Restoration by intratracheal gene transfer of bicarbonate secretion in cystic fibrosis mouse gallbladder

C. M. CURTIS, L. C. MARTIN, C. F. HIGGINS, W. H. COLLEDGE, M. E. HICKMAN, M. J. EVANS, L. J. MACVINISH, AND A. W. CUTHBERT. Restoration by intratracheal gene transfer of bicarbonate secretion in cystic fibrosis mouse gallbladder. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G1053–G1060, 1998.—Gallbladders from cystic fibrosis (CF) mice (Cftrtm1Cam and Cftrtm2Cam) were examined with the short-circuit current technique. The tissues failed to show any electrogenic anion transport in response to forskolin (cAMP stimulus) but responded to the Ca2+-ionophore ionomycin. Administration of the plasmid pTrial10-CFTR2 complexed with cationic liposomes (3:8-[N-(dimethylaminoethane)-carbamoyl]cholesterol and L-α-phosphatidylethanolamine dioleoyl) to the airways restored the phenotype of CF gallbladders to that of the wild type, but did not do so when given orally. Formation of human CFTR mRNA in gallbladders of transfected CF null mice was demonstrated. Using the reporter genes pCMV-luc and pCMV-LacZ, we showed that 1) the intratracheal route was more effective than the oral, intravenous, intramuscular, subcutaneous, or intraperitoneal routes in expressing luciferase activity in the gallbladder and 2) β-galactosidase staining after pCMV-LacZ was confined to the columnar epithelium lining the gallbladder without any discernible activity in its smooth muscle. The discovery of an unusual route for gene transfer to the biliary system may give useful insight into counteracting the consequences of biliary fibrosis in human CF patients.

Cystic fibrosis transmembrane conductance regulator; luciferase; β-galactosidase

The mouse gallbladder secretes HCO₃⁻ predominantly through a conductive anion pathway in response to a cAMP stimulus (14). To determine if the conductive anion channel was the cystic fibrosis transmembrane conductance regulator (CFTR), we used transgenic animals in which functional CFTR is not present to examine how this affects secretion. We used both cystic fibrosis (CF) null mice (Cftrtm1Cam), in which no CFTR is produced, and ΔF508 mice (Cftrtm2Cam), which produce a mutant protein that is not incorporated in the plasma membrane (3, 16). Using forskolin, we found that murine CF gallbladders show virtually no response to a cAMP stimulus. In humans, both the mRNA for CFTR and the protein itself are found in the intra- and extrahepatic biliary epithelium, including the gallbladder, where the protein is located at the apical face of the cells (2, 8). In CF there are primary abnormalities in hepatobiliary anion transport. Seventy percent of CF adults have focal biliary fibrosis, which may lead to multilobular biliary cirrhosis and portal hypertension (4). After a chance observation made using gallbladders from CF mice, we showed that the murine gallbladder readily takes up genetic material expressed in the columnar epithelial cells. When a plasmid-liposome complex (lipoplex) containing the cDNA for human CFTR is instilled intratracheally in CF animals, a phenotypic reversion occurs in the gallbladder and ion-transporting activity is restored. The demonstration of gene transfer in the mouse gallbladder in vivo may provide useful insights for humans.

MATERIALS AND METHODS

Animals

Wild-type mice as well as CF null (Cftrtm1Cam) (16) and ΔF508 CF mice (Cftrtm2Cam) (3), aged between 1 and 3 mo, were used in the study. For tracheal or oral transfection, 10 µg of pCMV-Luc and pCMV-LacZ, 5 µg of pCMV-Luc and pGL-3luc. For tracheal transfection, 10 µg of pCMV-Luc and pGL-3luc.

