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Mucociliary clearance in cystic fibrosis knockout mice infected with *Pseudomonas aeruginosa*

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Mucociliary clearance in cystic fibrosis knockout mice infected with Pseudomonas aeruginosa. E.A. Cowley, C-G. Wang, D. Gosselin, D. Radzioch, D.H. Eidelman. ©ERS Journals Ltd 1997.

ABSTRACT: In this study, we examined whether mucociliary clearance differed between cystic fibrosis (CF) knockout mice and wildtype controls. Additionally, we investigated whether infection with *Pseudomonas aeruginosa*, a common pathogen in the CF lung, affected this important host defence mechanism.

Ciliary beat frequency (f_{cb}) and particle transport (PT) were recorded using an *in vitro* lung explant preparation. Measurements were made from uninfected cystic fibrosis transmembrane conductance regulator (CFTR) knockout (-/-) mice and littermate controls (+/+) and compared to measurements from infected animals.

While there were no differences detectable in f_{cb} between CFTR -/- mice and their +/+ controls either in the presence or absence of *P. aeruginosa*, PT rates were different between these groups; interestingly, PT rates appeared dependent on both CFTR and infection status, with uninfected CFTR +/+ animals demonstrating higher rates of PT than their -/- littermates, while CFTR +/+ *P. aeruginosa*-infected mice demonstrated lower PT than knockout mice.

These data demonstrate differences in mucociliary clearance between cystic fibrosis transmembrane conductance regulator knockout mice and controls, and further that *Pseudomonas aeruginosa* infection affects mucociliary clearance in the peripheral airways of mice. Additionally, the observed differences in particle transport suggest that cystic fibrosis transmembrane conductance regulator knockout mice demonstrate different mucociliary responses to infection.

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Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic adenosine monophosphate (cAMP)-dependent chloride channel present on the apical surface of epithelial cells [1], including those in the airways [2]. Chronic bacterial infection is a characteristic feature of CF lung disease [3, 4]. Although the exact sequence of events that produces the tissue damage seen in CF remains unclear, it appears that initial infection of the lower respiratory tract by bacterial organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* results in an inflammatory response and the subsequent release of tissue-damaging oxidants and enzymes from recruited inflammatory cells [5, 6]. In normal lungs, such bacteria and particulate matter are cleared by mucociliary transport, an important component of primary host defence in the airways. This process moves foreign objects entrapped in mucus up the respiratory tract *via* the beating of cilia, and is dependent on ciliary function and both the quantity and quality of the airway surface fluid in which the cilia are bathed. In the CF lung, however, mucociliary clearance is severely impaired [7]. Absence of the CFTR chloride channel results in abnormal transepithelial salt and water transport [8], possibly leading to dehydration of mucus and a reduction in mucociliary clearance. As CF lung disease progresses and mucus hypersecretion and mucoid impaction become more prominent, the thick viscid mucus present in the airways further impairs clearance [9], while the rheological properties of mucus are also altered [10], further compromising mucociliary clearance. Although the end stages of CF lung disease are well characterized, the factors that initiate CF lung disease are much less well understood, and are difficult to study in humans. Ideally, therefore, we require a model to investigate the pathogenesis of early CF lung disease.

Since the genetic basis for CF was elucidated [11], several murine models of CF have been developed, based on either inactivation or disruption of the CFTR gene [12–18]. However, none of these mouse models has demonstrated the severe lung pathology seen in the human disease. Interestingly, there has been one report [19] that CFTR -/- mice demonstrate a reduced capacity to clear *S. aureus* and *Burkholderia cepacia*, resulting in an increase in lung pathology when compared to CFTR +/+ animals. This strongly suggests that at least one CFTR -/- mouse model demonstrates an important difference in its host defence mechanisms when challenged with bacteria.

