Administration of an adenovirus containing the human *CFTR* cDNA to the respiratory tract of individuals with cystic fibrosis

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We have administered a recombinant adenovirus vector (Ad*CFTR*) containing the normal human *CFTR* cDNA to the nasal and bronchial epithelium of four individuals with cystic fibrosis (CF). We show that this vector can express the *CFTR* cDNA in the CF respiratory epithelium *in vivo*. With doses up to 2×10^9 pfu, there was no recombination/complementation or shedding of the vector or rise of neutralizing antibody titres. At 2×10^9 pfu, a transient systemic and pulmonary syndrome was observed, possibly mediated by interleukin-6. Follow-up at 6–12 months demonstrated no long term adverse effects. Thus, it is feasible to use an adenovirus vector to transfer and express the *CFTR* cDNA in the respiratory epithelium of individuals with CF. Correction of the CF phenotype of the airway epithelium might be achieved with this strategy.

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Cystic fibrosis (CF), a common lethal hereditary disorder, is caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, a 250 kilobase (kb) gene on chromosome 7 (refs 1–4). *CFTR* codes for a transmembrane cAMP-regulated Cl^- channel on the apical surface of epithelial cells[5,6]. The disorder is characterized by airway and gastrointestinal disease, but the lung manifestations dominate, and 90% of the affected individuals die of respiratory complications[1]. The pathogenesis of the airway disease is clearly linked to the lack of sufficient *CFTR* function in the airway epithelium[7–9]. Within the first decade there is development of thick mucus, colonization with infectious bacteria and chronic airway inflammation[1,10]. Despite advances in therapy directed toward suppressing the infection and clearing the airways, CF almost invariably leads to respiratory insufficiency, with a median survival in the US of 29 years[8].

Theoretically, one approach to prevent the respiratory manifestations of CF is gene therapy, in which the normal *CFTR* cDNA would be transferred to the airway epithelium[11]. This strategy is based on the knowledge that *in vitro* transfer of the normal *CFTR* cDNA to CF epithelial cells enables these cells to secrete Cl^- in a normal fashion in response to cAMP[11–13]. Because of the complex, branching airway structure, it is not possible to use *ex vivo* gene therapy, in which the epithelium would be removed, corrected and returned to the patient[14,15]. Rather, gene therapy for CF must be carried out *in vivo*, by transferring the normal *CFTR* cDNA directly to the epithelium by airway administration. We believe that *in vivo* gene therapy could be effectively carried out in humans using a replication-deficient recombinant adenovirus (Ad) to transfer the *CFTR* cDNA, based on: (i) the ability to construct a replication deficient Ad vector to accommodate the 4.5 kb human *CFTR* cDNA[11]; (ii) the natural tropism of Ad for the respiratory epithelium[11,16–20]; (iii) the ability of an Ad vector to transfer a recombinant gene into the slowly proliferating cells of the airway epithelium with subsequent expression of the recombinant gene[11,17,18,21]; and (iv) the lack of an association of human Ad infection with malignancy[22]. Recombinant Ad vectors can transfer the normal *CFTR* cDNA to the respiratory epithelium of experimental animals *in vivo*, with expression of the transferred gene for at least six weeks[11] and to CF airway epithelial cells *in vitro* with correction of the "CF phenotype" in these cells[21]. These observations have been confirmed by several groups[23–31].

In 1992, we proposed a clinical trial to evaluate the administration of a *CFTR* cDNA Ad vector to the epithelium of the respiratory tract of individuals with CF[16]. Our primary goals were to evaluate safety and to demonstrate *in vivo* gene transfer following administration of an Ad vector to the respiratory epithelium, with escalating doses to different individuals. We began the first human gene therapy trial for CF on April 17, 1993. This report summarizes our experience with the first four patients.

Pre-clinical testing

With Ad vectors, experimental animal studies demonstrated[16,32]: (i) mild to moderate inflammation in the airways, blood vessels and alveoli (neutrophils dominated at 1–3 d, followed by a mononuclear cell infiltrate at 4–7 d, lasting at least 30 d); (ii) no change in clinical safety parameters attributable to the vector; (iii) limited shedding of the

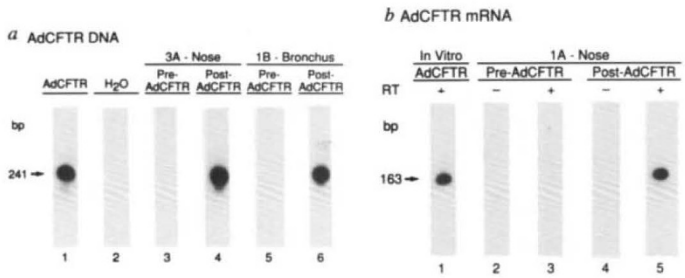

Fig. 1 Evidence for Ad*CFTR* DNA and Ad*CFTR*-derived *CFTR* mRNA in respiratory epithelial cells of study individuals following *in vivo* administration of Ad*CFTR*. *a*, Detection of Ad*CFTR* DNA, by Southern analysis of PCR amplified cell lysate using primers specific for Ad*CFTR* DNA and a ^{32}P-labelled nested probe. Lane 1, positive control with purified Ad*CFTR* genomic DNA; lane 2, negative control (H_2O); lane 3, nasal epithelial cells of individual 3A obtained before administration of Ad*CFTR*; lane 4, as lane 3, but 7 days after nasal administration of Ad*CFTR* (2×10^7 pfu); lane 5, bronchial epithelial cells of individual 1B obtained before administration of Ad*CFTR*; and lane 6, as lane 5, but 15 days after bronchial administration of Ad*CFTR* (2×10^7 pfu). *b*, Detection of Ad*CFTR*-derived *CFTR* mRNA. Respiratory epithelial cell total RNA was converted to cDNA with reverse transcriptase (RT), amplified by PCR using primers specific for Ad*CFTR*-derived mRNA, and evaluated by Southern analysis using a ^{32}P-labelled nested probe. + and − indicates the presence and absence of RT, respectively, in the cDNA synthesis reactions. Lane 1, positive control, nasal epithelial cells of individual 1A infected *in vitro* with Ad*CFTR*; lane 2, nasal epithelial cells of individual 1A obtained before nasal administration of Ad*CFTR*, without RT; lane 3, as lane 2, but with RT; lane 4, nasal epithelial cells obtained from the same individual nine days after nasal administration of Ad*CFTR* (2×10^5), without RT; and lane 5, as lane 4, but with RT. For *a* and *b*, the control and test samples for each individual are from the same autoradiographs.

vector (none in cotton rats; in 11/34 rhesus monkeys, shedding was observed, all within 7 d following administration of the vector except in 1 animal (14 d)); (iv) no detection of vector DNA in any organ other than the lung; (v) serum anti-Ad antibodies induced in cotton rats by 7 d, and low titre serum neutralizing immunity in rhesus monkeys.