Preparation of Lipoplexes

pTrial 10-CFTR2. For tracheal transfection, 10 µg of plasmid (pTrial10-CFTR2) containing the cDNA for human CFTR (12) were added to 25 µl of Krebs-HEPES buffer (KHB; pH 9) to which was added 100 nmol of 3:8-[N-(dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) and 6:2 phosphatidylethanolamine dioleoyl (DOPE) (3:2 molar ratio) liposomes mixed with 15 µl of KHB (pH 9). The plasmid and liposomes were mixed to give a total volume of 100 µl, and complexes were allowed to form at 37°C for 10 min in a polystyrene tube. Control plasmid pTrial 10 was prepared in the same way for transfection.

pCMV-luc and pGL-3-luc. For tracheal or oral transfection 10 µg of pCMV-luc plasmid mixed with 25 µl KHB (pH 9) were added to 100 nmol of DC-Chol and DOPE liposomes in 15 µl KHB. As described above, the complex was allowed to form at 37°C for 10 min in a total volume of 100 µl. Likewise, for other routes 133 µg of pCMV-luc plasmid were added to 560 nmol of
DC-Chol and DOPE in KHB and the complex was allowed to form. pGL-3-luc was used in the initial experiment in which nasal and oral routes were compared. pGL-3-luc (5 µg DNA) was mixed with liposomes in the same proportions as for pCMV-luc.

pCMV-LacZ. We mixed 133 µg of plasmid (pCMV-LacZ) with 560 nmol DC-Chol and DOPE liposomes with 5% glucose and allowed the complex to form at 37°C for 10 min in a polystyrene tube.

Administration of Lipoplexes to Mice

Mice were anesthetized with bromothiol (0.02 ml/g body wt), and the transfection mixture was instilled into the trachea, 20 µl at a time (for pTrial10-CFTR2 and pCMV-luc, 10 µg DNA for each) until the whole volume (100 µl) had been delivered. For nasal transfection, one-half of the tracheal dose (pTrial10-CFTR2, 5 µg) was given, divided between the two nostrils. Anesthetized mice were placed on their backs, and 2 µl transfection mixture were placed in turn over each nostril until all the mixture was given. For oral transfection, 5 or 10 µg DNA (as pTrial10-CFTR or pCMV-luc lipoplexes) were given directly into the stomach in anesthetized mice. We gave 133 µg DNA (as pCMV-luc lipoplex) by other routes in anesthetized mice as follows: intramuscularly into the hind-limb, into the peritoneal cavity, subcutaneously under the anesthetized mice as follows: intramuscularly into the hind-limb, into the peritoneal cavity, subcutaneously under the skin of the back, and intravenously. In the latter case, the lipoplex was administered over a 15-min period via the tail vein, using a pediatric cannula. pCMV-LacZ lipoplex was also given by the intravenous route as for pCMV-luc. With all routes, the mice were allowed to recover for 2 days before use.

Demonstration of Gene Transfer to the Gallbladder

Along with demonstrating function in transfected gallbladders, we investigated the formation of mRNA for CFTR. Furthermore, reporter genes were used to explore the location and extent of protein formation when different routes for transfection were used. Experiments with reporter genes (for β-galactosidase and luciferase) were performed with wild-type mice to conserve the supply of CF mice. After mice were euthanized, the gallbladders were removed as quickly as possible and processed as detailed below.

Detection of mRNA by RT-PCR. Total mRNA was extracted from a single gallbladder (3–5 mg) using the acid guanidinium thiocyanate method (1). cDNA was produced by reverse transcription from ~5 µg of total RNA with Moloney murine leukemia virus RT ( Gibco-BRL) using a pd(N)6 primer. PCR of the cDNA templates was performed using a pair of specific primers for human CFTR that amplified a fragment of 500 bp containing exons 7–10. The primers were X7H, 5′-ACAAAA-CATGGTAGCTCTTTGAGG-3′, and X10H, 5′-GGGTGCATGCTTTGTGAGCCGTCT-3′. The pTrial10-CFTR2 plasmid served as a positive control for the human primers. The PCR reactions were carried out in 20 µl of PCR buffer containing dATP, dTTP, dCTP, and dGTP at a concentration of 1 mM, 10 pmol of each primer, 1 U Taq polymerase (Advanced Technologies), and 5 µl of cDNA. The reaction mixtures were heated to 95°C for 5 min in a Techma-PCR machine and were then subjected to 40 cycles of denaturation (94°C for 30 s), primer annealing (60°C for 30 s), and extension (72°C for 45 s). PCR products were examined by electrophoresis on a 1% agarose gel stained with ethidium bromide. To ensure that the human CFTR signal from transfected gallbladders subjected to RT-PCR was not a result of amplifying residual plasmid pTrial10-CFTR2 cDNA, the extracted material was subjected to DNase treatment as described below.