In the present study, we wished to determine if mucociliary clearance might be altered in CF knockout mice. We reasoned that although limited evidence of lung disease has been found in CF animal models, more subtle changes

in physiology might be present, and could serve as surrogate markers of CF. Furthermore, there has been one report of decreased mucociliary clearance in mice that have a disrupted CFTR gene [20]. Additionally, we examined the effects of infection with *P. aeruginosa* upon mucociliary clearance, since this is the most common microorganism found in the airways of CF patients [21], and we wished to investigate whether CFTR -/- mice differ in their mucociliary clearance response to this pathogen. To investigate these questions, we used a technique that we have previously described in which ciliary beat frequency (f_{cb}) and particle transport (PT) rates can be measured in the intraparenchymal airways of mice [22].

Materials and methods

Mice

CFTR knockout mice, initially developed at the University of North Carolina [23], were established on a C57BL/6 background, previously shown to be susceptible to *P. aeruginosa* [24]. Heterozygous CFTR knockout (+/-) mice were backcrossed for several generations with C57BL/6 mice, and at each generation mice with the highest level of homozygocity for C57BL/6 were selected by single sequence length polymorphism analysis and backcrossed to C57BL/6.

All mice were 6–12 weeks old (weight 22.96±3.8g) and were divided into CFTR -/- or CFTR +/+ animals. Mice from both groups were then infected with *P. aeruginosa* beads and compared to uninfected animals. Additionally, there was a vehicle control group in which a sterile bead suspension was introduced to the mice.

Preparation of beads

A log-phase of *P. aeruginosa* 508 (a clinical mucoid isolate provided by J. Lagacé, University of Montreal, Canada) diluted in trypticase soy agar (TSA) at 52°C was added to heavy mineral oil (Fisher Scientific, Ottawa, Canada) and stirred vigorously for 6 min at 20°C. After cooling of this mixture with ice for 10 min, beads formed which contained the bacteria, and which were less than 200 μm in diameter. These beads were isolated by centrifugation at 9000×g for 20 min at 4°C, and suspended in phosphate-buffered saline (PBS). The density of viable *P. aeruginosa* within the beads was determined by plating serial dilutions of homogenized bead suspension onto plates containing TSA medium. Immediately prior to infection, the bacteria-bead suspension was diluted to a density of 2–10 $\times 10^5$ colony forming units (CFUs). A control (uninfected) bead suspension was also prepared using TSA diluted with PBS.

Infection protocol

Mice were anaesthetized with ketamine hydrochloride (75 mg·kg^{-1}) and xylazine (30 mg·kg^{-1}) intramuscularly. The trachea was exposed and a 22-gauge *i.v.* catheter (Criticon, Tampa, FL, USA) inserted, through which 50 μL of the bead suspension followed by 50 μL of air was inoculated. The incision was sutured after the inoculation. Animals did not develop wound infections, and healing occurred within 2–3 days.

Histological evaluation

To determine the location of the bacteria-containing beads within the lungs, mice were killed by cervical dislocation and excised lungs fixed in 10% buffered formalin. The tissue was embedded in paraffin and 5 μm sections cut for staining with haemotoxylin and eosin.

Figure 1 shows an example of *P. aeruginosa*-infected agarose beads within the airways of a mouse and the heterogeneous pattern of infection this protocol produces. An extensive inflammatory infiltrate is apparent surrounding the beads and within the airway immediately adjacent. The inflammatory response, however, is heterogeneous, and in areas further from the beads, less evidence of inflammation is apparent.

Preparation of explants

Lung explants were prepared as described previously [22]. Briefly, mice were anaesthetized with sodium pentobarbital (100 mg·kg^{-1} intraperitoneally), intubated by tracheotomy with polyethylene microtubing (PE-90, Intramedic, Becton Dickson, Parsippany, NJ, USA), and the lungs excised. For the *P. aeruginosa*-infected mice, explants were prepared 72 h after infection. A 1:1 mixture of buffered culture medium (BCM), prepared from

