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A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis

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Cystic fibrosis (CF) is a common, serious, inherited disease. The major cause of mortality in CF is lung disease, due to the failure of airway epithelial cells to express a functional product of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. A potential treatment for CF lung disease is the expression of CFTR in the airways following gene transfer. We have undertaken a double-blinded, placebo-controlled, clinical study of the transfer of the CFTR cDNA to the nasal epithelium of 12 CF patients. Cationic liposomes complexed with plasmid containing the human CFTR cDNA were administered to eight patients, whilst four patients received placebo. Biopsies of the nasal epithelium taken 7 days after dosing were normal. No significant changes in clinical parameters were observed. Functional expression of CFTR assessed by in vivo nasal potential difference measurements showed transient correction of the CF chloride transport abnormality in two patients (15 days after dosing in one patient). Fluorescence microscopy demonstrated CFTR function ex vivo in cells from nasal brushings. In total, evidence of functional CFTR gene transfer was obtained in six out of the eight treated patients. These results provide proof of concept for liposome-mediated CF gene transfer.

Keywords: *gene therapy; clinical trial; cationic liposome; CFTR; airway*

Introduction

Cystic fibrosis (CF) is an inherited condition affecting approximately one in every 2000 Caucasians. The condition is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-regulated chloride channel expressed on the surface of epithelial cells.[1] The CFTR chloride channel has an important role in regulating the transepithelial transport of salt and water. Abnormality or absence of CFTR can result in disease in many organs of the body, but the major cause of morbidity and mortality in CF is lung disease. Defective chloride secretion and elevated sodium absorption in the airways results in the development of thick mucus secretions in the lung and subsequent, chronic bacterial infection.[2] Despite advances in treatment, this condition still leads to an untimely death, often in early adult life.[3]

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The cloning of the *CFTR* gene has made gene replacement therapy a realistic possibility for CF. Heterozygotes with a mutation in the *CFTR* gene have a normal phenotype indicating that a single copy of the *CFTR* gene is sufficient for healthy lung function. Successful introduction of the CFTR cDNA into CF epithelial cells *in vitro* and into CF transgenic mice corrected the defective cAMP-regulated chloride secretion.[4–7]

Several phase I clinical studies have been performed to assess the transfer of the CFTR cDNA into the airways of CF patients. The results of four clinical trials utilising adenoviral vectors have been published. The findings from these studies were equivocal. Some evidence of efficacy was shown in three trials,[8–10] although none was observed in a fourth study.[11] In addition, inflammatory and immunological effects were observed at high doses. Alternative, nonviral, gene delivery systems are receiving increased attention, specifically cationic liposomes such as DC-Chol/DOPE (3β[N-(N',N'-dimethylaminoethane)-carbomoyl] cholesterol/dioleoylphosphatidylethanolamine).[12] In clinical trials, DC-Chol/DOPE liposomes have been shown to mediate gene transfer in patients with no evidence of inflammation, tissue damage or systemic immune response.[13,14]

Table 1 Patient details

Treatment	*Patient*	*Age*	*Sex*	*Genotype*	*FEV1 (litres)*	*FVC (litres)*	*Clinical score*
Low CFTR	3	19	M	ΔF508/ΔF508	4.80	6.30	90
Low CFTR	5	17	M	ΔF508/ΔF508	4.40	5.70	95
Low CFTR	6	21	M	ΔF508/ΔF508	3.10	4.60	75
Low CFTR	10	27	F	ΔF508/ΔF508	1.40	1.90	70
High CFTR	2	21	M	ΔF508/ΔF508	1.65	3.25	60
High CFTR	7	21	M	ΔF508/ΔF508	3.00	4.30	75
High CFTR	9	20	F	ΔF508/G551D	1.45	3.0	50
High CFTR	12	27	F	R553X/Q493X	2.30	3.15	45
Placebo-Vector	1	20	M	ΔF508/G551D	1.90	3.10	40
Placebo-Vector	8	19	M	ΔF508/R1162X	0.85	1.55	40
Placebo-Krebs	4	33	M	ΔF508/ΔF508	3.00	4.00	70
Placebo-Krebs	11	21	F	ΔF508/ΔF508	2.25	3.15	85

All patients were pancreatic insufficient.
FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; Clinical score, Shwachman–Kulczycki clinical score.[29]

Here, we describe a double-blinded, placebo-controlled study to test the safety and efficacy of DC-Chol/DOPE liposomes to deliver CFTR cDNA to the nasal epithelium of CF patients. The respiratory epithelium lining the nasal cavity was targeted by direct instillation of the DNA–liposome complexes. This enabled us to monitor closely for evidence of potential toxicity and correction of the CF ion transport defect. No inflammatory, immunological or other toxic effects were observed and CFTR function following gene transfer was demonstrated using two independent techniques. The results provide proof of concept for liposome-mediated gene transfer.

Results

Clinical evaluation

Twelve CF patients were enrolled in the study (Table 1) and attended the clinic as out-patients on 14 occasions (Table 2). All subjects completed the 4-week study and tolerated the procedures well. The subjects had no significant changes in their vital signs, blood chemistry, chest radiographs or respiratory function tests (Table 3). There was no increase in inflammatory markers. The immunoglobulin and complement levels did not increase and were frequently reduced at the end of the study. Two patients (2 and 12) had symptoms of an upper respiratory tract infection during the third week of the study and rhinovirus was cultured from the nasal lavage fluid of patient 12. Patients continued with their normal treatment, including the use of intravenous antibiotics by patients 6, 7, 9 and 10. No patient complained of any pain or discomfort in their nose following dosing.

White deposits were observed on the nasal epithelium of six patients (1, 2, 7, 9, 10 and 12) who each received DNA and liposomes (either CFTR or control vector plasmid). The deposits were most often seen on the nasal septum, occasionally the middle and inferior turbinates and were often removed by perfusion of fluids during

Table 2 Trial timetable

	Pre-Treatment			*Treatment*		*Post-Treatment*								
Day	*1*	*3*	*5*	*8*	*9*	*10*	*11*	*12*	*13*	*15*	*17*	*19*	*23*	*25*
Clinical exam	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Chest X-ray	X													X
Haematology	X					X		X			X			X
Immunology	X													X
Cultures	X					X				X			X	
Dosing				X	X									
Nasal biopsy										X				
Brush for SPQ								X						
Nasal PD	X	X	X			X	X		X	X	X	X	X	X

Timetable to show the days on which the following procedures were performed: Clinical exam: clinical examination of patient; X-ray: chest radiography; haematology: blood tests (see also Table 3); immunology: immunological tests (see also Table 3); cultures: nasal lavage, sputum and throat swabs taken for viral and bacterial culture; dosing: treatment administered; nasal biopsy: biopsy of nasal epithelium in patient's left nostril; brush for SPQ: nasal brushings and SPQ fluorescence measurements; PD: transepithelial potential difference measurement.

