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A second dose of a CFTR cDNA–liposome complex is as effective as the first dose in restoring cAMP-dependent chloride secretion to null CF mice trachea

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Phase I clinical trials have provided encouraging data suggesting that gene transfer could provide a treatment for cystic fibrosis (CF). However, for all the current viral and nonviral vectors used to deliver the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the duration of CFTR expression is limited, necessitating a repeat dosing regimen to provide a long-term treatment. This study was performed to determine whether a second delivery of a CFTR cDNA–liposome complex could result in a similar level of functional CFTR expression observed after a single delivery and to assess whether the deliveries produced adverse inflammatory responses. CFTR functional expression was assessed by short circuit current measurements of tracheas taken from CF null mice (Cftr^{tm1Cam}) treated with a CFTR cDNA–liposome complex in the upper airways. Mice receiving two deliveries of this complex, the second after the response to the first had declined, showed cAMP-stimulated chloride currents which were not significantly different from normal tracheas or tissues assayed after a single dose of the complex. This double treatment was well tolerated with no discernible inflammation of lung tissue.

Keywords: *gene therapy; cationic liposome; cystic fibrosis; mouse model*

Introduction

The primary physiological defect in cystic fibrosis (CF) is the failure of electrogenic chloride ion secretion across the epithelia of many organs, including the lungs, intestine and pancreas.[1] Current treatments are aimed at alleviating some of the symptoms of CF rather than providing a cure and none are able to abolish the cycle of recurrent airway infections which gradually destroy lung function resulting in premature death.

CF is caused by a variety of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-dependent chloride channel located in the apical membrane of epithelial cells.[2] Epithelial cells from CF patients fail to secrete chloride ions (Cl^-) in response to agents which increase intracellular cAMP. Since the problems arising in CF result from mutations in a single gene, the possibility exists that the introduction of a normal copy of the gene into the epithelia could provide a treatment for the disease, or affect a cure if the gene transfer was permanent.

Viral and nonviral approaches have been used to deliver a functional *CFTR* gene to airway epithelia. Neither method is designed to result in stable integration of the DNA into the genome and due to the natural turnover of epithelial cells, repeat applications will be necessary to provide a long-term treatment for CF. The repeated use of adenoviral vectors is limited by the antigenic nature of the adenoviral proteins.[3–8] Experiments in both cotton rats and Rhesus monkeys show that the delivery of adenovirus is associated with an inflammatory response due to expression of adenovirus early and late proteins in transfected cells. Several groups have tried to attenuate this inflammatory response by creating a new generation of adenoviral vectors. These are associated with more stable expression of the transfected gene but do not completely abolish the inflammatory response.[3,8]

Cationic liposomes represent an alternative to adenoviral vectors for the *in vivo* delivery of CFTR cDNA. Liposomes are nonimmunogenic, have no specific cell tropism and can be used to deliver DNA to many cells types including nondividing airway epithelial cells.[9,10] The ability of cationic liposomes to deliver the *CFTR* gene has been evaluated in CF mice.[11–13] These studies showed that after a single delivery of a plasmid containing the CFTR cDNA complexed with cationic liposomes a cAMP-dependent chloride secretion could be detected in tracheal epithelium. Gene transfer has also been detected in the nasal epithelia of CF patients after a single delivery of a CFTR cDNA–liposome complex, but only results in

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transient detection of cAMP-sensitive potential changes indicative of chloride secretion.[14,15] This study used a CF null mouse model (*Cftr*^{tm1Cam}) to assay the safety and efficacy of repeat delivery of a CFTR expression vector complexed with cationic liposomes to the trachea of these mice.

