Repeated Adeno-Associated Virus Serotype 2 Aerosol-Mediated Cystic Fibrosis Transmembrane Regulator Gene Transfer to the Lungs of Patients With Cystic Fibrosis*

A Multicenter, Double-Blind, Placebo-Controlled Trial

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Study objectives: **The primary objective was to determine the safety and tolerability of repeated doses of aerosolized adeno-associated serotype 2 vector containing cystic fibrosis transmembrane conductance regulator (CFTR) complementary DNA (cDNA) [tgAAVCF], an adeno-associated virus (AAV) vector encoding the complete human CFTR cDNA. Secondary objectives included evaluation of pulmonary function assessed by spirometry, lung abnormalities by high-resolution CT (HRCT), airway cytokines, vector shedding, serum neutralizing antibody to AAV serotype 2 (AAV2), and gene transfer and expression in a subset of subjects undergoing bronchoscopy with bronchial brushings.**

Design: **Randomized, double-blind, placebo-controlled, phase II trial.**

Setting: **Eight cystic fibrosis (CF) centers in the United States.**

Subjects: **CF patients with mild lung disease, defined as $FEV_1 \geq 60\%$ predicted.**

Interventions: **Subjects were randomized to inhale three aerosolized doses of 1×10^{13} deoxyribonuclease-resistant particles of tgAAVCF or matching placebo at 30-day intervals using the Pari LC Plus nebulizer (PARI; Richmond, VA).**

Measurements and results: **Of 42 subjects randomized, 20 subjects received at least one dose of tgAAVCF and 17 subjects received placebo. No difference in the pattern of adverse events or laboratory abnormalities was noted between the two treatment groups. Improvements in induced-sputum interleukin-8 ($p = 0.03$) and FEV_1 ($p = 0.04$) were observed at day 14 and day 30, respectively, in the group receiving tgAAVCF when compared to those receiving placebo. No significant differences in HRCT scans were noted. Vector shedding in sputum was observed at low levels up to 90 days after the third dose of vector. All subjects receiving tgAAVCF exhibited an increase (by at least fourfold) in serum AAV2-neutralizing antibodies and detectable levels in BAL fluid from five of six treated subjects undergoing BAL. Gene transfer but not gene expression was detected in a subset of six tgAAVCF subjects who underwent bronchoscopy.**

Conclusions: **Repeat doses of aerosolized tgAAVCF were safe and well tolerated, and resulted in encouraging trends in improvement in pulmonary function in patients with CF and mild lung disease.** ***(CHEST 2004; 125:509–521)***

Key words: adeno-associated virus; aerosol; cystic fibrosis; cytokine; gene therapy; gene transfer; high-resolution CT; inflammation; pulmonary function; vector

Abbreviations: AAV = adeno-associated virus; AAV2 = adeno-associated virus serotype 2; cDNA = complementary DNA; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator; DSMC = Data Safety Monitoring Committee; FDA = US Food and Drug Administration; $FEF_{25–75}$ = forced expiratory flow between 25% and 75% of vital capacity; HRCT = high-resolution CT; IL = interleukin; i.u. = infectious units; PCR = polymerase chain reaction; tgAAVCF = adeno-associated serotype 2 vector containing cystic fibrosis transmembrane conductance regulator complementary DNA

Cystic fibrosis (CF) is an autosomal recessive disorder with an incidence in white subjects of 1 in 2,000 to 4,500 live births.[1] It is due to defects in the CF transmembrane conductance regulator (CFTR) gene, located on chromosome 7.[2,3] The CFTR protein functions as a cyclic adenosine monophosphate-activated chloride channel and regulates the activity of other ion channels.[4–7] Through a mechanism that is not fully understood, CFTR defects strongly predispose affected individuals to chronic endobronchial infections with organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[8,9] Gene therapy holds the promise of addressing the primary defect in CF by reconstituting CFTR function in the lung.[10,11] When the intact CFTR complementary DNA (cDNA) is transferred to human respiratory epithelial cells in cell culture, CFTR messenger RNA is expressed, suggesting a potential method for correction of the defect.[4,12–14]

Delivery of the normal cDNA to the lungs of patients with CF has presented several challenges, including the identification of an acceptable vector. The adeno-associated virus (AAV)-based vector is suitable for this purpose. AAV is a replication-defective vector, capable of efficient and stable (up to 2 months) gene transfer and expression in a variety of cells, including respiratory epithelial cells from patients with CF.[15] Adeno-associated serotype 2 vector (AAV2) containing CFTR cDNA (tgAAVCF) is a recombinant AAV2 vector genetically engineered to contain the cDNA for the CFTR gene (Targeted Genetics Corporation; Seattle, WA). When administered to rabbits and monkeys, human CFTR cDNA and messenger RNA are recovered from lung tissue at necropsy.[14,16–19]

Early studies[20–23] of tgAAVCF showed that delivery of tgAAVCF to the sinus, nose, and single lobe of the lung was safe and well tolerated. Dose-dependent changes in sinus transepithelial potential difference were observed in a dose-escalation trial of intrasinus instillation of tgAAVCF to the maxillary sinus of CF subjects who had undergone maxillary antrostomies for chronic sinusitis.[20,21] Increases in the anti-inflammatory cytokine interleukin (IL)-10 and trends toward decline in IL-8 were observed in sinuses treated with tgAAVCF when compared to placebo in the double-blind, placebo-controlled, phase II portion of the study[22] in which tgAAVCF was administered to one sinus, and placebo to the contralateral sinus. No safety concerns arose in a study of direct intranasal and intrabronchial instillation of tgAAVCF in patients with CF.[23]

More recent studies have focused on delivery of tgAAVCF to the lungs of patients with CF via aerosol nebulization as an effective means to correct the genetic defect throughout the lungs. Dose-dependent gene transfer was demonstrated in a phase I, open-label trial[24] of aerosolized delivery of tgAAVCF to the lungs of 12 patients with CF. As in the early, proof-of-concept studies, aerosolized delivery of tgAAVCF to the entire lung was also shown to be safe and well tolerated. Based on these encouraging findings, we conducted a randomized, placebo-controlled, double-blind study of aerosolized tgAAVCF in patients with CF and mild lung disease.

Materials and Methods

Study Agent

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-globulin. This construct was flanked by the AAV2 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 Corporation.

Both tgAAVCF and matching placebo were administered using the Pari LC Plus nebulizer (PARI; Richmond, 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.

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Study Population

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

Entry criteria included a confirmed diagnosis of CF as determined by a sweat chloride result of > 60 mEq/L or presence of two identified CF alleles and symptoms suggestive of CF,[25] and a FEV_1 of $\geq 60\%$ predicted. Initial age for enrollment was limited to subjects ≥ 18 years old; the age requirement was reduced to ≥ 15 years old, then ≥ 12 years old following interim safety reviews by an independent Data Safety Monitoring Committee (DSMC) described below. Exclusion criteria included recent acute pulmonary infection, severe hemoptysis, receipt of gene-transfer agents in the preceding 6 months, pregnancy, lactation, cigarette smoking, and substance abuse.