Gallbladder total RNA (~5 µg) was treated with 40 U of RNase-free DNase (Promega) in RT buffer in the presence of 10 U RNase inhibitor (Promega) for 1 h at 37°C. Afterward, 0.1 vol of ice-cold 3 M sodium acetate and 2 vol of ice-cold ethanol were added, mixed, and left for 1 h at ~20°C, after which the mixture was spun at 20,000 g for 30 min. The supernatant was removed, and the RNA pellet was resuspended in diethyl pyrocarbonate water containing 20 U RNase inhibitor. The optical density at 260 nm (OD260nm) was measured, and 200 ng of RNA were taken and subjected to 40 cycles of PCR using human specific CFTR primers for exons 7–10, in the absence of a preceding RT reaction. The result was examined for the presence of plasmid amplified CFTR. In the absence of any signal the DNase treatment was deemed to have been efficient and the remaining RNA was reverse transcribed as described above.

Luciferase formation after transfection by different routes. To measure luciferase activity, the gallbladders were snap frozen on dry ice immediately after removal from the mouse. Each gallbladder was separately homogenized in 75 µl of reporter lysis buffer (Promega), using a Polytron homogenizer, and vortexed for 15 s. The homogenates were centrifuged at 4°C to give a clear supernatant, the latter being stored at ~70°C until assayed. To assay, 20 µl of each extract were warmed to room temperature and 100 µl of luciferase assay reagent (Promega) were added and gently mixed. Light emission was measured in a Turner luminometer (model 20) using a 3-s delay and 30-s integration time. The protein content of the extracts was measured at OD595nm, using a Bio-Rad protein assay system.

β-Galactosidase staining. Gallbladders were washed in PBS (0.1 M, pH 7.4) and fixed in 4% formaldehyde for 30 min at room temperature. After further washing in PBS, the gallbladders were stained overnight at 30°C in a solution of 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside and potassium ferri- and ferrocyanide. Gallbladders were then paraffin embedded, and 6-µm sections were cut and counterstained.

Statistical Treatment of Results

Student’s t-test was used to test for the significance of observed differences, with P < 0.05 considered significant. When F values indicated that the standard deviations of the two populations being compared were significantly different, a nonparametric test (Mann Whitney U-test) was used to compare means.

RESULTS

Ion Transport in Gallbladders from CF Mice

Gallbladders from CF mice, both null and those with the ΔF508 mutation, were examined in the same way as described previously for wild-type animals (14). Essentially, they showed little or no response to forskolin and hence no response to either furosemide or acetazolamide (Fig. 1, C and D). However, it could be shown that the tissues were viable, as they responded to the Ca2+ secretagogue ionomycin. Figure 2 shows a CF gallbladder that, although showing no response to forskolin, did respond to ionomycin. In comparison, wild-type gallbladders responded both to forskolin and to ionomycin. In both types of tissue there was an initial peak followed by a plateau of maintained current. CF gallbladders, similar to those from wild-type
animals, failed to respond to ionomycin in ~30% of preparations. Because of the lack of availability of CF mice it was not possible, as with wild-type animals, to euthanize mice solely to examine the responses to ionomycin. Consequently, gallbladders were recovered at the time of euthanasia from animals that had been used for other procedures. There is no reason to believe the behavior of the gallbladders was affected by this necessary economy. The responses to ionomycin were 25.4 ± 13.4 µA/cm² in CF null gallbladders (n = 9) and 12.3 ± 3.5 µA/cm² in ΔF508 CF gallbladders (n = 11). Neither of these values was significantly different from that found for wild-type gallbladders (14.8 ± 3.9 µA/cm²; n = 10).