Baseline and vehicle control

For all individuals, evaluation of the respiratory epithelium in the control periods demonstrated: (i) absence of Ad2/5 E1a sequences; (ii) no viral respiratory pathogens; and (iii) no replication of the Ad*CFTR* vector when cultured *in vitro* with the individual's respiratory epithelium. All had baseline low-titre serum anti-Ad antibodies. There were no changes in the safety parameters related to Ad administration. Typically as for CF[33], nasal and bronchial epithelial brushing samples in the baseline period contained a variable number of inflammatory cells, representing 2–51% (range) of the cells in the nasal samples and 30–73% of the cells in the airway samples. At both sites neutrophils dominated, representing 50–100% of the inflammatory cells recovered from the nasal epithelium (49–83% viable) and 88–100% from the bronchial epithelium (56–85% viable).

Ad*CFTR* administration

As in the control periods, the epithelial samples included large numbers of inflammatory cells (predominantly neutrophils), as well as mucus, bacteria and other debris and thus limited numbers of viable respiratory epithelial cells were available for assessment of Ad*CFTR*-directed gene transfer and expression. Of the cells recovered from the nasal epithelium in the week following Ad*CFTR* administration, 57–72% were viable, and 5–21% were inflammatory cells (20–100% neutrophils). Of the cells

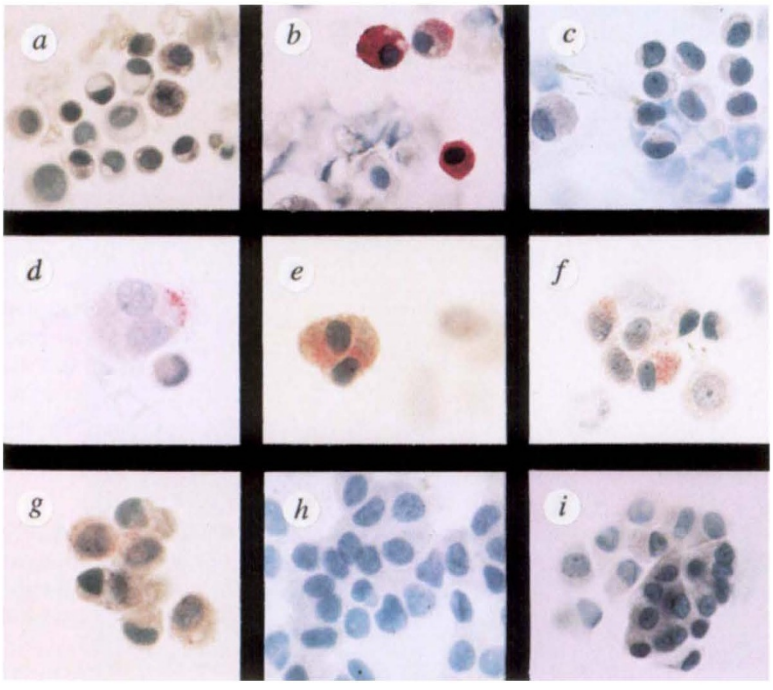

Fig. 2 Immunocytochemical detection of human CFTR in nasal epithelial cells before and after *in vivo* administration of Ad*CFTR* to the nasal epithelium. CFTR detected by the anti-CFTR antibody is indicated by a pink to red colour. The low level endogenous expression of CFTR in the nasal epithelium pre-therapy is not detected under the conditions used. *a*, Nasal epithelial cells from individual 1A recovered pre-therapy and maintained in tissue culture medium under conditions identical to *b*. *b*, as (*a*), but infected with Ad*CFTR* *in vitro* as a positive control. *c*, Fresh (not cultured) nasal epithelium from the same individual obtained pre-therapy during the baseline period prior to administration of Ad*CFTR*. *d*, The left nasal epithelium of the same individual 2 d after *in vivo* administration of Ad*CFTR* (2×10^5 pfu) to the left nasal epithelium. *e–g*, as (*d*), additional examples. *h*, The right nasal epithelium of the same individual 2 d after *in vivo* administration of Ad*CFTR* to the left nasal epithelium. *i*, as (*d*), but in the absence of the primary anti-CFTR antibody. Evaluation of nasal cells recovered in the pre-therapy period showed no positive cells. All of the positive cells following therapy are ciliated, non-ciliated columnar or basal epithelial cells; no alveolar macrophages, lymphocytes or polymorphonuclear leukocytes are positive. In all panels, the samples were counterstained with hematoxylin; all panels 630× except *d*, 1,000×.