Table 3 Details of blood and immunological tests

	Day	*Low*	*High*	*Placebo*
Total WCC	1	8.4 (0.8)	11.3 (2.6)	10.2 (0.8)
	25	6.9 (0.9)	9.1 (0.8)	8.0 (1.2)
Neutrophils	1	5.7 (0.8)	7.6 (2.3)	7.0 (1.0)
	25	4.3 (0.7)	5.8 (0.4)	5.3 (0.8)
PV	1	1.8 (0.1)	1.9 (0.1)	1.8 (0.1)
	25	1.7 (0.2)	1.9 (0.1)	1.7 (0.1)
ESR	1	17.2 (8.9)	14.2 (4.2)	16.2 (6.3)
	25	10.2 (5.1)	13.5 (2.1)	10.2 (3.2)
CRP	1	15.7 (6.5)	11.7 (3.5)	17.2 (12.9)
	25	10.7 (3.6)	14.5 (9.8)	5.0 (0.8)
AST	1	31.0 (4.6)	24.7 (1.6)	31.5 (7.1)
	25	26.0 (3.5)	24.0 (1.7)	33.5 (6.2)
LDH	1	330 (55)	319 (64)	240 (65)
	25	310 (45)	290 (24)	254 (76)
IL-6	1	6.9 (4.1)	15.2 (1.9)	18.8 (12.5)
	25	5.3 (2.2)	4.3 (1.8)	11.9 (3.3)
TNF-α	1	23.0 (1.1)	17.2 (2.1)	16.0 (1.9)
	25	18.2 (2.2)	16.2 (1.5)	15.7 (0.3)
IgG	1	11.6 (1.9)	16.6 (2.1)	14.5 (1.1)
	25	10.6 (1.5)	15.2 (2.1)	13.6 (1.1)
IgM	1	1.0 (0.2)	1.8 (0.4)	1.1 (0.2)
	25	0.8 (0.2)	1.8 (0.5)	1.0 (0.2)
IgA	1	1.5 (0.7)	2.4 (0.5)	1.7 (0.6)
	25	1.4 (0.6)	2.2 (0.4)	1.6 (0.6)
IgE	1	63.5 (53.6)	82.3 (43.1)	280 (224)
	25	64.2 (55.3)	75.0 (28.1)	261 (214)
C3	1	166 (13.7)	159 (5.2)	181 (17.4)
	25	164 (7.3)	148 (9.2)	163 (11.8)
C4	1	27.2 (1.1)	34.7 (7.2)	37.2 (4.1)
	25	28.5 (2.9)	33.5 (8.5)	35.0 (2.9)

Results are presented for the low CFTR dose ($n = 4$), high CFTR dose ($n = 4$) and placebo ($n = 4$) cohorts. Pre-treatment values (day 1) and post-treatment values (day 25) are shown for each cohort. All results are expressed as mean (s.e.m.). The values obtained on days 10, 12 and 17 (data not shown) were not significantly different from those obtained on day 25. All patients were negative for antibodies to double-stranded DNA, antinuclear antibodies and rheumatoid factor, before and after treatment.
Total WCC: total white cell count ($\times 10^9$/l); neutrophils: total neutrophil cell count ($\times 10^9$/l); PV: plasma viscosity (cP); ESR: erythrocyte sedimentation rate (mm/h); CRP: C reactive protein (mg/l); AST: aspartate transaminase (IU/1); LDH: lactate dehydrogenase (IU/1); IL-6: interleukin-6 (pg/ml); TNF-α: tumour necrosis factor-alpha (pg/ml); IgG, IgM, IgA, IgE: immunoglobulins (g/l); C3, C4: complement factors (mg/dl).

measurement of nasal PD. Brushing of the area did not reveal the presence of inflammatory cells. It is likely that these deposits were residual DNA–liposome complexes dried on to the nasal epithelium, and were a result of the direct instillation method of administration.

Patient 12 (high CFTR dose) experienced right earache lasting just over 1 h on the evening after the first dosing session (day 8). Examination of the right ear revealed an injected tympanic membrane which persisted until day 15, whilst the left tympanic membrane appeared normal. This patient also developed symptoms of an upper respiratory tract infection on day 14 and rhinovirus was cultured from nasal-lavage fluid on day 15. The cause of the earache was unclear.

Histology

Histopathology of nasal biopsies revealed no differences in the number of inflammatory cells in the epithelium or underlying tissue (data not shown). Seven of the biopsies showed mild, chronic inflammation. In one case (patient 10), there was a slightly increased number of polymorphs and eosinophils, indicating a very mild acute inflammation. These observations were similar to those seen in non-CF individuals and consistent with the biopsy site chosen, indicating that the levels of inflammation were within normal limits. There was no significant difference between the results obtained from the CFTR cohort (low and high dose) and the placebo cohort. In patient 9, the biopsy consisted of blood clot. In two cases (patients 1 and 8), insufficient epithelium was visualised but the underlying tissue was normal.

Evaluation of ex vivo electrophysiological measurements (SPQ analysis)

We and others have shown that cAMP agonist-stimulated halide secretion as measured by SPQ analysis (see Materials and methods) effectively discriminates between samples of CF and non-CF nasal epithelial cells.[15] SPQ analysis was performed on samples of patient nasal epithelial cells isolated on day 12 of the study (Table 2). Samples from three of the low dose CFTR cohort (Figure 1a, c and d; patients 3, 6 and 10) and two of the high dose CFTR cohort (Figure 1e and f; patients 2 and 7) showed changes in SPQ fluorescence following the addition of the cAMP agonists IBMX and Fsk, consistent with the presence of functional CFTR. No such changes were observed in the remaining samples from the CFTR treated patients (Figure 1b, g and h) or in any of the samples from the placebo cohort (Figure 1i, j, k and l).

Evaluation of in vivo electrophysiological properties (PD analysis)

Nasal PD was measured on three occasions before treatment and on eight occasions after treatment (Table 2). For comparative purposes, patients were grouped according to treatment cohort and the data were grouped into pre-treatment values (days 1–5) and post-treatment values (days 10–15 or days 10–25). On each occasion, the PD was measured in response to four solutions (see Materials and methods).

Basal PD was measured in response to perfusion with a physiological Kreb's HEPES solution. No significant post-treatment changes were observed in basal PD (Figure 2) in any of the dosing cohorts. However, basal PD was reduced in two individuals with clinically confirmed, upper respiratory tract infections (Figure 2b; patient 2 on days 15–25 and patient 12 on days 17–23).