Results

The mice used were aged between 22 and 100 days with a mean age of 47.8 ± 2.0 days. All the groups studied had a mean age close to this value with the exception of the group of untreated CF null −/− mice which were 36.5 ± 3.8 days old. The average weight of the CF null mice was lower than that of age-matched wild-type mice as CF mice thrive less well and are runted compared to their normal littermates.[16] However, the age of the animals is not as important as it was in earlier studies,[16,17] as this study used the protocol developed by MacVinish *et al*[13] designed to separate the calcium-dependent and cAMP-dependent chloride secretory currents. In certain tissues, forskolin has been shown to raise the level of intracellular calcium and cAMP, so part of the forskolin response may reflect calcium-mediated chloride secretion through non-CFTR channels.[18] MacVinish *et al*[13] used the calcium-ATPase inhibitor 2,5-di-tert-butyl-hydroquinone (TBHQ) to mobilise intracellular calcium stores from the endoplasmic reticulum,[19,20] while at the same time allowing calcium influx through the ionophore A23187. This allows maximum stimulation of calcium-mediated chloride secretion so that any further chloride secretion seen on the subsequent addition of forskolin is the result of cAMP-dependent chloride secretion via CFTR.

Basal SCC and the response to amiloride

Figure 1 shows the basal short circuit current measurements (BSCC) and the amiloride-sensitive short circuit currents (ASSCC) for the six experimental groups of mice. Amiloride was added in a concentration which completely blocks electrogenic sodium absorption, which if present will reduce the electrochemical gradient for chloride exit through the apical face of the tracheal epithelium cells. As the extent of sodium absorption varies in different tissues, blocking sodium transport will hyperpolarise the apical membrane to a level where all epithelia experience the same condition for measuring the chloride secretory response. No significant difference in the mean basal short circuit currents was seen between any of the groups. Only one group, receiving two doses of pTRIAL10CFTR2:DC-Chol/DOPE, showed a significant difference in the mean response to amiloride compared with the untreated wild-type and CF null tracheas ($P < 0.05$).

Calcium-dependent chloride secretion

The calcium-ATPase inhibitor TBHQ and the calcium ionophore A23187 cause a steep increase in SCC which usually peaks at approximately 10 min and then declines to a plateau after 20 min (Figure 2). Only the group given two deliveries of pTRIAL10CFTR2:DC-Chol/DOPE showed a significant difference in the mean maximal response to TBHQ/A23187 compared with untreated wild-type and CF null tracheas ($P < 0.01$) (Figure 1). However, the TBHQ/A23187 response of this group did not differ significantly from the group given two mock transfections (Figure 1).