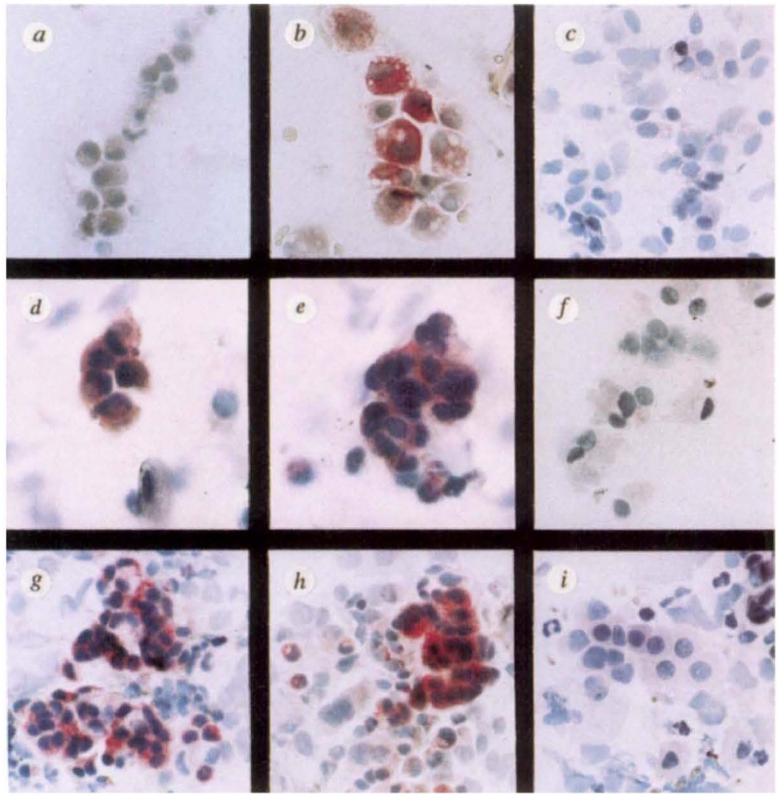

Fig. 3 Immunocytochemical detection of human CFTR in bronchial epithelial cells before and after *in vivo* administration of Ad*CFTR* to the bronchial epithelium. Low level endogenous expression of *CFTR* in the bronchial epithelium pre-therapy (*a–e*) is not detected; for *f–i*, the cells were exposed to the substrate for a longer period, and minimal pink staining is observed in the control (*f*). *a*, Bronchial epithelial cells from individual 3A recovered pre-therapy and maintained in culture under conditions identical to *b*. *b*, as (*a*), but infected with Ad*CFTR in vitro* as a positive control. *c*, Fresh (not cultured) bronchial epithelial cells of the same individual obtained pre-therapy immediately prior to intrabronchial administration of Ad*CFTR*. *d* and *e*, Left bronchial epithelium 4 d after intrabronchial administration of Ad*CFTR* (2×10^6 pfu) to the airway epithelium of the left lower lobe. *f* and *g*, same individual as in (*c–e*), but with the cells exposed to the colorimetric substrate for a longer time to increase the sensitivity of the assay. *f*, Bronchial epithelial cells obtained prior to intrabronchial administration of Ad*CFTR*. *g* and *h*, Evaluation similar to (*f*) of the left bronchial epithelium 4 d after intrabronchial administration of Ad*CFTR*. In (*h*), the morphology of one "positive" cell (at 9 o'clock, far left) is indeterminate. *i*, as (*g*) and (*h*), but with an irrelevant isotype control antibody. The positive epithelial cells following therapy include ciliated, non-ciliated columnar or basal cells. For *a–e*, the time of exposure to the alkaline phosphatase substrate was 9 min; for *f–i*, the time was 17 min. For the short exposure to the substrate, quantification of the "% positive cells" demonstrated in the pre-therapy period 0% epithelial cells were positive (n=800 cells), and 0% of inflammatory cells were positive (n=200), while in the post-therapy period 5.0% of epithelial cells were positive (n=500) and 0% inflammatory cells were positive (n=500). For the longer exposure, in the pre-therapy period 0% epithelial cells were positive (n=500), and 0% inflammatory cells were positive (n=200), while in the post-therapy period 14.0% epithelial cells were positive (n=500), and 0.4% inflammatory cells were positive (n=500). In all panels, the samples were counterstained with hematoxylin; all panels 630× except *d* and *e*, 1,000×.

recovered from the bronchi in the week following Ad*CFTR*, 72–81% were viable, and 55–78% were inflammatory cells (84–100% neutrophils).

Despite these limitations, Ad*CFTR* DNA could be detected in some respiratory epithelial samples (Fig. 1*a*) as could Ad*CFTR*-directed *CFTR* mRNA (Fig 1*b*). Furthermore, CFTR protein was detected in some samples by immunohistochemistry with an anti-CFTR antibody. As previously described[11], CFTR was not detected in freshly obtained nasal epithelial cells (pre-therapy control, Fig. 2*c*). After Ad*CFTR* administration however, CFTR was detectable with the anti-*CFTR* antibody (Fig. 2*d–g*), but not in the nasal epithelium on the side not receiving the vector (Fig. 2*h*), nor on the administered side in the absence of the primary anti-CFTR antibody (Fig. 2*i*). In the bronchial epithelium, no CFTR was detectable in freshly isolated cells pre-therapy (Fig. 3*c,f*) but was detectable in the epithelium after administration of the vector (Fig. 3*d,e,g,h*), but not with an irrelevant isotype control antibody (*i*).

It is difficult to estimate the numbers of cells "corrected", since the methodology is insensitive (for example, it cannot detect CFTR in the normal airway epithelium), and thus the positives are representative of overexpression compared to normal. Furthermore, it is difficult to administer reproducibly the vector and sample the bronchial epithelium in the identical site. With these caveats, quantitative assessment of individual 3A demonstrated 0% positive epithelial cells pre-therapy, 5.0% positive epithelial cells post-therapy (short exposure time to the alkaline phosphatase substrate) and 14.0% positive post-therapy (a longer exposure time).

Other than individual 1A, all nasal mRNA and protein samples were negative. For individuals 1A and 1B, no bronchial epithelial samples showed Ad*CFTR*-directed mRNA or protein expression and for individual 3A, no bronchial epithelial samples showed Ad*CFTR*-directed mRNA. No bronchial epithelial samples were obtained from individual 2A because of the potential risks of the bronchoscopy procedure (see below). No epithelial samples taken from any individual 10 or more days after administration of the vector demonstrated expression.

We did perform measurements of the nasal potential difference at the site of administration of Ad*CFTR*. However, as there was significant day-to-day variation in baseline measurements, variation secondary to prior sampling of epithelial cells, and a possibility that the vector *per se* modulated the electrical properties of the cells, we cannot make a definitive statement about these data until various issues regarding controls are resolved.

Safety of Ad*CFTR*

No adverse effects were noted in association with Ad*CFTR* administration to the nasal epithelium. For individuals 1A, 1B and 3A, there were no adverse effects attributable to the vector following administration to the bronchial epithelium (Table 1). In individual 1B (dose 2×10^7 pfu), there was transient leukocytosis, fever and a minimal,

transient infiltrate observed in the chart X-ray (roentgenogram) following bronchoscopy, similar to that observed after bronchoscopy pre-therapy.

For individual 2A (dose 2×10^9 pfu, 20 ml, right lower lobe bronchus), starting 12 to 24 h after Ad*CFTR* administration, a systemic and local syndrome developed, including headache, fatigue, fever, tachycardia, hypotension (not requiring vasopressor therapy) and dyspnea. There were bronchial breath sounds (without wheezing) in the region of the right lower and middle lobes, a decrease in vital capacity (VC), total lung capacity (TLC), forced expiratory volume in 1 s (FEV1) and diffusing capacity (DLCO) (but with the FEV1/forced VC at baseline levels), hypoxemia, and roentgenographic infiltrates of parts of the right lower (including the superior segment) and middle lobes (Fig. 4). Scattered small infiltrates were observed in some roentgenograms during this period on the opposite side. No leukocytosis or leukopenia were observed. There was a decrease in the haemoglobin/haematocrit and a transient increase in the serum creatinine. Because of the large number of blood samples taken to evaluate the aetiology of the syndrome and because aminoglycosides and semi-synthetic penicillins were administered, it was not possible to determine if the anaemia and/or creatinine increase was related to the Ad*CFTR* therapy. Broad spectrum antibiotics, antipyretics, nasal oxygen and intravenous fluids were administered, with resolution of all objective clinical signs and symptoms by 14 d (fever 6 d, hypotension 3 d, bronchial breath sounds 14 d). The chest roentgenogram and CT scan returned to baseline after 25 d (Fig. 4) and lung function after 30 d.

As specified in the protocol[16], the NIH Recombinant DNA Advisory Committee (RAC), the Food and Drug Administration (FDA), and the local regulatory committees were informed of these clinical findings. In addition, as this study represented the first experience with Ad vectors administered to humans for gene therapy, the principal investigator (R.G.C.) informed the principal investigators of other proposed Ad vector protocols of our observations.