When the PD response to perfusion with Kreb's HEPES solution containing the sodium channel blocker amiloride (ΔPDamil) was measured, there were no significant post-treatment changes observed. The mean values for the low CFTR cohort were -16.17 ± 1.66 mV on days 1–5; -21.45 ± 2.21 mV on days 10–25 ($P = 0.20$); and -22.9 ± 1.53 mV on days 10–15 ($P = 0.08$). The mean values for the high CFTR cohort were -30.76 ± 3.48 mV on days 1–5; -26.08 ± 3.21 mV on days 10–15 ($P = 0.43$); and -23.98 ± 2.74 mV on days 10–25 ($P = 0.20$). The mean values for the placebo cohort were -26.91 ± 3.39 mV on days 1–5; -23.69 ± 2.06 mV on days 10–15 ($P = 0.27$); and -25.21 ± 1.74 mV on days 10–25 ($P = 0.49$).

$\Delta PDCl^-$ was measured in response to perfusion with low chloride Kreb's HEPES buffer in which chloride ions had been replaced with gluconate. Measurement of

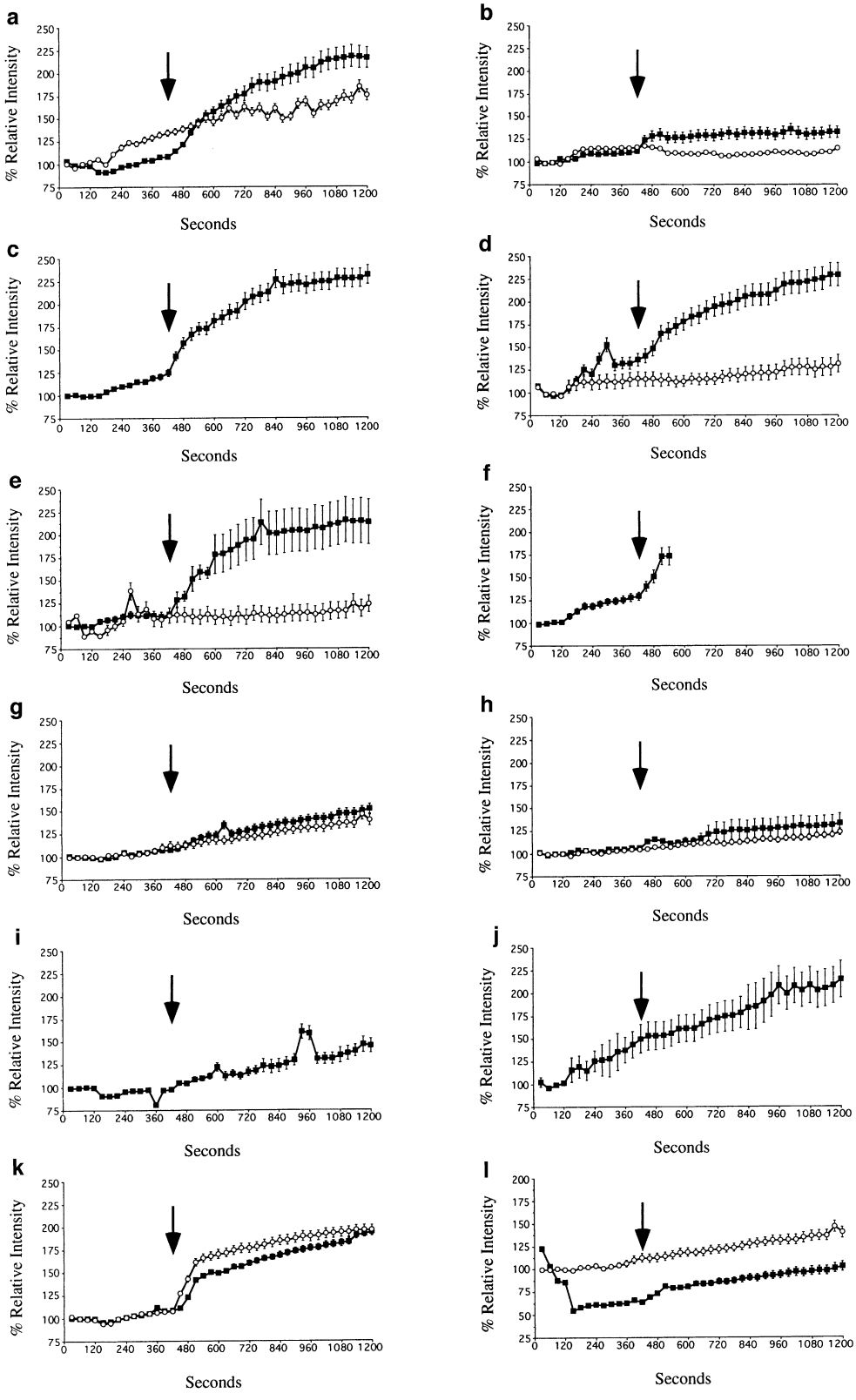

Figure 1 *SPQ analysis of cells collected from patients by nasal brushing. Percentage relative intensity of fluorescence (see Materials and methods) is plotted against time (seconds) for each patient. Where possible, a comparison of the response to cAMP agonists (■) compared with no addition of drugs (○) is shown. In the event of insufficient cell harvest the response to cAMP agonists only was investigated. The arrow indicates the time at which the cAMP agonists (Fsk/IBMX), where appropriate, were added. An increase in the rate of change in percentage relative intensity after the addition of cAMP agonists (particularly when compared to no addition) is indicative of CFTR activity. (a, b, c and d) Low CFTR dose cohort: patients 3, 5, 6 and 10 respectively. (e, f, g and h) High CFTR dose cohort: patients 2, 7, 9 and 12 respectively. (i, j, k and l) Placebo dose cohort: patients 1, 4, 8 and 11 respectively. (f) Cells from patient 7 detached from the cover slip during the assay. (k) In cells from patient 8, the increase in SPQ fluorescence under both experimental and control conditions is unexpected. Importantly, addition of cAMP agonists did not increase the rate of change in percentage relative intensity compared with the control conditions. Error bars represent s.e.m.*

