S13.4
INFLUENCE OF LUNG TRANSPLANTATION

James R. Yankaskas, M.D.
Cystic Fibrosis/Pulmonary Research and Treatment Center,
The University of North Carolina, Chapel Hill, NC, USA

Respiratory failure due to airway infection, inflammation and bronchiectasis is almost universal in CF. Hypoxemic failure is primarily caused by ventilation-perfusion mismatching and alveolar hypoventilation, and can often be treated effectively with supplemental oxygen. Hypercapnic failure, caused by alveolar hypoventilation, can be treated by standard therapy for airway infection and inflammation, while the resultant acidosis is initially compensated by metabolic alkalosis. Treatments eventually loss effectiveness, and death ensues. Preparing the patient, family and care team for this clinical course requires empathy, skill and experience.

Conventional mechanical ventilation thorough endotracheal tubes has rarely been indicated for CF. A 1978 review of 46 CF patients with respiratory failure treated by mechanical ventilation demonstrated poor immediate and long term survival, as most died during or shortly after the episode (1). However, a 1989 review of 5 infants with acute respiratory failure demonstrated 100% survival, and long term survival comparable to age and gender matched CF controls who did not have respiratory failure (2). Thus, mechanical ventilation is often appropriate for CF patients with acutely reversible complications. For most CF patients with respiratory failure that progresses despite maximal standard therapy, mechanical ventilation may prolong life for a short time, but is unlikely to improve survival or quality of life significantly.

The development of effective lung transplantation techniques provided a dramatic therapeutic option for end-stage CF lung disease. The risks of surgery, immunosuppression, and infectious and other complications are significant, but many CF patients have enjoyed dramatic improvements in their pulmonary function, survival, and quality of life. The supply of donor organs is much less than the demand, and the average time from acceptance as a lung transplant candidate to actual transplantation is about two years. The indications, contraindications, complications, and other aspects of lung transplantation were summarized in a CFF Consensus Statement (3).

Lung transplantation adds a new dimension to end-of-life care and affects decision-making in numerous ways. First, transplantation causes end-of-life decision-making to begin earlier. Donor organs are allocated on the basis of blood type and time on the waiting list. The waiting time is currently two or more years (because of low donor organ availability), and requires that transplantation be considered when natural survival is about this long. The variable course of CF lung disease makes it difficult to identify this point. The transplantation of lung lobes from living related donors can provide with shorter waiting times, but this procedure can be more complicated. In general, transplantation and end-of-life care decisions must be considered when respiratory and overall functions are relatively well preserved. This is an advantage. Second, to minimize operative and post-transplant risks, the care of transplant candidates is intensified to maximize lung function, nutrition, fitness, and general medical health prior to surgery.

Third, ventilatory support for CF patients with hypoxemic and/or hypercapnic respiratory failure is appropriate in several settings. Assisted ventilation by nasal or face masks can improve gas exchange and respiratory muscle function (4, 5). Fourth, mechanical ventilation through cuffed endotracheal tubes is feasible, but compromises the ability to cough and clear secretions. Thus, life can be sustained, but at the cost of worsening lung function, impaired communication, and increased risk of major complications. Despite several notable exceptions, experience at the University of North Carolina suggests that CF patients with severe hypercapnic respiratory failure ($PaCO_2$>100) can be sustained on conventional ventilators for only several weeks. Therefore, we consider instituting time-limited conventional ventilation for CF lung transplant candidates who are at the top of the candidate list and who select this option after thoroughly understanding the risks and benefits.

Fifth, terminal phase of care is affected. Patients who decline or do not qualify for transplantation, who are removed from transplant waiting list because of complications, or who have severe respiratory failure when the waiting time for donor organs is still too long, should receive standard terminal care as summarized in the other sections of this symposium. Individuals who opt for mask or conventional ventilatory support may have several weeks or months of continued hope for a successful operation. These efforts may be met with dramatic success or tremendous disappointment. The later situation accentuates the loss, and can make grieving and acceptance more difficult. Finally, death after failure of transplanted lungs requires care and coping approaches similar those summarized in the other sections of this symposium. Most patients and their families have been pleased with the clinical improvements following lung transplantation, however brief, and would select the procedure again, even after experiencing serious complications firsthand.

In summary, lung transplantation offers a major opportunity for improved life for CF patients, and sever-

al advantages to end-of-life decision-making and care. It provides a reason to address end-of-life issues when general health is good, and decisions can be considered without severe time-pressure. It provides hope for a dramatic improvement in function. It defines some therapeutic choices more clearly. Transplantation also introduces a new set of potential complications. Although it does not remove the discomfort of death for the individual or their loved ones, it can extend productive life and help to organize some end-of-life processes.

1. Davis PB, di Sant'Agnese PA. Assisted ventilation for patients with cystic fibrosis. JAMA 1978; 239:1851-1854.
2. Garland JS, Chan YM, Kelly KJ, Rice TB. Outcome of infants with cystic fibrosis requiring mechanical ventilation for respiratory failure. Chest 1989; 96:136-138.
3. Yankaskas JR, Mallory GB Jr. Lung transplantation in cystic fibrosis: consensus conference statement. Chest 1998;113:217-226.
4. Hodson ME, Madden BP, Steven MH, Tsang VT, Yacoub MH. Non-invasive mechanical ventilation for cystic fibrosis patients—a potential bridge to transplantation. Eur Respir J 1991; 4:524-527.
5. Piper AJ, Parker S, Torzillo PJ, Sullivan CE, Bye PT. Nocturnal nasal IPPV stabilizes patients with cystic fibrosis and hypercapnic respiratory failure. Chest 1992; 102:846-850.

S14.1
NEW CASES OF PSEUDOMONAS AERUGINOSA: RISK FACTORS AND OUTCOMES

J. Wagener[1], S. Butler[2], H. Rabin[3], W. Regelmann[4], C. Johnson[2]
for the Epidemiologic Study of Cystic Fibrosis Advisory Committee, [1]Univ. of Colorado, Denver, CO, [2]Genentec, S. San Francisco, CA, [3]Univ. of Calgary, Calgary, CANADA, [4]Univ. of Minnesota, Minneapolis, MN

The majority of cystic fibrosis (CF) patients are colonized with Pseudomonas aeruginosa (PA) by seven years of age and following colonization they are at increased risk for subsequent hospitalization, worsening pulmonary function, and possibly death.[1-6] Risk factors for colonization are numerous, including age, gender, lung function, having a sibling with CF, the age at diagnosis, and previous antibiotic use.[6,7] Whether or not a patient is cared for in a CF center and the the way the center isolates patients may also be a risk factor for PA acquisition.[8,9] Additionally, early therapy may reduce the chance of the PA persisting and result in an improved prognosis.[11,12]

The Epidemiologic Study of Cystic Fibrosis (ESCF) includes serial data from over 20,000 CF patients collected over four years. We designed this study to address the specific questions: 1) What are the risk factors for new detection of Pseudomonas aeruginosa?, 2) For patients with newly detected PA, what are the predictors of treatment and follow-up within 3-12 months?, and 3) What is the probability of and predictors for persistence of PA in the year following new detection?

Patient Selection: Patients were included if they had at least three years of active follow-up in ESCF, they had at least two cultures negative for PA during the year prior to and the year following study enrollment, and had at least one culture reported during the second year following enrollment. Patients with cultures for B. cepacia and S. maltophilia were excluded.

Methods: Patients were divided into PA positive and

Table 1.
New Pseudomonas aeruginosa cases separated by age. (number in cell and % of age group)

	0-5 years	6-12 years	13-17 years	18-24 years	≥25 years
Negative	528 (81%)	600 (83%)	176 (77%)	76 (75%)	64 (85%)
Positive	123 (19%)	122 (17%)	53 (23%)	26 (25%)	11 (15%)

Table 2.
New Pseudomonas aeruginosa cases separated by baseline percent predicted FEV_1.*

	$FEV_1 \geq 80\%$	$60 \leq FEV_1 < 80\%$	$40 \leq FEV_1 < 60\%$	$FEV_1 < 40\%$
Negative	550 (84%)	247 (76%)	117 (79%)	14 (78%)
Positive	101 (16%)	77 (24%)	32 (21%)	4 (22%)

*number of patients in cell and % of FEV_1 group

PA negative groups based on whether a culture at any time during the second year following enrollment was positive for PA. Baseline data included: age, gender, genotype, pulmonary function, disease severity, nutritional state, prior infection and therapy for other bacteria, frequency of clinic visits and bacteria cultures, and several variables describing the center (i.e. size, microbiology protocol, etc.). Therapy response to the new PA detection included whether or not the patient was treated with anti-pseudomonas antibiotics in the three months following the culture. Finally, follow-up measures included the frequency of clinic visits and re-culture during the year following new detection.

Results: 1779 patients had at least two negative PA cultures during enrollment and the first study year plus three years of data. 335 (19%) of these patients had their first recorded positive PA culture during the second study year. The risk of a new positive culture was unrelated to gender (female 19%, male 18%), was similar for all age groups (Table 1), and did not appear related to lung function (Table 2).

Following a new positive culture, only 46% of patients received PA specific antibiotic therapy within the subsequent three months. Older patients were slightly more likely to be treated.

These preliminary results suggest that the risk of a new positive culture is not related to age, gender, or lung function. This suggests that all PA culture negative patients are at similar risk of being infected by environmental exposure. Additionally, it appears that their is no consensus about whether or not patients with a new positive culture for PA should be treated. Whether or not early treatment will effect outcome requires longer patient follow-up.

References

1. Kerem E, et.al. Pulmonary function and clinical-course in patients with cystic fibrosis after pulmonary colonization with Pseudomonas aeruginosa. J Pediatr 1990; 116:714-9.
2. Kulczycki LL, et.al. Pseudomonas colonization in cystic fibrosis. A study of 160 patients. JAMA 1978; 240:30-4.
3. Hudson VL, et.al. Prognostic implications of initial oropharyngeal bacterial flora in patients with cystic fibrosis diagnosed before the age of two years. J Pediatr 1993; 122:854-60.
4. Pamukcu A, et.al. Effects of pseudomonas aeruginosa colonization on lung function and anthropometric variables in children with CF. Pediatr Pulmonol 1995; 19:10-5.
5. Henry RL, et.al. Mucoid Pseudomonas aeruginosa is a marker of poor survival in cystic fibrosis. Pediatr Pulmonol 1992; 12:158-61.
6. Kerem E, et.al. Risk factors for Pseudomonas aeruginosa colonization in cystic fibrosis patients. Pediatr Infect Dis J 1990; 9;494-8.
7. Demko CA, et.al. Gender differences in cystic fibrosis: Pseudomonas aeruginosa infection. J Clin Epidemiol 1995; 48:1041-9.
8. Hoiby N, Pedersen SS. Estimated risk of cross-infection with Pseudomonas aeruginosa in Danish cystic fibrosis patients. Acta Paediatr Scand 1989; 78:395-404.
9. Farrell PM, et.al. Acquisition of Pseudomonas aeruginosa in children with cystic fibrosis. Pediatrics 1997; 100:E2.
10. Szaff M, et.al. Frequent antibiotic therapyt improves survival of CF patients with chronic Pseudomonas aeruginosa infection. Acta Paediatr Scand 1983; 72:651-7.
11. Valerius NH, et.al. Prevention of chronic Pseudomonas aeruginosa colonisation in cystic fibrosis by early treatment. Lancet 1991; 338:725-6.

S14.2
MEASURING OUTCOMES IN ESCF: CAN WE DO IT?

Mary Ellen Wohl, MD.[*] Steven M Butler, PhD.[#] Charles A Johnson, MB. ChB[#]
[*]*CF Center, Children's Hospital Medical Center, Boston Massachusetts,*
[#]*Department of Medical Affairs, Genentech Inc., South San Francisco,California*

The management of cystic fibrosis patients is generally carried out by a small group of care centers following guidelines produced by the Cystic Fibrosis Foundation[1]. Although care is generally uniform, factors such as center size, geographic location, variations in physician practice and the influence of third party payers might affect practice and outcomes. Two recent developments underscore the importance of reviewing CF care; the justifiable concern over the increasing cost of providing appropriate healthcare and, in the field of CF, the intriguing data from Europe suggesting that aggressive and costly management of *Pseudomonas* colonization can improve survival[2].

In a review of outcomes in CF practice, the ideal outcome would be mortality. However since relatively few patients die in the short term, pulmonary function has been validated as a surrogate[3]. In patients too young for reliable spirometry, weight for age might be a useful substitute.

We studied the lung health and nutritional status of a large cohort of patients to determine if there were consistent association of outcomes with practice at CF care

Table 1.
Characteristics of patients in the upper (U) and lower (L) quartile ranking centers by age group. P-values for FEV1 and Wt. for age are from two sample t-tests within each age group.

Age	0-6		6-12		13-17		≥18	
Ranking	U	L	U	L	U	L	U	L
Sites (n)	22	22	29	29	22	22	27	27
Patients (n)	415	554	1132	774	572	350	865	919
FEV1 (% pr.)	—	—	92	75	85	64	64	50
p=	—		<0.001		<0.001		<0.001	
Wt. for age (%)	46	29	35	31	31	20	36	30
p=	<0.001		<0.001		<0.001		<0.001	

sites. We hypothesized that 1) detectable differences in lung health existed between centers, 2) detectable differences in nutrition existed between centers, and 3) associations existed between practice patterns and lung health. Such differences would represent associations, and not necessarily causation. For example, differences among centers could result from practice or from unexplained differences in the patients available to the centers.

Comparisons were done separately for four different age groups (<6, 6-12,13-17,>18). For each age group, sites were included if 10 or more patients in the age group had been followed from Jan 1995 to Dec 1996. For each site and age group, lung health at a center was defined as the median of FEV_1 values for the patients at that site. Nutritional status at a center was defined as the median of weight for age percentile. Patients from sites with a total enrollment of less than 50 were pooled. For each age group, the median values at eligible centers were ranked in order from the highest to lowest value. Rankings of centers tended to be consistent across all age groups and from the beginning and end of the two year reporting period. Pooled sites with less than 50 patients tended to show results similar to those in the middle of the rank order for larger sites.

The mean FEV_1 and weight for age percentile for all patients in the 25% of centers reporting the highest median values was compared to the mean values for the patients in 25% of centers with the lowest ranked medians. Surprisingly large differences were observed between the centers ranked in the upper 25% compared to the lower 25%. (Table 1)

To examine these inter site disparities further we reviewed center size, geographic location, visit frequency, use of routine therapies and types of antibiotic used for prophylaxis and exacerbation.

The results suggest that sites reporting higher values for FEV1 and weight for age monitored patients more frequently, reviewed spirometry more often and treated patients more aggressively. Data to be presented in this seminar will include a detailed review of the monitoring and use of specific therapies at different centers, focusing particularly on the use of antibiotic interventions.