Study Procedures

After completion of screening procedures, eligible subjects were randomized to inhale three doses of either 1×10^{13} deoxyribonuclease-resistant particle tgAAVCF or placebo at 30-day intervals. Dosing was based on dose-escalation results of the phase I aerosol study.[24] The first eight subjects were randomized in a 3:1 ratio of tgAAVCF to placebo, in order to minimize bronchoscopies in subjects not receiving active agent while yielding valuable control data. The remaining subjects were subsequently randomized in a 3:4 ratio of tgAAVCF to placebo, so that the final overall proportion of subjects randomized to tgAAVCF and placebo would be 1:1. Subjects were evaluated at days – 14, 0, 14, 30, 45, 60, 75, 90, 120, and 150. Study drug was administered on days 0, 30, and 60. Safety was evaluated by serial medical history and physical examination, CBC count with WBC differential, blood chemistries, urinalysis, AAV2-neutralizing antibody testing, and sputum for vector shedding. Efficacy was evaluated through serial pulmonary function tests, high-resolution CT (HRCT) scans, IL-8 content and microbiologic cultures derived from induced sputum, and the number of courses of IV antibiotics received during the study period. The first eight randomized subjects underwent fiberoptic bronchoscopy either 30 days (first four subjects) or 60 days (second four subjects) after the third dose of study drug to obtain samples by bronchial brushing for molecular analysis of gene transfer and expression, and bronchial wash fluid for evaluation of IL-8 and IL-10 levels, and neutralizing antibodies. Bronchoscopies were limited in this study to adults in the first two cohorts, and largely replaced by induced-sputum analysis, on the basis of concern for protection of subjects, especially children, arising from prior experience in the phase I trial where adverse events and proinflammatory effects related to bronchoscopy were noted.[24]

Safety Evaluations

DSMC: The study was overseen by a DSMC of the Cystic Fibrosis Foundation. The DSMC was an independent multidisciplinary group of physician subspecialists and a statistician who, collectively, had experience in both treating patients with CF and in the conduct of randomized clinical trials. The DSMC convened four times during the course of the study. The DSMC convened after the first four subjects had received the second dose of study agent, and allowed subsequent subjects to receive further doses of vector, and reduced the enrollment age to from 18 to 15 years. The DSMC convened again 2 weeks after the fourth subject underwent bronchoscopy 30 days after receiving the third dose of study agent, and allowed enrollment to continue and the next four subjects to undergo bronchoscopy. The DSMC convened a third time 30 days after the first eight subjects had received all three doses of study drug, and allowed the entry criteria to be modified to include subjects ≥ 12 years old. The DSMC convened a fourth time 2 weeks after bronchoscopy of the final subject in the second cohort, and recommended that enrollment continue. In this fashion, the written study protocol was executed under independent safety oversight.

Adverse Event Monitoring: Adverse event monitoring followed FDA guidelines. Adverse events were followed up until resolution or stabilization.

AAV Neutralizing Antibody Testing: Serum and bronchial wash fluid-neutralizing antibodies to AAV2 were evaluated using an infectivity bioassay as described.[26] In brief, cells of the 293 epithelial cell line were seeded into standard, 96-well tissue culture plates and allowed to attach. Cells were infected with adenovirus type 5 at a multiplicity of infection of 5 for 1 h. Wild-type AAV2 was first preincubated with twofold serial dilutions of test sera for 1 h at 37°C. Preincubated AAV2 was then added to the 293 cells in triplicate and cultured for 2 to 3 days to allow for replication of infectious AAV2. Presence of replicating AAV2 was determined by hybridization. An increased titer of anti-AAV2 antibodies is determined by measuring the inhibition of infectivity. Inhibition of infectivity is demonstrated by the absence of hybridizing signal. In this assay, neutralizing antibody titer is defined as the reciprocal of the dilution for each sample where all three replicates are negative for hybridizing signal. Seroconversion defined as a fourfold or greater change in titer was considered significant.

Vector Shedding in Sputum: To determine if vector was present in induced sputum, samples were inoculated into adenovirus-infected C37 cells (a HeLa cell line derivative that can express AAV rep protein and allow vector amplification) and cultured for 2 days as described.[26] Samples were split into two parts, and a portion of each sample was spiked with tgAAVCF to assess the potential inhibition of the sample matrix on vector amplification and detection; inhibition was detected in one sample. Lysates of infected cells were prepared, and a portion of the lysate was amplified using vector specific primers for 35 cycles under standard polymerase chain reaction (PCR) conditions. The limit of detection was 100 infectious units (i.u.) per 10-μL sputum sample.

Samples that were positive by this limit of detection assay were then tested in a quantitative assay. Positive sputum samples were treated with Sputolysin (Caldon BioTech; Carlsbad, CA), and then the samples and standards were inoculated into adenovirus-infected C37 cells and cultured for 2 days to allow for replication of infectious vector. After denaturation, cell lysates were transferred to a nylon membrane, and quantification of infectious tgAAVCF vector was determined by hybridization with a vector-specific probe.

Efficacy Evaluations

Pulmonary Function Tests: Spirometric pulmonary function tests were performed at screening (days – 14 to − 7), baseline (day 0), and days 30, 60, 90, and 150. FEV_1, FEV_1 percentage of predicted, forced expiratory flow between 25% and 75% of vital capacity ($FEF_{25–75}$), and FVC were obtained according to American Thoracic Society guidelines.

HRCT Chest Scans: Study subjects underwent HRCT scans at baseline and day 90. The HRCT protocol used 1-mm sections at 10-mm intervals from apices to lung bases obtained during

voluntary breath holding after a full inspiration, and 1-mm sections at six levels from 0.5 cm above the aortic arch to 1.5 cm above the diaphragm following a full exhalation. Images were obtained at 80 mA and 120 kilovolt peak. The HRCT scans were filmed at window 1,500, level − 600 on a standardized format. These hard-copy films were sent to a central reading area where all identifying information was removed and a randomly generated identifying number applied. HRCT scans were scored by two independent radiologists blinded to study treatment assignment and the time point at which the HRCT was performed. The radiologists read each HRCT scan individually, and assigned scores grading extent and degree of bronchiectasis, mucous plugging, peribronchial thickening, parenchymal abnormalities (opacity, ground glass, and cysts/bullae), and hyperinflation in each of the lobes of the lung with the lingula evaluated as a separate lobe producing six lobar scores. These scores were then evaluated according to an algorithm developed for the protocol (Table 1) to form subscores for each of these measures. The subscores were summed over all six "lobes" of the lung, and then combined to form an overall score, which was normalized to a scale from 0 to 100.

Following completion of the individual scores for the entire set of HRCT scans, the films were then sent to the radiologists as pairs. The radiologists performed paired comparisons of the HRCT scans by reviewing the baseline and day-90 HRCT scans for each subject in random order and determining if the first HRCT scan was much worse, slightly worse, unchanged, slightly better, or much better than the second HRCT scan with respect to bronchiectasis, mucous plugging, peribronchial thickening, parenchymal changes (opacity, ground glass, cysts/bullae), and hyperinflation for each of the six lobes. These blinded readings were converted into scores taking the time points into account as follows: day 90, much worse than baseline (− 2); day 90, slightly worse than baseline (− 1); no change (0); day 90, slightly better than baseline (1); or day 90, much better than baseline (2). These scores were summed over all six lobes of the lung and then combined to form an overall score.