Cultures of blood, urine and pharynx were negative for pathogenic organisms, and the sputum cultures continued to demonstrate *P.aeruginosa* as in the pre-therapy period. No replication-competent or deficient virus was observed in nasal, pharyngeal, rectal, blood or urine samples. Re-evaluation of the vector demonstrated no contaminating adventitious agents. Repeat cotton rat and rhesus monkey

Table 1 Study population, pre-therapy parameters, doses and sites of administration of the AdCFTR vector, and safety parameters

	Study individual			
Parameters	1A	1B	2A	3A
Age (yr)	23	33	24	23
Sex	M	M	F	M
CFTR genotype	ΔF508/ΔF508	ΔF508/ΔF508	ΔF508/ΔF508	ΔF508/ΔF508
AdCFTR therapy[a]				
Nasal Site	L	R	R	L
Dose (pfu)	2×10^5	2×10^5	2×10^7	2×10^7
Volume (ml)	0.2	0.2	0.2	0.2
Airway site	LLL	RLL	RLL	LLL
Dose (pfu)	2×10^7	2×10^7	2×10^9	2×10^6
Volume (ml)	20	20	20	5
Safety parameters[b]				
Haematologic	0	1[c]	1[d]	0
Coagulation	0	0	0	0
Hepatic	0	0	0	0
Renal	0	0	1[e]	0
Gastrointestinal	0	0	0	0
Neurologic	0	0	1[f]	0
Systemic	0	1[c]	2[g]	0
Cardiovascular	0	0	1[h]	0
Pulmonary	0	1[c]	1[i]	0

[a]Site of administration: nasal, inferior turbinate, L=left, R=right; airway, left lower lobe (LLL) or right lower lobe (RRL) bronchus, just distal to the orifice for the superior segment.
[b]Graded toxicity scale[16]: 0=none, 1=mild, 2=moderate, 3=severe, 4=intolerable.
[c]Transient leukocytosis, fever, X-ray infiltrate following bronchoscopy; also observed in control period.
[d]Transient anaemia relative to baseline values, coincident with multiple blood sampling for diagnostic purposes.
[e]Transient increase in creatinine (<2.0 mg dl^{-1}) following administration of aminoglycosides and semi-synthetic penicillins.
[f]Headache.
[g]Fever and fatigue.
[h]Tachycardia and hypotension (not requiring vasopressor therapy).
[i]Decreased vital capacity, forced expiratory volume in 1 s, total lung capacity and diffusing capacity compared to baseline, but with forced expiratory volume in 1 s/forced vital capacity similar to baseline; mild hypoxemia; bronchial breath sounds over the right lower and middle lobes; evidence on chest roentgenogram and CT scan of infiltrates of parts of the right lower and middle lobes.

studies with doses 100- to 1,000-fold greater (by weight) than that administered to the patient showed dose-dependent inflam–mation in the alveoli and airways, but no changes in the safety parameters (as in the pre-clinical testing). While complement fixing and ELISA anti-adenovirus antibodies were observed in the serum of this individual, the syndrome appeared before a rise in the anti-adenovirus titres (Fig. 5*a*,*b*).

Serum samples from before and after administration of the vector demonstrated no detectable interleukin (IL)-1α, tumour necrosis factor-α (TNF), IL-2 or IL-4. However, there were significant levels of IL-6 detectable in serum, with high levels (peak 75 pg ml^{-1}) associated with the systemic and local syndrome (Fig. 6*a*). Consistent with the increase in IL-6 levels in this individual, there was a rise in blood platelets, fibrinogen levels and the erythrocyte sedimentation rate (ESR) and a transient increase in body temperature (Fig. 6*B*). For individuals 1A, 1B and 3A, serum samples showed low levels of IL-6 detected transiently following Ad*CFTR* (Fig. 6*a*), suggesting a dose-dependent association of vector administration and the presence of IL-6 in the serum, with a threshold for symptomatic responses to the level of IL-6.

All four individuals, including 2A, were discharged on time according to the protocol schedule, and have returned for monthly evaluations up to 1 year. In all individuals, all safety parameters have been similar to the pre-therapy periods, including lung function (Fig. 7).

Evoked immunity

Following administration of Ad*CFTR*, there were no changes compared to baseline in serum total IgG, A or M, nor in serum complement levels (CH50, C3, C4). For individual 1A, the serum IgE titre increased following Ad*CFTR*, and remained elevated for 12 months. For 2A, the IgE titre increased transiently (9 d) with return to baseline at 2 wk. In 1A, 2A and 3A, there was an increase in titre of anti-Ad antibodies detected by complement fixation and/or ELISA (Fig. 5*a*,*b*). The highest titres were observed in 2A, the individual receiving the highest dose. For this individual, evaluation of T-cell proliferation *in vitro* in response to Ad5 demonstrated a proliferation index (ratio of the amount of Ad5 to the total number of mononuclear cells, 100 pfu $cell^{-1}$) of 85 pre-therapy, that rose to 330 at 14 d after Ad*CFTR* administration, and fell to 45 at 27 d. No increase in titres of anti-Ad neutralizing antibodies above pre-therapy levels were detected in any individual following Ad*CFTR* administration (Fig. 5*c*).

Discussion

We have shown that it is feasible to use an Ad vector to express the normal human *CFTR* cDNA in the epithelium of the respiratory tract of CF individuals. Our data suggest that there is a 1,000-fold dose range (2×10^6 to 2×10^9 pfu) in which such vectors can be administered to the bronchial epithelium without permanent adverse effects, production of replication-competent Ad, shedding of the vector or induction of neutralizing immunity. This study does not address whether such therapy will be successful in preventing the respiratory manifestations of the disease, chronicity of expression or whether repeat administration will yield expression of the normal *CFTR* cDNA. However, in the context of the observation of electrical correction of the CF-biologic phenotype in the nasal epithelium with a