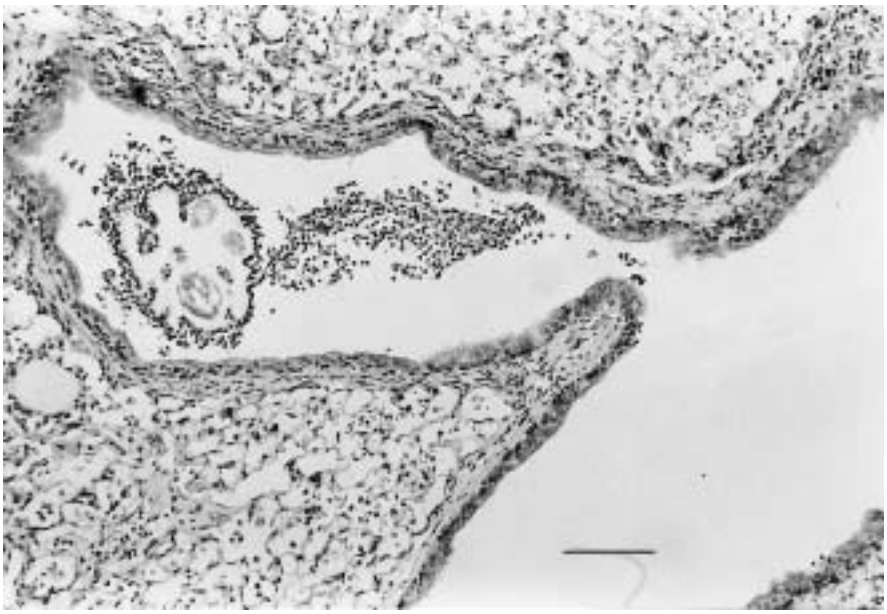

Fig. 1. – Agarose beads infected with *Pseudomonas aeruginosa* within a peripheral airway of a mouse. This infection protocol produces a heterogeneous pattern of infection, with some areas demonstrating a pronounced inflammatory response, and other areas appearing more normal. Five micrometre paraffin section stained with Haematoxylin and eosin. (Internal scale bar =100 μm).

minimal essential medium powder plus Earle's salts and L-glutamine (GIBCO Canada, Burlington, Canada) and agarose type VII (Sigma Chemicals, St Louis, MO, USA) at 37°C was then instilled into the lungs to an inflation volume of 0.05 mL·g body weight^{-1}. The agarose-filled lungs were then placed at 4°C for 30 min to gel the agarose, after which they were placed in a 35 mL syringe and surrounded with a 4% agarose solution. Again, the lungs were placed at 4°C for 30 min which resulted in the formation of an agarose-lung block, which was then clamped horizontally and cut into 0.5–1.0 mm slices using a hand-held microtome blade (model 818, Cambridge Instruments, Buffalo, NY, USA). Explants were then cultured overnight in BCM at 37°C in 5%CO_2/95% air. Explants which included either an entire airway cut in cross section or an example of a tangentially sliced airway were selected for measurements of f_{cb} or PT, respectively. All measurements were performed at 22°C.

Measurements of f_{cb}

Explants which contained examples of airways cut in cross section were selected for measurements of f_{cb}. Two or three explants were studied from each animal and the values summed to give an overall measure of f_{cb} for that animal. Explants were placed in 100 μL BCM and placed on the stage of an inverted microscope (Olympus, Tokyo, Japan). Ciliary activity was observed at 100× magnification in an area of the epithelium free from tissue debris and agar. Images were captured using a charged coupled device (CCD) camera (Video Scope International, Washington, DC, USA) and displayed on a video monitor during the experiment (see [22] for details). They were then recorded at an effective sampling rate of 60 frames·s^{-1} on an optical disc recorder (Matsushita Electric Industrial, Osaka, Japan) and could be played back frame by frame to be digitized using an 8-bit frame grabber (model PIP 1024, Matrox, Montreal, Canada) for later digital analysis using customized software (Galileo, Inspiraplex, Montreal, Canada). At each sampling location, the power spectrum of fluctuations in light intensity was calculated using fast Fourier transformation, and this was taken to reflect ciliary action. The f_{cb} was taken as the mean frequency in the power spectrum.