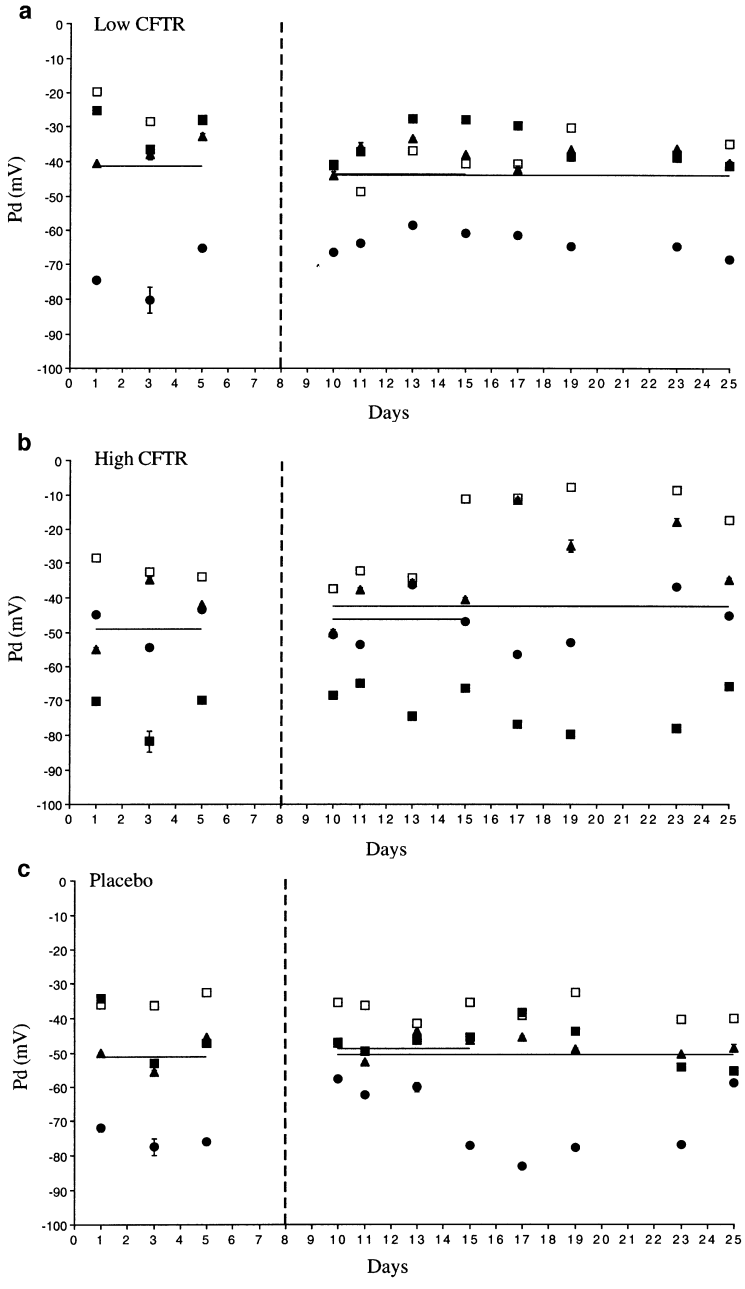

Figure 2 *Basal PD in each patient cohort. Individual measurements were made three times before and eight times after treatment. Each data point represents the mean of recordings from both nostrils. Error bars represent s.e.m. Time of treatment is indicated by dashed vertical lines. Horizontal lines indicate mean values for days 1–5, 10–15 and 10–25. (a) Low CFTR dose cohort. Patients 3, 5, 6 and 10 are represented by the symbols* □, ■, ● *and* ▲ *respectively. Mean values are: –41.4 ± 5.9 mV on days 1–5; –42.8 ± 2.8 mV on days 10–15 (P = 0.26); and –44.0 ± 2.2 mV on days 10–25 (P = 0.19). (b) High CFTR dose cohort. Patients 2, 7, 9 and 12 are represented by the symbols* □, ■, ● *and* ▲ *respectively. Mean values were: –49.3 ± 5.0 mV on days 1–5; –46.3 ± 4.1 mV on days 10–15 (P = 0.78); and –42.7 ± 3.9 mV on days 10–25 (P = 0.44). (c) Placebo dose cohort. Patients 1, 4, 8 and 11 are represented by the symbols* □, ■, ● *and* ▲ *respectively. Mean values were: –51.3 ± 4.7 mV on days 1–5; –48.9 ± 2.5 mV on days 10–15 (P = 0.85); and –50.6 ± 2.3 mV on days 10–25 (P = 0.94).*

$\Delta PDCl^-$ readily distinguishes between CF and non-CF individuals (Figure 3a).[16,17] Typical recordings from a patient who received placebo (Figure 3b), showed no changes after treatment. Certain individuals receiving CFTR treatment also showed no evidence of any change in $\Delta PDCl^-$ recordings (Figure 3c). In contrast, Figure 3d shows recordings from a CFTR-treated individual whose post-treatment $\Delta PDCl^-$ values were corrected into the normal range and were indistinguishable from a non-CF response.

One measurement from one patient receiving a low CFTR dose (Figure 4a) and three measurements from one patient receiving high CFTR dose (Figure 4b) were restored into the range for non-CF subjects. Correction of $\Delta PDCl^-$ into the non-CF range was seen for up to 7 days after treatment in patient 7 (Figure 4b; days 11, 13 and 15) and up to 15 days post-treatment in patient 5 (Figure 4a; day 23). The low CFTR dose cohort showed a slight (approximately 2 mV), but statistically significant ($P = 0.03$, days 10–15 and $P = 0.01$, days 10–25) increase in