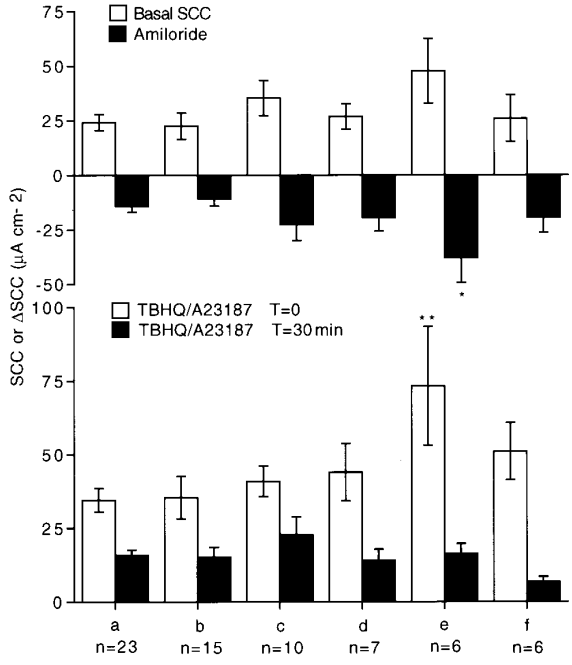

Figure 1 *Basal, amiloride and TBHQ/A23187 short circuit current (SCC) responses in the trachea. Mean basal SCC values and responses to amiloride are shown in the upper panel and the mean responses to TBHQ/A23187, initially and after 30 min, are shown in the lower panel for each of the different groups of mice. The number of measurements for each of the six experimental groups is shown. Only two measurements were significantly different from the values found in wild-type and CF null tracheas, shown by *($P < 0.05$) and **($P < 0.01$) (Mann–Whitney U test). However, neither of the significant values were different when compared with those animals which had received two doses of pTRIAL10 (group f). The error bars represent the standard error of the mean. The six experimental groups are: (a) untreated wild-type; (b) untreated CF null; (c) CF null killed 2 days after transfection with pTRIAL10CFTR2; (d) CF null killed 10 days after transfection with pTRIAL10CFTR2; (e) CF null killed on day 12 after transfection with pTRIAL10CFTR2 on day 0 and day 10; (f) CF null killed on day 12 after transfection with pTRIAL10 on day 0 and day 10.*

cAMP-dependent chloride secretion

Following the addition of amiloride and TBHQ/A23187, the forskolin-sensitive SCC response (FSSCC) can clearly distinguish between wild-type (12.9 ± 1.4 μA cm^{-2}, n = 23) and CF null (−/−) tracheas (4.0 ± 0.9 μA cm^{-2}, n = 15), $P < 0.0001$ (Figure 3). Two separate groups of CF null mice were transfected with a single dose of pTRIAL-10CFTR2:DC-Chol/DOPE. Mice killed 2 days after transfection had a forskolin response of 13.7 ± 2.1 μA cm^{-2} (n = 10). This was significantly greater than the FSSCC of untreated CF null mice ($P < 0.0001$) and not significantly different from the FSSCC of untreated wild-type mice (Figure 3). Mice killed 10 days after transfection had a forskolin response which had returned to 6.0 ± 1.5 μA cm^{-2} (n = 7), significantly less than that of the wild-type tracheas ($P < 0.05$) but not significantly different from that of the CF null tracheas (Figure 3).

A third group of mice was transfected with two doses of pTRIAL10CFTR2:DC-Chol/DOPE, on day 0 and again on day 10, and killed on day 12. The forskolin response of these mice was 16.3 ± 3.4 μA cm^{-2} (n = 6). This was not