Cytokine Assays (IL-8 and IL-10): Induced-sputum samples obtained on days 14, 45, and 75 were analyzed for proinflammatory cytokine IL-8, and bronchial wash fluids were analyzed for IL-8 and anti-inflammatory cytokine IL-10 as described.[22] Briefly, 96-well Nunc Immunoplates (Nalge-Nunc; Copenhagen, Denmark) were prepared with the relevant monoclonal antihuman IL-8 or IL-10 antibodies (R&D Systems; Minneapolis, MN). Plates were washed three times between each step. Nonspecific activity was blocked with 1% bovine serum albumin (Sigma; St. Louis, MO), 5% sucrose, in phosphate buffered saline solution at room temperature. Samples, samples plus spike, and standards were diluted in 0.1% bovine serum antigen, 0.05% Tween-20, in 1X Tris-buffered saline solution (diluent buffer), and incubated for 1 to 1.5 h with shaking at room temperature. The plates were washed three times with wash buffer. Detection of captured human IL-8 or IL-10 was achieved by the addition of the appropriate biotinylated anticytokine antibody (R&D Systems). Colorimetric quantitation was performed using streptavidin-

Table 1—*Individual HRCT Scoring System*

Bronchiectasis (range 0 to 12)	(Extent of bronchiectasis in central lung 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	+	Extent of bronchiectasis in peripheral lung) 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	×	Average bronchiectasis size multiplier Average size multiplier 0.5 = 0; 1 = 1 1.5 = 1.25 2.0 = 1.5 2.5 = 1.75 3.0 = 2
Where average bronchiectasis	(Size of largest dilated bronchus 1 = < 2x 2 = 2x to 3x 3 = > 3x	+	Average size of dilated bronchi) 1 = < 2x 2 = 2x to 3x 3 = > 3x	÷	2
Mucous plugging (range 0 to 6)	Extent of mucous plugging in central lung 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	+	Extent of mucous plugging in peripheral lung 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe		
Peribronchial thickening (range 0 to 9)	(Extent of peribronchial thickening in central lung 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	+	Extent of peribronchial thickening in peripheral lung) 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	×	Severity of peribronchial thickening 1 = mild 1.25 = moderate 1.5 = severe
Parenchyma (range 0 to 9)	Extent of dense parechymal opacity 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	+	Extent of ground-glass opacity 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	+	Extent of cysts or bullae 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe
Hyperinflation (range 0 to 9)	Extent of air trapping 0 = none 1 = 1/3 of lobe 2 = 1/3 to 2/3 of lobe 3 = > 2/3 of lobe	×	Appearance of air trapping 1 = subsegmental 1.5 = segmental or larger		

horseradish peroxidase. Tetramethybenzidine and hydrogen peroxide (1:1) [Pierce; Rockford, IL] was added and incubated at room temperature without shaking for 15 to 20 min. The reaction was stopped by the addition of 1 mol/L H_2SO_4. Optical density was determined at 450 nm (with background subtraction at 540 nm) with a microplate reader (SpectraMax 250 or SpectraMax PLUS^{384}; Molecular Devices; Sunnyvale, CA). Cytokine levels were quantified using internal dilutions of recombinant standards.

Sputum Cultures: Quantitative microbiology of induced-sputum samples was performed at the microbiology laboratory at Children's Hospital and Regional Medical Center (Seattle, WA) as previously described.[27] The 1-mL aliquots removed for microbiologic cultures were plated within 2 h of addition of dithiothreitol, frozen, and sent to the centralized microbiology laboratory. Solubilized samples were serially diluted and plated directly onto the following media: MacConkey, oxidation-fermentation polymyxin bacitracin lactose agar, deoxyribonuclease, Streptococcus selective, mannitol salt, Haemophilus selective, and Mycosel agars. MacConkey, deoxyribonuclease, and mannitol salt plates were incubated in a 35°C ambient air incubator for 48 h. Oxidation-fermentation polymyxin bacitracin lactose agar plates were incubated in a 35°C ambient air incubator for 72 h. Selective Streptococcus and Haemophilus agar plates were incubated anaerobically at 35°C for 48 h. Mycosel plates were incubated in a 30°C ambient air incubator for 5 days. Colony-forming units per gram of sample were determined by quantitating the number of bacterial colonies of each organism growing on selective media.[27,28] Standard biochemical testing identified organisms.

Gene Transfer: Gene transfer was detected in bronchial brushings using a DNA PCR assay. DNA was isolated from brushed airway specimens using Qiagen QIAamp kit (Qiagen; Valencia, CA) following the instructions of the manufacturer for tissue isolation. DNA was quantified using spectrophotometric analysis. A multiplex PCR analysis was performed to determine copy number of tgAAVCF in samples of brushed cells. The 3′ primer specific for tgAAVCF is located in the synthetic polyA region of the vector, and the 5′ primer is located approximately 100 base-pairs upstream in the CFTR reading frame. Primers for the endogenous β-actin gene were used as a control for total amplifiable DNA from each sample. Reactions were performed in a total volume of 50 μL using Universal Master Mix PCR buffer (PE Applied Biosystems; Foster City, CA) and 0.14 μmol/L of the forward tgAAVCF primer, 0.2 μmol/L of the reverse tgAAVCF primer, 0.1 μM of the FAM-labeled TaqMan CF-specific hybridization probe, 0.05 μmol/L of each β-actin primer, and 0.1 μM of the VIC-labeled TaqMan β-actin–specific hybridization probe. Quantitative PCR was performed using TaqMan technology and the ABI PRISM Sequence Detection System (PE Applied Biosystems; Foster City, CA). Quantitation was determined by detection and quantitation of the fluorescent reporters of the TaqMan probes.

Gene Expression: Gene expression in bronchial brushings was assessed using a real-time RNA-specific reverse transcriptase-PCR.[29] RNA was isolated from brushed airway specimens using Qiagen RNeasy kit (Qiagen) following instructions of the manufacturer for tissue isolation. RNA was quantified using spectrophotometric analysis. Briefly, first-strand cDNA was synthesized from polyadenylated messenger RNA using an oligo-d(T/U) primer with an X-linker sequence attached at the 5′ end. Following ribonuclease H treatment, second-strand cDNA was synthesized using a CFTR-specific primer. This second-strand cDNA is the template for both the tgAAVCF PCR and the endogenous CF PCR described below.

Qualitative PCR was performed using TaqMan technology and the ABI PRISM Sequence Detection System (PE Applied Biosystems). To detect tgAAVCF, forward and reverse PCR primers were specific for tgAAVCF and X-linker sequence, respectively, with a tgAAVCF-specific TaqMan probe. In a separate PCR, reverse transcriptase-PCR performance was monitored via the endogenous CF gene. Forward and reverse primers were specific for endogenous CF and X-linker sequence, respectively, with an endogenous CF-specific TaqMan probe. Each sample was evaluated for tgAAVCF messenger RNA in duplicate PCR reactions, while endogenous CF was amplified in a single PCR. The tgAAVCF limit of detection for the assay was 18 copies of vector-derived CFTR messenger RNA per 100 ng of total RNA, which is the equivalent of 1 copy per 370 cell equivalents. Detection of the sequence of interest was determined by detection and quantitation of the fluorescent reporters of the TaqMan probes. Samples without detectable endogenous CF gene were reported as inconclusive.