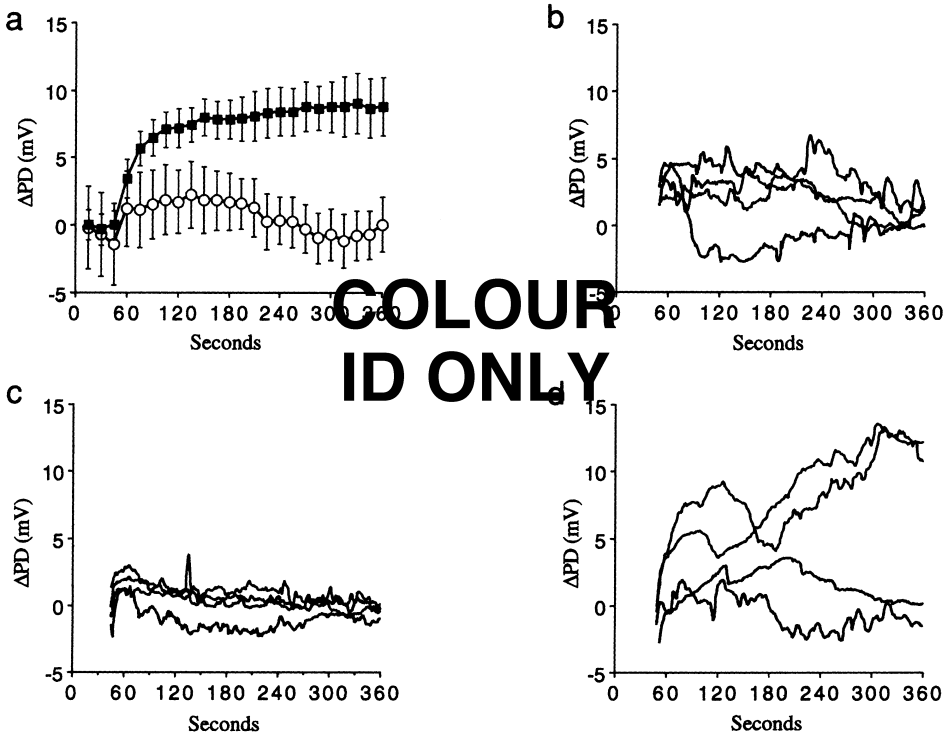

Figure 3 *Representative ΔPDC1⁻ recordings from individual patient nostrils, for both pretreatment (red: days 1, 3 or 5) and post-treatment (green: days 10, 11 or 13). (a) Comparison of typical CF (n = 15) and non-CF (n = 15) recordings. The traces represent the mean of n = 30 nostrils from each group with the data pooled every 15 s. Error bars represent s.e.m. This graph illustrates our CF and non-CF ranges for ΔPDC1⁻ recordings. (b) Representative pre- and post-treatment recordings from patient 11 (placebo cohort). (c) Representative pre- and post-treatment recordings from patient 2 (high CFTR dose cohort). (d) Representative pre- and post-treatment recordings from patient 7 (high CFTR dose cohort). The data presented are selected to illustrate the best response seen after treatment (d), and examples of individuals who showed no change in recordings after treatment (b and c).*

$\Delta PDCl^-$, consistent with CFTR gene transfer and efficacy (Figure 4a). No significant post-treatment changes were observed in $\Delta PDCl^-$ measurements in the high CFTR dose or placebo cohorts (Figure 4b and c). ΔPDIso measurements were obtained in response to perfusion with a low chloride Kreb's HEPES solution containing the cAMP agonist isoprenaline. No significant post-treatment changes were observed in ΔPDIso measurements in any of the dosing cohorts (data not shown).

Discussion

Gene therapy is being considered as a treatment for cystic fibrosis. Preclinical animal experiments have demonstrated the feasibility of this approach; however, the development of a safe and effective gene therapy for CF patients will only become available after a series of clinical trials.[6,7,18,19] The use of recombinant adenovirus as a delivery system for CF gene therapy has been assessed in several clinical studies.[8–11,20] A meaningful comparison of the results has proved difficult due to extensive differences in individual trial design. The efficacy observed in these studies was variable and some potentially serious safety issuses were raised.[21] In a placebo-controlled study, inflammation of the nasal epithelium was reported in two of three patients receiving the highest dose of adenovirus.[11] Evidence of systemic and local toxicity was observed after adenovirus vector was delivered to the lower airways.[20] The development of future adenoviral vectors with reduced immunogenicity may lessen the immune response in the patient. However, alternative nonviral delivery systems such as cationic liposomes are now receiving increased attention.

To assess the safety and efficacy of DC-Chol/DOPE liposome-mediated CFTR gene transfer, we used the respiratory epithelium of the nasal cavity as a surrogate tissue for the lung. This offered simple, noninvasive access to an epithelium with a histology and function similar to the tracheobronchial epithelium, and similar CF-associated electrolyte abnormalities.[2] In this double-blinded, placebo-controlled study a single dose of DNA-liposomes was instilled directly on to the nasal epithelium. No serious adverse effects were observed. There was no significant change in any of the clinical parameters measured and no evidence of an immunological response. This is consistent with findings from other studies in which DC-Chol/DOPE liposomes were administered to patients.[13,14] Although histopathology of epithelial biopsies taken 7 days after treatment showed individuals with mild chronic inflammation and one individual with mild acute inflammation, these results were all within the normal range for the biopsy site chosen. Thus topical administration of DNA–liposomes by direct instillation did not result in inflammation or irritation of the nasal epithelium. One patient who received a high CFTR dose (patient 12) described a short period of earache after the first treatment day. In an adenoviral, CFTR gene transfer study, Knowles *et al*[11] also reported a patient with earache. Both studies employed a similar instillation method to deliver the treatment solution to the nose, although whether the cause of the earache was the result of the treatment administration, or