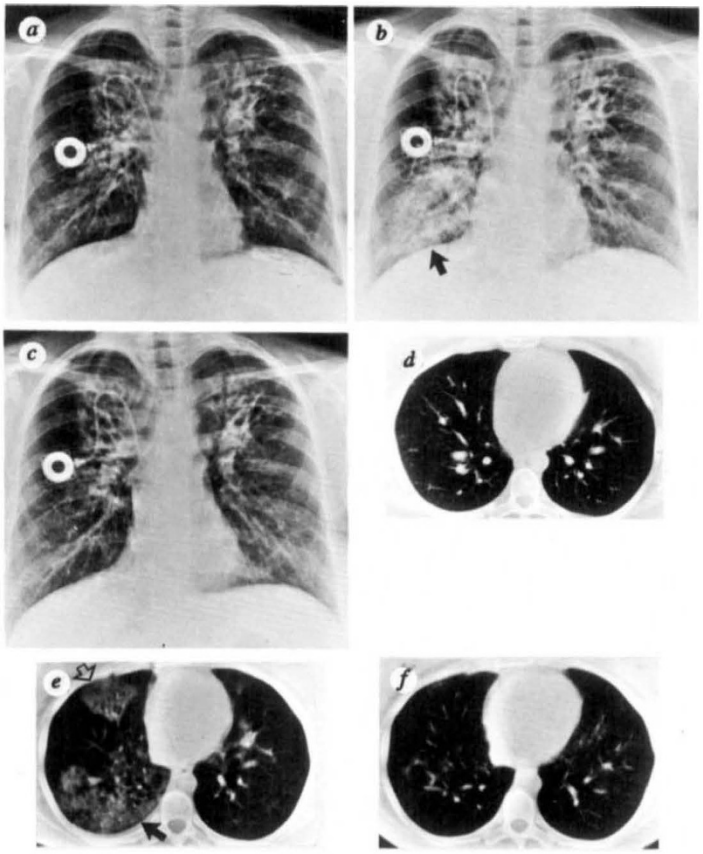

Fig. 4 Chest roentgenogram and computed tomography (CT) scans of individual 2A before and after receiving Ad*CFTR*. The vector (2×10^9 pfu in 20 ml) was administered via a bronchoscope inserted into the right lower lobe bronchus distal to the orifice of the superior segment. *a*, X-ray taken during the baseline period, 5 wk prior to therapy, showing chronic bilateral changes typical of CF, more prominent in the upper zones; a permanent venous access catheter is present. *b*, One day following administration of Ad*CFTR* there is opacification of the right lower lobe (solid arrow) and middle lobe. *c*, X-ray taken 25 d after administration showing return to baseline. *d–f*, CT scans (6 mm cut, 6 cm below the level of the carina). *d*, Pre-Therapy. *e*, Five days after administration of Ad*CFTR*; the opacification of the right lower lobe (solid arrow) and right middle lobe (open arrow) are identified. *f*, Twenty-five days after administration of Ad*CFTR* showing return to baseline.

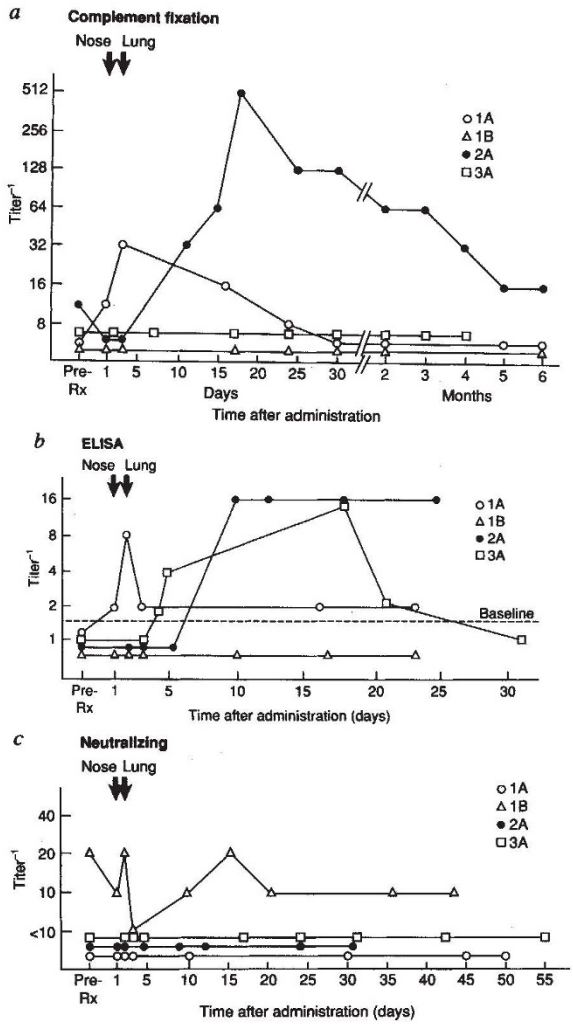

Fig. 5 Serum anti-Ad antibodies elicited by administration of Ad*CFTR* to the respiratory tract. Serum was evaluated for anti-Ad antibodies before and after administration of Ad*CFTR*. *a*, Complement fixing antibodies against adenovirus. The ordinate is presented as the inverse of the maximum dilution of serum yielding a positive value; intermediate values (for example, <1:8, 1:8-1:16) are indicated as such. The pre-rx value indicates the highest value observed in the baseline and/or control period. For individuals 1A, 1B, values for months 7–11 were similar to month 6; the same was true for 2A through month 9. *b*, Anti-Ad*CFTR* antibodies detected by enzyme-linked immunosorbent assay (ELISA). The ordinate is presented as the inverse of the maximum dilution of serum yielding a value higher than the highest value observed during the baseline and/or vehicle control period ("Pre-Rx"). *c*, Neutralizing antibodies against adenovirus. No increase in neutralizing antibody titres was detected after Ad*CFTR* administration in the study individuals. For individual 2A, sampling was limited after 12 d because of anaemia (see text). For all panels, the study individuals are indicated (1A, ○; 1B, △; 2A, ●; 3A, □).

CFTR cDNA-containing Ad vector[34], our study suggests that correction of the CF phenotype of the airway epithelium might be achieved by delivering *CFTR* using an Ad vector.

Long term safety. All individuals were discharged as scheduled, with no detectable chronic adverse effects observed over the subsequent 6–12 months. Thus, despite the transient syndrome observed in individual 2A, and with the caveats that this study is limited to four individuals and the vector was administered to the bronchus of only one lobe, a single administration of 2×10^6 to 2×10^9 pfu of virus directly into the airways seems not to be associated with permanent adverse effects in individuals with CF.

Replication, recombination, complementation and shedding. The Ad*CFTR* vector is missing the E1a sequence, required to direct the production of replication-competent virus[11,20]. However, it is theoretically possible that Ad type 2/5 E1a sequences in the epithelium, or a subsequent respiratory tract Ad infection, could provide E1a function *in trans*. Furthermore, the vector could replicate by recombining with epithelial Ad 2/5 sequences, or E1a-like information could be provided by other viruses or genes expressed in the airway epithelium[16,35–37]. It should be noted that it requires inhalation of only 10^3 pfu of replication-competent Ad can cause a clinically apparent infection of the respiratory tract[16,38], while we administered 2×10^6 to 2×10^9 pfu of the vector directly to the airways.