Statistical Considerations: Enrollment of 18 subjects per treatment group provided adequate power to test differences between treatment groups with respect to the primary and secondary protocol end points defined as follows. The power to detect differences in the proportions of subjects experiencing adverse events (the primary end point) ranged from 74%, if 12 of 18 subjects experienced it in the tgAAVCF group and 3 of 18 subjects experienced it in the placebo group, to 95%, if 18 of 18 subjects experienced it in the tgAAVCF group and 9 of 18 subjects experienced it in the placebo group, using a two-sided Fisher exact test with a significance level of 0.05. The power to detect a 6% difference between the two groups with respect to the change in FEV_1 percentage of predicted from baseline to day 90 (the secondary end point) was 80% with a two-sided significance level of 0.05.

Descriptive statistics and graphical displays were used to summarize data by treatment group. Univariate comparisons between treatment groups were performed using two-sample *t* tests or nonparametric Wilcoxon statistics for continuous variables, and χ^2 statistics or Fisher exact test for categorical or ordinal variables.

Results

Baseline Characteristics

Forty-six subjects underwent screening procedures between October 2000 and April 2002. Four subjects did not meet entry criteria because the baseline FEV_1 was ≤ 60% of predicted. Forty-two subjects were randomized. After the first two subjects were randomized, the FDA imposed a 2-month hold in December 2000 on all clinical trials involving any AAV vector to review findings from an animal study of a vector unrelated to tgAAVCF.[30,31] The first subject, who had received one dose of study medication, was withdrawn from the study as a result of this FDA hold. The second subject was withdrawn prior to dosing, then later was rescreened and was rerandomized, but withdrew consent prior to dosing. Three other subjects withdrew consent after randomization but prior to starting treatment because of heavy work schedule, concern about potential risks, and a change of mind, respectively.

Thirty-seven subjects received at least one dose of study agent. The demographics of the 20 subjects

who received tgAAVCF and the 17 subjects who received placebo are summarized in Table 2. More female subjects were randomized to tgAAVCF than placebo, but the difference was not significant ($p = 0.16$). Seventy-seven percent of subjects randomized to placebo were homozygous for the ΔF508 genotype compared to 25% of those randomized to tgAAVCF ($p = 0.01$); however, all but one subject (homozygous for R334W, randomized to placebo) were pancreatic insufficient, and the age distribution, height, weight, sweat chloride test results, and baseline spirometric lung function were similar between the two groups.

Safety Evaluations

Adverse Event Monitoring: Thirty-five of 37 subjects who started treatment received all three doses of study agent. No subjects withdrew from the study due to adverse events. One subject randomized to tgAAVCF withdrew after one dose of study agent because of the FDA hold. A second subject randomized to tgAAVCF withdrew consent after receiving two doses of study agent.

All subjects experienced at least one adverse event. A total of 284 adverse events were reported by the subjects randomized to tgAAVCF, and 254 by the subjects randomized to placebo. As seen in Table 3, which summarizes respiratory and other common adverse events, the pattern and frequency of adverse events were similar among subjects randomized to tgAAVCF and those randomized to placebo. The most frequent adverse events (experienced by > 40% of subjects in either treatment group) were increased sputum (experienced by 65% tgAAVCF subjects vs 71% of placebo subjects), increased cough (60% vs 71%), rhinitis (55% vs 59%), pharyngitis (45% vs 41%), fatigue (40% vs 41%), fever (40% vs 47%), and chest pain (30% vs 41%). No events were considered definitely related to study drug.

Ten subjects experienced 14 adverse events categorized as serious because they resulted in hospitalization. Six of 20 subjects randomized to tgAAVCF were admitted to hospital for CF pulmonary exacerbation, and one for dehydration. Four of 17 subjects randomized to placebo were admitted for pulmonary exacerbation and one each for abdominal pain, hyperglycemia, and arthralgia. The incidence of clinically significant abnormal laboratory tests was low, and similar between treatment groups (data not shown).

AAV Neutralizing Antibodies: Sera for neutralizing antibodies to AAV2 were obtained at baseline and days 14, 45, and 75. Twelve subjects (6 randomized to tgAAVCF and 6 to placebo) had positive serum neutralizing antibodies at baseline. Fourfold or greater increases in serum neutralizing antibodies from baseline were noted in all subjects randomized to tgAAVCF. Neutralizing antibodies were measured in bronchial wash fluids in the first eight subjects, who underwent bronchoscopy 30 or 60 days after third study drug administration. Samples were positive in the bronchial wash fluid of five of six subjects receiving tgAAVCF, and were negative in the two subjects receiving placebo.

Table 2—*Demographics of Study Subjects**

Demographics	tgAAVCF (n = 20)	Placebo (n = 17)	p Value†
Sex			0.16
Male	6 (30)	9 (53)	
Female	14 (70)	8 (47)	
Age, yr	24.2 ± 8.7	23.2 ± 11.1	0.76
12–14	2 (10)	3 (18)	
15–17	5 (25)	3 (18)	
≥18	13 (65)	11 (65)	
Height, cm	167 ± 11	165 ± 10	0.64
Weight, kg	60 ± 11	60 ± 12	0.99
CF genotype			0.01
ΔF508 homozygous	5 (25)	13 (77)	
ΔF508 heterozygous	12 (60)	2 (12)	
Other/unknown	3 (15)	2 (12)	
Sweat chloride, mEq/L	101 ± 17	107 ± 22	0.35
FEV_1, % predicted	82.2 ± 19.3	84.4 ± 15.1	0.70

*Data are presented as No. (%) or mean ± SD.
†Two-sided χ^2 test for categorial variables; two-sided t test statistic for continuous variables.

Table 3—*Incidence of Respiratory and Other Common Adverse Events**

Events	tgAAVCF (n = 20)		Placebo (n = 17)	
	All	Related	All	Related
Abdominal pain	6 (30)	0 (0)	6 (35)	0 (0)
Asthma	4 (20)	1 (5)	2 (12)	0 (0)
Chest pain	6 (30)	3 (15)	7 (41)	1 (6)
Cough	3 (15)	2 (10)	0 (0)	0 (0)
Cough increased	12 (60)	3 (15)	12 (71)	2 (12)
CF pulmonary exacerbation	6 (30)	1 (5)	3 (18)	3 (18)
Dyspnea	6 (30)	1 (5)	4 (24)	1 (6)
Fatigue	8 (40)	1 (5)	7 (41)	1 (6)
Fever	8 (40)	1 (5)	8 (47)	0 (0)
Headache	7 (35)	2 (10)	6 (35)	0 (0)
Hemoptysis	5 (25)	2 (10)	4 (24)	0 (0)
Lung disorder	6 (30)	0 (0)	4 (24)	0 (0)
Lung function decreased	3 (15)	0 (0)	4 (24)	0 (0)
Pharyngitis	9 (45)	1 (5)	7 (41)	0 (0)
Rhinitis	11 (55)	1 (5)	10 (59)	0 (0)
Sinusitis	7 (35)	0 (0)	5 (29)	1 (6)
Sputum increased	13 (65)	2 (10)	12 (71)	0 (0)

*Data are presented as No. (%). Common adverse events are defined as events occurring in ≥ 30% of subjects in either treatment group.