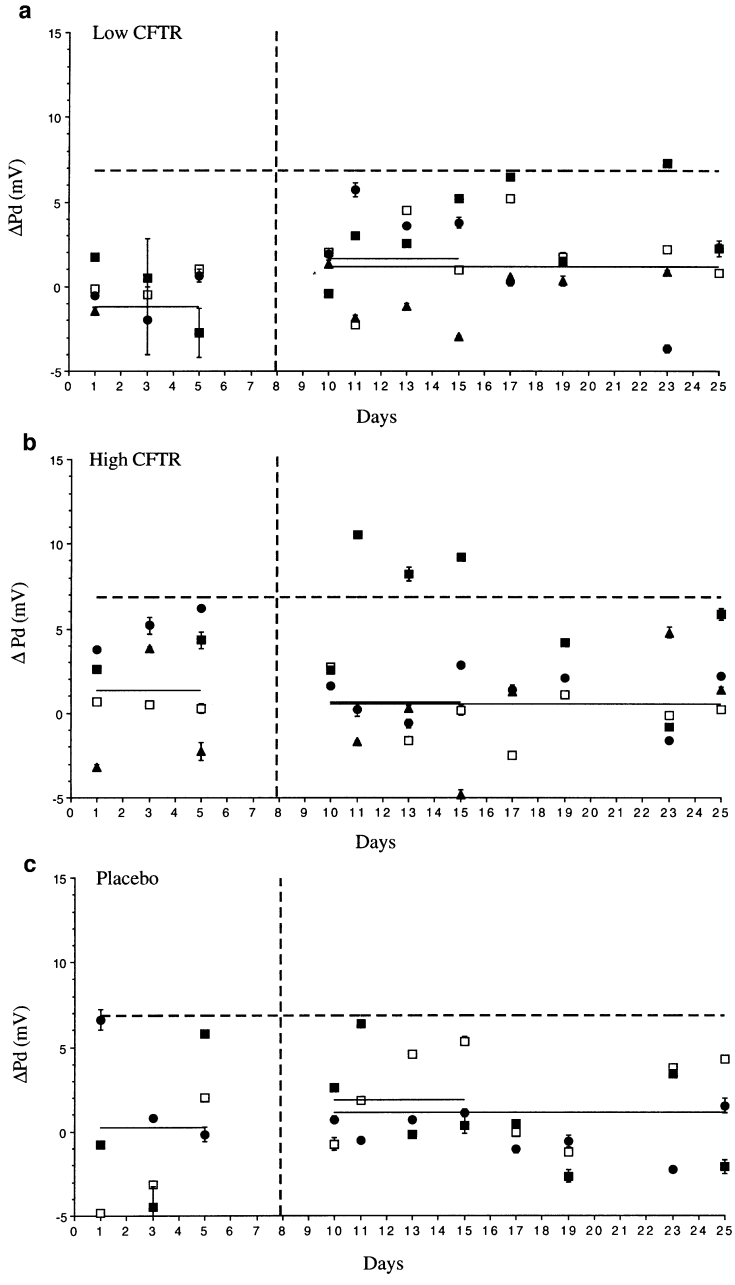

Figure 4 *Transepithelial ΔPDC1⁻ in each patient cohort. Details are as in legend to Figure 2. The non-CF range (>6.8 mV and data not shown) is delineated by a dashed horizontal line.[14,17] (a) Low CFTR dose cohort: patients 3, 5, 6 and 10 are represented by the symbols □, ■, ● and ▲ respectively. Mean values are: –1.2 ± 0.7 mV on days 1–5; 0.8 ± 2.8 mV on days 10–15 (P = 0.03); and 1.1 ± 0.7 mV on days 10–25 (P = 0.01). (b) High CFTR dose cohort: patients 2, 7, 9 and 12 are represented by the symbols □, ■, ● and ▲ respectively. Mean values are: 1.3 ± 1.1 mV on days 1–5; 0.6 ± 1.5 mV on days 10–15 (P = 0.52); and 0.5 ± 0.9 mV on days 10–25 (P = 0.44). (c) Placebo cohort: patients 1, 8 and 11 are represented by the symbols □, ■ and ● respectively. Mean values are: 0.2 ± 1.7 mV on days 1–5; 1.8 ± 0.8 mV on days 10–15 (P = 0.50); and 1.1 ± 1.4 mV on days 10–25 (P = 0.35). For ease of presentation, nine out of the 121 individual data points which fall below the abscissa (–5 mV) are not shown in the graphs, although they are reflected in the mean values. The comparatively large s.e.m. values observed for pre-treatment ΔPDC1⁻ values obtained for patients 5, 6, 7 and 8 on day 3, and patient 5 on day 5, reflect the use of manually recorded data on those occasions. The individual measurements of ΔPDC1⁻ in patient 4 (placebo cohort) were omitted from the group analysis due to extreme variation in ΔPDC1⁻ pre- and post-treatment; this patient demonstrated the unique finding in a CF patient of ΔPDC1⁻ values in the non-CF range (on one occasion 19.28 ± 0.27 mV). However, there was no significant difference between ΔPDC1⁻ measurements in this individual before and after treatment (6.54 ± 4.80 mV on days 1–5; 9.62 ± 0.95 mV on days 10–15 (P = 0.25) and 9.63 ± 0.46 mV on days 10–25 (P = 0.15)). The genotype of this individual (ΔF508 homozygous) was reconfirmed experimentally by deletion-based and amplification refractory mutation-based allele-specific PCR assays.*

due to a subsequent viral infection is unclear. In conclusion, and in contrast to current adenoviral vectors, DC-Chol/DOPE appears to provide a safe delivery vehicle for gene transfer. However, the long-term safety of repeated administration can only be assessed by further clinical trials.

To assess functional CFTR gene transfer, we exploited differences in electrolyte transport between CF and non-CF individuals. The nasal epithelium of CF individuals shows increased sodium absorption and decreased chloride secretion compared to non-CF individuals.[17,22] The functional effects of CFTR cDNA transfer were evaluated

in vivo, by measurement of transepithelial PD, and *ex vivo*, using SPQ fluorescence microscopy.

The electrophysiological measurement of transepithelial PD to evaluate efficacy in CF gene transfer clinical studies is well documented.[9,11,14] Together the four nasal PD parameters (basal PD, ΔPDamil, $\Delta PDCl^-$ and ΔPDIso) indicate the movement of sodium and chloride ions across the nasal epithelium.[17,23,24] The $\Delta PDCl^-$ and ΔPDIso measurements are both indicators of chloride transport and are reduced in CF individuals. We (Figure 3a) and others have shown that $\Delta PDCl^-$ as measured along the floor of the nose is the most consistent single discriminator between CF and non-CF, whilst ΔPDIso measurements are more variable.[17] Therefore, an increase in $\Delta PDCl^-$ after treatment would be consistent with CFTR function following successful gene transfer.

In this study, the low CFTR dose cohort showed a slight (approximately 2 mV), but statistically significant increase in $\Delta PDCl^-$ ($P = 0.03$, days 10–15 and $P = 0.01$, days 10–25), consistent with a limited degree of CFTR gene transfer and efficacy (Figure 4a). Inspection of post-treatment $\Delta PDCl^-$ measurements from individual patients revealed that the response to CFTR treatment was variable. However, one measurement from a patient receiving a low CFTR dose (Figure 4a) and three measurements from a patient receiving a high CFTR dose (Figure 4b) were in the range for non-CF subjects. The best post-treatment recordings in an individual receiving CFTR treatment showed complete correction of the CF chloride abnormality and were indistinguishable from a non-CF response (Figure 3d). The correction of $\Delta PDCl^-$ into the non-CF range was of approximately 7 days duration in patient 7 (Figure 4b; days 11, 13 and 15) although patient 5 showed correction into the non-CF range 15 days after treatment (Figure 4a). Thus, correction following liposome-mediated CFTR gene transfer is transient. The reasons for this are unknown, but could be due to decreased CFTR expression from the plasmid, the loss of the plasmid from the cells, or the loss of transfected cells from the nasal epithelium.

No change was observed in sodium-related measurements (basal PD and ΔPDamil) in the CFTR or placebo cohorts, indicating that despite successful correction of the chloride defect there was no correction of the sodium defect by CFTR gene transfer. These results are consistent with previous experiments in cultured cells which demonstrated that whereas only 5% of cells need to express CFTR for normal chloride secretion, a far greater proportion are required to restore normal sodium absorption.[25,26] Results from a clinical study to assess adenoviral CFTR gene transfer showed that although no evidence of correction was observed in ΔPDamil, changes in basal PD reflected epithelial damage at high viral doses.[11] In this study, the absence of basal PD changes after treatment supports our toxicological findings that even at the highest dose administered, delivery of DNA–liposomes did not damage the nasal epithelium.