These theoretical risks were minimized by requiring pre-therapy: (i) no Ad type 2/5 E1a sequences in the

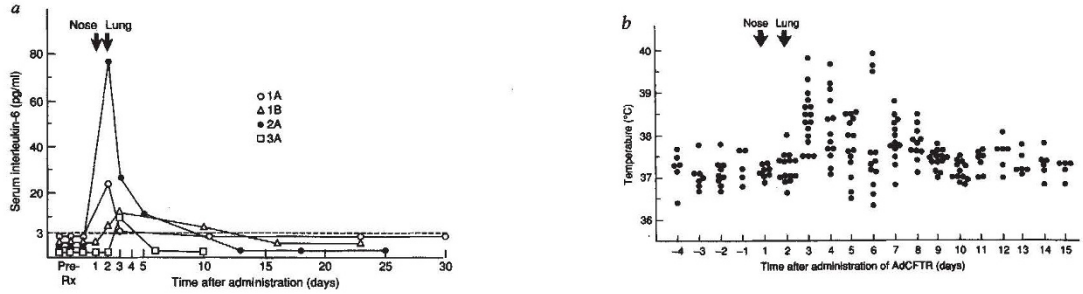

Fig. 6 Dose-dependent serum IL-6 levels following administration of Ad*CFTR* to the respiratory tract and relationship to fever observed at the highest dose administered to the bronchi (individual 2A, 2×10^9 pfu in 20 ml). *a*, Serum IL-6 levels. For individuals 1A, 1B and 2A the lung administration was on d 2; for 3A it was on d 3. The detection limit is > 3 pg ml^{-1} (dashed line). "Pre-rx" refers to the baseline and vehicle control periods; samples were measured on a minimum of 3 different days in the control periods. Each data point represents the average of triplicate determinations. *b*, Temperature of individual 2A after administration of Ad*CFTR* to the airways of the right lower lobe. The abscissa indicates the time after administration of the vector (– indicates time points pre-rx). Each data point indicates the temperature taken at different times throughout the 24 h period.

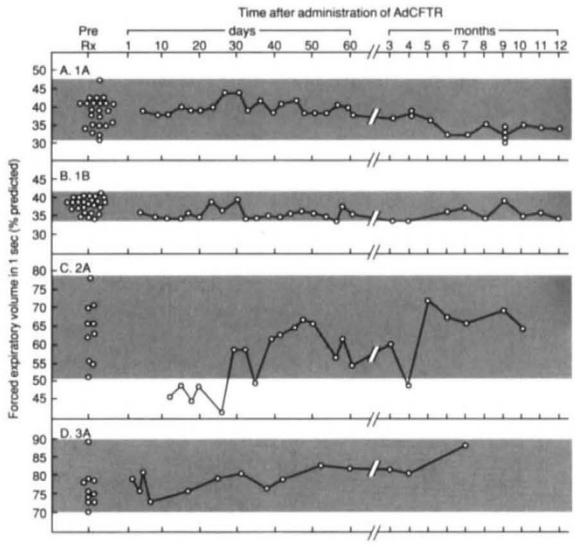

Fig. 7 Lung function following administration of the Ad*CFTR* vector. Shown is the forced expiratory volume in 1 s (FEV1) pre-therapy (baseline and vehicle control periods) and following administration of the vector (nasal administration on d 1, followed by bronchial administration on d 2 for individuals 1A, 1B, 2A) or d 3 (for individual 3A). The data are presented as the % predicted value. Each data point represents an independent measurement of the FEV1; for the post-vector administration period, the individual time points are indicated. The shaded areas represents the pre-therapy range of values for each individual. *a*, Individual 1A; *b*, 1B; *c*, 2A; and *d*, 3A. Except for the transient mild decrease in FEV1 for individual 2A in the 30 d following administration of the vector, all values following administration of the vector are within the pre-therapy range.

respiratory epithelium; (ii) absence of respiratory viruses in respiratory cultures; (iii) no recent respiratory viral illness; and (iv) no replication of the Ad*CFTR* vector in the patient's own cells *in vitro*. Furthermore, since respiratory tract Ad-mediated illness is attenuated by pre-existing anti-Ad antibodies, evidence for humoral immunity against Ad was required for inclusion[16,20,39]. Importantly, despite the fact that shedding is common following natural Ad infection in humans[40] and was observed in the pre-clinical studies, no vector or replication-competent Ad were found in any individual following Ad*CFTR* administration. This should minimize apprehension regarding contamination of the environment.

Immunity and safety. Ad infection induces both humoral and cellular immunity[20,22,40,41]. Since all individuals had anti-Ad antibodies pre-therapy, there was a risk for an immediate anaphylactic-type immune reaction to administration of Ad antigens directly to airways. However, consistent with administration of Ad vectors to animals with anti-Ad immunity[16,29,42], no immediate anaphylactic-type reaction was observed. The increase in serum IgE titres in some individuals following Ad*CFTR* was not expected; whether this is relevant to the Ad vector is unknown. Cytotoxic T cells are induced by infection of replication-competent Ad and vectors like Ad*CFTR* likely induce cytotoxic T cells secondary to minimal early and/or late gene transcription or the viral antigens *per se*[20,22]. It is not known if cytotoxic T cells were induced in our study population.

Chronicity of expression. The exogenous DNA transferred by Ad vectors functions in an epichromosomal site, and chromosomal integration is rare, if at all[22]. This has the advantages of minimal risk for induction of malignancy, and expression will eventually wane, an important safety feature if the presence of the vector DNA is associated with toxicity. The disadvantage, however, is that the vector will have to be re-administered to maintain adequate *CFTR* expression.

Studies in cotton rats with Ad*CFTR* suggest a half-life of expression of the *CFTR* cDNA in the lung of 6 wk[11]. Chronicity of gene expression may be limited by cytotoxic T cells that recognize viral antigens expressed on the target cells[16,21,43]. Our study does not clearly define the pharmacodynamics of expression of the *CFTR* cDNA, but samples obtained ≥10 d after vector administration showed no Ad*CFTR*-directed mRNA nor CFTR protein. This may represent a limitation of the sensitivity of our methods and/or numbers of epithelial cells recovered, induction of cytotoxic T cells or other mechanisms, but it is consistent with the concept that current Ad vectors do not permanently transfer the *CFTR* cDNA into the genome of the target cells and/or the modified target cells are short lived. Thus, to maintain chronic expression, Ad vectors will probably have to be administered repeatedly. In this respect, it is relevant that we[16,32,42] and others[23–26,29,44] have shown that in rodents and primates, Ad vectors induce anti-Ad humoral immunity, including neutralizing antibodies, and that repeat administration to these animals may be associated with reduced expression of the exogenous gene[29,42,44]. It is thus important that there was no increase in serum titres of anti-Ad neutralizing antibodies in any individual. We do not know if antibodies were generated in the lung, but this observation is different from findings in animals, and has significant implications for gene therapy with repeat vector administration.

Safety. While there were no changes in safety parameters related to administration of 2×10^5–2×10^7 pfu of the Ad vector to the nasal epithelium or 2×10^6–2×10^7 pfu to the bronchial epithelium, there was a transient systemic and local syndrome observed at a dose of 2×10^9 pfu to the bronchial epithelium. This resolved with symptomatic therapy and prophylactic antibiotics, and had no permanent sequelae. Such a transient syndrome could be acceptable if it was associated with restoration of normal CFTR function in the airway epithelium.