References

1. Cystic Fibrosis Foundation Center Committee, Guidelines Subcommittee. Cystic fibrosis Foundation guidelines for patients services, evaluation and monitoring in cystic fibrosis centers. AJDC 1990; 144: 1311.
2. Frederiksen B, et al. Improved Survival in the Danish Center-Treated Cystic Fibrosis Patients: Results of Aggressive Treatment. Ped Pulm 1996; 21:153-158.
3. Kerem E, Reisman J, Corey M, Canny GJ, Levison H. Prediction of mortality in patients with cystic fibrosis. N Eng J Med 1992; 326: 1187-1191

S14.3
PREDICTORS OF PROGRESSION TO SEVERE PULMONARY STATUS IN CYSTIC FIBROSIS

D. Schidlow[1], S. Butler[2], C. Johnson[2], A. Rao[2], J. Wagener[3], W. Regelmann[4], W. Morgan[5],
for the investigators of the Epidemiologic Study of Cystic Fibrosis (ESCF).
[1]St. Christopher's Hospital for Children, Philadelphia, PA; [2]Genentech Inc., S. San Francisco, CA;
[3]Univ. of Colorado, Denver, CO; [4]Univ. of Minnesota, Minneapolis, MN; [5]Arizona Health Sciences Center, Tuscon, AZ.

The need to match medical intervention with projected clinical course, especially because of the availability of lung transplantation, demands that clinicians treating individuals with cystic fibrosis (CF) refine to a maximum their ability to accurately predict progression of lung disease. Epidemiologic studies like ESCF allow the

characterization of clinical phenomena in large populations and, hopefully, improve the ability of care givers to extrapolate to individual patients in a more informed manner. The present analysis will examine the predictive factors of short term decline to "severe status" in pulmonary function, defined here as having an FEV1 of less than 30% of predicted. A parallel analysis using FVC in place of FEV1, with a cutoff of 40% of predicted, will also be performed.

Previous studies have focused on decline in lung function or mortality in CF, and identified important risk factors[1,2,3,4,5]. The Epidemiologic Study of Cystic Fibrosis (ESCF) includes longitudinal data from over 20,000 CF patients collected over four years, and thus presents an opportunity to study risk factors in a very large population. In this analysis, patients are included if they had at least 3 years of potential follow-up, if they had at least two stable PFTs in a "baseline" period defined as 3 months before enrollment to 12 months after enrollment, and if all stable FEV1 measurements in the baseline period were above 30% of predicted. In the first set of analyses (Case 1) the "observation period" was the second year after enrollment (12 to 24 months), and patients must have had at least one stable spirometry during that period. The patient is considered to have "progressed to severe lung disease" if and when they first had a stable FEV1 below 30% in the observation period. For the patients who did not progress, the point of reference is their last stable PFT in the observation period.

Patients who progressed will be compared to patients who did not progress, focusing on their characteristics one year prior to progression (for those who progressed) or the last stable PFT in the observation period (for those who did not progress). These characteristics will include PFT scores and indicators of clinical, nutritional and microbiological status collected in ESCF. Independent associations with progression will be determined using logistic regression modeling. In addition, subsequent outcomes in the 12 months following progression or the last stable PFT will be compared, including death, transplantation, and repeated low FEV1 scores.

In a second, parallel set of analyses (Case 2) the third year after enrollment (24 to 36 months) will be considered the observation period, and patients will be included whose stable FEV1 measurements were all above 30% predicted until at least two years after enrollment. This will provide an opportunity for confirmation of the findings of the first analysis, and to compare patient characteristics two years prior to progression or the last stable PFT.

The following summarizes a preliminary descriptive analysis for Case 1. Findings for Case 2 were qualitatively similar, as were the findings for FVC. The comparisons are all univariate, and describe how the characteristics are individually associated with progression. A systematic multiple logistic regression analysis will be performed in order to select the important independent risk factors from a larger list, including the above characteristics plus additional microorganisms and medical conditions collected in ESCF. Statistical inference will be deferred to the presentation of the complete multivariate analysis.

Table.
Various characteristics at 12 months prior to progression or the last stable PFT, for patients who progressed or did not progress in the observation period, for each of three FEV1 groups (Groups 4 and 5 were excluded due to the small number of patients who progressed).

	FEV1 Group					
	30-39		40-49		50-59	
Progressed:	Yes	No	Yes	No	Yes	No
N	120	224	61	480	28	606
Age (mean, SD)	23 (10)	24 (10)	21 (10)	23 (10)	18 (7)	19 (9)
Male (%)	58	57	46	50	54	53
Pseudomonas aeruginosa (%)	81	83	80	79	79	78
Burkholderia cepacia (%)	12	6	8	4	11	5
Crackles (%)	66	67	72	53	54	40
Weight for age %tile (mean, SD)	16 (23)	22 (24)	20 (22)	24 (25)	14 (19)	26 (25)
% of ideal body weight (mean, SD)	92 (14)	94 (14)	95 (13)	97 (16)	92 (13)	98 (14)

There were 5880 eligible patients for whom PFTs were available at 12 months (plus or minus 3 months) prior to progression or the last stable PFT. Grouping patients by their FEV1 score at that time, we found that the number of patients who declined to severe status was 120 (35%) out of the 344 patients with FEV1 between 30-39% predicted (Group 1); 61/542 patients (11%) with FEV1 between 40-49% predicted (Group 2); 28/633 patients (4%) with FEV1 between 50-59% predicted (Group 3); 2/730 patients (0.3%) with FEV1 between 60-69% predicted (Group 4); and 6/3,631 patients (0.02%) with FEV1 at least 70% predicted (Group 5).

Univariate comparisons for various potential risk factors were stratified by FEV1 group. Relative to the date 12 months prior to progression or the last stable PFT, these characteristics were assessed at the most recent clinic visit or microbiological culture. A few examples are reported in the table. Progression does not appear to be associated with age, sex, detection of *Pseudomonas aeruginosa*, insulin use, or any hemoptysis in the previous six months. Progression does appear to be associated with detection of *Burkholderia cepacia*. Patients who progressed appear to have been lighter and smaller, as expressed by weight for age percentile, percent of ideal body weight, and height for age percentile. Patients who progress appear to be more likely to have had crackles, but not daily sputum, daily cough, or clubbing. The differences for weight for age percentile, percent of ideal body weight, and crackles appear to be greater for patients who declined from a higher value.

The risk factors discussed here for decline in lung function have been identified previously in ESCF and other studies, but the present study presents a unique opportunity to identify risk factors for progression to severe status in the short term. The apparent associations shown here between potential risk factors and

progression to severe status are suggestive, but the detailed statistical analysis will reveal the significant independent risk factors.

References

1. Karem E, et al. Prediction of Mortality in Patients with Cystic Fibrosis. N Engl J Med 1992; 326:1187-1191.
2. Corey M, Edwards L, Levison H, Knowles M. Longitudinal analysis of pulmonary function decline in patients with cystic fibrosis. J Pediatr. 1997; 131:1809-1814.
3. Kerem E, et.al. Pulmonary function and clinical course in patients with cystic fibrosis after pulmonary colonization with Pseudomonas aeruginosa. J Pediatr 1990; 116:714-9.
4. Milla CE, Warwick WJ. Risk of Death in Cystic Fibrosis Patients with Severely Compromised Lung Function. Chest. 1998; 113 (to appear).
5. Rosenfeld M, et al. Gender Gap in Cystic Fibrosis Mortality. Am J Epid 1997; 145: 794-803.

S14.4
ASSESSMENT OF PULMOZYME EFFECTIVENESS OVER TWO YEARS

Charles A. Johnson MB.ChB[*], Steven M. Butler PhD[*], Michael W. Konstan MD[#]
[*]Department of Medical Affairs, Genentech Inc. , South San Francisco,California. [#]CF Center, Rainbow Babies and Children's Hospital, Cleveland, Ohio.

Randomized placebo controlled trials are considered essential to determine the efficacy of any new therapy(1). Patients meeting strict eligibility requirements are enrolled into a study with a protocol which determines physician behavior and frequency of evaluation. The study of how drugs work in practice is more challenging but can be approached by using clinical epidemiologic databases. Determination of clinical benefit in these "real life" studies is termed effectiveness(2). Pulmozyme® (dornase alfa recombinant) in conjunction with standard therapies is indicated in the management of cystic fibrosis patients to improve pulmonary function. Fuchs et al demonstrated in a large randomized controlled study of six months duration that patients had significant improvements in pulmonary function relative to controls(3) The purpose of the study was to determine whether a large epidemiologic database could be used to assess drug effectiveness using Pulmozyme as an example.

This presentation will focus on effectiveness after 24 months of treatment with Pulmozyme. Only patients aged 6 years or older and with a baseline FEV1 of at least 40% predicted will be considered. In an earlier analysis of Pulmozyme effectiveness after 12 months, the control group consisted of 2382 patients who had never received Pulmozyme compared to 283 who had received the treatment. These patients had a baseline spirometry and a second spirometry recorded 12 months later. A baseline observation period of six months preceded the initial spirometry. The treated patients must have started Pulmozyme therapy during the course of the study, and there must have had a baseline spirometry within a 3 month window before receiving Pulmozyme. Furthermore, we required a 6 month observation period

prior to the baseline spirometry.

The challenge of assessing effectiveness in a non-randomized, observational study is exemplified by the baseline differences between the patients who were treated and those who were not treated with Pulmozyme. As expected, on average the treated patients were sicker, although a wide variation in severity was represented in both groups. Patients treated with Pulmozyme had lower mean baseline values for FEV_1 (76.1%) compared to the untreated comparison group (87.5%) despite a similar mean age of 14.1 and 13.9 years respectively. They were more likely to have reported positive respiratory cultures for Pseudomonas aeruginosa (64.1% vs 46.7%). Pulmonary exacerbations during the pretreatment baseline period of six months were reported more frequently (56.4%) in Pulmozyme treated patients compared to untreated patients (22.2%). Despite these marked differences at baseline, mean values of FEV_1 for patients treated with Pulmozyme improved. To estimate the true benefit, covariate adjustment was performed with 14 characteristics known to affect pulmonary function decline. This analysis provided an estimated benefit of 4.3% of FEV_1 to Pulmozyme over the untreated patients after 12 months ($p<0.0001$).

A secondary analysis was also performed. This included, in the actively treated group, those patients who had not completed Pulmozyme treatment through the twelve month spirometry. This was considered an "intent to treat" analysis and included an additional 191 patients. Following covariate adjustment the estimate of treatment benefit relative to the control group was 3.7% of predicted FEV_1($p<0.0001$).

This analysis provides an estimate of the benefit of Pulmozyme in practice using methods to adjust for significant selection bias in terms of disease severity. The results are consistent with the Phase III controlled study. Selection of patients with continuos treatment for the primary analysis could preferentially select those patients who respond with a short term improvement in pulmonary function. However the intent to treat analysis suggests that a "responder" selection effect is not the cause of the observed benefit. The intent to treat analysis suggest that patients who have incomplete therapy still derive benefit. The initial goals of the study have been met, since we have been able to use this large database to assess drug effectiveness in the setting of current CF practice. As these methods are refined it will become possible to examine other therapies using similar methods.

Reference

1. Ramsey B, Boat T, Outcome measures for clinical trials in cystic fibrosis. J Pediatr 1994, 124:177-92.
2. Wolfe F. The epidemiology of drug treatment failure in rheumatoid arthritis. Baillieres Clinical Rheumatology. 1995;9(4):619-32.
3. Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, Rosenstein BJ, Smith AL, Wohl ME. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. N Engl J Med 1994;331:637-642.

S15.1A
ROLE OF AIRWAY SURFACE LIQUID IONIC COMPOSITION IN CF

E.A. Cowley, K. Govindaraju and D.H. Eidelman
Meakins-Christie Laboratories, Montreal Chest Institute Research Centre, McGill University Health Centre, McGill University, Montreal, Canada

The ionic composition of airway surface liquid (ASL) remains a controversial topic within the CF world. According to the traditional hypothesis of CF lung disease pathogenesis, absence of CFTR function leads to a reduction in ASL volume that impairs mucociliary clearance which in turn consequent development of airway infection and CF lung disease. Under this hypothesis, the sodium and chloride concentrations of ASL are predicted to be close to those of plasma. This point of view was challenged when Smith et al. proposed a novel hypothesis to explain development of CF lung disease (1). They carried out a set of elegant experiments using cultured human epithelial cells. The "ASL" produced in culture was found to possess important antibacterial properties, mediated by a low molecular weight protein active against several types of bacteria, including Pseudomonas aeruginosa. This antibacterial activity was observed to be highly dependent on the NaCl concentration of the ASL. In this culture system, epithelial cells lacking CFTR produced ASL with higher sodium and chloride concentrations that inhibited the antibacterial effect of the ASL. This loss of antibacterial activity could be reversed by transfecting functional CFTR into the cells or by reducing the NaCl concentration of the fluid bathing the cells. These observations led to the development of the hypothesis that CF is associated with a failure to maintain hypotonicity of the ASL. The consequent high concentrations of Na and Cl in ASL interfere with its normal antibacterial function, permitting the proliferation of bacteria and eventually the development of bronchiectasis and CF lung disease.

A key prediction of this hypothesis is that ASL is

hypotonic in normals but less than in CF patients. The true nature of ASL composition is thus a critical issue in understanding the pathogenesis of CF. If, as predicted by the classical electrophysiological studies the ASL is isotonic, then the innate antibacterial properties of ASL may not be central to the pathogenesis of CF lung disease. Alternatively if ASL is hypotonic in normals but not in CF patients, then CF may be a disorder of innate immunity in which the loss of ASL antibacterial properties results in the initiation of infection and inflammation.

ASL is composed of at least two layers, typically modelled as sol and gel phases, whose thickness has been estimated to lie between 10 to 40 microns depending on the location in the airways. ASL is therefore very difficult to sample in vivo without disturbing the underlying airway epithelium. Furthermore, the source of the ASL at any given point in the airway tree is problematic as well. ASL may be produced by the action of the airway epithelial cells themselves, by the mucous glands in the trachea and bronchi, and at least in the lung periphery, ASL may include contributions from the alveolar liquid as well. This makes prediction of ASL composition based on physiological studies on isolated or cultured epithelium difficult.

Attempts to harvest ASL in humans have largely focused on the use of small pieces of filter paper to collect the fluid. The ionic composition has then been analyzed by a variety of techniques. Joris et al. (2) using energy dispersive X-ray analysis, found evidence that ASL is hypotonic in normal subjects and asthmatics but increased in patients with airway diseases including cystic fibrosis (2). On the other hand, Knowles and coworkers (3) have presented evidence that nasal ASL in both normal and CF patients is isotonic at least when the mucous glands were carefully blocked pharmacologically. Although they also found bronchial ASL to be hypotonic, they argued that this was an effect of gland secretion. They found no difference between CF and control ASL. A potential limitation of these studies is the use of filter paper to harvest the ASL. The filter paper technique yields relatively large volumes of fluid compared to the thickness of the ASL, raising the possibility that the samples do not reflect the physiological characteristics of ASL.

Our own work has focused on developing methods for measurement of ASL composition in living rats (4) and mice. We have employed capillary electrophoresis (CE) to measure inorganic and organic ion composition of the ASL because of its ability to work with samples as small as a few nanolitres. This makes it possible to directly inject ASL into the CE device without pretreatment or dilution. Another advantage of this approach is that by adapting the analytical conditions, it is possible to quantitate the concentrations of any charged molecule including large molecular weight proteins. Harvesting ASL from small animals presents a number of challenges, however. After considerable experimentation, we have found that direct placement of a narrow bore capillary at the distal end of the trachea results in the collection of 100-200 nanolitres over a few minutes (3 minutes in rats; 30 minutes in mice). ASL measured from both rats and mice using this approach are consistently hypotonic, although there appear to be important species differences. The Na and Cl content of mice is quite close to that previously reported in humans. Although have found no differences so far in the ionic composition in control and cftr-/- knockout mice, Pseudomonas aeruginosa infection in mice leads to increased concentrations of Na, Cl and K. Although these results support the notion that rodent ASL is hypotonic, we have also obtained indirect evidence that the liquid we harvest is at least partly glandular in origin. Inbred strains of mice differ in the ease with which ASL may be harvested and our success in harvesting fluid varies among strains in a manner which correlates with the volume of the tracheal mucous glands in each strain.