Measurements of PT

PT was measured in suitable examples of tangentially sectioned airways [22], to which a small amount (<5 μL) of a 0.1% weight/volume mixture of finely ground charcoal (Fisher Scientific, Montreal, Canada) in BCM was added. Movement of charcoal particles was observed at ×40 magnification and recorded through the CCD camera onto videocassette. The rate of PT was calculated as the average rate of translocation of five particles in each experiment, and one explant was studied per animal.

Experimental protocols

Measurements of f_{cb} and PT were made in CFTR +/+ or CFTR -/- animals divided into three groups: uninfected mice; those inoculated with *P. aeruginosa*-filled beads; and an additional vehicle-treated group consisting of mice (CFTR +/+ only) inoculated with sterile beads. After baseline measurements were recorded, the β-adrenergic agonist isoproterenol (Sigma Chemicals), which has previously been demonstrated to stimulate f_{cb} and PT [22], was administered at 10^{-6}, 10^{-4} or 10^{-2} M.

Statistical analysis

All data are expressed as mean±standard error of the mean (SEM). A difference was considered statistically significant when the p-value was less than 0.05, analysed by one-way analysis of variance.

Results

f_{cb} and PT in CFTR+/+ and CFTR -/- mice

All groups of mice examined demonstrated isoproterenol dose-dependent stimulation of f_{cb} and PT, which has previously been reported in this system [22], and which reflects β-adrenergic mediation of respiratory cilia activity.

A comparison of f_{cb} from CFTR +/+ and -/- mice (fig. 2a) revealed no difference between these groups at

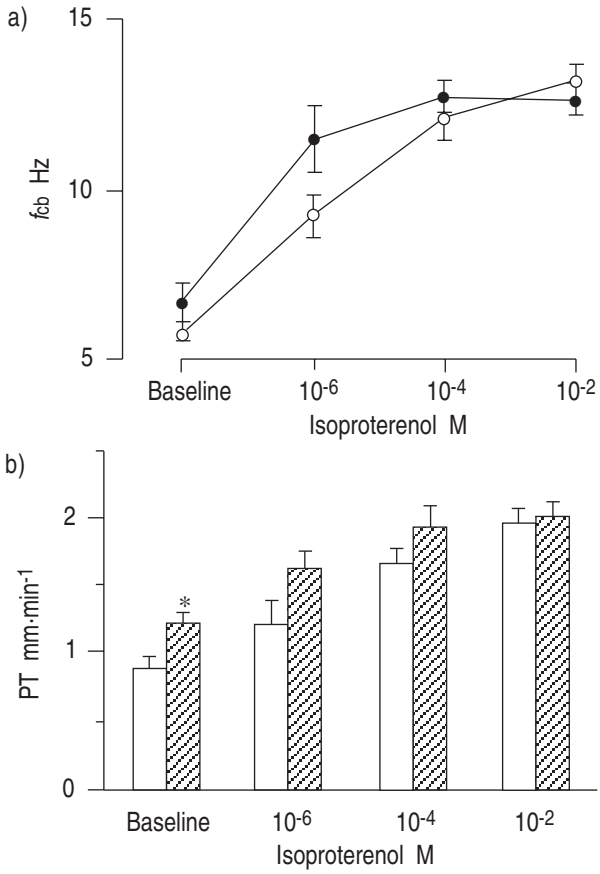

Fig. 2. – a) Ciliary beat frequency (f_{cb}) and b) particle transport (PT) in uninfected cystic fibrosis transmembrane conductance regulator (CFTR) -/- mice compared to CFTR +/+ littermate controls. While no difference was apparent in f_{cb} between these groups, there was a significantly higher rate of PT in the CFTR +/+ mice at baseline. —○—, □ : CFTR -/-; —●—, ▨ : CFTR +/+. *: $p<0.05$ as determined by analysis of variance (ANOVA).

baseline (*i.e.* prior to stimulation with isoproterenol). While f_{cb} did increase with increasing doses of isoproterenol in both groups of mice, no differences were detectable between CFTR knockouts and their littermate controls.