This syndrome was most likely caused by vector-induced inflammation of the lower respiratory tract. Consistent with this hypothesis was the finding of high levels of IL-6 in the serum in this patient. IL-6 is a multifunctional, single chain, glycosylated cytokine[45,46]. Since the serum half-life of IL-6 in humans is only 4.2 h[47], and the IL-6 remained elevated for several days in individual 2A, it is likely that there was continued production of IL-6 following vector administration. Although we have no direct evidence that IL-6 was produced in the lung in this individual, IL-6 can be produced by a variety of cells present in the CF lung, including parenchymal cells and inflammatory/immune cells[45,46,48–50]. Moreover, IL-6 is found in sputum and lung epithelial lining fluid in CF[51,52], IL-6 mRNA levels are increased in macrophages associated

with respiratory tract Ad infections[53], and IL-6 is found in lung and serum of mice following administration of adenovirus to the respiratory tract[54].

Infusion of IL-6 into experimental animals and humans has shown that IL-6 is a pyrogen, thrombopoietin, and acute phase reactant[45–47,55]. Individual 2A developed a fever lasting several days, associated with the high serum levels of IL-6. There were fatigue and headache, symptoms associated with the administration of IL-6 to humans[47]. Furthermore, high serum levels of IL-6 are commonly observed in conjunction with the hypotension associated with sepsis[56]. Increase in platelet number, ESR and fibrinogen are induced by IL-6[45–47,55]. Although the transient anaemia may have resulted from frequent phlebotomy, administration of IL-6 to humans is also associated with anaemia[47]. Also, while the increased serum creatinine may have resulted from the antibiotics administered, IL-6 has been implicated in the pathogenesis of proliferative glomerulonephritis, and transgenic mice overproducing IL-6 develop a similar lesion[45,46]. Finally, some IL-6 was detected following Ad*CFTR* administration to all four individuals. Together, these observations suggest that IL-6 played a significant role in the syndrome observed in individual 2A.

Despite the theoretical risk that high levels of IL-6 might induce the replication of an E1a-deficient Ad vector by inducing the transcription factor NF-IL6 (refs 36,37), no replication-competent or deficient Ad was detected in the upper respiratory tract of individual 2A (detection is likely if there was replicating Ad in the lung). It is therefore unlikely that IL-6 supported replication of the Ad*CFTR* vector in the respiratory epithelium. While no increases for IL-1α, TNF, IL-2 and IL-4 were seen, it is conceivable that some cytokines were released and remained in the local environment, or that the amounts released into the serum may have escaped detection, or that release may have been transient and not observed[54,57].

Implications for CF gene therapy. While extensive pre-clinical animal studies showed a dose-dependent inflammatory process in the lung, the animals did not exhibit clinical manifestations of the inflammation, even at equivalent doses much greater than the dose administered to individual 2A. Based on the pre-clinical data, our protocol originally planned for dose escalation of 2×10^7, 2×10^9, 2×10^{10} and 2×10^{11} pfu to the airways[16], other Ad vector *CFTR* gene therapy protocols[24,26] proposed starting at doses of 10^{10} pfu to the airways. Following our experience with individual 2A and subsequent communications with CF gene therapy investigators, the RAC and the FDA, such initial doses were reduced to 10^6 pfu with half-log dose increments in subsequent patients.

We chose a volume of 20 ml to instill the vector based on experience with bronchoalveolar lavage suggesting that this volume reaches airways, but not alveoli[58]. From the evidence that the pulmonary syndrome in individual 2A was primarily alveolar-based, it is likely that some vector did reach the alveoli. Furthermore, the radiographs suggested "spillover" of the vector beyond the site of administration. We therefore reduced the volume of vector for subsequent studies to minimize the possibility of the vector spreading, as have other investigators[59]. However, because of derangements of the anatomy and frequent coughing in CF, "spread" of the vector remains a problem.

An important lesson from patient 2A is that despite extensive planning, animal studies and thorough review, pre-clinical studies do not necessarily predict the response of humans (particularly individuals with disease) to gene therapy vectors. Of the animal models available, none mimic the human lung in CF. Thus, despite the lack of clinically evident toxicity observed in animal studies, human studies with viral vectors such as Ad*CFTR* must be approached with caution. Despite this caveat, only human studies will permit the definition of the "efficacy–toxicity" window relevant to gene therapy. This is an important concept in a disease like CF, where the therapy will be directed towards some individuals with significant pre-existing lung disease.

In this regard, it is of interest that the patient receiving the lowest dose (2×10^6 pfu in 5 ml) demonstrated expression of CFTR in the airway epithelium at a dose 1,000-fold less than was associated with induction of a systemic and local clinical syndrome. Thus, while these observations must be expanded into larger trials to make definitive statements about the relationship between efficacy and toxicity, our early data suggest that there may be a window in which Ad-mediated gene therapy could be used to treat the respiratory manifestations of cystic fibrosis.

Methodology

The clinical protocol was approved by the NHLBI Institutional Clinical Review Subpanel on September 21, 1992, the NIH Biosafety Committee on August 21, 1992, the RAC on December 3, 1992, and the FDA on April 16, 1993. Copies of this document are available (Office of Recombinant DNA Activities, Bldg. 31, Room 4B11, NIH, 9000 Rockville Pike, Bethesda, MD 20892).

Vector. Ad*CFTR* is a replication-deficient, recombinant Ad vector (the majority of E1 and E3 regions have been deleted) containing the Ad type 2 major late promoter and tripartite leaders followed by the entire coding region of the normal human *CFTR* cDNA and the SV40 early polyadenylation signals[11]. Details of the production, purification and storage of the vector are available[16]. The clinical grade vector preparation (concentration 8×10^{10} pfu ml^{-1}, particle to pfu ratio 20:1) was free of endotoxin, replication competent Ad or other infectious agents. The vector was administered in "vehicle" (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 3.3% glycerol).