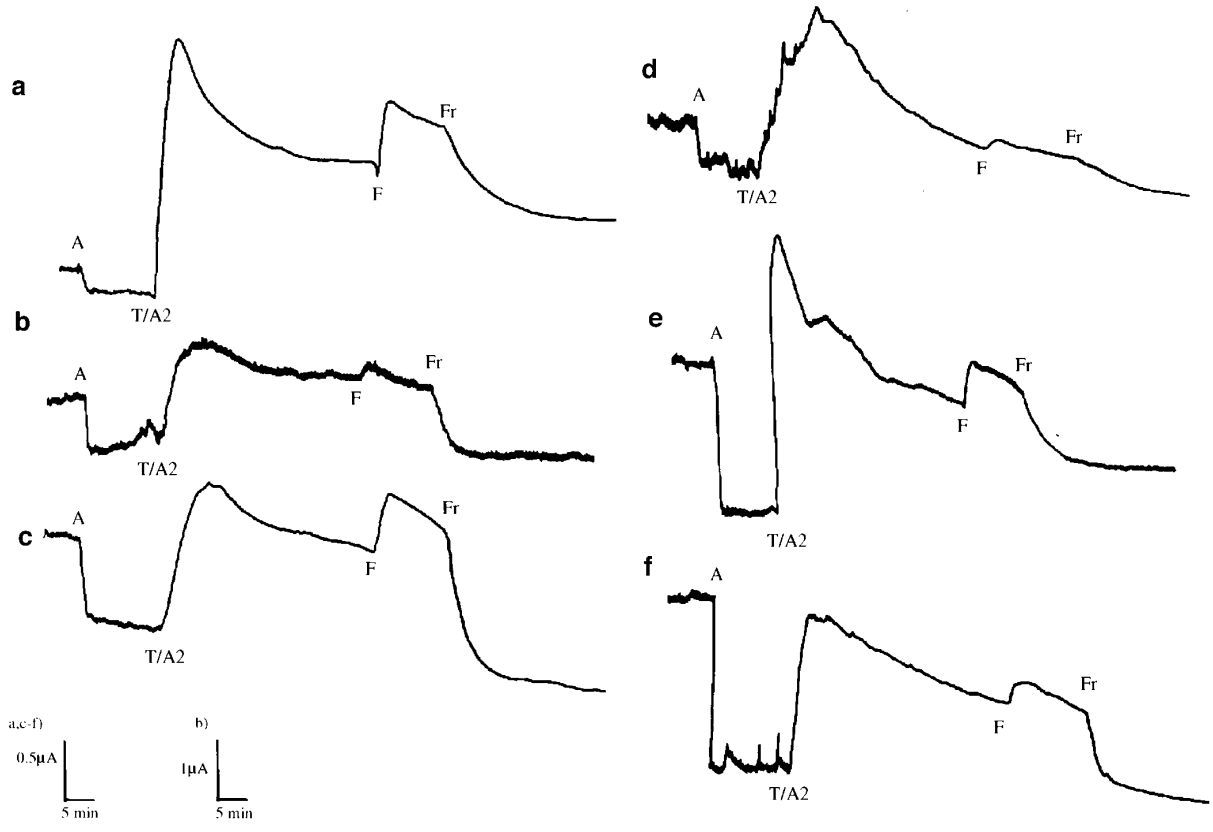

Figure 2 *Short circuit current records from tracheal epithelia. The responses to amiloride (A) (100 μm added apically), TBHQ/A23187 (T/A2) (25 μm and 1 μm respectively added to both sides), forskolin (F) (10 μm added to both sides) and frusemide (Fr) (1 mm added basolaterally). (a–f) As described in the legend to Figure 1.*

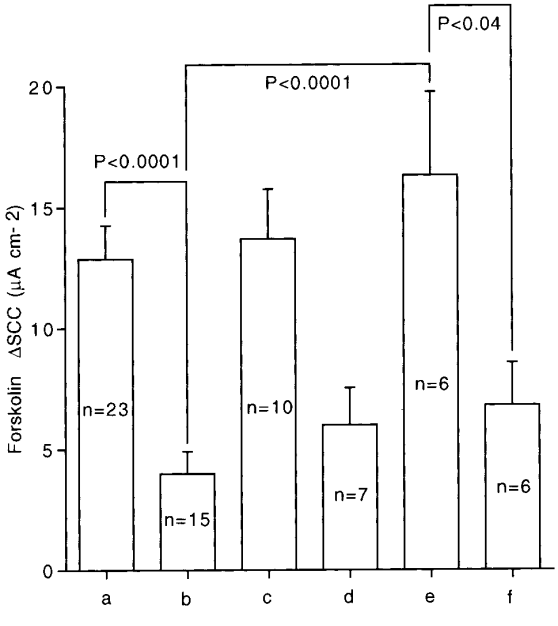

Figure 3 *Responses to forskolin (10 μm) in tracheas after pretreatment with amiloride and TBHQ/A23187. The columns represent the mean forskolin response seen in each of the experimental groups of mice. The error bars represent the standard error of the mean. The number of observations are indicated on each column. Statistical tests were made by using a two-tailed Mann–Whitney U test; the levels of significance for the comparisons made are indicated. (a–f) As described in the legend to Figure 1.*

significantly different from the forskolin response of the group assayed 2 days after a single transfection or that of untreated wild-type mice (Figure 3).

A fourth group of CF null mice were transfected twice with a control plasmid, pTRIAL10, which did not contain the CFTR cDNA. The mean response to forskolin when assayed on day 12 was 6.8 ± 1.8 μA cm^{-2} ($n = 6$), significantly less than that of mice treated with the expression plasmid ($P < 0.05$) and not significantly different from the forskolin response of untreated CF null mice or mice assayed 10 days after a single transfection (Figure 3).

In all instances the FSSCC was sensitive to the addition of frusemide which blocks entry of Cl^- into the cell across the basolateral membrane via the co-transporter (Figure 2).

