector was safe and well tolerated. Manipulations of AAV vectors to enhance transduction, and

potentially efficacy, include adding an intron, changing vector serotype, and coadministering proteasome inhibitors such as doxorubicin, all of which are currently under investigation (Carter, 2004; Zhang *et al.*, 2004; Liu *et al.*, 2006). Further studies on the potential effect of viral capsid-induced neutralizing antibodies, well documented in our studies, and even potential antibody responses to CFTR, are needed. Computational modeling suggests the possibility of cellular immune responses against putative CFTR T cell epitopes, including ΔF508 (Figueredo *et al.*, 2007). Other potential nonviral vector delivery systems for correction of the genetic defect in the cystic fibrosis airway include compacted DNA technology and plasmid DNA–liposomes (Griesenbach *et al.*, 2006). Finally, the role of innate and adaptive immune responses to gene delivery to the airway surface needs ongoing research to better define obstacles and to develop methods of safely overcoming them.

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AUTHOR DISCLOSURE STATEMENT

Barrie Carter, Dana Martin and Alison Heald are employees of Targeted Genetics, Inc., manufacturers of the study agent employed in this trial. No competing financial interests exist for the other authors.

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