***In vivo* pre-clinical studies.** *In vivo* efficacy studies in cotton rats (*Sigmodon hispidus*, the species most closely resembling humans in response to pulmonary infection by human Ad[60]) and non-human primates (rhesus monkeys) showed that Ad*CFTR* administered to the airways resulted in expression of human *CFTR* mRNA and protein in the airway epithelium[16,32]. In cotton rat safety studies, a total of 220 animals were administered Ad*CFTR* (or similar Ad vectors) to the respiratory tract (doses 10^6 to 4×10^9 pfu; equivalent human doses (by weight) 5×10^8 to 2×10^{12} pfu; intratracheal (n=32) or intranasal (n=188) administration was used to deliver the vector) and were evaluated up to 28 d for general well being, weight and histologic assessment of the lungs. Evidence for shedding of the vector (or a recombinant form), was determined by culturing pharyngeal and rectal swabs for replication competent and replication deficient Ad[16,32]. Systemic immunity against Ad*CFTR* was evaluated in sequential serum samples by ELISA[16]. In rhesus monkey safety studies, a total of 34 animals were administered Ad*CFTR* (or similar Ad vectors; intratracheal (n=8); intra-bronchial (n=13); or intranasal (day 1) followed by intrabronchial (day 2 as in the human protocol, n=13)), in doses from 2×10^7 to 10^{11} pfu (approximate equivalent human doses 2×10^8 to 10^{12} pfu). The animals were evaluated up to 585 d for changes in general well being, physical examination, vital signs, weight, complete blood count, serum chemistries, arterial blood gases, chest roentgenograms and histologic assessment of the lungs. Vector shedding was assessed as described for the cotton rats. In a random subgroup (n=6), multiple organs, including gonads, were evaluated for the presence of Ad*CFTR* DNA using PCR amplification[16,32].

Systemic immunity against the vector was evaluated in serial serum samples by testing for neutralizing antibodies[61].

***In vitro* pre-clinical studies.** *In vitro* studies demonstrated that Ad*CFTR* corrected the abnormality in cAMP-directed Cl^- secretion in epithelial cells derived from individuals with CF[11,16,21]. As a positive control, pre-therapy evaluation demonstrated the clinical lot of the Ad*CFTR* vector-directed *CFTR* mRNA and protein in freshly isolated respiratory epithelium. To minimize the possibility of Ad*CFTR* replicating *in vivo* secondary to recombination or complementation, for each individual to be treated, respiratory epithelial cells were recovered[62] and evaluated for the presence of Ad type 2/5 E1a sequences[63], and infected with Ad*CFTR* (at a multiplicity of infection estimated to be >100-fold higher than anticipated *in vivo*) to demonstrate that the vector would not replicate, but would direct the production of *CFTR* mRNA and/or protein[16,21]. Finally, the respiratory epithelium of all individuals was evaluated for occult infection with respiratory viruses that might influence Ad*CFTR* replication[16].

Study population. The selection criteria used to choose the study population are available[16]. The 4 participating individuals all had moderate lung disease typical of CF, including bronchiectasis and chronic colonization with Pseudomonas species (Table 1). All provided informed consent.

Human protocol. The clinical protocol included three periods: baseline, vehicle control, and Ad*CFTR* administration[16]. The baseline included a minimum of 1 wk evaluation to insure the individual fulfilled the selection safety and efficacy parameters. The vehicle control included a minimum of 1 wk evaluation of the safety and efficacy parameters following administration of the vehicle used to suspend the virus. On day 1, 200 μl of the vehicle was administered to an area of the nasal epithelium beneath the inferior turbinate in a fashion identical to the administration of the Ad*CFTR* vector (see below). For patients 1A, 1B and 2A, 20 ml of the vehicle was administered 24 h later to the lower lobe bronchus via a catheter inserted through a fibreoptic bronchoscope. For patient 3A, the volume of vehicle was 5 ml, and the time of administration was 48 h after the nasal administration. Prior to the administration of Ad*CFTR*, the patient was isolated for a minimum of 2 d to minimize the risk for development of intercurrent infection. To minimize the risk of shedding the vector and consequent environmental contamination, the vector was administered in a negative pressure room and the individual remained in that room until demonstrated not to be shedding the vector or replication competent Ad on at least three different days. On day 1, the vector was administered to the nasal epithelium in liquid form via a syringe and catheter over 1 to 2 min at a site in one nostril beneath the inferior turbinate. The individual was kept supine during the procedure and for 20 min thereafter to minimize vector spread. On day 2 or 3, the vector was administered to the bronchial epithelium in liquid form to the lower lobe bronchus (d 2 for 1A, 1B, 2A; d 3 for 3A) via a fibreoptic bronchoscope with a catheter positioned just distal to the orifice of the superior segment of the lower lobe bronchus of one lung over a period of 8 to 10 min (Table 1). The individual was then kept supine for 20 min to minimize vector spread.

***In vivo* biologic efficacy parameters.** Parameters to evaluate Ad*CFTR*-directed gene transfer and expression were assessed in samples of nasal and airway epithelium obtained by brushing before and after therapy[62]. The cell differentials were evaluated on cytocentrifuge preparations stained with modified Wright-Giemsa; cell viability was determined by trypan blue exclusion. Epithelial samples were evaluated for the presence of the Ad*CFTR* genomic DNA by Southern analysis of PCR amplified cell lysate[64] using Ad*CFTR*-specific primers (tripartite leader sense primer 5'–AGCTGTTGGGGCTCGCGGTTGAGG–3', *CFTR* exon 4 antisense 5'–AGTGTCCTCACAATAAAGAGAAGG–3', 35 cycles, followed by an aliquot amplified with same sense primer and exon 1 antisense 5'–CTGAAAAAAAGTTTGGAGACAACG–3', 40 cycles) and a ^{32}P-labelled nested probe[16]. The presence of AdCTR-directed *CFTR* mRNA was examined by using 3' rapid amplification of cDNA ends[65] using a primer containing a linker sequence and oligo (dT)(5'–GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT–3'); an aliquot of the cDNA was then amplified by PCR using Ad*CFTR*-specific and linker primers (sense 5'–CAGTAGCGGG-ATGCTCGGAA–3', antisense 5'–GACTCGAGTCGACATCGA–3', 40 cycles) and a ^{32}P-labelled nested oligonucleotide probe[16]. The presence of CFTR was evaluated by immunohistochemistry using an anti-human CFTR monoclonal antibody with an isotype-matched antibody or absence of the antibody as controls[11,21]. The electrical potential difference between the surface of the nasal epithelium beneath the inferior nasal turbinate and the subcutaneous tissues was measured during the baseline, vehicle control and Ad*CFTR* administration periods (minimum of three measurements at each time point; the three most negative values were averaged)[66].

Safety parameters. Full details of the safety parameters are available[16]. In brief, they included: general assessment (history, physical examination, vital signs, weight); routine blood studies (complete blood count, ESR, coagulation parameters, serum chemistries); general immunity parameters (anti-nuclear antibodies, rheumatoid factor, complement components and immunoglobulins); blood, urine and sputum cultures; urinalysis; electrocardiogram; chest roentgenogram and CT; and lung function tests [FVC, TLC, FEV1, DLCO, FEV1/FVC and arterial blood gases].

Other parameters. Anti-Ad immunity was evaluated in serum by determining the titre of complement fixing (Ad2 as the antigen), ELISA (Ad*CFTR* as the antigen), and neutralizing anti-adenovirus antibodies using conventional methods[16,61]. T-cell proliferation in response to Ad5 was tested in some samples[67]. Cytokine levels (IL-1α, IL-2, IL-4, IL-6, and TNF) were measured in serum by ELISA (Quantikine R & D, Minneapolis, MN).

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