Table 4—*Vector Shedding*

Day	tgAAVCF Positive/ Total Samples, No. (%)	Median, i.u./mL
0	18/20 (90)	6.79×10^5
14	1/20 (5)	< 4,000
60	6/17 (35)	< 4,000
75	2/16 (13)	< 6,000
90	0/6 (0)	Not available
150	3/17 (18)	< 4,000

Vector Shedding: Sputum samples for vector shedding were obtained immediately after dosing on day 0, then on days 14, 60, and 75. When samples from early enrolled subjects were still positive at day 75, the protocol was amended at the request of the DSMC to collect further sputum samples at days 90 and 150. Vector shedding in sputum was detected in 90% of samples with a median value of 7×10^5 i.u./mL 2 h after dosing on day 0 in subjects randomized to tgAAVCF. Vector shedding was detected in 0 to 18% of samples with median levels below the limit of quantitation (< 4,000 i.u./mL) up to day 150, 90 days after the third dose of tgAAVCF (Table 4), possibly due to epithelial cell turnover.

Efficacy Evaluations

Pulmonary Function: Spirometric pulmonary function tests were performed at screening, baseline, and days 30, 60, 90, and 150. Pulmonary function was comparable between treatment groups at baseline. The mean change in FEV_1, FEV_1 percentage of predicted, FVC, and $FEF_{25–75}$ over time for each treatment group is illustrated in Figure 1. Results were consistent among all four parameters. A trend in improvement in all measures of pulmonary function at day 30 was observed in subjects who received

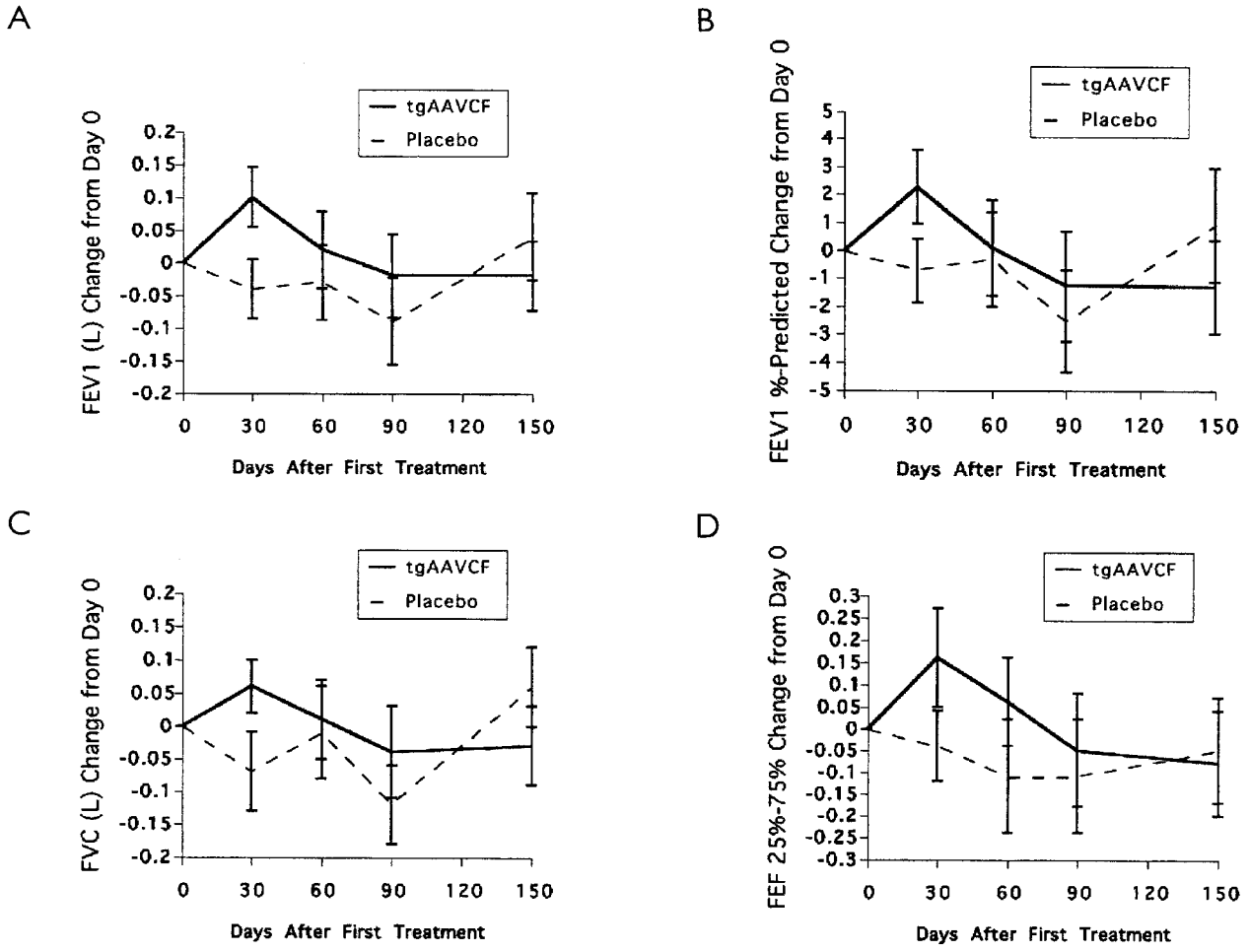

FIGURE 1. Change in pulmonary function over time. *Top left*, *A*: change in FEV_1 from day 0; *top right*, *B*: change in FEV_1 percentage of predicted from day 0; *bottom left*, *C*: change in FVC from day 0; *bottom right*, *D*: change in $FEF_{25–75}$ from day 0.

tgAAVCF when compared to those who received placebo; this effect was statistically significant for FEV_1 ($p = 0.04$). Differences between tgAAVCF and placebo subjects at days 60, 90, and day 150 (90 days after the final dose of study medication) were not statistically significant.