Regardless of whether the Smith hypothesis is ever confirmed, there is little doubt that the composition of ASL plays a key role in innate host defense of the airways. Techniques like CE have the potential to permit the detailed analysis of ASL in order to elucidate its regulation. As our understanding of the biology and physiology of ASL improves, it should become possible to advance new therapeutic strategies which can contribute to the care of CF patients.

References

1. Smith, J.J, Travis, S.M., Greenberg, E.P., and Welsh, M.J. (1996). Cystic Fibrosis Airway Epithelia Fail To Kill Bacteria Because of Abnormal Airway Surface Fluid. *Cell,* **85**, 229-236.
2. Joris, L., Dab, I., Quinto, P.M. (1993). Elemental Composition of Human Airway Surface Fluid in Healthy and Diseased Airways. *Am. Rev. Respir. Dis.,* **148**, 1633-1637.
3. Knowles, M.R., Robinson, J.M., Wood, R.E., *et al.* (1997). Ion Composition of Airway Surface Liquid of Patients with Cystic Fibrosis as Compared with Normal and Disease-control Subjects. *J. Clin. Invest.* **100**, 2588-2595.
4. Cowley, E.A., Govindaraju, K., Lloyd, D.K., and Eidelman, D.H (1997). Airway Surface Fluid Composition in the Rat Determined by Capillary Electrophoresis. *Am. J. Physiol.,* **273**, L895-L899.

S15.1B
ROLE OF AIRWAY FLUID IONIC COMPOSITION IN CF DISEASE

John W. Hanrahan, Ph.D.
Department of Physiology, McGill University, Montréal, Québec, Canada

Despite considerable progress towards understanding the structure and function of CFTR, fundamental questions remain concerning its role in transepithelial transport, airways defense and pathogenesis of cystic fibrosis. It is important to understand the transport mechanisms and abnormalities that are crucial to the disease process when devising new therapies and testing their efficacies. Two views of salt and fluid absorption across airway epithelium have emerged. In both models, Na^+ absorption is mediated by amiloride-sensitive channels and Cl^- is the counterion which moves across the epithelium to maintain electroneutrality. However in one scheme (model #1), Cl^- ions diffuse through tight junctions between the cells and water rapidly follows so that osmotic gradients do not develop. In the other scheme (model #2), Cl^- ions follow Na^+ by diffusing transcellularly through CFTR channels in the apical membrane, and water absorption does not keep up with salt absorption.

In principle, distinguishing between these hypotheses should be straightforward because they make very different predictions. Model #1 predicts that the NaCl concentration of airway surface liquid (ASL) should resemble that of plasma and should be unchanged in CF. Lack of CFTR protein in the apical membrane should upregulate both sodium channel activity (1) and isotonic fluid absorption, thereby reducing the volume of ASL and hindering mucociliary clearance. Model #2 predicts that the NaCl concentration of ASL should be lower than that of plasma, and this concentration difference between ASL and plasma should be diminished in CF because salt absorption requires CFTR. According to this hypothesis, the lack of CFTR should lead to elevated salt concentration and reduced antimicrobial activity on the epithelial surface (2).

In practice, there has been little consensus concerning the composition of ASL or the route of transepithelial Cl^- absorption. The periciliary sol layer which covers the cells is only microns deep, therefore the volumes available are small and difficult to collect and analyze. Absorption onto filter paper and aspiration into fine capillaries have been used successfully to obtain samples *in vivo*, but such methods could potentially trigger local fluid secretion that modifies the ASL being sampled. This is particularly true for the large airways, where submucosal glands may elaborate a fluid which differs from that normally present on the airway surface. Small samples are also susceptible to evaporation, which could lead to overestimation of the salt concentration in ASL. An alternative approach is to place fluid onto the surface of normal or CF airway epithelial cultures and monitor its composition with time. It remains uncertain if such cultures fully recapitulate the transport processes occurring *in vivo*. Despite these caveats, many studies have reported salt concentrations of ASL collected *in vivo* and *in vitro*.

Surface liquid absorbed onto dry filter paper from the large airways of normal humans and studied by X-ray microanalysis yielded ~83 mM Na^+ in normal individuals and 125 mM in CF (3). The estimates from normal individuals agree with those reported by Wager et al. (4). Using direct aspiration, Gilljam et al. (5) reported that Cl^- concentration was elevated in liquid from CF airways (170 mM vs. 85 mM in normals). ASL collected by aspiration and analyzed by capillary electrophoresis yielded values of 41 mM (rat; ref. 6) and 87 mM (mouse; 7). These reports are most compatible with model #2, but high salt concentrations have also been reported for ASL from dog and ferret trachea (158 - 172 mM Na^+; 8), and horse trachea (130-140 mM Na^+; 9). Using a modified filter paper method, Knowles et al. (10) found ~115 mM Na^+ and no difference between normal and CF individuals, consistent with model #1. Until technical problems are overcome and there is consensus regarding the ionic composition *in vivo*, it will not be possible to use ASL composition as a criterion for distinguishing between models #1 and #2. The site of collection may be important since both models may be correct, but only for particular regions of the airways. Finally, submucosal glands must eventually be incorporated into a model since they express CFTR at relatively high levels and contribute to ASL in the large airways.

If measuring ASL composition *in vivo* is problematic, which *in vitro* experiments might be useful for distinguishing between the models? One direct test would be to measure net Na^+ absorption (using radiotracers) and net fluid absorption (using a volume marker) under open circuit conditions, that is, in the presence of a spontaneous transepithelial voltage. If model #1 is correct, Na^+ and fluid absorption rate should both be *increased* in CF due to elevated Na^+ channel activity, and to the fact that Cl^- absorption does not require CFTR. A more precise test of model #1 would be to examine the effects of anion substitutions on net Na^+ flux (again measured using radioactive sodium). Amiloride-sensitive Na^+ absorption should only be inhibited by Cl^- removal if the replacement anion cannot pass through the tight junctions. Anions that permeate readily through the tight

junctions should have little effect on sodium fluxes under these conditions even if they are impermeant in CFTR. If model #2 is correct, Na^+ and fluid absorption should both be *decreased* in CF since CFTR channels are required for Cl^- ions to follow Na^+ in model #2, and absorption would stop without a permeant counterion. Amiloride-sensitive Na^+ should be blocked by replacing luminal Cl^- with any anion that is impermeant in the CFTR channel.

How would water permeability impact these models? The airways have significant osmotic water permeability; lower than leaky epithelia such as the renal proximal tubule, but higher than some other urinary epithelia that maintain osmotic gradients. The osmotic pressure of ASL depends on the rate of active salt transport and on transepithelial osmotic permeability, in other words, the ability of water to follow salt absorption. Moderate water permeability would be expected to diminish NaCl concentration gradients between the ASL and plasma, but would not exclude model #2 if ASL failed to reach osmotic equilibrium. If osmotic equilibration is rapid and the ASL is hypotonic, then some other force must oppose the osmotic effect of NaCl absorption. The force of surface tension generated by cilia is in the right direction (11), but its contribution may be reduced by the presence of a mucus layer.

Studies of the small distal airways are needed since these may be most relevant to CF disease. Accurate measurements of ASL composition *in vivo* are essential not only to understand the role of luminal salt (and salt-sensitive antimicrobials) in airways defense, but also to design physiologically-relevant solutions for *in vitro* studies, and to validate cell cultures. A culture preparation that produces fluid which is identical to ASL in normal or CF patients is probably good for transport studies. The most difficult part may be obtaining the gold standard; the ionic composition of ASL *in vivo*.

References

1. R. C. Boucher, C. U. Cotton, J. T. Gatzy, M. R. Knowles, and J. R. Yankaskas. Evidence for reduced Cl^- and increased Na^+ permeability in cystic fibrosis human primary cell cultures. *J.Physiol.(Lond.)* 405:77-103, 1988.
2. J. J. Smith, S. M. Travis, E. P. Greenberg, and M. J. Welsh. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85 (2):229-236, 1996.
3. L. Joris, I. Dab, and P. M. Quinton. Elemental composition of human airway surface fluid in healthy and diseased airways. *Am.Rev.Respir.Dis.* 148:1633-1637, 1993.
4. G.N. Wager, N. Church, J.T. Gatzy, R.C. Boucher, and M.R. Knowles. Airway surface liquid (ASL) composition in normal humans. *Am. Rev. Respir. Dis.* 141:A106, 1990.(abstract)
5. H. Gilljam, A. Ellin, B. Strandvik. Increased bronchial chloride concentration in cystic fibrosis. *Scand. J. Clin. Lab. Invest.* 49:121-124, 1989.
6. E.A. Cowley, K. Govindaraju, D.K. Lloyd, and D.H. Eidelman. Airway surface fluid composition in the rat determined by capillary electrophoresis. *Am. J. Physiol.* 273: L895-899, 1997.
7. E.A. Cowley, K. Govindaraju, D.K. Lloyd, and D.H. Eidelman. Is mouse airway surface fluid hypotonic? *Ped. Pulmonol. Suppl.* 14, 233, 1997. (abstract)
8. N.P. Robinson, H. Kyle, S.E. Webber, and J.G. Widdicombe. Electrolyte and other chemical concentrations in the tracheal airway and mucus. *J. Appl. Physiol.* 66:2129-2135, 1989.
9. L. Joris and P.M. Quinton. Filter paper equilibration as a novel technique for in vitro studies of the composition of airway surface liquid. *Am. J. Physiol.* 263: L243-L248.
10. M.R. Knowles, J.M. Robinson, R.E. Wood, et al. Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *J. Clin. Invest.* 100:2588-2595, 1997.
11. J.H. Widdicombe and J.G. Widdicombe. Regulation of human airway surface liquid. *Respir. Physiol.* 99:3-12, 1995.

S15.2
WATER TRANSPORT BY THE LUNG

Alan S. Verkman
Cardiovascular Research Institute & and Cystic Fibrosis Research Center, University of California, San Francisco, San Francisco CA 94143-0521

Substantial quantities of fluid move across epithelial and endothelial barriers in lung. In the perinatal lung, fluid absorption from the airspace occurs in preparation for alveolar respiration. In the adult lung, movement of salt and water between the airspace and capillary compartments is involved in the control of airspace hydration and water content of the airway surface liquid layer, and in the formation and resolution of pulmonary edema. Several potential barriers exist for the movement of fluid in lung. The trachea and large airways contain an epithelial cell layer, but represent a small fraction of the total surface area available for fluid movement. The more

numerous smaller airways also contain an epithelial cell layer, but still represent a relatively small surface area. In human lung, the total airway surface area is ~1.4 square meters, which represents only about one percent of the alveolar surface area of 143 square meters. The alveolar epithelium thus provides the major surface for the movement of salt and water. The alveolar epithelium contains two cell types: type I cells, which make up the majority of the alveolar epithelial surface but whose function is relatively poorly understood, and type II cells, which produce surfactant and transport salt actively. Movement of water between the airspace and capillary compartments also encounters potential permeability barriers in the interstitium and capillary endothelium.

The general paradigm for the transport of fluid across epithelia in lung is that active salt transport drives osmotic water movement. Our lab has developed biophysical methods to quantify osmotic water permeability of various barriers in lung. Using a conventional instill/sample method in sheep lung (1) and a pleural surface fluorescence method in mouse lung (2), it was found that osmotic water permeability between the airspace and capillary compartment was very high, with a water permeability coefficient (P_f) of ~0.02 cm/s. P_f was weakly temperature dependent and inhibited by $HgCl_2$, suggesting the involvement of molecular water channels (see below). P_f across alveolar microvessels in intact lung was estimated to be even higher (0.03-0.05 cm/s) by a pleural surface fluorescence method involving filling the airspace with an inert perfluorocarbon (3). Using a stopped-flow light scattering technique, P_f in immunoisolated type I alveolar epithelial cells was found to be exceptionally high (0.07 cm/s), which accounted for the high transalveolar water permeability (4). Water permeability across isolated microperfused distal airways was found to be substantially lower (P_f~0.004 cm/s) than that across alveoli (5). Together these results establish the barrier properties of lung tissues to water movement.

There have been recent advances in understanding the molecular mechanisms of water movement in lung. A family of related molecular water channels (aquaporins) has been identified that currently number 10 in mammals. The aquaporins (AQP) are small hydrophobic membrane proteins (Mr ~30 kDa) with homology to the Major Intrinsic Protein of lens fiber (MIP). Three aquaporins have been localized in lung: AQP1 in microvascular endothelia and some pneumocytes (1), AQP4 in the basolateral membrane of airway epithelium (6), and AQP5 in the apical membrane of type I alveolar epithelial cells. Aquaporin-type water channels have not yet been identified at the basolateral surface of alveolar epithelium or the apical membrane of airway epithelia. The specific localization of aquaporins to endothelial and epithelial cells suggests a possible role in lung water movement. Other indirect evidence supporting a physiological role for aquaporins in lung include the increase in aquaporin expression and lung water permeability around the time of birth (7), and the high water permeability described above.

To study the physiological role of aquaporins, we have generated transgenic null mice lacking AQP1 (8) and AQP4 (9), and knockouts for several other aquaporins are in progress. The AQP1 knockout mice manifest a severe urinary concentrating defect, which was shown to involved defective isosmolar fluid reabsorption in proximal tubule (10) and low water permeability in the descending limb of Henle. The AQP1 knockout mice also have reduced survival at birth, are mildly growth retarded and have defective dietary fat absorption. The AQP4 knockout mice have a mild urinary concentrating defect but no neuromuscular abnormalities. Initial evaluation of lung phenotype of these mice was done (11). Airspace-capillary P_f was reduced 10-fold by AQP1 deletion, but not affected by AQP4 deletion. The reduced water permeability of microvessels in AQP1 null mice was shown to slow the formation of hydrostatically-induced interstitial edema. However, active isosmolar reabsorption of alveolar fluid was not affected by AQP1 or AQP4 deletion. Additional phenotype studies in aquaporin null mice are in progress to determine the role of molecular water channels in lung fluid balance.