In all experiments in which CF mice were used, a single colonic epithelial preparation was used as a check on the genotyping of the animals. In the murine CF colon, a small K⁺ secretory response to forskolin is revealed, which is reversed by furosemide (6). Reference has already been made to the economy required in the use of CF mice, a strategy that on one occasion led to unexpected serendipity. A gallbladder taken from a CF null animal was found to have a perfectly normal wild-type phenotype, whereas the colonic epithelium from the same animal had a characteristic CF profile (Fig. 3A). On this occasion, the mouse had been transfected with pTrial10-CFTR2 complexed with cationic liposomes by intratracheal instillation 2 days earlier. The transfection was carried out as part of another study (12). In all, four similarly treated animals were examined in the same way, and the pooled results are depicted in Fig. 1B, where a phenotypic fingerprint remarkably similar to wild-type animals is revealed. Three other CF mice were transfected by the nasal route, and the gallbladders of two of the three animals showed a wild-type phenotype, one of which is shown (Fig. 3B). Finally, three CF mice were transfected with pTrial10-CFTR2 lipoplex (10 µg DNA) given by the intragastric route. No indication was found that the phenotype of the gallbladders was other than expected from the genotype in these three, one of which is depicted in Fig. 3C. Finally, two CF null mice receiving pTrial10 (i.e., plasmid without CFTR cDNA) by the tracheal route yielded gallbladders with a CF phenotype.

Demonstration of Human CFTR mRNA in Transfected Murine Gallbladders

Total RNA from one gallbladder transfected via the trachea 2 days previously was subjected to RT-PCR as described. A band corresponding with the positive human CFTR control was found in the transfected sample (Fig. 4, left). Although it seemed unlikely that plasmid DNA could be present in the gallbladder 2 days after transfection with pTrial10-CFTR2 in the airways, a further experiment was devised to test for this possibility. A fresh gallbladder from a null mouse, transfected 2 days before, was extracted and treated with DNase as described above, before being subjected to PCR. The absence of a signal corresponding to the positive control indicated that either no plasmid DNA was present or that the DNase treatment had been effective. The remaining RNA was then subjected to RT-PCR and gave a product corresponding to the positive control, with no signal when either the RT step was omitted or no cDNA was added. To confirm the integrity of the mRNA, the same sample was subjected to RT-PCR, using primers for the ubiquitous proliferat-
ing cell nuclear antigen (PCNA), which gives a 720-bp fragment encompassing exons 1–4 of mouse PCNA.

Luciferase Expression in Gallbladders Transfected with pCMV-luc or pGL-3-luc

To further investigate the route from the lungs to the gallbladder, we investigated whether the gallbladder could be transfected by the oral route using pGL-3-luc. The same amount of plasmid was given by the nasal and oral routes, the latter being equivalent to the whole of the airway dose being swallowed. There was no significant difference between the background signal from animals receiving liposomes only and the light emitted by gallbladder extracts from orally transfected animals, whereas the intranasal route was effective in stimulating luciferase activity in the gallbladder (Fig. 5A). In a more elaborate experiment, groups of wild-type mice were transfected with pCMV-luc lipoplex by five different routes, namely tracheal, intravenous, intramuscular, intraperitoneal, and subcutaneous, as described in MATERIALS AND METHODS. Control animals were given liposomes without plasmid. Two days later the mice were killed, the gallbladders were removed, and tissue extracts were prepared for assay by light emission. The results from this experiment are shown in Fig. 5B. Light emission from tissue extracts from animals given the plasmid via the intramuscular, intraperitoneal, or subcutaneous routes showed no increase over background values, whereas values from the intravenous recipients were over five times that of the background. However, even these values were small compared with the values that were obtained when the plasmid was delivered intratracheally, which were 100 times greater than the background value.

Histological Demonstration of Gene Transfer to the Gallbladder

To further explore gene transfer to the gallbladder, the plasmid pCMV-LacZ (10 µg), complexed with cationic liposomes, was given intratracheally, but no staining for β-galactosidase was seen in the gallbladder. However, gross visible staining is one of the least sensitive methods for detecting successful transfection and protein formation. It was not possible to deliver a much greater dose by the intratracheal route, but when a larger dose of pCMV-LacZ (133 µg), again complexed with cationic liposomes, was given intravenously via the tail vein, the gallbladder and even more so the bile ducts were deeply stained when exposed to substrate for β-galactosidase. The sections of stained gallbladder shown in Fig. 6 depict the following features: staining is uniform and confined to the columnar epithelium of the gallbladder, without staining of the underlying connective tissue or smooth muscle cells (Fig. 6A); the pres-
ence of β-galactosidase in the epithelium is apparent even when counterstaining with hematoxylin and eosin is used (Fig. 6B); and no staining is apparent when liposomes alone are delivered (Fig. 6, C and D).