However, when rates of PT were compared (fig. 2b), CFTR -/- mice demonstrated a significantly reduced PT at baseline ($p<0.05$) compared to wild type (0.89±0.1 mm·min^{-1}, n=6 mice compared to 1.23±0.08 mm·min^{-1}, n=9). However, this difference was not apparent following isoproterenol treatment.

Effect of Pseudomonas aeruginosa *infection on f_{cb} and PT*

When similar recordings of f_{cb} were made from CFTR +/+ and -/- mice inoculated with beads containing *P. aeruginosa*, no differences between groups were apparent, either at baseline or following isoproterenol stimulation (fig. 3a).

The baseline PT rate between these groups was again different (fig. 3b). This time, CFTR +/+ mice demonstrated a significantly reduced PT at baseline compared to CFTR -/- mice (0.68±0.05 mm·min^{-1}, n=6, compared to 1.07±0.17 mm·min^{-1}, n=7; $p<0.05$).

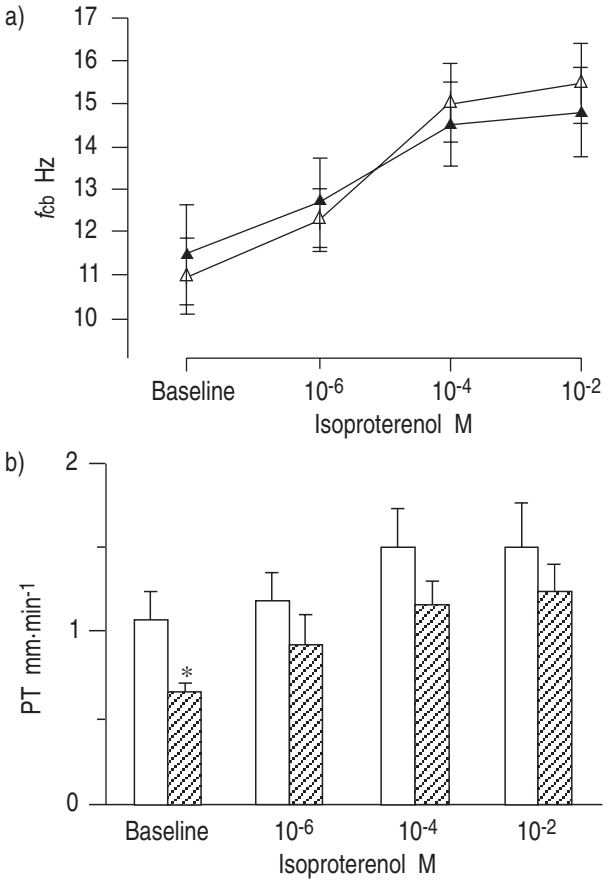

Fig. 3. – a) f_{cb} and b) PT in CFTR -/- mice compared to CFTR +/+ littermate controls following infection with *Pseudomonas aeruginosa*. No significant differences in f_{cb} were found between the groups of mice at either baseline or following isoproterenol stimulation. However, there was a significantly lower rate of PT in the CFTR +/+ mice at baseline. *: $p<0.05$ as determined by ANOVA —△—, □ : infected CFTR -/-; —▲—, ▨ : infected CFTR +/+. For definitions, see legend to figure 2.

Effect of Pseudomonas aeruginosa *infection in CFTR +/+ mice*

When f_{cb} in normal and *P. aeruginosa*-infected CFTR +/+ mice was compared (fig. 4a), a significant increase in baseline f_{cb} was observed ($p<0.05$) in the infected mice (11.44±1.15 Hz, n=6) *versus* uninfected controls (6.73±0.63 Hz, n=9). There was also a significantly higher rate of baseline f_{cb} in mice inoculated with *P. aeruginosa* beads compared to those inoculated with sterile agar beads (11.44±1.15 Hz, n=6 *versus* 7.50±0.88, n=8; $p<0.05$). Following isoproterenol stimulation, comparisons between *P. aeruginosa*-infected and uninfected mice did not show any significant differences; however, *P. aeruginosa* bead inoculated mice showed significant increases in f_{cb} when compared to the sterile bead vehicle controls.