The percentage change in FEV_1 from day 0 at days 30, 60 and 90 of individual patients is depicted in the

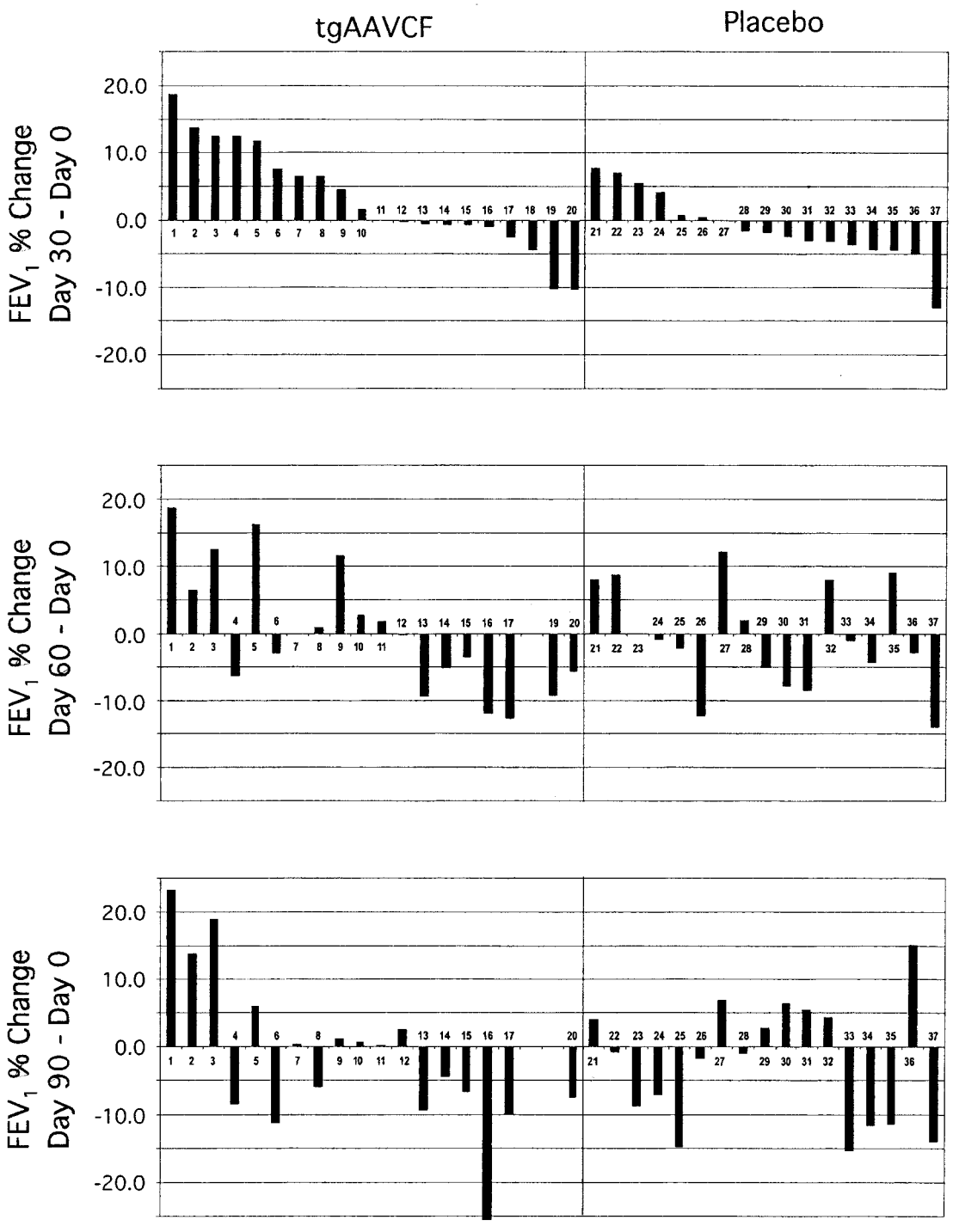

FIGURE 2. Percentage change in FEV_1 from day 0 for each subject at days 30, 60, and 90. Subjects randomized to tgAAVCF are numbered from 1 to 20 and are arranged in descending order of percentage change in FEV_1 at day 30. Subjects randomized to placebo are numbered from 21 to 37, and are arranged in a similar fashion.

histograms in Figure 2. In the tgAAVCF group, five subjects (25%) had > 10% improvement from baseline FEV_1 value at day 30 vs none receiving placebo; at day 60, this improvement was seen in four tgAAVCF patients and 1 placebo group subject, and at day 90 in three tgAAVCF group and one placebo group subject.

HRCT Scans: There were no significant differences in the individual HRCT scores at baseline between the two treatment groups when compared using the Mann-Whitney *U* test. No statistically significant differences in the differences in HRCT scores between baseline and day 90 were observed, but there were trends toward worsening of the parenchymal opacity score in the placebo group and worsening of the bronchiectasis and hyperinflation scores in the tgAAVCF group (Table 5). Similarly, no significant differences in the comparison scores summed over all six lobes were noted between the two treatment groups, with trends toward improvement in the opacity score and a worsening of the hyperinflation score in the tgAAVCF group when compared to the placebo group (Table 6).

Cytokines (IL-8 and IL-10): Levels of IL-8 were measured in induced sputum from samples obtained at baseline, and at days 14, 45, and 75. At Day 14, sputum IL-8 levels decreased 0.09 ± 0.19 $\log_{10}$ ng/mL in subjects randomized to tgAAVCF, and increased 0.12 ± 0.27 $\log_{10}$ ng/mL in subjects randomized to placebo ($p = 0.03$) [Fig 3]. No differences in sputum IL-8 levels were observed at days 45 and 75. No difference in BAL IL-10 levels were seen in the eight subjects undergoing bronchoscopy (tgAAVCF, 6; placebo, 2). We were unable to detect IL-10 in induced-sputum samples, possibly due to technical limitations.

Sputum Microbiology: The number and type of microorganisms were determined in expectorated sputum at day 0 and day 90. The recovery of pathogens was similar at both time points in the two treatment groups (Table 7). The number of subjects who were positive for *P aeruginosa* or *Burkholderia cepacia* at baseline and day 90 were similar between treatment groups, while there was a trend toward less *S aureus* recovery in the tgAAVCF group at day 90. No quantitative differences were seen (data not shown). There were also no significant differences in courses of IV antipseudomonal antibiotics in the two groups (data not shown).

Gene Transfer and Expression: The first eight subjects, all adults (six subjects randomized to tgAAVCF and two subjects randomized to placebo), underwent bronchoscopic brushings either 30 days or 60 days after the third dose of study drug to obtain samples of bronchial cells for testing for gene transfer and expression. Gene transfer was documented in all six subjects randomized to tgAAVCF. A median of 29 to 100 copies of vector DNA per diploid genome was detected, depending on the site of brushing. Gene transfer was documented in seven of nine specimens at levels ranging from 13 to 2,303 copies per diploid genome in subjects who underwent bronchoscopy with bronchial brushings 30 days after their third dose of study drug, and in four of nine specimens at levels ranging from 17 to 184

Table 6—*Comparison of HRCT Scan Scores**

Scores	tgAAVCF (n = 18)	Placebo (n = 17)	p Value†
Bronchiectasis	− 0.3 ± 1.5	0.2 ± 1.2	0.65
Mucous plugging	− 0.4 ± 1.9	0.2 ± 1.4	0.57
Peribronchial thickening	− 0.0 ± 1.9	0.1 ± 1.8	0.39
Opacity	0.3 ± 0.5	− 0.1 ± 0.7	0.08
Ground glass	0.0 ± 0.4	− 0.1 ± 0.4	0.58
Cysts/bullae	− 0.1 ± 0.2	− 0.0 ± 0.3	0.23
Hyperinflation	− 0.4 ± 2.2	0.8 ± 1.5	0.08

*Data are presented as mean ± SD.
†Based on a two-sided Wilcoxon rank-sum test for two-sample data.