References

1. Folkesson, H.G., M.A. Matthay, H. Hasegawa, F. Kheradmand and A.S. Verkman (1994). Transcellular water transport in lung alveolar epithelium through mercurial-sensitive water channels. *Proc. Natl. Acad. Sci. USA* 91:4970-4974.
2. Carter, E.P., M.A. Matthay, J. Farinas and A.S. Verkman (1996). Transalveolar osmotic and diffusional water permeability in intact mouse lung measured by a novel surface fluorescence method. *J. Gen. Physiol.* 108:133-142.
3. Carter, E.P., B.P. Ölveczky, M.A. Matthay and A.S. Verkman (1998). High microvascular endothelial water permeability in mouse lung measured by a pleural surface fluorescence method. *Biophys. J.* 74:2121-2128.
4. Dobbs, L., R. Gonzalez, M.A. Matthay, E.P. Carter, L. Allen and A.S. Verkman (1998). Highly water-permeable type I alveolar epithelial cells confer high water permeability between the airspace and vasculature in rat lung. *Proc. Natl. Acad. Sci. U.S.A.* 95:2991-2996.
5. Folkesson, H., M. Matthay, A. Frigeri and A.S. Verkman (1996). High transepithelial water permeability in microperfused distal airways: evidence for channel-mediated water transport. *J. Clin. Invest.* 97:664-671.
6. Frigeri, A., M. Gropper, C.W. Turck and A.S. Verkman (1995). Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. *Proc. Natl.*

Acad. Sci. USA 92:4328-4331.
7. Carter, E.P., F. Umenishi, M.A. Matthay and A.S. Verkman (1997). Developmental changes in alveolar water permeability in perinatal rabbit lung. *J. Clin. Invest.* 100:1071-1078.
8. Ma, T., B. Yang, A. Gillespie, E.J. Carlson, C.J. Epstein and A.S. Verkman (1998). Severely impaired urinary concentrating ability in transgenic mice lacking aquaporin-1 water channels. *J. Biol. Chem.* 273:4296-4299.
9. Ma, T., B. Yang, A. Gillespie, E.J. Carlson, C.J. Epstein and A.S. Verkman (1997). Generation and phenotype of a transgenic knock-out mouse lacking the mercurial-insensitive water channel aquaporin-4. *J. Clin. Invest.* 100:957-962.
10. Schnermann, J., C.L. Chou, T. Ma, T. Traynor, M.A. Knepper and A.S. Verkman (1998). Defective proximal tubular reabsorption in transgenic aquaporin-1 null mice. *Proc. Natl. Acad. Sci. U.S.A.* 95: In press.
11. Bai, C., M.A. Matthay, T. Ma and A.S. Verkman (1998). Role of aquaporin water channels in lung fluid transport: phenotype analysis of aquaporin 1 and 4 knockout mice. Submitted.

S15.3
GENOTYPE/PHENOTYPE IN RELATION TO LUNG DISEASE

P.R. Durie, E. Tullis, M. Corey, L. Ellis, S. Martin, K. Jarvi, J. Zielenski, M. Gilljam and L-C. Tsui
The Research Institute, The Hospital for Sick Children and St. Michael's Hospital; and from Departments of Pediatrics, Medicine, Public Health Sciences and Molecular and Medical Genetics, University of Toronto, Toronto, Canada

Since the CF gene was cloned a large number of CFTR gene mutations (more than 700) have been identified. There have been extensive efforts to establish correlations between genotype and phenotype. Surveys of large patient populations confirm a close association between genotype and pancreatic function (pancreatic sufficiency or pancreatic insufficiency). In general, patients with "mild" mutations that confer the pancreatic sufficient phenotype have less severe pulmonary disease. Nevertheless, lung involvement shows a wide range of severity even in patients with identical CFTR gene mutations such as individuals who are homozygous for the most common gene mutation (ΔF508). Furthermore, over the past nine years, the ability to detect CF mutation and to determine the bioelectric properties of the nasal epithelium (nasal PD) has greatly expanded the CF clinical spectrum. Many of these patients have monosymptomatic findings at presentation which may include electrolyte abnormalities, pancreatitis, liver disease, sinusitis and in males some form of obstructive azoospermia. In such cases demonstration of CF causing gene mutations or the *in vivo* demonstration of abnormal ion transport across the nasal epithelium, can be used for diagnostic purposes.

This presentation will highlight our ongoing efforts to define nasal potential difference measurements and the CF phenotype in patients who present with "atypical" or "monosymptomatic" forms of cystic fibrosis (Supported by the Canadian Cystic Fibrosis Foundation and NIH).

References

1. Rosenstein, B.J., Cutting, G.R., for the Cystic Fibrosis Foundation Consensus Panel (Boat, T.F., Cantin, A.M., Dorkin, H.L., **Durie, P**., FitzSimmons, S., Knowles, M., Saiman, L., Tullis, E.). The diagnosis of cystic fibrosis: A consensus statement. J. Pediatr. 132:589-595, 1998.
2. Wilson, D.D., Ellis, L., Zielenski, J., Corey, M., Ip, W., Tsui, L-C., Tullis, E., Knowles, M.R., **Durie, P.R.** Uncertainty in the diagnosis of cystic fibrosis: Possible role of *in vivo* nasal potential difference measurements. J. Pediatr. 132:596-599, 1998.

S15.4
CF LUNG CLINICAL PATHOBIOLOGY: UNANSWERED QUESTIONS

Pamela B. Davis, M.D., Ph.D.
Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland OH

A critical issue in the design of CF therapeutics is how the basic defect in CF, which is clearly understood to be the malfunction of a cAMP-regulated chloride channel in the apical membrane, translates into the lung disease which ultimately claims the lives of the patients and which is characterized by persistent infection with particular bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* and persistent neutrophilic infiltrate. This problem is central, because if we fail to correct the aspect of the CF basic defect which leads to these particular downstream consequences, we may not ultimately benefit the patient. It is already clear that for various con-

sequences of the CF defect to be corrected, we need different degrees of correction. For example, the dose of gene therapy reagent required to correct the increased epithelial sodium reabsorption is much greater than is required to correct the chloride transport defect, and the abnormal sulfation patterns of CF mucins are corrected by lipid-DNA complexes which barely affect chloride transport in xenografts. Thus, it is crucial to understand the connection between defective CFTR and CF lung pathobiology for successful treatment of the basic defect.

The initial vulnerability to infection in CF is still unexplained, although several compelling hypotheses have been advanced in the last few years. One of them, the salt-sensitivity of nonspecific antibacterial defenses, postulates that CF patients have increased salt content of their airway surface fluid, and suggests that this abnormality gives rise to abnormal function of nonspecific antibacterial reagents, such as defensins or lysozyme, in this milieu. A variant of this hypothesis, which does not require that CF airway surface fluid have abnormal salt content, proposes that some of the nonspecific airway defenses, such as defensins or NO, are deficient in CF. Another hypothesis is that CFTR itself is the receptor for certain pathogenic bacteria, including pseudomonas, and failure of CFTR function leads to failure of binding, ingestion, and killing of bacteria by sloughing the infected epithelial cell. A third hypothesis suggests that binding of certain bacteria to CF epithelial cells is increased, and increased adherence makes for initiation of infection as well as an overexuberant inflammatory response. Another simple possibility is that the abnormal quantity of airway surface liquid interferes with normal mucociliary clearance, allowing bacteria to be trapped in the CF lung. There is evidence for and against each of these hypotheses, but there seems little doubt that bacteria are able to establish residence in the CF lung where they are cleared from the normal lung.

It also seems quite likely that there is a connection between abnormal CFTR function and an excessive inflammatory response in the airways. Clinical studies and animal models indicate that in the CF lung disease, the inflammatory response to bacterial infection is excessive; anti-inflammatory therapy can be administered without harm, and actually with considerable benefit, to the patient. In particular, in vitro, excessive release of neutrophil chemoattractants occurs from CF cells when they are stimulated with CF pathogens, and CF mice are more vulnerable to death from *Pseudomonas aeruginosa*, mounting an exuberant inflammatory response compared to normal littermates even though the bacterial burden is comparable in the two groups of animals.

In order to investigate these two pathophysiologic features of CF, it will be important to understand in which specific cells CFTR is expressed, how its dysfunction leads to various Asecondary@ manifestations of CF (via changes in Cl- transport, direct interaction with other proteins, other cellular functions of CFTR?), and which of the primary or secondary manifestations of CF is (are) crucial for the initiation of infection and the exuberant inflammatory response.

These two abnormalities, vulnerability to the initial bacterial infection and the propensity to respond with excessive inflammation, are the most important aspects of lung pathobiology to be related to the basic defect. Evidence currently in hand does not permit us to choose among the various hypotheses which have been advanced to explain these phenomena, which need not be mutually exclusive. This particular aspect of CF lung pathobiology must be understood to make intelligent choices in design of definitive therapeutic agents.

S16.1
REGULATION OF THE MUC2 MUCIN GENE

J. R. Gum, Jr.[1,2], J. W. Hicks[1,2], A. M. Gillespie[2], E. Carlson[2],
L. Komuves[2], S. Karnik[1,2], J. C. Hong[1,2], S. Kuan[3], C. Epstein[2], and Y. S. Kim[1,2].
VA Medical Center[1] & UCSF[2], San Francisco, CA, and the University of Chicago[3], Chicago, Il, USA

Mucins are the principal structural components of the mucus gels that coat and protect the delicate epithelia of the gastrointestinal, reproductive, and respiratory tracts (1). Mucin genes are expressed only in specific tissues and cells, their transcription being exquisitely regulated. Inappropriate and/or over expression of mucin genes occurs in several diseases and in cystic fibrosis the inappropriate expression of increased quantities of *MUC2* may lead to significant pathology in both the small intestine and airways. As part of an effort to understand the regulation of mucin gene expression in normal and diseased tissues, the 5^{l}-flanking region of the *MUC2* gene was isolated (2,3). DNA segments containing the 5^{l}-flanking region of *MUC2* have been shown to have promoter activity in cultured cells, and several elements that appear to participate in its regulation have been identified (3-6). Studies utilizing cultured cells may not accurately model all aspects of *MUC2* transcriptional control however, in part because transfectable, differentiated cell lines expressing the high levels of gene product found in goblet cells *in vivo* are non-existent. To circumvent this problem and to gain further insight into *MUC2* gene regulation, we have examined the expression of a *MUC2*-human growth hormone (hGH) promoter-reporter construct in transgenic mice. The transgene was prepared by fusing bases -2864 to +17 of the human *MUC2* 5^{l}- flanking region into the 5'-untranslated region of a human growth hormone (hGH) reporter gene. Eight transgenic strains were obtained and four were

found to express reporter. Reporter expression levels were variable and not strictly dependent upon transgene copy number. hGH message expression was found to be highest in the distal small intestine, only one strain expressed comparable levels of reporter in the colon. This contrasts with endogenous *MUC2* expression, which is expressed at its highest levels in the colon. All non-*MUC2* expressing tissues examined also failed to express reporter. RNase protection analysis indicated a similar transcription start site for the transgene as for endogenous *MUC2* message. Immunohistochemical analysis indicated goblet cell specific expression of reporter initiating deep in the crypts, also similar to endogenous *MUC2* gene expression. Immunoelectron microscopic analysis detected hGH expression only in granular goblet cells in the small intestine. These results indicate that bases -2864 to +17 of the *MUC2* 5'-flanking sequence contain elements sufficient for the appropriate expression of *MUC2* in the small intestine while elements located outside this region appear to be required for colonic expression, implying that a different set of regulatory factors effect expression in the two tissues. The -2864 to +17 region is also sufficient for the goblet cell specific expression of *MUC2*. Also, the marked integration site dependence for transgene expression between pedigrees suggests a possible regulatory significance for the clustering of secretory mucin genes found at chromosome 11p15. Thus many, but not all, of the elements necessary for controlling *MUC2* expression appear to reside in the initial 2864 bases of its 5'-flanking sequence. Work in progress includes the examination of mice containing promoter/reporter constructs initiating at base -8112 and base -343 of the *MUC2* 5'-flanking sequence. These additional pedigrees may help identify specific promoter elements important for *MUC2* regulation in individual tissues.

Reference

1. Gum, J. R. (1995). Human mucin glycoproteins: varied structures predict diverse properties and specific functions. Biochem. Soc. Trans. **23**, 795-799.
2. Gum, J. R., Hicks, J. W., Toribara, N. W., Siddiki, B. and Kim, Y. S. (1994). Molecular cloning of human intestinal mucin (*MUC2*) cDNA: Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. J. Biol. Chem. **269**, 2440-2446.
3. Gum, J. R., Hicks, J. W. and Kim, Y. S. (1997). Identification and Characterization of the MUC2 (Human Intestinal Mucin) Gene 5'-Flanking Region: Promoter Activity in Cultured Cells. Biochem. J. **324**, 259-267.
4. Velcich, A., Palumbo, L., Selleri, L., Evans, G. and Augenlicht, L. (1997). Organization and regulatory aspects of the human intestinal mucin gene (MUC2) locus. J. Biol. Chem. **272**, 7968-7976.
5. Li, J. D., Dohrman, A. F., Gallup, M., Miyata, S., Gum, J. R., Kim, Y. S., Nadel, J. A., Prince, A. and Basbaum, C. B. (1997). Transcriptional activation of mucin by Pseudomonas aeruginosa lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. Proc. Natl. Acad. Sci. USA **94**, 967-972.
6. Li, J. D., Feng, W., Gallup, M., Kim, J. H., Gum, J. R., Kim, Y. and Basbaum, C. (1998). Activation of NF-kB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for Pseudomonas aeruginosa-induced mucin overproduction in epithelial cells. Proc. Natl. Acad. Sci. USA **95**, 5718-5723.

S16.2
AIRWAY STEM CELLS

John F. Engelhardt

Department of Anatomy and Cell Biology, University of Iowa Medical Center, Iowa City, Iowa 52242

The conducting airways of the adult human lung can be grossly divided into tracheal/bronchial and bronchiolar epithelia. The proximal airways, which include the trachea and bronchi, are generally thought to be composed of five major cell types including: basal, intermediate, goblet, non-ciliated columnar, and ciliated cells. In contrast, the bronchioles are predominantly composed of Clara and ciliated cells. The theoretical considerations of a stem cell compartment in the conducting airways of the adult lung has long eluded concrete scientific confirmation. It is generally accepted that both secretory and basal cells have the capacity to divide within the proximal surface airway epithelium. In contrast ciliated cells are thought to be terminally differentiated and non-dividing . Several approaches have been used to identify pluripotent progenitor cells in the tracheas of rats including radiolabeling with ^{3}H-thymidine and cell purification strategies followed by reconstitution in denuded tracheal grafts. In addition, a third approach utilizing recombinant retroviruses to genetically track lineage fates has also been used in rat and human bronchial xenografts . However, the pluripotent capacity of basal and secretory cells remains subject to general debate.

In the bronchiolar airways, Clara cells are generally considered to be the regenerating compartment which give rise to non-dividing ciliated cells . However, studies which have demonstrated heterogeneity in the gene expression patterns of Clara cells, suggest that this population may in turn be composed of more than one phenotype . It is currently less clear whether basal cells or secretory cells are the stem cell compartment in the proximal conducting airways of the lung. The concept of a discrete self-renewing compartment of stem cells in the proximal airway is based on other well defined systems such as blood, intestine, and epidermis. In continuously renewing epithelial tissues such as the intestine, a subcompartment of cells are

thought to contain undifferentiated stem cells capable of indefinite self-renewal and maintenance of transiently amplifying cells which ultimately give rise to differentiated cell populations . Similar hypotheses have been proposed for the airway . Using retroviral lineage marking, studies in human bronchial xenografts suggests that a subpopulation of basal cells may represent airway stem cells which are capable of giving rise to diverse subcompartments of progenitor cells in the airway with defined capacity for differentiation. In accordance with the theoretical considerations of stem cell biology, this basal cell type appears to have a large capacity for pluripotent regeneration and is in low abundance in the airway . In contrast to hypotheses of programmed cellular differentiation, others have proposed more plastic models of self-renewing airway epithelia where both basal and goblet cells have the capacity for pluripotent differentiation . However, given the diversity of epithelial cell types composing the conducting airways between different species, caution should be used when comparing results between different model systems.