P. aeruginosa-infected CFTR +/+ mice demonstrated a significant decrease in baseline PT compared to uninfected mice (0.65±0.05 mm·min^{-1}, n=6 *versus* 1.23±0.08 mm·min^{-1}, n=9; $p<0.05$). This decrease in PT with *P. aeruginosa* infection was significant at all isoproterenol doses measured. While there were no differences between PT rates in uninfected mice compared to those

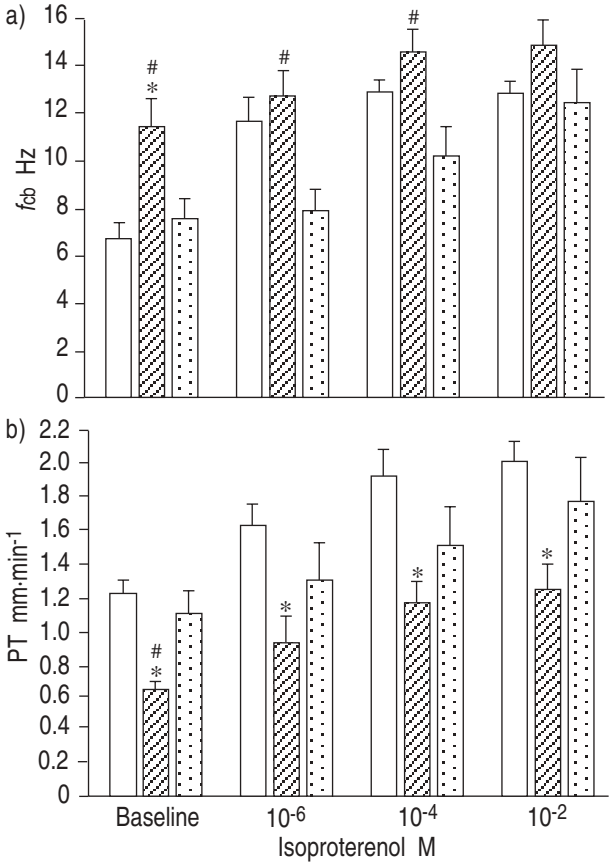

Fig. 4. – Mucociliary function in CFTR +/+ mice with and without infection with *Pseudomonas aeruginosa*. a) f_{cb} of the infected CFTR +/+ mice was significantly increased at baseline while sterile agar beads had no effect. b) PT of the infected CFTR+/+ mice was significantly reduced when compared to uninfected mice. Again, addition of sterile beads had no effect. □ : uninfected CFTR +/+; ▨ : infected CFTR +/+; ▭ : vehicle CFTR +/+. *: $p<0.05$, uninfected *versus* infected mice; #: $p<0.05$, infected *versus* sterile beads control mice, as determined by ANOVA. For definitions, see legend to figure 2.