Histology

Examination of lung sections stained with haematoxylin and eosin did not reveal any inflammatory responses, even after two deliveries of pTRIAL10CFTR2:DC-Chol/DOPE. Lungs taken from treated and untreated mice were indistinguishable and showed no histological differences. Figure 4 shows representative sections of lungs taken from treated (a) and untreated mice (b). Sections of the lung were studied from all transfected mice used in this study and in all sections the bronchi showed a normal pseudostratified columnar epithelium. There was no evidence of peribronchial inflammation and no pus, mucus or foreign material could be detected in the bronchial lumina. The alveolae throughout the lungs

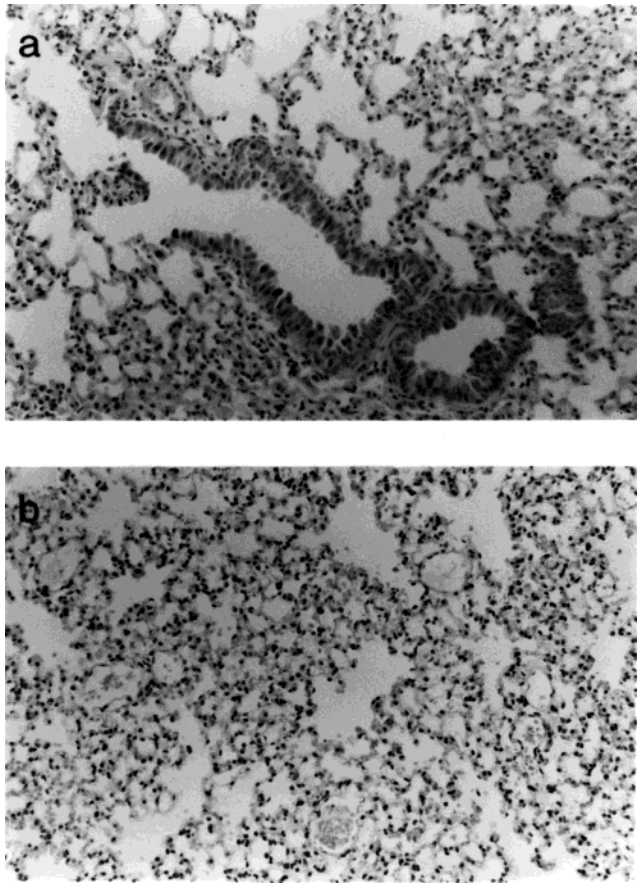

Figure 4 *Histological examination (haematoxylin–eosin staining) of the lungs of mice. (a) lung section from a mouse killed on day 12 after transfection with pTRIAL10CFTR2 complexed with DC-Chol/DOPE on day 0 and day 10. (b) Lung section from an untreated CF null mouse.*

showed a normal inconspicuous flat pneumocyte lining with no inflammatory cells or oedema fluid in the alveolar spaces. Lungs were chosen to assess the inflammatory responses since the transfection mixture will be in longer contact with this tissue than the trachea and it was speculated that greatest damage would be expected at this site.

Discussion

Phase I clinical trials using either adenoviral or liposome vectors to deliver the *CFTR* gene to the airways of CF patients have been completed in both the USA and Europe.[14,15,21–26] These trials have provided data suggesting that gene transfer can be used to introduce a functional copy of *CFTR* into human airway epithelial cells. However, *CFTR* function has only been measurable for up to 4 weeks following gene delivery in these trials. Many factors contribute to the length of gene expression; for example, turnover of epithelial cells, promoter inactivation or vector degradation. Since these plasmids are not capable of autonomous replication, it will be necessary to give CF patients multiple deliveries of *CFTR* cDNA in order to treat their condition, at least until vectors are developed which can be maintained within a transfected cell.