Table 5—*Individual HRCT Scan Scores**

	tgAAVCF (n = 18)			Placebo (n = 17)			
Scores	Baseline	Day 90	Baseline − Day 90	Baseline	Day 90	Baseline − Day 90	p Value†
Bronchiectasis	16 ± 11	18 ± 10	− 2 ± 3	14 ± 10	14 ± 9	0 ± 3	0.07
Mucous plugging	11 ± 6	13 ± 14	− 2 ± 13	10 ± 8	10 ± 7	0 ± 3	0.92
Peribronchial thickening	15 ± 9	15 ± 8	0 ± 4	15 ± 11	16 ± 10	− 1 ± 7	0.49
Parenchyma	5 ± 3	5 ± 2	0 ± 3	5 ± 3	6 ± 4	− 1 ± 2	0.07
Hyperinflation	29 ± 12	31 ± 17	− 2 ± 11	38 ± 15	35 ± 15	1 ± 3	0.09
Summary score	14 ± 7	15 ± 7	− 1 ± 3	35 ± 16	35 ± 17	0 ± 6	0.31

*Data are presented as mean ± SD.
†Based on a two-sided Mann-Whitney *U* test for the day 90 minus baseline scores between the two groups.

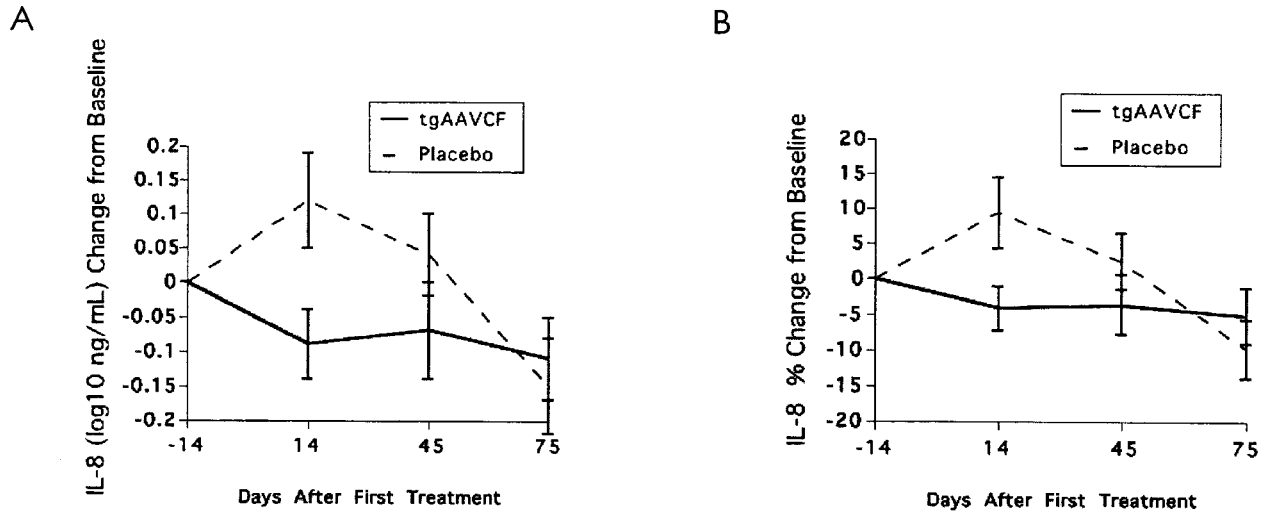

FIGURE 3. IL-8 levels in induced sputum. *Left*, *A*: change in induced sputum IL-8 levels from day 0; *right*, *B*: percentage change in induced-sputum IL-8 levels from day 0.

copies per diploid genome in subjects who had bronchoscopy 60 days after their third dose of study drug. Gene expression was not detected in these bronchial brushing samples.

Subgroup Analysis: A *post hoc* exploratory subgroup analysis was performed to address differences between treatment groups at baseline despite randomization, and to examine the effect of elevated AAV2 neutralizing antibody titers at baseline or after treatment. Among 20 subjects who received tgAAVCF, male subjects had a greater increase in FEV_1 at day 30 than female subjects ($p = 0.04$). No difference was seen between subjects who were homozygous for the ΔF508 mutation and those who were not, in the mean change in FEV_1 at day 30 between subjects who were seropositive at baseline and those who were not, or between those who had fourfold or greater rises in neutralizing antibody titers by day 14 and those who did not. Neither the magnitude of baseline neutralizing antibody titer at baseline nor the magnitude of the increase in titer at day 14 correlated with the percentage change in FEV_1 from baseline at day 30. However, lacking adequate statistical power, this exploratory analysis could easily have missed an effect in a subgroup.

DISCUSSION

This study represents the first clinical evaluation of repeated aerosol dose delivery of CFTR DNA to the lower respiratory tract of patients with CF using an AAV vector. The study was designed with safety and tolerability as the primary end points. Thus, the trial was powered and sample size calculated to detect differences in adverse events between subjects re-

Table 7—*Microorganisms in Expectorated Sputum by Treatment Group**

Organisms	tgAAVCF (n = 20)		Placebo (n = 17)		p Value†
	Day 0 (n = 16)	Day 90 (n = 14)	Day 0 (n = 8)	Day 90 (n = 12)	
Alcaligenes xylosoxidans	1 (6)	1 (7)	0 (0)	0 (0)	1.00
B cepacia	0 (0)	0 (0)	1 (13)	1 (8)	0.46
B cepacia Genomovar III		0 (0)		1 (8)	0.46
P aeruginosa	11 (69)	10 (71)	4 (50)	8 (67)	1.00
Small colony variant					
S aureus		0 (0)		1 (8)	0.46
S aureus	6 (38)	3 (21)	3 (38)	7 (58)	0.11
Stenotrophomonas maltophilia	2 (13)	1 (7)	1 (13)	1 (8)	1.0

*Data are presented as No. (% positive).

†Fisher exact test used to compare the treatment groups; p values displayed are for the comparison between the treatment groups at day 90.

ceiving tgAAVCF and placebo. The reason for this emphasis on safety lies in clinical toxicity encountered in previous attempts to deliver CFTR in adenoviral or liposomal vectors to the lungs of patients with CF.

While an initial single-dose adenoviral vector reported by Bellon et al[32] was well tolerated, the doses involved resulted in poor DNA transfer as measured by PCR of bronchial brushings for vector DNA. When Crystal et al[33] administered higher doses of an adenoviral vector by direct installation into a lower lobe bronchus, acute systemic and respiratory illness with alveolar infiltrates resulted. A subsequent adenoviral vector study[34] employing up to four applications of localized endobronchial spraying did not result in adverse effects but only a small portion of airway was exposed. Toxicity including fever, myalgia, and pulmonary infiltrates using an adenoviral vector delivered by either bronchial instillation or aerosolization was also reported by Joseph et al.[35] In general, first- and second-generation adenoviral vectors have produced a safety record that precludes their development in present form. Helper-dependent adenoviral vectors have not been evaluated in clinical trials.