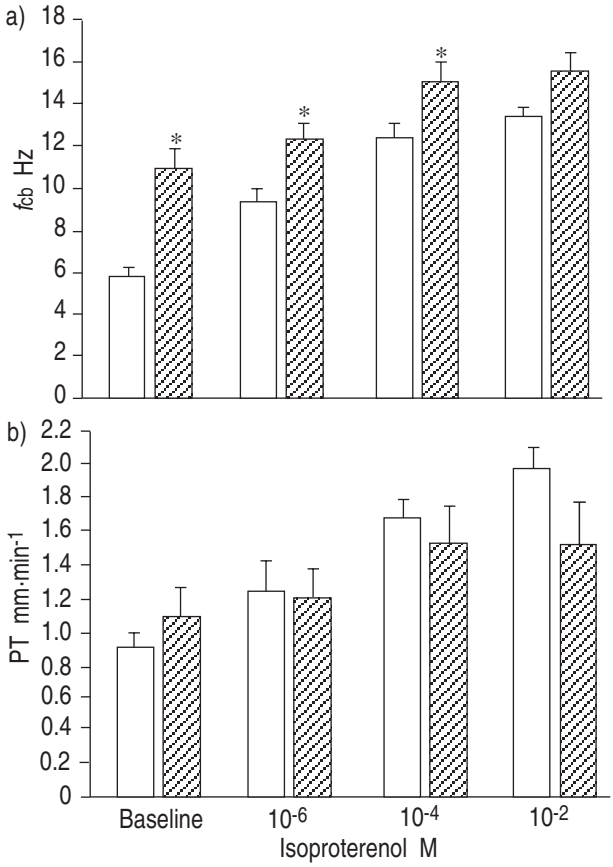

Fig. 5. – Mucociliary function in CFTR -/- mice with and without infection with *Pseudomonas aeruginosa*. a) f_{cb} of the infected CFTR -/- mice was significantly increased when compared to controls. b) No significant differences were apparent in rates of PT ☐ : uninfected CFTR -/-; ▨ : infected CFTR -/-. *: $p<0.05$, as determined by ANOVA. For definitions, see legend to figure 2.

inoculated with sterile beads, baseline PT was significantly reduced when *P. aeruginosa*-infected mice were compared to the vehicle control (fig. 4b).

Effect of Pseudomonas aeruginosa *infection in CFTR -/- mice*

P. aeruginosa-infected mice had a significantly higher baseline f_{cb} than uninfected animals (5.79±0.43 Hz, n=6 *versus* 10.92±0.88 Hz, n=7). This increase was maintained following isoproterenol stimulation (fig. 5a). However, when rates of PT were compared, there were no differences between uninfected and infected mice (fig. 5b).

Discussion

Using an *in vitro* lung explant system, we found a difference in the rate of PT between CFTR knockout mice and their littermate controls, although there was no difference in f_{cb}. Additionally, measurements of these parameters in mice chronically infected with *P. aeruginosa* revealed that infection with this agent leads to an increase in f_{cb}, while PT rates appeared dependent on both CFTR and infection status.

This finding, that PT is altered in CFTR knockout mice while f_{cb} is normal, demonstrates that mucociliary clearance mechanisms are in some way dependent on the presence of the CFTR gene product. Our work supports an earlier report which appeared in abstract form [20], in which *in vivo* PT measurements were significantly reduced in CF mice compared to controls. It is interesting that the overall conclusions of this work and ours are similar, especially given that different murine CF models were used. One of the major criticisms of CF mouse models has been that they do not develop the gross lung pathology seen in the human disease. Indeed, it has been demonstrated that an alternative calcium-dependent chloride channel exists in mice [25], leading to the hypothesis that the activity of this channel somehow protects the mouse lung from the deleterious effects of knocking out CFTR. However, our finding of reduced clearance clearly demonstrates that physiological differences do exist between the lungs of CFTR knockout mice and normals, and that the hypothesized presence of an alternative chloride conductance is not enough to compensate fully for the loss of the CFTR chloride channel.

The mechanism by which the CFTR gene product affects PT rate is not apparent from this study. While CFTR could affect ciliary function directly, it is more probable that the differences relate somehow to the alterations in transepithelial salt and water fluxes associated with loss of this channel. One important component of mucociliary clearance relates to the quantity and quality of the airway surface fluid (ASF) in which cilia are bathed. ASF composition is altered in CF [26], in which the Cl^- concentration is elevated. While there have been no reports of the composition of normal or CF mouse ASF, it is possible that important differences in the ionic composition could exist as a result of the altered transepithelial Cl^- flux, and that subsequent differences in the composition or depth of the ASF could affect mucociliary clearance mechanisms.

The effects of bacterial infection on mucociliary clearance have not been previously investigated in a murine CF model, despite the fact that CF patients demonstrate chronic bacterial colonization with organisms such as *P. aeruginosa*. While we found no difference between f_{cb} in *P. aeruginosa*-infected knockout and control mice, PT was again different: this time however, CFTR -/- mice demonstrated a significantly higher rate of PT at baseline than normals. f_{cb} was clearly higher when mice inoculated with *P. aeruginosa* entrapped agar beads were compared to uninfected controls, whether CFTR knockout mice or normals are considered. This increase in f_{cb} in the presence of *P. aeruginosa* in CFTR +/+ mice was significant over rates recorded from mice inoculated with sterile beads, strongly suggesting that the increased rate we see is due to the presence of this bacterial load rather than a result of the introduction of foreign particles. Furthermore, *P. aeruginosa*-infected CFTR +/+ mice have significantly lower PT than uninfected animals (or vehicle controls at baseline); however, this difference is not seen in knockout mice. One limitation of the murine CF model is the limited number of CFTR -/- mice available for study. We were therefore unable to obtain sufficient animals to study knockout mice inoculated with sterile beads alone.