Repeat dose clinical trials using adenovirus vectors have shown that, although the first delivery can correct the CF defect, subsequent deliveries are less able to generate a response especially at high doses of vector.[24] It appears that this is due to induction of acute T cell-mediated inflammatory reactions against adenoviral proteins,[3,4,8] which destroys cells which have been successfully infected by adenovirus leading to reduced expression of CFTR. Second generation adenoviral vectors which express fewer viral proteins give reduced inflammatory responses but still contribute to the formation of neutralising antibodies which block subsequent gene transfer.[27–29] These humoral responses are compounded by the finding that over 96% of CF patients carry at least one antibody subtype against adenovirus.[30] Gene expression can be prolonged by the addition of interleukin-12 (IL-12) or interferon gamma (IFN-γ) at the time of transfection as they diminish the activity of the subgroup of T helper cells involved in the inflammatory response and the production of neutralising antibody.[31] Co-administration of immunosuppressive drugs (cyclosporin) can also be used to reduce the immune response.

To date, clinical trials using a liposome vector to deliver a single dose of *CFTR* to the nasal epithelium have produced no evidence of adverse clinical effects or immunohistological changes in CF patients.[14,15,26] Due to the problems associated with using adenoviral vectors in a multidosing regimen, this study was undertaken to establish whether a second application of a DNA plasmid complexed with liposome would result in a similar level of cAMP-stimulated Cl^- current, after the response to the first application had subsided.

CF null tracheas should not respond to forskolin (cAMP) as no CFTR is present. Forskolin has been shown to increase the concentration of intracellular calcium as well as cAMP in some tissues, and this residual forskolin response could be due to Cl^- secretion through calcium-activated channels,[18] or by another route. The previous addition of TBHQ and A23187 was used to maximise intracellular calcium concentrations eliminating further increases by forskolin which could lead to non-CFTR-dependent Cl^- secretion. Whatever the reason for the residual forskolin sensitive currents in CF mice the data show that tracheas from wild-type mice have a significantly higher FSSCC response than those from CF mice.

It has been demonstrated that sodium absorption (measured as the response to amiloride) is reduced in the trachea of CF null mice.[11,13,17,32] Here the response was smaller in the CF group but was not significantly different to the amiloride response of untreated wild-type tracheas. Reference to Figure 1 shows that the response to amiloride and to TBHQ/A23187 in the group receiving two doses of CFTR plasmid (group e) were significantly larger than those in untreated controls (group a). However, neither result was significantly different when compared with the more correct control, that is the group given two doses of the control plasmid, pTRIAL10 (group f). It is also seen that the TBHQ/A23187 response in group e falls to normal levels at 30 min. Therefore we do not consider the increased response to amiloride in group e is of significance and is a result of the greater variance in this group. However the finding indicates the importance of removing all sodium transport before the ability to activate CFTR-dependent chloride secretion is measured.

Earlier studies have shown that 2 days after the delivery of a single dose of CFTR cDNA complexed with liposomes to the upper airway a cAMP-stimulated Cl^- ion current can be detected in the tracheal epithelium of CF null mice.[11,13] The data here confirm these findings and extends them to show that 10 days after delivery of a single dose the forskolin response (a measure of CFTR-dependent Cl^- secretion) had returned to the level seen in CF null tracheas. When such mice were transfected with a second dose of CFTR on day 10 and killed 2 days later it was found that the forskolin response was restored such that it was not significantly different from that of wild-type mice or CF null mice given a single delivery. In the future it will be important to show, by adjusting the frequency of the administration, that the gene transfer can permanently sustain the forskolin response within the wild-type range.

Examination of lung sections after a single or double delivery of pTRIAL10CFTR2:DC-Chol/DOPE revealed no indication of neutrophil infiltration or signs of cellular damage. The alveolar pneumocyte lining is very thin and even minor injury to the lung would be expected to result in damage to this barrier and permit fluid to enter the alveolae. More serious injury can result in proteinous exudate entry and changes in the lining cells such as swelling and proliferation of type 2 pneumocytes. In the bronchi, inhalation of irritants leads to increased mucus production and inflammatory changes which are then followed by conversion of the columnar epithelium to a squamous epithelium. The absence of any of the above signs of damage argue in favour of no damage to the lungs of the transfected mice. This evidence for the lack of toxicity of DC-Chol/DOPE is encouraging for its use as a delivery vehicle in repeat dosing strategies.