The analysis of transepithelial PD is currently the standard method for studying CFTR gene transfer; however, it has several limitations. One limitation is that sodium-related PD measurements are changed toward the non-CF range in patients with an upper respiratory tract infection.[23] This can complicate the interpretation of PD measurements and negate data obtained during a clinical trial. Two individuals in this study had clinical evidence of upper respiratory tract infection around day 15 and subsequently showed reduced basal PD measurements (Figure 2b; patients 2 and 12). It was therefore impossible to determine if these patients showed signs of correction using this technique.

In order to circumvent these problems, this study included the *ex vivo* technique of SPQ fluorescence microscopy as a complementary assay of efficacy. Halide secretion stimulated by cAMP-agonists is decreased in CF cells compared to non-CF cells, and SPQ analysis can readily distinguish between them.[15] SPQ analysis was performed *ex vivo* on cells taken from the nasal epithelium. Cells taken from three patients in the low CFTR dose cohort and two patients in the high CFTR dose cohort (Figure 1) showed an increase in fluorescence, consistent with the presence of functional CFTR. Since a change in SPQ fluorescence is a measurement of CFTR function in a single cell, whilst PD analysis reflects CFTR function across the intact epithelium, it is likely that SPQ analysis is a more sensitive assay of functional CFTR. However, patient 5 showed correction of the $\Delta PDCl^-$ measurement, but no increase in SPQ fluorescence. A possible explanation is that gene transfer in the nasal cavity was uneven, and whilst PD was measured along the floor of the nose, SPQ analysis was performed on cells taken from above the inferior turbinate. This illustrates one difficulty in reliably assessing CFTR function following gene transfer. Importantly, SPQ analysis was not affected by infections of the upper respiratory tract. Evidence of CFTR function could still be detected using SPQ analysis in patient 2, whilst PD measurements were severely affected by viral infection (Figure 1 and 2b). This study provided the first use of SPQ fluorescence analysis in a CF clinical gene transfer study and it has proved to be a valuable technique. It may also be possible to further improve the method to make SPQ analysis a quantitative assay.[27]

The combined results of $\Delta PDCl^-$ correction (patients 5 and 7) and changes in SPQ fluorescence (patients 2, 3, 6, 7 and 10) showed evidence of functional CFTR gene transfer in six out of the eight CFTR-treated patients. In addition, post-treatment changes in $\Delta PDCl^-$ reached statistical significance for the low CFTR dose cohort (Figure 4). These electrophysiological data are encouraging.

Both in this study and that of Caplen *et al*,[14] the response to isoprenaline during nasal PD measurements in CFTR-treated patients was variable. The activation of CFTR via the cAMP pathway is essential for the discrimination of CF and non-CF cells by the SPQ assay;[15] but the switch to low chloride alone is sufficient to discriminate between CF and non-CF individuals by the PD assay.[17] This difference may reflect the differing physiology of intact and isolated epithelial cells.

The results from this study compare favourably with those obtained in the only other completed, liposome-mediated CFTR gene transfer trial, in which limited gene transfer was also obtained.[14] A direct comparison can be made between the two studies, both of which used DC-Chol/DOPE liposomes to deliver plasmid DNA to the nasal epithelium. There were minor differences in gene transfer formulations, such that a DNA:lipid (wt:wt) ratio of 1:6, with a constant DNA:volume ratio was used in this study, whereas a DNA:lipid ratio of 1:5 with a changing DNA:volume ratio was used by Caplen *et al*.[14] Other

differences include the use in this study, of an alternative CFTR expression plasmid containing the Rous sarcoma virus 3' long terminal repeat (RSV 3'LTR) rather than a simian virus 40 promoter sequence, and the inclusion of a vector plasmid which did not express CFTR as a further control. This additional control was important in this study to eliminate possible non-CFTR-specific effects of DNA–liposomes. Although only two patients received the control vector dose, we observed no toxicological effects and no interference in the PD efficacy measurements. In addition, we used direct instillation, rather than aerosolisation, to target the DNA–liposome complexes to the nasal epithelium. This method limited deposition of the gene transfer reagents in the lungs and maximised contact time with the nasal epithelium.

This study shows that the use of cationic liposomes to facilitate CFTR gene transfer to the nasal epithelium is safe, with no adverse effects observed even at the highest dose administered. This is in contrast to results from clinical studies using adenovirus. In addition, this study confirms that liposome-mediated gene transfer is feasible. Six out of the eight CFTR-treated patients showed evidence of CFTR function by transepithelial PD measurement or by SPQ analysis. Although the correction of the CF ion transport abnormalities observed using these methods was transient, in one patient it was observed 15 days after treatment. Nevertheless, if gene transfer proves successful in the lower airways, repeat dosing at relatively short intervals may be required for long-term, therapeutic benefit. It is expected that the efficiency of this gene transfer system will improve as the mechanism of liposome-mediated transfection is better understood and DNA–liposome formulations are optimised.

Materials and methods

Subjects

Twelve CF patients (eight male and four female) with defined CF genotypes were enrolled in the study (Table 1). All patients were over 16 years of age and gave written informed consent. The patients continued with their normal treatment during the 4-week study.

Study design

The study was approved by the UK Gene Therapy Advisory Committee, the Medicines Control Agency and the appropriate Hospital Research Ethics Committees. Subjects attended the clinic as out-patients on 14 occasions (Table 2). In addition to the routine clinical examination at each visit, patients were asked to report any symptoms. The subjects were randomly assigned a treatment regimen, such that both patients and investigators were blinded to the treatment each subject received, and remained blinded until after data analysis was completed.

Gene transfer reagents

Two plasmids, pTRIAL10 and pTRIAL10CFTR2 were used in this study.[19] Both plasmids contained an expression unit consisting of the RSV 3'LTR promoter and the late polyadenylation signal from simian virus 40; and the tetracycline-resistance gene incorporating a mutation to minimise expression in mammalian cells.[19] The plasmids were identical except that pTRIAL10CFTR2 contained the human CFTR cDNA. This plasmid was shown to express functional CFTR protein in cells grown *in vitro* and in an animal model.[19] Plasmid DNA was prepared at the Imperial Cancer Research Fund's Biotherapeutics and Hybridoma Development Unit in London. The plasmid DNA was supplied sterile and essentially free of endotoxins (<1 EU/mg DNA), in single-use vials at a concentration of 0.667 mg/ml in Kreb's HEPES buffer (pH 9.0).