The ability to conclusively identify a stem cell compartment in the airway has been hindered by the lack of identifiable marker genes for this population of cells. With an interest in characterizing progenitor cells which give rise to submucosal glands in the airway, our laboratory has recently identified a HMG-transcription factor called Lef1 which is expressed in a subset of surface airway epithelial cells just prior to gland bud formation in the ferret trachea . Functional studies in Lef1 deficient mice and human bronchial xenografts have demonstrated that expression of this transcription factor is necessary but not sufficient for gland development in the airway. Based on retroviral marking studies, this population of gland progenitor cells appears to have pluripotent capacity for both surface airway epithelial and submucosal gland differentiation as well as a high capacity for regeneration . Hence, this compartment of gland progenitor cells may represent a potential stem cell compartment in the airway. Current efforts are focused on identifying what genes may control the transcriptional switch of Lef1 gene expression and hence progenitor cell commitment in the formation of submucosal glands.

The identification of airway stem cells is highly relevant to the development of effective gene targeting strategies to the lung in cystic fibrosis. The use of integrating vectors to target airway stem cells capable of reconstituting both the surface airway epithelium and submucosal gland regions in the lung will lead to more persistent transgene expression and prolonged correction of disease pathology. Additionally, an increased knowledge of progenitor/stem cell differentiation in the airway will also enhance our understanding of mechanisms responsible for goblet cell metaplasia/hyperplasia and submucosal gland hypertrophy/hyperplasia associated with the hypersecretory condition seen in the lungs of cystic fibrosis patients.

References

1. Boers, J.E., A.W. Ambergen, and F. Thunnissen. Number and proliferation of basal and parabasal cells in normal human airway epithelium [In Process Citation]. *Am J Respir Crit Care Med* 157:2000-6, 1998.
2. Breuer, R., G. Zajicek, T.G. Christensen, E.C. Lucey, and G.L. Snider. Cell kinetics of normal adult hamster bronchial epithilium in the steady state. *Am J Respir Cell Mol Biol* 2: 51-8, 1990.
3. Donnelly, G.M., D.G. Haack, and C.S. Heird. Tracheal epithelium: cell kinetics and differentiation in normal rat tissue. *Cell Tissue Kinet* 15: 119-30, 1982.
4. Duan, D.A. Sehgal, J. Yao, and J.F. Engelhardt. Lef1 Transcription Factor Expression Defines Airway Progenitor Cell Targets for In Utero Gene Thereapy of Submucosal Gland in Cystic Fibrosis. *Am J Respir Cell Mol Biol* 18: 750-8, 1998.
5. Engelhardt, J.F., E.D. Allen, and J.M. Wilson. Reconstitution of tracheal grafts with a genetically modified epthelium. *Proc Natl Acad Sci* USA 88: 11192-6, 1991.
6. Engelhardt, J.F., H. Schlossberg, J.R. Yankaskas, and L. Dudus. Progenitor cells of the adult human airway involved in submucosal gland development. *Development* 121: 2031-46, 1995.
7. Engelhardt, J.F., M. Zepeda, J.A. Cohn, J.R. Yankaskas, and J.M. Wilson. Expression of the cystic fibrosis gene in adult human lung. *J Clin Invest* 93: 737-49, 1994.
8. Evans, M.J., S.G. Shami, L.J. Cabral-Anderson, and N.P. Dekker. Role of nonciliated cells in renewal of the bronchial epithelium of rats exposed to NO2. *Am J Pathol* 123: 126-33, 1986.
9. Inayama, Y., G.E. Hook, A.R. Brody, G.S. Cameron, A.M. Jetten, L.B. Gilmore, T. Gray, and P. Nettesheim. The differentiation potential of tracheal basal cells. *Lab Invest* 58: 706-17, 1988.
10. Inayama, Y., H. Kitamura, T. Shibagaki, Y. Usuda, T. Ito, Y. Nakatani, and M. Kanisawa. In vivo growth and differentiation potential of tracheal basal cells of rabbits in vitamin A deficiency. *Int J Exp Pathol* 77: 89-97, 1996.
11. Nettesheim, P., A.M. Jetten, Y. Inayama, A.R. Brody, M.A. George, L.B. Gilmore, T. Gray, and G.E. Hook. Pathways of differentiation of airway epithelial cells. *Environ Health Perspect* 85: 317-29, 1990.
12. Potten, C.S., C. Booth, and D.M. Pritchard. The intestinal epithelial stem cell: the mucosal governor. *Int J Exp Pathol* 78: 219-43, 1997.
13. Randell, S.H., Progenitor-progeny relationships in airway epithelium. *Chest* 101: 11S-16S, 1992.
14. Randell, S.H., C.E. Comment, F.C. Ramaekers, and P. Nettesheim. Properties of rat tracheal epithelial cells separated on expression of cell surface alpha-galactosyl end groups. *Am J Respir Cell Mol Biol* 4: 544-54, 1991.
15. Zepeda, M.L., M.R. Chinoy, and J.M. Wilson. Characterization of stem cells in human airway capable of reconstituting a fully differentiated bronchial epithelium. *Somat Cell Mol Genet* 21: 61-73, 1995.

S16.3
HUMAN AIRWAY EPITHELIAL TIGHT JUNCTIONS

RWA Godfrey and PK Jeffery
Lung Pathology Unit, Imperial College School of Medicine at the Royal Brompton Hospital, London SW3 6NP, UK

Airway epithelial tight junctions form a selectively permeable barrier to the flux of fluid, ions, macromolecules and inflammatory cells across the epithelium by regulating movement through the paracellular pathway (the fluid-filled spaces between cells)[1-3]. The barrier characteristics of the tight junctions are thought to be determined by a transmembrane component that extends from the membrane bilayer of one cell into the paracellular channel to form contact with a similar structure from the adjacent cell thus limiting movement through the paracellular pathway. A 65kD membrane protein, occludin, is reported to be a principal component of this transmembrane structure[4]. A number of proteins found in the cell cytoplasm have been shown to be associated with the tight junction, in particular Z0-1, Z0-2, Z0-3, cingulin, p130, symplekin, ZA-1TJ and the 7H6 antigen[5,6]. At present exactly how these transmembrane and cytoplasmic components regulate and modify paracellular permeability is unclear[5].

In human airways, the majority of data on the ultrastructure of tight junctions has been gained by applying freeze-fracture electron microscopy, a specialised technique which splits membranes along their hydrophobic core enabling the internal architecture to be viewed *en face*. Applying this technique, the tight junctional belt appears as a interconnecting network of strands and grooves. The number of strands comprising this belt is hypothesised to correlate with the permeability characteristics of the junction[7]. To date only three groups of workers have published data on tight junction morphology in normal human airways[8-10]. The largest quantitative study was undertaken by Godfrey et al (1992) who studied tight junction morphology in main (airway level I) and lobar bronchi (airway level II), where, on average, the junctional belt was found to be 0.5mm deep and to be comprised of 11 strands with 1.4 interconnections per mm strand length. Limited functional data has been published, reflecting the difficulty in gaining such measurements *in situ*. The most effective way to obtain such information is to measure the transepithelial resistance generated by the epithelium (TER). The results are lumen negative[11,12] and the high proximal negative values have been demonstrated to became progressively less negative peripherally[12].

In the airways of patients with cystic fibrosis abnormalities in tight junction morphology have been consistently reported. A basal extension of the junctional elements comprising the junctional belt and the presence of isolated junctional elements distant from the apico-lateral belt have been described[13-15]. Godfrey et al (1993) reported similar changes in control human postmortem tissue (ie from patients dying of non-respiratory causes), however the abnormalities found in cystic fibrosis were found to occur before postmortem degradation, (ie in tissue removed at transplantation) confirming a disease-related abnormality. These abnormalities in tight junction structure are likely to be secondary changes linked to the chronic infection and inflammation found in the airways of patients with cystic fibrosis and not due directly to the gene defect. The proliferation of junctional elements found in cystic fibrosis may reduce the passage of water and ions through the paracellular pathway and so confound the problem of the lack of hydration of the airway secretions characteristic to cystic fibrosis[15].

References

1. Schneeberger EE, Lynch RD. Structure, function, and regulation of cellular tight junctions. J Appl Phys 1992; 262:L647-L661.
2. Godfrey RWA. Human airway epithelial tight junctions. Micr Res Tech 1997; 38:488-499.
3. Madara JL. Regulation of the movement of solutes across tight junctions. Ann Rev Physiol 1998; 60:143-159.
4. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S. Occludin: A novel intetgral membrane protein localizing at tight junctions. J Cell Biol 1993; 123:1777-1788.
5. Mitic LL, Anderson JM. Molecular architecture of tight junctions. Ann Rev Physiol 1998; 60:121-142.
6. Haskins J, Gu L, Wittchen ES, Hibbard J, Stevenson BR. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. J Cell Biol 1998; 141:199-208.
7. Claude PC. Morphological factors influencing transepithelial permeability: a model for the resistance of the cannula occludens. J Membrane Biol 1978; 39:219-232.
8. Carson JL, Collier AM, Knowles MR, Boucher RC. Ultrastructural characterization of epithelial cell membranes in normal human conducting airway epithelium: a freeze fracture study. Am J Anat 1985; 173:257-268.
9. Elia C, Bucca C, Rolla G, Scappaticci E, Cantino D. A freeze-fracture study of tight junctions in human bronchial epithelium in normal, bronchitic and asthmatic subjects. J Submic Cytol Pathol 1988; 20:509-517.
10. Godfrey RWA, Severs NJ, Jeffery PK. Freeze-fracture morphology and quantification of human bronchial epithelial tight junctions. Am J Respir Cell Molec Biol 1992; 6:453-458.

11. Knowles M, Gatzy JT, Boucher R. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. N Engl J Med 1981; 305:1489-1494.
12. Alton EWFW, Khagani A, Taylor RFH, Logan-Sinclair R, Yacoub M, Geddes DM. Effect of heart-lung transplantation on airway potential difference in patients with and without cystic fibrosis. Eur Respir J 1991; 4:5-9.
13. Severs NJ, Jeffery PK. Freeze-fracture demonstration of tight junction abnormalities in airway epithelium of cystic fisbrosis (CF) in patients. (IOP Publishing Ltd). Inst Phys Conf Ser 1988; 3:141-142.
14. Carson JL, Collier AM, Gambling TM, Knowles MR, Boucher RC. Ultrastructure of airway epithelial cells among patients with cystic fibrosis. Hum Pathol 1990; 21:640-647.
15. Godrey RWA, Severs NJ, Jeffery PK. Structural alterations of airway epithelial tight junctions in cystic fibrosis: Comparison of transplant and post-mortem tissue. AmJ RespirCellMolBiol 1993;9:148–156.

S16.4
DYNAMICS OF AIRWAY EPITHELIUM WOUND REPAIR

E. Puchelle[1], JM. Zahm[1], S. de Bentzmann[1], J. Jacquot[1], JM. Tournier[1], R. Tirouvanziam[2], B. Péault[2].
[1]*Unité INSERM 314, IFR 53, Reims, France.*
[2]*Institut d'Embryologie Cellulaire et Moléculaire, CNRS 9064, Nogent sur Marne, France.*

The airway epithelium not only represents a mechanical barrier to the environmental agressions (bacteria, viruses, oxidants…) ; the diverse cells lining the airways are also able to respond to injury by producing a variety of defense factors that operate to protect the respiratory tract. Moreover, airway epithelial cells can rapidly shift their phenotype in order to adapt to changes in the local environment or to repair the injured airway epithelium. The barrrier function is maintained at the apex of the airway epithelial cells by junctional complexes including ZO1 (*Zona occludens*) tight junctional proteins which closely interact with actin microfilaments. The disruption of actin filaments by cytochalasin B or D results in the opening of tight junctions, decreased transepithelial resistance and increased epithelial permeability (1). Other junctional complexes, like desmosomes and gap junctions, situated at the interface between basal cells and columnar cells, also play a critical role in the maintenance of the airway epithelium integrity. After ATP depletion, which occurs following oxidative stress or ischemia during lung transplantation, loss of airway epithelium integrity is associated with the disappearance of junctional complexes in particular, desmosomes and gap junctions. Moreover, depolymerization of F-actin filaments by ATP depletion is accompanied by a loss of epithelial cell polarity and inhibition of CFTR apical distribution (2). We also observed that c-AMP dependent Cl^- secretion and intracellular diffusion capacity of a low molecular weight diffusible fluorescent dye through gap junctions were completely inhibited following ATP depletion. Repair of the wounded airway epithelium involves several major mechanisms including spreading and migration of the cells at the margin of the wound as well as proliferation of the cells located behind the wound. Airway epithelial cells have the capacity by themselves, without the contribution of any other non epithelial cells, to migrate at a speed ranging between 10 and 30 μm/h and to re-establish a barrier junction within 48-72 hours according to the size of the wound. Different molecules are directly involved in the airway epithelium repair (3) : fibronectin, α 5 β1 integrin, matrix metalloproteinases of epithelial origin (gelatinase B and stromelysin 3). Cell spreading and migration induce an apical redistribution of fibronectin and its cellular receptor, the $\alpha 5\beta 1$ integrins, which have been shown to represent specific sites of *Pseudomonas* adherence (4). Virulence factors associated with bacterial binding to these dedifferentiated spreading and migrating cells, may impair epithelium wound repair. In CF, *Pseudomonas aeruginosa* (Ps.a) plays a major role on the airway mucosal damage. Ps.a produces a number of virulence factors, which have been demonstrated, in airway cell cultures and in animal models or even in human airway epithelium, capable of increasing the airway mucosal permeability (5). Changes in ionic environment of the airway surface fluid due to CFTR defect are also capable of inducing an upregulation of Ps.a virulence factors and increased IL-8 content in ASL (6), leading to an efflux of inflammatory cells. Virulence factors produced by Ps.a such as elastase and exotoxin A diminish the epithelial barrier function by damaging, or inhibiting the synthesis of associated proteins. Such virulence factors open the junctions sufficiently to allow bacteria to cross the epithelial barrier and may render cells unable to repair damaged tight junctions. We have recently investigated the *in vitro* effect of virulence factors secreted by Ps.a (PAO1 strain) on airway epithelium wound repair. Incubation of wounded cell cultures with PAO1 bacteria-free culture supernatant significantly decreased the wound repair speed and the proliferation rate of the cells engaged in the repair process. The delay in the wound repair was associated with an alteration of the epithelial integrity which was not restored 24 hours after the wound closure in contrast to control experiments. During the wound repair process, activated forms of matrix metalloproteinases (MMP 2: 72kDa gelatinase and of MMP9 : 92 kDa gelatinase) increased, in a dose-dependent manner, in presence of the PAO1 virulence factors.

Nevertheless, the *in vitro* analysis of epithelium wound repair does not get an insight into the *in vivo* barrier defense capacity of the airway epithelium, particularly in CF. Recent studies using the *in vivo* model of well-differ-

entiated human fetal tracheal xenografts in the SCID mouse (7) suggest that, prior to infection, the leukocyte burden is increased in both the intra-and sub-epithelial compartments of CF airways as compared to non-CF. Concomitantly, we observed an up to 8-fold increase in the IL-8 content of the airway luminal liquid collected in CF vs non-CF xenografts. Following Ps.a infection, CF xenografts showed a rapid and major exfoliation of apical cells, which was associated with the intraepithelial infiltration of leucocytes of both human and murine origins. This marked alteration of the epithelial integrity allowed Ps.a to gain access to critical adherence sites, at the level of basal cells and the basal lamina. These results and others are thus in favour of an increased wounding process of CF airway epithelium after Ps.a infection, due at least in part to a native inflammatory disorder. The increased susceptibility of the respiratory epithelium to colonization by Ps.a and the greater severity of infection have also recently been reported (8).