In summary, we show that a second delivery of pTRIAL10CFTR2 complexed with DC-Chol/DOPE is as effective as the initial dose in restoring a functional cAMP-stimulated chloride channel to the tracheal epithelium and that no airway inflammation is associated with this repeat dose. The plasmid used in this study is identical to that used in a recent phase I clinical trial in which six of the eight patients receiving the plasmid showed evidence of functional change.[15] Based on the results of this clinical trial, and the data obtained in these murine experiments, permission has been granted for a second phase I clinical trial involving multiple dose delivery of pTRIAL10CFTR2 complexed with DC-Chol/DOPE to the nasal epithelium of CF patients.

Materials and methods

Delivery of DNA

Mice anaesthetised with avertin (0.5 g of 2,2,2-tribromoethanol dissolved in 0.63 ml of tertiary amyl alcohol and then diluted 1:50 in PBS) at a dose of 25 μl/g body weight were transfected with 10 μg of plasmid DNA complexed with 100 nmoles of DC-Chol/DOPE in a final volume of 100 μl (Kreb's HEPES buffer pH 9.0 was used as the diluent) via tracheal instillation.[13] The ratio of plasmid: liposome (1:6 w/w) and the preparation of materials is equivalent to that used in a single-dose clinical study and for a proposed multiple-dose clinical trial.[15] The charge ratio of the transfection complex is 1.98.[33] Two plasmids were used, pTRIAL10CFTR2 which contains the human *CFTR* cDNA under the control of the Rous sarcoma virus 3′ long terminal repeat (RSV 3′LTR) promoter and pTRIAL10 which lacks the *CFTR* cDNA as a negative control.[13]

Several groups of mice were studied in this experiment.

Controls: (a) Untreated wild-type mice (n = 23). Weight, 22.9 ± 0.9 g; age, 53.0 ± 4.0 days (note: this cohort combined data from homozygote +/+ (n = 9) and heterozygote +/− (n = 14) mice as the SCC responses of either group are indistinguishable).[16] (b) Untreated CF null mice (−/−) (n = 15); weight, 13.4 ± 1.7; age, 36.5 ± 3.8.

Transfected mice: (c) CF null mice transfected with pTRIAL10CFTR2 on day 0 and killed on day 2 (n = 10); weight, 17.7 ± 1.1 g; age, 48.1 ± 4.8 days. (d) CF null mice transfected with pTRIAL10CFTR2 on day 0 and killed on day 10 (n = 7); weight, 15.9 ± 2.8 g; age, 46.4 ± 3.9 days. (e) CF null mice transfected with pTRIAL10CFTR2 on day 0 and day 10 and killed on day 12 (n = 6); weight, 19.4 ± 1.5 g; age, 50.7 ± 3.5 days. (f) CF null mice transfected with pTRIAL10 on day 0 and day 10 and killed on day 12 (n = 6); weight, 17.5 ± 1.4; age, 53.0 ± 2.6 days.

Measurement of ion transporting properties of the tracheal epithelium

Gene transfer and *CFTR* expression were assessed *ex vivo* by measuring short circuit current (SCC) in tracheas, voltage clamped at zero potential, following the protocol of MacVinish *et al*.[13]

Statistical analysis

To compare the SCC responses of wild-type, CF null and transfected tissues a nonparametric Mann–Whitney U test with two tails was used, $P < 0.05$ being considered significant.

Toxicity

To assess potential toxicity, the lungs from treated and untreated animals were fixed in 10% buffered formol-saline, processed and embedded in paraffin wax. Seven micron sections were cut along the sagittal plane so that all lobes could be examined and stained with haematoxylin and eosin.

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