However, the results seen with the administration of sterile beads to their CFTR +/+ littermate controls strongly suggests that the effects we see are due to the presence of *P. aeruginosa* rather than the administration of the beads.

Our data indicating that *f*cb is increased in the presence of *P. aeruginosa* is in contrast with earlier work demonstrating that a variety of *P. aeruginosa*-derived exoproducts either disrupt ciliary function or decrease *f*cb [27–29]. While production of such compounds could in part account for the reduction in PT rate in wildtype infected airways, they do not explain the increases in mean *f*cb we observe. It is possible therefore that this difference may be due to sampling variation, since *f*cb has not previously been recorded from infected mouse peripheral airways. Additionally, since we are sampling from intact airways, it is possible that *P. aeruginosa* infection could induce the production of an agent which stimulates *f*cb, such as nitric oxide [30]. A potential limitation of our method is that the overnight culture of explants at 37°C may permit *P. aeruginosa* to grow *ex vivo*, thus releasing exoproducts different to those seen *in vivo*. Nevertheless, previous studies of the effects of Pseudomonas exoproducts have also relied upon *in vitro* culture of bacteria, making this an unlikely explanation for our findings.

The reasons why CFTR knockout mice do not demonstrate the decrease in PT which accompanies *P. aeruginosa* infection is not obvious; however, it does demonstrate another difference between CF and normal mouse lung function in terms of their ability to handle a bacterial load. However, our findings that PT rates can be altered independently of *f*cb support the earlier report of SEYBOLD *et al.* [31], who found that an increase in one parameter of mucociliary clearance does not necessarily lead to an increase in another. The observation that PT differs from normal in CFTR knockout mice, and that the direction of this difference is dependent upon infection status, implies that infection somehow uncouples the normal relationship between *f*cb and PT.

Our infection protocol involved the introduction of *P. aeruginosa*-infected beads into the airways. While this system has the advantage of permitting efficient and reproducible infection because the agarose beads protect the bacterial inoculum from the rapid bacterial clearance seen in rodents [32], this approach has the limitation that infection can be regionally heterogeneous, particularly in the lung parenchyma (fig. 1). Furthermore, the presence of beads in some areas restricted sampling of those airways for measurements of mucociliary function. As we carefully confined our measurements to areas with an intact epithelium and where the airway was clear of debris, our findings probably represent bronchial regions with relatively low levels of infection. Despite restricted sampling, we nevertheless found evidence of an effect of *P. aeruginosa* infection on mucociliary clearance as reflected in higher *f*cb rates and lower PT, effects not induced by the simple addition of sterile beads (fig. 4). We therefore believe that, despite the restrictions imposed by our sampling approach, our measurements provide a useful estimate of *f*cb and PT rates in this model.

In conclusion, using an *in vitro* lung explant technique, we have measured two parameters of mucociliary clearance in a murine model of cystic fibrosis. While there were no differences in ciliary beat frequency rates between cystic fibrosis transmembrane conductance regulator knockout mice and their littermate controls, particle transport rates appeared dependent upon both cystic fibrosis transmembrane conductance regulator and infection status. We also describe the first study of mucociliary clearance rates in mice following bacterial infection. These results indicate that absence of the cystic fibrosis transmembrane conductance regulator gene product affects mucociliary clearance mechanisms in the peripheral airways of mice. Furthermore, infection with *Pseudomonas aeruginosa* clearly modulates mucociliary clearance in this model, and cystic fibrosis transmembrane conductance regulator knockout mice demonstrate a different mucociliary response to infection when compared to controls.

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