All these studies underline the higher susceptibility of CF airway epithelium to bacterial infection. Moreover, dynamics of airway epithelium wound repair appear to be severely disturbed after *Pseudomonas* infection. It can be hypothesized that in CF, impairment of epithelial integrity will favour the adherence of bacteria to receptors exposed on the baso-lateral membranes of epithelial cells and partly explains the adherence of Ps.a to the repairing airway epithelium. The development of motogenic molecules able to accelerate or stimulate wound repair such as epidermal growth factor (9), represents future therapeutic strategies designed to restore the integrity of the wounded airway epithelium in CF.

Supported by A.F.L.M.

References

1. Puchelle, E., and Zahm J.M. (1996). Repair processes of the airway epithelium. In: Airways and environments: from injury to repair; M Dekker. Inc. Publish., Series Lung Biology in Health and Disease. Edited by J. Chretien and D. Dusser. 93, chap. 7 : 157-182.
2. Brezillon, S., Zahm, J.M., Pierrot, D., Gaillard, D., Hinnrasky, J., Klossek, JM., Tümmler, B., Puchelle E. (1997). ATP depletion induces a loss of respiratory epithelium functional integrity and down-regulates CFTR expression. J. Biol. Chem. 272 : 27830-27838.
3. Herard, A.L., Pierrot, D., Hinnrasky, J., Kaplan, H., Sheppard, D., Puchelle, E., Zahm, J.M. (1996). Fibronectin and its α5 β1-integrin receptor are involved in the wound-repair process of airway epithelium. Am. J. Physiol. 271 : L726-733.
4. de Bentzmann, S., Plotkowski, M.C., Puchelle, E. (1996). Receptors in the *Pseudomonas aeruginosa* adherence to injured and repairing airway epithelium. Am. J. Respir. Crit. Care Med. 154 : S155-162.
5. Azghani, A.O. (1996). *Pseudomonas aeruginosa* and epithelial permeability. Role of virulence factors elastase and exotoxin A.. Am. J. Respir. Cell. Mol. Biol. 15 : 132-140.
6. DiMango, E., Zar, H.S., Bryan, R., Prince, A. (1995). Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukine-8. J. Clin. Invest. 96 : 2204-2210.
7. Tirouvanziam, R., Desternes, M., Saari, A., Puchelle, E., Peault, B., Chinet, T. (1998). Bioelectric properties of human cystic fibrosis and non-cystic fibrosis fetal tracheal xenografts in SCID mice. Am. J. Physiol. 274: C875-882.
8. Cohn, L., Philips, T., Kaplan, H., Morlin, G., Ramphal, R., Cony, S., Smith, A.L. (1997). Susceptibility to and severity of *Pseudomonas aeruginosa* infection by CF respiratory epithelium. Pediatr. Pulmonol. suppl. 14, 251.
9. Kim, S.J., McKinnis, V.S., Nawrocki, B., White, S.R. (1998). Stimulation of migration and wound repair of guinea-pig airway epithelial cells in response to epidermal growth factor. Am. J. Respir. Cell. Mol. Biol., 18 : 66-74.

S17.1
CPX: ACTIVATION OF CFTR CHANNELS AND REPAIR OF ΔF508-CFTR TRAFFICKING DEFECT

Harvey B. Pollard, Ofer Eidelman, Michal Razin, Linda Fossom, Meera Srivastava and Nelson Arispe
Institute for Molecular Medicine & Department of Anatomy and Cell Biology, USU School of Medicine, USUHS, Bethesda, MD. 20814

Cystic Fibrosis (CF) is the most common, fatal autosomal recessive disease affecting the U.S. population. The disease is due to mutations in the CFTR gene which interfere with cAMP-activated CFTR chloride channel activity in epithelial cells of lung, pancreas, intestine, and other affected tissues. The mutation responsible for more than 70% of CF cases in the caucasian population is ΔF508. CFTR molecules bearing this mutation appear to traffick poorly through the endoplasmic reticulum (ER) and are proteosomically destroyed before reaching their native destination in the apical plasma membrane. However, ΔF508-CFTR does have chloride channel activity, and a few mutant ΔF508-CFTR molecules can apparently escape the ER and reach the membrane where they can be activated to some extent either by endogenous signals or by drugs such as the xanthine CPX.

CPX was initially discovered as a compound capable of activating chloride efflux from a variety of cells expressing ΔF508-CFTR (Eidelman, et al, 1992; Schweibert, et al, 1993; Guay-Broder et al, 1995; Jacobson et al, 1995;

Haws, et al, 1996). More recently we found that CPX can bind directly to the NBF-1 domain of CFTR (Cohen et al, 1997). The latter experiments were performed directly with recombinant NBF-1, and it was noted that ΔF508NBF-1 could bind CPX with higher affinity than could wildtype NBF-1. We have interpreted these data to indicate that the mechanism of action of CPX could involve direct interaction of CPX with the mutant protein in the region of the ΔF508 mutation. Consistent with this interpretation is the fact that CPX can directly activate CFTR channels in planar lipid bilayers (Arispe et al, 1998; *Ped. Pulm.*, 1998).

In addition to activating CFTR channels directly, CPX is apparently able to correct the trafficking defect of ΔF508-CFTR in cultured cells. We noted previously that CPX could increase the occurrence and intensity of higher molecular weight immunoreactive ΔF508CFTR in Western blot analysis of L-cells expressing the mutant gene. More recently we have extended these studies to include immunocytochemistry of these cells using confocal microscopy to image the data. Relatively low concentrations of CPX induce a dramatic increase in perinuclear immunoreactivity in cells expressing the ΔF508 mutation. A more radially distributed signal can also be frequently observed. Images of effects on L cells expressing mutant CFTR appear similar to images of L-cells bearing the wildtype CFTR, qualitatively and quantitatively. We have interpreted these data to indicate that the mechanism of CPX action in correcting the CF phenotype involves direct interaction of CPX with CFTR, with consequences for enhanced trafficking efficiency and activation of those mutant channels that do now manage to reach the plasma membrane.

CPX is a compound which has quite low toxicity to cells and organisms, and the concentrations of the drug needed to achieve enhancing effects on both channel activity and trafficking are quite low. For that reason, CPX, or a drug with CPX-like properties, might be attractive as candidates for CF therapy in patients. In the case of CPX, Phase I trials have concluded successfully, and Phase II trials are now in the planning stage.

References:

Arispe, N., Ma, J., Jacobson, K.A., and Pollard, H.B. (1997) Direct activation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by the alkylxanthines 8-cyclopentyl-1,3-dipropylxanthine (CPX) and 1,3-diallylcyclohexyl xanthine (DAX). *J.Biol.Chem.* 273:5727-5734.

Cohen, B.E., Lee, G., Jacobson,K.A., Kim, Y-C., Huang, Z., Sorscher, E.J. and Pollard, H.B. (1997) CPX (1,3-dipropyl- 8-cyclopentyl xanthine) and other alkyl-xanthines differentially bind to the wild type and ΔF508 mutant First Nucleotide Binding Fold (NBF-1) domains of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). *Biochemistry*, 36:6455-6461.

Eidelman, O., Guay-Broder, C., van Galen, P.J.M., Jacobson, K.A., Fox, C., Turner, R.J., Cabantchik, Z.I., and Pollard, H.B., (1992) A1-adenosine-receptor antagonists activate chloride efflux from cystic fibrosis cells. *Proc. Natl. Acad. Sci. USA*. 89, 5562-5566.

Guay-Broder, C., Jacobson, K.A., Barnoy, S., Cabantchik, Z.I., Guggino, W.B., Zeitlin, P.L., Turner, R.J., Vergara, L., Eidelman, O., and Pollard, H.B., (1995) A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine selectively activates chloride efflux from human epithelial and mouse fibroblast cell lines expressing the cystic fibrosis transmembrane regulator DF508 mutation. *Biochemistry* 34, 9079-9087.

Haws,C.M., Nepomuceno,I., Krouse,M.E., Wakelee,H., Law,T., Xia,Y., Nguyen,H., & Wine, J.J.(1996). □F508-CFTR channels: kinetics, activation by forskolin and potentiation by xanthines. *Am.J.Physiol.* 270,1544-1555

Jacobson, K.A., Guay-Broder, C., van Galen, P.J.M., Gallo-Rodriguez, C., Melman, N.K.A. Jacobson, M.A., Eidelman, O., Pollard, H.B., (1995) Stimulation by alkylxanthines of chloride efflux in CFPAC-1 cells does not involve A_1 adenosine receptors. *Biochemistry* 34, 9088-9094.

Schweibert. E., Gruenert, D., and Stanton, B. (1992) G proteins inhibit cAMP activated chloride channels in normal and CF airway epithelia. *Pediatric Pulm.* S8, p257.

S17.2
PHENYLBUTYRATE THERAPY FOR CYSTIC FIBROSIS: BASIC MECHANISMS AND CLINICAL APPLICATIONS

Pamela L. Zeitlin[*], Ronald C. Rubenstein[#], Michael P. Boyle[*] and Shijian Chu.[*]
[*]*The Johns Hopkins University School of Medicine, Baltimore, MD and*
[#]*The Children's Hospital of Pennsylvania, Philadelphia, PA*

The most common CF mutation, ΔF508, encodes a cAMP-activated chloride channel which becomes misprocessed and retained in the endoplasmic reticulum of the epithelial cell.[1,2,3] The mutant protein is still functional when induced in the oocyte expression system,[4] a planar lipid bilayer,[3] or in high level expression systems that allow a small fraction to reach the cell surface.[5] Growth of CF epithelial cells at reduced temperature[6,7] or exposure to chemical chaperone compounds[8,9] can also overcome the trafficking defect in vitro. We are studying a new class of chemical agents that repair the ΔF508 trafficking defect through a pharmacologic strategy. Our goal is the restora-

tion of normal chloride conductance using a transcriptional regulator to correct the biosynthetic trafficking defect associated with ΔF508 expression and enhance the mutant protein chloride transport.

The new drug Buphenyl (4-phenylbutyrate or 4-PBA) is currently approved for the inherited urea cycle disorders where it functions to scavenge excess ammonia. Buphenyl is in Phase 2 trials for the inherited hemoglobinopathies (sickle cell disease[10,11] and thalassemias[12,13]) and Phase I trials in cancer.[13,14] 4-PBA may act as either the prodrug for phenylacetate or as an independent chemotherapeutic agent with effects distinct from phenylacetate.[15] Ammonia generated in the urea cycle disorders is scavenged by conjugation of phenylacetate (generated during beta oxidation of 4-PBA) with glutamine and excreted as phenylacetylglutamine (PAG).[16] Ninety percent of administered 4-PBA is excreted as PAG in the urine. There is little toxicity[17] beyond a slightly bitter taste, mild stomach discomfort or mild peripheral edema if severely anemic.

Remarkably, 4-PBA has a second potent ability to regulate gene and/or protein expression in a number of physiologic processes. Although it is now established that 4-PBA administration increases fetal hemoglobin levels, the mechanism is incompletely understood. [10,11,18] Fetal hemoglobin levels and percent F cells increase, and it is thought that transcriptional upregulation of γglobin may be explained by the observation that butyrate promotes regulation of gene expression via inhibition of histone deacetylase.[19,20] Inhibition of histone deacetylation by the butyrates is associated with tumor cell differentiation and is the rationale for the use of phenylbutyrate as an adjunct chemotherapeutic agent. In general, the butyrate class of chemical agents may be thought of as a new kind of "gene drug" that acts by transcriptional regulation. Transcriptional regulators can be harnessed to up or down-regulate redundant gene pathways that normally are relatively quiescent.

We chose to study the butyrate analog, 4-PBA, as a potential upregulator of ΔF508 expression. 4-PBA promotes functional correction of cAMP-mediated chloride transport in CF airway epithelial cells[21] and increases chloride transport in nasal potential difference measurements of homozygous ΔF508 patients taking the drug for 1 week.[22] These early studies have identified at least two potential mechanisms of action. The first involves the endoplasmic reticulum quality control pathway for removal of misfolded or mutant proteins. Evidence for this mechanism is derived from ongoing studies with immortalized or primary CF cells in culture which suggest that the Hsc70 chaperone protein is selectively down-regulated by 4-PBA treatment in a time- and dose-dependent relationship.

The 70 kD heat shock protein family consists of Hsp70 (sometimes called Hsp72) which is inducible by heat shock and/or the presence of denatured intracellular proteins[23] and Hsc70 (sometimes called Hsp73), the 70 kD heat shock cognate protein which is constitutively expressed and is involved in the uncoating of clathrin-coated endosomes.[23] Hsc70 also has a role in the lysosomal degradation of intracellular proteins,[23,24] and was recently shown to be required for the ubiquitin-dependent degradation of a number of cellular proteins.[24] Since the rapid intracellular degradation of ΔF508 can be disrupted by the addition of ATP,[25] which is known to regulate the association of proteins with Hsc70, we asked whether Hsc70 was affected by the butyrates.

IB3-1 cells were incubated with increasing concentrations of 4-PBA in culture for 2 days. Total Hsc70 immunoreactivity in whole cell lysates declined with increasing concentrations of 4-PBA as detected by an Hsc70-specific rabbit polyclonal antisera (gift of W Welch) or with an Hsc70-specific monoclonal antibody (gift of A. Laszlo). These data are consistent with a dose-dependent reduction of Hsc70. Similar immunoblots were prepared using antisera specific for Hsp90 (a molecular chaperone that is required for correct folding and function of a number of cellular proteins [23]), Hsp70 (the inducible heat shock protein), and Hsp40 (a protein which regulates Hsp70 interactions with unfolded peptides). None of the other 3 chaperones were regulated by 4-PBA. We have recently confirmed that 4-PBA also down regulates Hsc70, but not Hsp70 in primary CF cells. Immunoprecipitation of Hsc70 with polyclonal antisera co-immunoprecipitates CFTR. At 4-PBA concentrations less than 1 mM for 48 hrs, there was a dose-dependent reduction in immunoprecipitable Hsc70/CFTR complexes. Between 1 and 5 mM, Hsc70 and CFTR were both undetectable. These observations are consistent with disruption of the Hsc70/CFTR complex through a diminution in Hsc70 protein, and a coincident escape by CFTR of the ER quality control mechanism. These concentrations of 4-PBA correlate with enhanced production of band C CFTR in our previous experiments; the highly glycosylated band C CFTR is a marker for the CFTR that has traversed the ER and through the Golgi apparatus.

A second mechanism of action may also be important. 4-PBA and the butyrates inhibit histone deacetylation, thereby uncovering repressed genes and promoting gene expression.[19] Alternative chloride channel genes are potential targets of the butyrates and can be screened in vitro for butyrate sensitivity. Our laboratory has been examining the pH- and voltage-sensitive ClC-2 chloride channel gene which is highly expressed in mammalian fetal lung during the period of fetal lung chloride and fluid secretion, and which becomes repressed immediately at birth. Using a series of promoter-luciferase constructs, we observed a three-fold stimulation by 4-PBA of reporter gene expression driven by the full length ClC-2 promoter. Over-expression of ClC-2 cDNA in CF epithelial cell lines by a transient transfection methodology complements the CFTR chloride transport defect in vitro.[26] Overcoming perinatal down- regulation of alternative chloride channel genes may provide an additional strategy to restore the normal ionic milieu to the airways.