Cationic liposomes complexed with CFTR-expressing plasmids have also been evaluated in aerosol trials and have also been found to cause clinical toxicity. Alton et al[36] reported occurrence of influenza-like symptoms in seven of eight CF patients so treated, and patients treated with lipid alone also showed some toxicity, indicating probable proinflammatory effects of the lipid in patients with CF. Interestingly, this toxicity may be limited to patients with CF, as it was not seen in normal subjects, suggesting the proinflammatory bias of the CF lung may present additional difficulties for aerosol gene transfer.[37]

We previously utilized tgAAVCF in both single- and multiple-dose studies involving instillation into the maxillary sinuses of patients with CF and in a phase I aerosol single-dose study, and found tgAAVCF to be well tolerated and without discernable toxicity.[20–24] These results gave us the confidence to conduct a multiple dose aerosol tgAAVCF trial using a dose (1×10^{13} deoxyribonuclease-resistant particles) and interval (monthly) suggested by prior data to be both safe and of potential therapeutic benefit as judged by degree and persistence of DNA transfer.[20–24] The results reported here confirm that repeated high-dose aerosolization of tgAAVCF is safe and well tolerated.

This study is also the first CF gene transfer trial to incorporate standardized chest CT scores as both an additional safety and also a new efficacy outcome measure. Two different radiologist visual scoring analyses of the CT data were performed. Each CT scan was scored individually using one scoring system, and a paired comparison was performed using a different scoring system. Both analyses produced similar results. We found no statistically significant changes in the CT scores between days 0 and 90. The lack of increasing parenchymal opacities in the treated group suggests that this therapy is well tolerated without evidence of inflammation such as that seen in several subjects in trials using adenoviral vectors.[33,35]

Although this study was not powered for efficacy, a number of established and exploratory outcome measurements were evaluated. The traditional efficacy outcome measure most commonly employed in studies of new CF therapies is spirometry, in particular absolute and percentage of predicted values of FEV_1. In this trial, a significant improvement in FEV_1 ($p = 0.04$) was noted in subjects receiving tgAAVCF compared to placebo at 30 days (Fig 1). Generally higher spirometric values were noted for FEV_1 and also FVC and $FEF_{25–75}$ in subjects receiving tgAAVCF through day 90, but these differences were not statistically significant, perhaps due to lack of adequate power. Although the randomization process resulted in more placebo subjects homozygous for the ΔF508 mutation, baseline pulmonary function was similar in the two groups. We believe these results suggest that tgAAVCF may confer a clinically relevant benefit of improved pulmonary function in patients with CF patients and mild lung disease, meriting further study using an adequately powered sample size based on a clinically relevant (spirometric) efficacy end point.

Other potential intermediary measures of efficacy include changes in cytokines that may be relevant to disease pathogenesis and/or activity in CF, including the proinflammatory cytokine IL-8 and the anti-inflammatory cytokine IL-10. Previously we demonstrated a significant increase in IL-10 and a trend toward reduced IL-8 in sinus lavage fluids of patients with CF treated with a single dose of instilled tgAAVCF.[22] Alton et al[36] reported a decrease in sputum IL-8 following liposomal delivery of CFTR to the lungs despite inability to detect vector-specific CFTR messenger RNA in bronchial mucosa. In the present study, induced-sputum samples from subjects receiving tgAAVCF contained significantly less IL-8 than placebo samples at the 14 day time point that may have persisted, but the trial lacked power to show this at later time points (Fig 3). Our interest in IL-10 was stymied by technical difficulties in measuring IL-10 in sputum, perhaps due to inhibitory substances (unpublished data). We believe that sentinel cytokines such as IL-8 and IL-10 show promise as potential biochemical intermediary surrogate out-

come measures that warrant further study in CF gene therapy trials. In our study, quantitative sputum microbiology did not reveal differences in treated and placebo groups.

In this trial, robust DNA transfer was demonstrated by PCR in brushed bronchial cells from all six subjects receiving tgAAVCF who underwent bronchoscopy 30 to 60 days after the last dose. Despite this, evidence of vector-specific CFTR messenger RNA was not demonstrable in these samples. Gene expression has been difficult to verify in virtually all CF gene therapy trials. There are a number of possible confounders to consider when evaluating the meaning of these negative results. Delivered and deposited dose may play a role. In a primate study,[38] similar dose and aerosolization of AAV vector resulted in airway deposition. In addition, vector-specific CFTR messenger RNA after tgAAVCF aerosolization was found at necropsy in lung tissue with lymph nodes dissected away from five of eight rhesus monkeys but only in one animal at one time point in bronchial brushings, suggesting that cell yield and/or quality may influence likelihood of finding vector-specific CFTR messenger RNA (unpublished data). Other factors playing a role in failure to detect vector-derived gene expression could include more distal deposition in small airways following aerosolization not detected with the central airways sampled by bronchial brushings; the different volume of cells obtained; differences in composition of cell types obtained; insufficient sensitivity of the PCR assay; and the potential role of inhibitory substances that may be more prevalent in larger airways.[39] Since tgAAVCF is a single-stranded DNA vector, RNA-specific PCR was used to ensure a high degree of specificity. However, in comparison with control RNA assays, CF patient lung brushings yielded approximately 10-fold less amplifiable messenger RNA on extraction, limiting sensitivity (data on file, Targeted Genetics Corporation; Seattle, WA). The relative insensitivity of PCR-based expression assays is also suggested by studies[22,36] showing changes in transepithelial potential difference measurements in patients receiving CFTR-containing vectors suggestive of improved Cl^- transport in the absence of detectable messenger RNA expression.

Delivery of tgAAVCF was associated with a rise in AAV2-neutralizing antibodies, but in addition to no observable adverse effects of these antibodies, *post hoc* subgroup analysis suggested there was no correlation between the magnitude of baseline AAV2-neutralizing antibody or the increase in titer with the change in FEV_1 from baseline. In the primate animal model, neutralizing antibodies were not associated with inflammation or blockage of gene transfer (data on file, Targeted Genetics Corporation). The potential role of such antibodies in limiting potential efficacy of gene transfer clearly requires further study since the *post hoc* analysis lacks adequate statistical power. Shedding of viral vector in sputum was common immediately after dosing but detected in only a minority of samples at low levels at day 150, 90 days after the final dose of tgAAVCF.

In conclusion, this double-blind, placebo-controlled, randomized trial of multiple high-dose aerosolization of tgAAVCF in CF patients $\geq$ 12 years of age with mild pulmonary disease demonstrated a good safety and tolerability profile. Suggestions of clinical effect were seen in short-term surrogate measures of pulmonary function and sputum inflammatory cytokine. Induction of AAV2-neutralizing antibodies did not seem to correlate with spirometric effect or adverse events, and sputum vector shedding was of generally limited duration and magnitude. In a small subset of subjects undergoing bronchial brushings, gene transfer but not expression was demonstrated. Results of this study are promising, and a further trial has started that is adequately powered to detect pulmonary function effect sizes observed in this study and to confirm these observations. In addition, if the early improvement in lung function is confirmed, the questions of carryover effect beyond the first dose and potentially limiting role of rising antibody titers, and the role of improved pulmonary function vs stabilization of decline, need to be addressed if tgAAVCF is to continue to move toward clinical application.

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