DISCUSSION

Gallbladders from CF mice, either in CF null (16) (producing no CFTR) or homozygous ΔF508 mice (3) (producing a nonfunctional mutant form of CFTR), are incapable of generating an electrogenic anion secretory response to forskolin, in contrast to wild-type gallbladders (Fig. 1). This finding, together with those by Martin et al. (14), indicates that CFTR is essential for electrogenic HCO₃⁻ secretion in the murine gallbladder. Very similar conclusions were made for HCO₃⁻ secretion in normal and CF human airway epithelia (17). Here HCO₃⁻ secretion via an apical conductive pathway was demonstrated in response to cAMP, which was absent in CF epithelia.

Both types of CF gallbladders showed Iₑ increases that were often transient and followed by an elevated plateau to the Ca²⁺ ionophore ionomycin, a finding again consistent with the study on CF airway epithelia by Smith and Welsh (17). The normal mouse gallbladder also shows responses to the Ca²⁺ ionophore ionomycin, but we have not been able to demonstrate statistically clear differences between wild-type and CF tissues. However, similar to the human gallbladder (8), the mouse epithelium is sensitive to both cAMP and Ca²⁺ signals.

In summary, the anion secretory current in the gallbladder is CFTR dependent, consistent with earlier studies (15, 18), in which Cl⁻ was identified as the transported species. In these earlier studies (15, 18), reliance was placed on the failure to elicit a secretory response in normal gallbladders when all Cl⁻ was removed from both bathing solutions. It is not extraordinary to suggest that CFTR can allow ions other than Cl⁻ to permeate, as there is ample evidence from patch-clamp studies that CFTR channels are permeable to HCO₃⁻. For example, in the pancreatic duct the permeability ratio of HCO₃⁻ to Cl⁻ is 0.13 (9), whereas in the cortical collecting duct the ratio reaches 0.67 (11).

The limited availability of CF mice encouraged us to examine gallbladders from mice that as part of another study had been transfected intratracheally or nasally with pTrial10-CFTR2 complexed with cationic liposomes. Mice are genotyped by extracting the DNA from tail clips before experiments, but we always examine the colonic epithelium to confirm this. The expectation was that the gallbladders of transfected mice would behave as tissues with the CF phenotype, yet a normal phenotype was observed, with responses to forskolin...
and acetazolamide. Meanwhile, the colonic epithelia were unchanged, presenting the classic CF phenotype (6). No responses to forskolin were seen in gallbladders from mice transfected intragastrically. Although this latter result does not preclude the possibility that the oral route may occasionally be successful, it seems unlikely that the reversal after airway transfection was caused by the mice swallowing plasmid introduced into the airways. While functional studies alone provided a powerful piece of evidence for the pulmonary route for successful transfection of the biliary tract, the demonstration of the presence of human CFTR mRNA in gallbladder tissue added a vital confirmation.

Using wild-type mice and the pCMV-luc lipoplex, we further explored the transfer of genetic information to the gallbladder. Again, as with pTrial10-CFTR2, it was necessary for the plasmid to be both transcribed and translated to give luciferase expression and hence light