Finally, our CF research center is conducting a random-

ized, double-blind Phase I trial of 4-PBA in 24 homozygous ΔF508 patients at three dose levels. The objectives are to establish the safety of 20 gm, 30 gm, and 40 gm divided t.i.d, the pharmacokinetics of absorption, metabolism, and excretion in CF patients, and the dose- and time-dependent characteristics of nasal epithelial chloride transport as measured by the nasal potential difference technique. At the time that this summary was submitted, the 20 gm dose cohort was completed and patient enrollment for the 30 gm cohort was in progress.

References

1. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE: Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 63:827-834, 1990
2. Lukacs GL, Mohamed A, Kartner N, Chang XB, Riordan JR, Grinstein S, FelX: Conformational maturation of CFTR but not its mutant counterpart (delta F508) occurs in the endoplasmic reticulum and requires ATP. Gastroenterology 109:282-284, 1995
3. Li C, Ramjeesingh M, Reyes E, Jensen T, Chang X, Rommens JM, Bear CE: The cystic fibrosis mutation (delta F508) does not influence the chloride channel activity of CFTR. Nat.Genet. 3:311-316, 1993
4. Drumm ML, Wilkinson DJ, Smit LS, Worrell RT, Strong TV, Frizzell RA, Dawson DC, Collins FS: Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. Science 254:1797-1799, 1991
5. Cheng SH, Fang SL, Zabner J, Marshall J, Piraino S, Schiavi SC, Jefferson DM, Welsh MJ, Smith AE: Functional activation of the cystic fibrosis trafficking mutant delta F508-CFTR by overexpression. Am.J.Physiol. 268:L615-L624,1995
6. Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, Welsh MJ: Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive [see comments]. Nature 358:761-764, 1992
7. Egan ME, Schweibert EM, Guggino WB: Differential expression of ORCC and CFTR induced by low temperature in CF airway epithelial cells. Am.J.Physiol.(Cell Physiol) 268 (37):C243-C251,1995
8. Brown CR, Hong-Brown LQ, Welch WJ: Correcting temperature-sensitive protein folding defects. J.Clin.Invest. 99:1432- 1444, 1997
9. Sato S, Ward CL, Krouse ME, Wine JJ, Kopito RR: Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. J.Biol.Chem. 271:635-638, 1996
10. Dover GJ, Brusilow S, Charache S: Induction of fetal hemoglobin production in subjects with sickle cell anemia by oral sodium phenylbutyrate. Blood 84:339-343, 1994
11. Dover GJ, Brusilow S, Samid D: Increased fetal hemoglobin in patients receiving sodium 4- phenylbutyrate [letter]. N.Engl.J.Med. 327:569-570, 1992
12. Collins AF, Pearson HA, Giardina P, McDonagh KT, Brusilow SW, Dover GJ: Oral sodium phenylbutyrate therapy in homozygous beta thalassemia: a clinical trial. Blood 85:43-49, 1995
13. Smigel K: Non-toxic drug being tested to treat cancer and anemias [news]. J.Natl.Cancer Inst. 84:1398-1398, 1992
14. Wood CG, Lee C, Grayhack JT, Kozlowski JM: Phenylacetate and phenylbutyrate promote cellular differentiation in human prostate cancer systems (Meeting abstract). Proc.Annu.Meet.Am.Assoc.Cancer Res. 35:A2404 1994-A2404 19941994
15. Piscitelli SC, Thibault A, Figg WD, Tompkins A, Headlee D, Lieberman R, Samid D, Myers CE: Disposition of phenylbutyrate and its metabolites, phenylacetate and phenylacetylglutamine. J.Clin.Pharmacol. 35:368-373, 1995
16. Brusilow SW: Phenylacetylglutamine may replace urea as a vehicle for waste nitrogen excretion. Pediatr.Res. 29:147-150, 1991
17. Batshaw ML, Brusilow SW: Evidence of lack of toxicity of sodium phenylacetate and sodium benzoate in treating urea cycle enzymopathies. J.Inherit. Metab. Dis. 4:231-231, 1981
18. Stamatoyannopoulos G, Blau CA, Nakamoto B, Josephson B, Li Q, Liakopoulou E, Pace B, Papayannopoulou T, Brusilow SW, Dover G: Fetal hemoglobin induction by acetate, a product of butyrate catabolism [see comments]. Blood 84:3198-3204, 1994
19. Candido EP, Reeves R, Davie JR: Sodium butyrate inhibits histone deacetylation in cultured cells. Cell 14:105-113, 1978
20. Lea MA, Tulsyan N: Discordant effects of butyrate analogues on erythroleukemia cell proliferation, differentiation and histone deacetylase. Anticancer Res. 15:879-883, 1995
21. Rubenstein RC, Egan ME, Zeitlin PL: In vitro pharmacologic restoration of CFTR-mediated chloride transport with sodium 4-phenylbutyrate in cystic fibrosis epithelial cells containing delta F508-CFTR. J.Clin.Invest. 100:2457-2465, 1997
22. Rubenstein RC, Zeitlin PL: A pilot clinical trial of sodium 4-phenylbutyrate (Buphenyl) in delta F508-homozygous cystic fibrosis patients: evidence of restoration of nasal epithelial CFTR function. Am.J.Resp.Crit.Care Med. 157:484-490, 1998
23. Gething MJ, Sambrook J: Protein folding in the cell. Nature 355:33-45, 1992
24. Chiang HL, Terlecky SR, Plant CP, Dice JF: A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 246:382-385, 1989
25. Strickland E, Qu BH, Millen L, Thomas PJ: The molecular chaperone hsc70 assists the in vitro folding of the

N- terminal nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator J.Biol.Chem. 272:25421-25424, 1997

26. Schweibert EM, Cid LP, Stafford D, Carter M, Blaisdell CR, Zeitlin PL, Guggino WB, Cutting GR: Analysis of ClC-2 channels as an alternative pathway for chloride conduction in cystic fibrosis airway cells. Proc.Natl.Acad.Sci.U.S.A. 95:3879-3884, 1998

S17.3
PHARMACOLOGICAL MODULATION OF WILD-TYPE AND MUTANT CFTR

Tzyh-Chang Hwang, Fei Wang, Layla Al-Nakkash, and Shenghui Hu
Department of Physiology and Dalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, MO 65211

CFTR is activated by protein kinase A (PKA)-dependent phosphorylation and gated by ATP binding/hydrolysis. Both phosphorylation/dephosphorylation and ATP-dependent gating affect CFTR activity in an intact cell. By using whole-cell, cell-attached, and excised inside-out modes of the patch-clamp technique, we investigate regulation and gating of wild-type (wt) and ΔF508-CFTR expressed in NIH3T3 cells and examine the effects of genistein, IBMX, and NS004 on CFTR activation.

In cell-attached patches under a maximal cAMP stimulation (i.e., 10 μM forskolin), the open probability (Po) of ΔF508-CFTR is ~10% of wt-CFTR. But the behavior of wt-CFTR under a submaximal cAMP stimulation (e.g., 50 nM forskolin) mimics that of ΔF508 channels. However, in the presence of genistein and forskolin, the Po's of wt- and ΔF508-CFTR are very similar. In excised inside-out patches, addition of PKA and ATP activates macroscopic wt- and ΔF508-CFTR currents with different rates. The activation of ΔF508-CFTR is slower than that of wt channels by at least one order of magnitude. However, when ΔF508-CFTR is fully activated with PKA and ATP, the Po of ΔF508-CFTR is close to that of wt-CFTR, suggesting little differences in the ATP-dependent gating for wt- and ΔF508-CFTR. Application of genistein together with PKA and ATP dramatically increases the rate of activation for ΔF508-CFTR. These results suggest that ΔF508-CFTR is defective in PKA-dependent phosphorylation activation and that genistein rectifies this functional abnormality with the ΔF508 mutation.

Using the whole-cell patch-clamp technique, we determine the dose-response relationships of genistein and NS004 on wt-CFTR. Macroscopic CFTR current is first elicited by application of 10 μM forskolin. Different concentrations of genistein or NS004 are subsequently added. The concentration-dependent effect of genistein shows a biphasic response: the potentiation effect of genistein reaches a peak at 20 - 30 μM with a $K_{1/2}$ of ~ 5 μM; CFTR current decreases as the concentration of genistein is further increased above 50 μM. These results suggest two binding sites for genistein, one stimulatory and the other inhibitory. Surprisingly, effects of NS004 can be observed even at a femtomolar (10^{-15} M) range. A complete dose-response relationship shows a $K_{1/2}$ of ~ 50 fM and a saturating effect at ~100 pM. This effect of femtomolar NS004 is further confirmed in cell-attached patches where femtomolar NS004 increases wt-CFTR current activated with 10 μM forskolin. Experiments testing the effect of NS004 on ΔF508-CFTR is currently in progress.

Effects of IBMX are examined with the cell-attached patch clamp technique. Micromolar IBMX increases wt-CFTR current only in the presence of submaximally effective concentrations of forskolin (e.g., 100 nM). In the presence of 10 μM forskolin, neither CPT-cAMP (200 μM), a membrane permeant cAMP analog, nor IBMX (200 μM) increases CFTR current. However, millimolar IBMX enhances CFTR current activated by 10 μM forskolin. This effect of millimolar IBMX is accompanied with a decrease of the single channel amplitude. At 5 mM IBMX, the effect of IBMX on the Po of wt-CFTR approximates that of 20 μM genistein. These results suggest that multiple mechanisms are involved in IBMX modulation of CFTR activity and that effects of millimolar IBMX may not involve an increase in cellular cAMP concentrations.

Genistein, NS004, and IBMX seem to activate CFTR through a common mechanism since once CFTR is maximally activated by either compound, other reagents cannot further increase CFTR activity and CFTR channel kinetics are very similar in the presence of either compound. However, their effective concentrations differ by several order of magnitude. The simplest interpretation of our data is that these reagents act at the same binding site with different affinities. Understanding the structure-activity relationship of these chemicals could provide information for future development of therapeutic reagents.

S17.4
AMINOGLYCOSIDE REPAIR OF PREMATURE TRANSLATION TERMINATION MUTATIONS

David M. Bedwell[1], Anisa Kaenjak[1], Kim Keeling[1], Marina Manouvakhova[1], Zsuzsa Bebok[2], Dale Benos[2], James Bubien[2], Erik Schwiebert[2], J.P. Clancy[3], Fadel Ruiz[4], and Eric Sorscher[4]
Departments of Microbiology[1], Physiology and Biophysics[2], Pediatrics[3] and Medicine[4], The University of Alabama at Birmingham, Birmingham, AL USA

Low concentrations of aminoglycoside antibiotics have been shown to cause translational misreading in bacteria, which can result in the suppression of premature stop mutations (1). However, this approach has not previously been examined as a means of treating diseases caused by premature stop mutations in humans. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause the common genetic disease cystic fibrosis (CF). Premature stop mutations in the *CFTR* gene represent approximately 5% of all CF mutations and may occur in 10% of all CF patients. Furthermore, premature stop mutations in the *CFTR* gene are found at a much higher incidence (60% of all mutant alleles) in the Ashkenazi jewish population (2). In this study, we asked whether aminoglycoside antibiotics can suppress premature stop mutations in the *CFTR* gene.

We found that low concentrations of the aminoglycoside antibiotics G-418 and gentamicin in the culture medium can suppress premature stop mutations in a human *CFTR* cDNA, resulting in the synthesis of full length, functional CFTR when expressed in HeLa cells (3). We also found that these compounds can suppress a genomic *CFTR* nonsense mutation in IB3-1 cells, a bronchial epithelial cell line derived from a CF patient (4). Our results indicate that G-418 and gentamicin restore cAMP-activated chloride currents, CFTR protein at the apical cell surface, and the abundance of the *CFTR* nonsense mRNA in IB3-1 cells. Experiments using a mammalian cell free translation system indicate that several different aminoglycosides can suppress stop codons with differing efficiencies, and suppression of translation termination occurs in a codon and context-specific manner. Interestingly, our results indicate that each of the four most common premature stop mutations found in CF patients are susceptible to suppression by aminoglycosides. A clinical trial is currently testing the ability of gentamicin to restore CFTR function in CF patients carrying *CFTR* premature stop mutations.

When taken together, our results suggest that aminoglycosides can overcome many premature stop mutations in human cells, resulting in the restoration of a low but significant level of CFTR protein function. We conclude that aminoglycoside-mediated suppression of premature stop mutations may represent a viable approach to treating genetic diseases such as cystic fibrosis in patients carrying these mutations.

References

1. Davies, J., Gorini, L. & Davis, B.D. (1965). Misreading of RNA codewords induced by aminoglycoside antibiotics. Mol. Pharmacol. **1**, 93-106.
2. Shoshani, T., Augarten, A., Gazit, E., Bashan, N., Yahav, Y., Rivlin, Y., Tal., A., Hagit, S., Yaar, L., Kerem, E., and Kerem, B.-S. (1992). Association of a nonsense mutation (W1282X), the most common mutation in the Ashkenazi jewish cystic fibrosis patients in Israel, with presentation of severe disease. Am. J. Hum. Genet. **50**, 222-228.
3. Howard, M., Frizzell, R. and Bedwell, D. (1996) Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. Nature Med. **2:** 467-469.
4. Bedwell, D., Kaenjak, A., Benos, D., Bebok, Z, Clancy, J., Hong, J., Tousson, A., Bubien, J., and Sorscher, E. (1997) Suppression of a *CFTR* premature stop mutation in a bronchial epithelial cell line. Nature Med. **3**: 1280-1284.

S17.5
PHOSPHODIESTERASE INHIBITOR-STIMULATED CHLORIDE SECRETION FROM GUT AND RESPIRATORY EPITHELIA

Mitchell L. Drumm, Wendy K. Steagall, Thomas J. Kelley, Laura J.H. Milgram and Michael Konstan
Departments of Pediatrics and Genetics, Case Western Reserve University, Cleveland, Ohio

Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator, CFTR, a cyclic-AMP regulated chloride channel, and accordingly a hallmark of the disease is altered ion permeability of various epithelia. It is still not clear how dysregulation of ion transport contributes to the pathophysiology of the disease, but a reasonable hypothesis is that correcting a primary defect in the disease, such as chloride permeability, may have clinical impact on the disease. To test this hypothesis, we have focused on the protein kinase A (PKA) pathway in the airways and intestines with the goal of identifying potent and

somewhat specific compounds for activation of CFTR chloride channels. We have found that inhibition of specific classes of cyclic nucleotide phosphodiesterase (PDE) is an effective way to activate CFTR, not only *in vitro* in cultured cells and in mouse intestines, but also *in vivo* in mouse nasal epithelium. To assess the relative potencies of phosphodiesterase inhibitors in airway epithelium, we have monitored the change in nasal potential difference in CF mice exposed to various inhibitors. Mice homozygous for a null mutation (S489X or Y122X) were used as negative controls, and compared to mice homozygous or heterozygous for wild type CFTR, homozygous for ΔF508 or homozygous for a relatively mild allele, R117H/neo. The assays revealed that inhibitors of class III phosphodiesterases, such as milrinone and amrinone, are most effective at hyperpolarizing the amiloride-insensitive component of the mouse nasal potential difference. The null genotypes showed no hyperpolarization, while the ΔF508 and R117H/neo mice were indistinguishable from each other, hyperpolarizing ~6 mV at 2 minutes after application of drugs. In contrast, wild type mice hyperpolarize >15 mV simply by imposing a chloride gradient to the nasal epithelium (lumenal low chloride).