DC-Chol/DOPE cationic liposomes were prepared at the University of Pittsburgh under conditions approved by the US Food and Drug Administration.[12] The DC-Chol/DOPE liposomes (6:4 molar ratio of DC-Chol to DOPE) were made by a microfluidisation procedure, to a final concentration of 2 μmol/ml (equivalent to 1.2 mg/ml of total lipid) in endotoxin-free water.[28] The transfection efficiency of the liposomes was assayed using HeLa cells and a plasmid expressing the chloramphenicol acetyl transferase reporter gene.[19] The DNA and liposomes were equilibrated to room temperature and mixed approximately 5 min before patient administration.

Dosing

Four treatment regimens were compared in the trial. To ensure that the absolute concentration of DNA–liposomes was equivalent in each dose, different volumes of the gene transfer reagents were administered. Four patients received a low CFTR dose, such that each nostril received 0.8 ml containing 40 μg pTRIAL10CFTR2 DNA complexed with 0.4 μmol DC-Chol/DOPE. Four patients received a high CFTR dose (8 ml) containing 400 μg CFTR DNA complexed with 4 μmol DC-Chol/DOPE. Two patients received control vector dose (8 ml) containing 400 μg pTRIAL10 DNA complexed with 4 μmol DC-Chol/DOPE and two patients received Kreb's HEPES Buffer (0.8 ml). The last two regimens constituted the placebo doses.

The treatment solutions were delivered to the patients in four sessions spread over 2 days, such that a quarter of the total dose (0.2 ml or 2.0 ml as appropriate) was administered at each treatment session. The treatment solutions were instilled directly on to the nasal septum using an MS16A syringe driver from Graseby Medical (Watford, UK) via a soft, infant, 4F, 50 cm feeding catheter from Portex (Hythe, UK). The patients lay on their left side with their head tilted down and the tube was placed 2 cm into the right nostril. The solution was delivered over a period of 15 min following which the patient remained in the same position for a further 15 min. After sitting upright for a few minutes, the patient was repositioned on their right side to instil the dose into the left nostril. Radioisotope studies demonstrated that whilst maximising the delivery to the nasal septum, this method gave a good spread of solution (using both 0.2 ml and 2.0 ml volumes) throughout the nasal cavity with minimal mucociliary clearance into the nasopharynx over 30 min (data not shown).

Nasal biopsy

An epithelial biopsy was taken from the left nostril of each patient 6 days after dosing (day 15). The biopsy was taken using local anaesthesia. Cotton wool soaked with 5% cocaine solution was placed in the left nostril for 5 min. Cocaine paste (25%) was then applied directly to the

nasal septum. The biopsy (3–5 mm^2) was taken approximately 3 cm back from the anterior nares on the nasal septum, the site of maximal exposure to the treatment solution. Histology of this region from control individuals revealed both transitional and ciliated, respiratory epithelia. The tissue was stored in formalin fixative and the tissue processed and stained with haematoxylin and eosin for histological examination. Adjacent sections were immunostained with peroxidase-labelled monoclonal antibodies against either neutrophil elastase (By87a; anti-neutrophil elastase), T cells (CD3; anti-CD3), B cells (JCB117; anti-CD79a) or macrophages (PGM1 or KP1; anti-CD68) according to standard pathology procedures and examined for evidence of inflammation.

Ex vivo electrophysiological measurements (SPQ analysis)

CFTR function was assessed in freshly isolated, nasal epithelial cells collected from the region between the middle and inferior turbinates by gentle brushing with a 3 mm cytology brush. Both nostrils were brushed and the cells from each nostril combined. A proportion of the resulting cell suspension consisted of ciliated epithelial cells which we and others have shown to possess ion transport properties characteristic of the airway epithelium.[15] Cells were loaded with the halide-sensitive fluorophore 6-methoxy-N-(3-sulphopropyl)-quinolium (SPQ) from Cambridge Bioscience (Cambridge, UK) by hypotonic shock. Halide efflux stimulated by the addition of 100 μM 3-isobutyl-1-methyl-xanthine (IBMX) and 25 μM forskolin (Fsk) from Sigma Chemical (Poole, UK), was measured essentially as described.[15] In the presence of functioning CFTR protein, the addition of cAMP agonists results in a reduction in the intracellular halide concentration. This reduction can be measured by an increase in SPQ fluorescence. Fluorescence images (excitation 350 nm, emission >430 nm) of the cells were captured every 30 s using Leica DMIRBE fluorescence microscope (Milton Keynes, UK) in conjunction with an Intracellular Ion Imaging System from Improvision (Coventry, UK). Studies were at room temperature. Wherever possible the cell sample was divided and one half used as a negative control in which the cAMP agonists were omitted. Where insufficient cells were obtained for division of the sample, the cells were subjected to the experimental conditions only. Fields of cells (typically 25–500 cells) were visualised at ×100 magnification. The brightest regions of interest were selected for quantification of fluorescence from the last image without knowledge of the rate of fluorescence change. The number of regions of interest analysed for each individual varied between 20 and 100. To correct for cell-to-cell differences in absolute fluorescence intensity (due to differences in cellular concentration of SPQ, cell size and cell clumping) the data are presented as percentage relative intensity which is $100 \times (F_t/F_o)$ where F_t is fluorescence intensity at time t and F_o is the mean of fluorescence intensity for the initial 120 s. Error bars indicate standard errors of the mean.

In vivo electrophysiological measurements (PD analysis)

CFTR function was assessed *in vivo* by measuring the potential difference (PD) across the nasal epithelium on the floor of the nasal cavity on 11 occasions during the study (Table 2). Measurements were performed essentially as described.[17] Baseline PD values in response to perfusion (4 ml per min) of Krebs' HEPES solution (pH 7.4) were allowed to stabilise and were subsequently recorded for 2 min. The perfusing solution was then changed sequentially to Kreb's HEPES plus 100 μM amiloride for 4 min, low chloride Kreb's HEPES (chloride replaced by gluconate) plus 100 μM amiloride for 6 min, and finally low chloride Kreb's HEPES plus 100 μM amiloride and 10 μM isoprenaline for 5 min. All solutions were at room temperature. PD was recorded every second by a custom-built, battery-powered, data logger (Logan-Sinclair, Rochester, UK) except for patients 5, 6, 7 and 8 on day 3, and patient 5 on day 5, where data were recorded manually. Measurements were taken from both nostrils on each measurement day. Values of basal PD, the change in PD after amiloride treatment (ΔPDamil), the change in PD after low chloride substitution ($\Delta PDCl^-$) and the change in PD after isoprenaline treatment (ΔPDIso) were obtained by averaging the PD values for the last 60 s in each solution from both nostrils. For discussion purposes, increases and decreases in PD refer to absolute magnitude of the PD. The Mann–Whitney U test for comparison between data sets was used as in Caplen *et al*[14] and the null hypothesis of no difference was rejected at $P < 0.05$. Error bars indicate standard error of the mean.

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