Fig. 4. Expression of human CFTR mRNA in CF null gallbladders after tracheal transfection with pTrial10-CFTR2 lipoplex. Left: mRNA from a single gallbladder was subjected to RT-PCR, amplifying specific gene fragments of exons 7–10 using human CFTR primers X7H and X10H. Lane 1, human CFTR signal (500 bp) from transfected gallbladder. Lane 2, positive control generated with plasmid DNA. Lane 3, negative control where no cDNA was present in the reaction mix. Right: a similar experiment using a single, tracheally transfected CF null gallbladder. mRNA was subjected to DNase treatment before RT-PCR. Lane 2, signal from the gallbladder. Lane 4, positive control with plasmid DNA (500 bp). Lane 1, no RT negative control, confirming the absence of plasmid DNA. Lane 3, negative control for PCR reaction, as no cDNA was present. Expression of proliferating cell nuclear antigen (PCNA) mRNA (720 bp) from the same gallbladder was achieved using primers to amplify gene fragments of exons 1–4 (lane 5) to control for mRNA integrity. Signal for PCNA mRNA from a wild-type gallbladder is shown in lane 6. For both gels, 1-kb ladders (GIBCO-BRL) are shown. The oligonucleotide primers for mouse PCNA were 5’-GTTGTTAGT-TGTCGCTGTA (sense) and 5’-CAGGCTCATTCATCTCTATCG (antisense).

Fig. 5. Luciferase activity in wild-type mouse gallbladders transfected via the airways 2 days previously with pGL-3-luc or pCMV-luc lipoplex. A: mice were transfected with pGL-3-luc (5 µg DNA) via the nasal (N) or oral (O) route or with liposomes alone by the nasal route (C). Means ± SE are shown where appropriate. B: means ± SE are given for gallbladder extracts from mice receiving pCMV-luc intratracheally (10 µg DNA) or by other routes (133 µg DNA). Control animals were given liposomes alone by the intratracheal route (C). IT, intratracheal; IV, intravenous; IM, intramuscular; IP, intraperitoneal, SC, subcutaneous. P determined with Mann Whitney U-test.
emission. Strikingly, only the intratracheal route gave good transfection of the gallbladder, although there was an increase in activity after intravenous infusion, while other routes were ineffective. Although transfection of the gallbladder by the intratracheal route was significantly better than with the other routes, there was considerable variability between animals, as evidenced by the large standard error (Fig. 5). This seems to indicate that there are many factors determining gene transfer, which are, at present, unknown and uncontrolled. It was disappointing not to be able to demonstrate the transfer of the pCMV-LacZ lipoplex to the gallbladder via the intratracheal route. It has been estimated from staining and immunologic studies that reliance on the appearance of a blue stain is 43 times less sensitive than detecting the presence of β-galactosidase using immunostaining (5). Thus the demonstration of β-galactosidase staining is a rather insensitive method, while phenotypic correction measured by bioassay requires only low level expression. For instance, CF “knockout” mice expressing only 10% of the normal CFTR have higher survival rates compared with CF null mice and show minor functional responses (7). By increasing the dose of pCMV-LacZ plasmid some 10-fold, staining was demonstrated after delivery by the intravenous route and confined to the gallbladder epithelium. No staining of the underlying smooth muscle was discernible, although it cannot be concluded that it was completely absent.

Others have shown (10) that it is possible to transfect human CF intrahepatic biliary epithelial cell lines with adenoviral vectors and restore cAMP-dependent halide efflux. Furthermore, biliary epithelial cells in the rat can also be transfected by retrograde infusion of adenoviral vectors into the common bile duct (19). The latter approach involves an invasive procedure and a more efficient gene delivery system. Transfection with liposomes, either in vivo or in vitro, is generally considered to be an inefficient method for gene transfer even though, for human gene therapy, it is less likely to result in immunologic consequences on repeated application (13). The serendipitous finding that the gallbladder is relatively easily transfected by material given via the airways is not only an important finding in relation to gene therapy for CF, but when the mechanism is unraveled it may have important lessons for improving the efficiency of transfection in other situations.

In summary, it is found that the cAMP-dependent anion secretory current, due primarily to the secretion of HCO₃⁻, is absent in murine CF gallbladders. It is concluded that CFTR is essential for the HCO₃⁻ secretory current and that gene transfer via an airway transfection route can restore the secretory activity in CF gallbladders.

This work was supported by a Sir Henry Wellcome Commemorative Award for Innovative Research to A. W. Cuthbert. Supplies of mice, plasmids, and other materials were supported by grants from the Medical Research Council and the Cystic Fibrosis Trust.
REFERENCES