The same concept has been applied to humans with CF, in which nasal PDs were recorded before and during exposure to 100μM milrinone. A cohort of 13 CF patients were analyzed by nasal PD, in which a baseline PD in physiologic saline was established, then amiloride was added until a steady state was reached, and then amiloride in a chloride-free solution was used to obtain a steady-state reading. A final solution containing either 10 μM isoproterenol or 10 μM isoproterenol + 100 μM milrinone was perfused onto the epithelium and the change in PD recorded. No statistically significant differences were seen between PD changes stimulated by isoproterenol or isoproterenol + milrinone, or between PDs before and after isoproterenol + milrinone. Preliminary experiments in which the dose of milrinone is increased to 1 mM show a suggestion of small (1-3 mV) changes not observed at 100 μM.

Because mortality in the CF mouse is due to intestinal obstruction in the area of the distal ileum and cecum, the small intestine is a potentially useful site in which to test the concept of pharmacotherapies for CF. To identify candidate compounds for the mouse effective for the intestinal tract, short circuit currents across jejunum have been recorded in response to application of the various phosphodiesterase inhibitors. The rank order of effect is class III > class I > class V ≥ class IV. Combinations of the various compounds shows that class III and class I act additively and possibly synergystically, unlike other combinations. The response of jejunum from the various genotypes to class III or class III and class I inhibitors shows a slightly different profile than the nasal potential differences. When exposed to 100 μM milrinone alone, the null genotypes and ΔF508 mice show no response, but the R117H/neo mice show a modest increase of ~3 $\mu A/cm^2$. When the combination of milrinone and 8-methoxymethyl-3-isobutyl-1-methylxanthine (class I phosphodiesterase inhibitor) is used, the null genotypes again do not respond, but the ΔF508 mice show an increase of ~2.5 $\mu A/cm^2$ and the R117H/neo mice increase by ~6 $\mu A/cm^2$. The R117H/neo mice survive the pre-weaning stage of life better than the null or ΔF508 animals. The small difference between the R117H/neo animals and the ΔF508 or null mice may account for the difference in survival. As the goal of these studies is to determine if manipulations of CFTR activity can increase the longevity of humans afflicted with CF, CF mice are being exposed to these compounds systemically to assess the effects on survival as well as transepithelial short circuit currents across intestines.

References

Kelley, T.J., Thomas, K., Milgram, L.J., and Drumm, M.L. (1997). In vivo activation of the cystic fibrosis transmembrane conductance regulator mutant ΔF508 in murine nasal epithelium. Proceedings of the National Academy of Sciences of the United States of America *94,* 2604-8.

Zeiher, B.G., Eichwald, E., Zabner, J. et al. (1995). A mouse model for the ΔF508 allele of cystic fibrosis. Journal of Clinical Investigation *96,* 2051-64.

S18.1
INHALED CORTICOSTEROIDS IN CYSTIC FIBROSIS—IN FAVOUR OF THEIR USE

Ian M Balfour-Lynn

Department of Paediatric Respiratory Medicine, Royal Brompton & Harefield NHS Trust, London SW3 6NP, United Kingdom. [E-mail: i.balfourlynn@ic.ac.uk]

Is it a good idea in principle?

It is well established that inflammation has an important role in CF lung disease. This inflammation is often present early in infancy and is a persistent feature, regardless of the clinical state of the patient.[1,2] Although probably secondary to chronic bacterial and viral infections, the lung inflammation may even be an inherent part of CF. A degree of lung inflammation is a beneficial part of the host defence system, but the exaggerated response seen in CF contributes to the morbidity and ultimately the mortality associated with the disease. There has been interest in anti-inflammatory strategies

for CF that initially focussed on oral corticosteroids. Long term systemic corticosteroids have been shown to have some efficacy but at quite a price in terms of adverse effects (recently reviewed [3]). Logically, inhaled corticosteroids would then be the drug of choice as they are delivered to the site of inflammation and long term side effects are less problematic.

Are they safe?

Inhaled corticosteroids have been used to treat asthma for over thirty years in many thousands of children. They have an excellent safety record, especially at standard doses, which is enhanced further by administering them through a spacer device to reduce mouth deposition.[4,5] Although high doses can affect short-term growth, final adult height is unaffected by even 7-11 years of continuous inhaled corticosteroids.[6] The issue of developing cataracts is not fully resolved in children although is a concern in adults; regular ophthalmoscopic examination of all patients on high dose inhaled corticosteroids is advised.[7] A recent abstract has been published in which a trial of 500 mcg/day fluticasone propionate was halted early due to concerns over an increased incidence of *Pseudomonas aeruginosa* acquisition in the treatment group.[8] Although caution is certainly warranted, further evidence is required to determine whether this is a genuine finding.

Have they been shown to work in CF?

To date, there have been 5 published studies on the use of inhaled corticosteroids in CF (table).[9-13] Although some have been encouraging, they have failed to provide convincing evidence of significant benefit using outcomes such as lung function, inflammatory markers or symptomatology. There have also been 3 abstracts reported but not yet published;[14-16] none showed improved lung function but there were decreased inflammatory markers in sputum [14] and bronchoalveolar lavage fluid [16] in two studies. A Cochrane review of corticosteroid use in CF is currently being undertaken in the UK.

BDP - beclomethasone dipropionate, BUD – budesonide, FP - fluticasone propionate

BHR - bronchial hyperreactivity, TGV - thoracic gas volume, FEV_1 – forced expiratory volume in 1 second

Why has benefit not yet been proven conclusively?

Rather than supposing inhaled corticosteroids simply do not have any beneficial effect in CF, the likeliest reason for lack of proof of their efficacy lies in the trials carried out so far. Problems include small subject numbers, short treatment periods, and inadequate drug dosages. There are also difficulties with outcome measures, especially of inflammatory markers.[12] The main problem to overcome, however, is that the drug has to penetrate thick viscid mucus so the method of delivery is important.

What are we doing in practice?

In the UK,[17] approximately 40% of children with CF are prescribed inhaled corticosteroids which compares with 11% in United States,[18] 10% in France and 12% in Germany.[19] From our UK survey, the commonest indication for their use was to control symptoms of wheezing, but almost two-thirds of centres also prescribed them to combat excessive lung inflammation.[17]

What should be done next?

The case for using inhaled corticosteroids in CF still needs to be conclusively proven with large trials. We have recently started a multicentre trial in the UK and Ireland but are having difficulties with recruitment as so many potentially eligible children are already on inhaled corticosteroids. A trial has also started in Belgium and Holland using the same high dose (1000 mcg fluticasone propionate per day). Whilst in the UK it may already be too late to successfully complete such a trial, hopefully work in other European centres or the USA will provide the answer.

Conclusions

There is little doubt that the use of inhaled corticosteroids in CF is a good idea. Unfortunately, their benefit has not yet been proven, although it is probably just a matter of time. It is likely high doses will be needed to overcome the barrier of mucus in the lungs, so careful monitoring for adverse effects will be mandatory. If, however, it is shown that inflammation is reduced but there is no change in lung function or how the patient feels, compliance to treatment is likely to be poor.

	No.	Age (years)	Drug	Daily dose (mcg)	Time	Inflammation	Lung function
Schiøtz et al 1983[9]	26	4 – 29	BDP	400	16w	No change	No change
Van Haren et al 1995[10]	12	16 – 45	BUD	1600	6w	–	↑ BHR
Nikolaizik et al 1996[11]	49	20 ± 7	BDP	1500	4w	–	↑ TGV
Balfour-Lynn et al 1997[12]	23	6 – 17	FP	400	6w	No change	No change
Bisgaard et al 1997[13]	55	9 – 29	BUD	800	26w	–	↓ FEV_1

BDP - beclomethasone dipropionate, BUD – budesonide, FP - fluticasone propionate
BHR - bronchial hyperreactivity, TGV - thoracic gas volume, FEV_1 – forced expiratory volume in 1 second

References

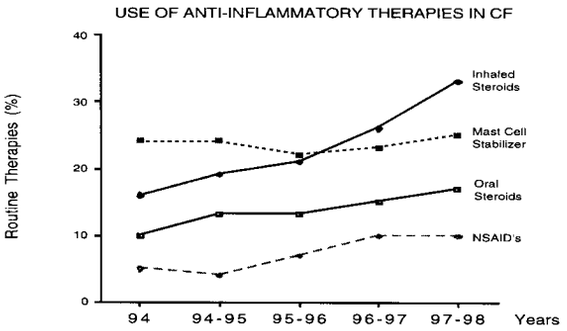
1. Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DWH. Early pulmonary inflammation in infants with cystic fibrosis. Am J Respir Crit Care Med 1995;**151**:1075-1082.
2. Konstan MW, Hilliard KA, Norvell TM, Berger M. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. Am J Respir Crit Care Med 1994;**150**:448-454.
3. Balfour-Lynn IM, Dinwiddie R. Role of corticosteroids in cystic fibrosis lung disease. J Roy Soc Med 1996;**89(Suppl 27)**:8-13.
4. Pedersen S, O'Byrne P. A comparison of the efficacy and safety of inhaled corticosteroids in asthma. Allergy 1997;**52 (Suppl 52)**:1-34.
5. Russell G. Inhaled corticosteroid therapy in children: an assessment of the potential for side effects. Thorax 1994;**49**:1185-1188.
6. Agertoft L, Pedersen S. Final height of asthmatic children treated for 7-11 years with inhaled budesonide. AM J Resp Crit Care Med 1998;**157**:A711.
7. Chylack LT. Cataracts and inhaled corticosteroids. New Engl J Med 1997;**337**:47-48.
8. Schmidt J, Davidson AGF, Seear M, Wong LTK, Peacock D, Gravelle A, Menon K, Cimolai N, Speert DP. Is the acquisition of Pseudomonads in cystic fibrosis patients increased by use of inhaled corticosteroids? Unexpected results from a double blind placebo controlled study. Ped Pulmonol 1997; **Suppl 14**:293-294.
9. Schiøtz PO, Jørgensen M, Flensborg EW, Faerø O, Husby S, Høiby N, Jacobsen SV, Nielsen H, Svehag SE. Chronic Pseudomonas aeruginosa lung infection in cystic fibrosis. Acta Paediatr Scand 1983;**72**:283-287.
10. Van Haren EHJ, Lammers J-W J, Festen J, Heijerman HGM, Groot CAR, van Herwaarden CLA. The effects of the inhaled corticosteroid budesonide on lung function and bronchial hyperresponsiveness in adult patients with cystic fibrosis. Resp Med 1995;**89**:209-214.
11. Nikolaizik WH, Schöni MH. Pilot study to assess the effect of inhaled corticosteroids on lung function in patients with cystic fibrosis. J Pediatr 1996;**128**:271-274.
12. Balfour-Lynn IM, Klein NJ, Dinwiddie R. Randomised controlled trial of inhaled corticosteroids (fluticasone propionate) in cystic fibrosis. Arch Dis Child 1997;**77**:124-130.
13. Bisgaard H, Pedersen SS, Nielsen KG, Skov M, Laursen EM, Kronborg G, Reimert CM, Høiby N, Koch C. Controlled trial of inhaled budesonide in patients with cystic fibrosis and chronic bronchopulmonary Pseudomonas aeruginosa infection. Am J Respir Crit Care Med 1997;**156**:1190-1196.
14. Wojnarowski C, Götz M, Eichler HG, Frischer T, Renner S, Koller DY, Eichler I. Inhaled corticosteroids suppress inflammation in patients with cystic fibrosis. Proceedings of 20th European CF Conference 1995.
15. Nieman R, Williams S, Maden C, Knight R, Hodson M. A double-blind placebo-controlled study comparing the effects of the inhaled corticosteroid fluticasone propionate 500 mcg BID with placebo in adults with cystic fibrosis. Am J Respir Crit Care Med 1996;**153**:A72.
16. Wojtczak HA, Wagener JS, Kerby G, Gotlin R, Accurso F. Effect of inhaled beclomethasone dipropionate on airway inflammation in children with cystic fibrosis. Pediatr Pulmonol 1996;**Suppl 13**:323-24.
17. Balfour-Lynn IM, Dezateux C. Anti-inflammatory therapy for lung disease in cystic fibrosis: current paediatric practice in UK. Ped Pulmonol 1997;**Suppl 14**:298.
18. Oermann CM, Sockrider MM. The use of anti-inflammatory medications in cystic fibrosis: trends and physician attitudes. Ped Pulmonol 1997;**Suppl 14**:298.
19. Koch C, McKenzie SG, Kaplowitz H, Hodson ME, Harms HK, Navarro J, Mastella G. International practice patterns by age and severity of lung disease in cystic fibrosis: Data from the Epidemiologic Registry of Cystic Fibrosis (ERCF). Ped Pulmonol 1997;**24**:147-154.

S18.2
INHALED CORTICOSTEROIDS IN CF - CON

Bruce C. Marshall, M.D.
Intermountain CF Center, University of Utah and Primary Children's Medical Centers, Salt Lake City, Utah, USA

Airways inflammation and infection begin very early in CF and dominate the clinical picture as the disease progresses. There is evidence that anti-inflammatory therapy may attenuate the course of the pulmonary disease. However, in practice this knowledge has translated into the widespread use of therapies that have not been rigorously validated. The figure below, derived from the Epidemiologic Study of Cystic Fibrosis (ESCF), documents the extent to which anti-inflammatory therapies are prescribed in North America. Note the striking increase in the use of inhaled corticosteroids over this several year time frame. Data from the Epidemiologic

Registry of Cystic Fibrosis (ERCF) demonstrate that this therapy is also commonly prescribed in Europe. Overall, 25.5% of European patients were using inhaled steroids in 1994-95 (10% in France, 12% in Germany and 36% in the United Kingdom). This practice cannot be justified by the currently available data.



Before outlining the rationale against the routine use of inhaled steroids in CF, I will briefly review the benefits and risks of systemic steroids in CF. Greally et al performed a double-blind, placebo controlled trial in 24 patients, 5 to 20 years of age. Subjects were treated with oral prednisolone at 2mg/kg/day for 2 weeks followed by 1mg/kg every other day for 10 weeks. The steroid treated group showed a modest improvement in pulmonary function and a decrease in several inflammatory markers as compared to the placebo group. The Cystic Fibrosis Foundation Prednisone Trial also demonstrated that alternate day steroids may have some role in the treatment of CF. This 4 year double-blind, placebo controlled multi-center trial involved 285 children with CF, 6 to 14 years of age. Patients infected with *Pseudomonas aeruginosa* at the outset of the study showed a treatment benefit with respect to pulmonary function. Unfortunately this benefit was counterbalanced by the occurrence of significant adverse events. The 2 mg/kg group was aborted at the 2 year point because of an increased frequency of cataracts, glucose abnormalities and growth retardation. The 1mg/kg group also showed evidence of significant growth retardation at the completion of the trial. In addition, the steroid-treated groups became infected with *Pseudomonas aeruginosa* more frequently than the placebo group (placebo-treated, pre-56% and post-59% versus steroid-treated, pre-53% and post-74%, $p<0.05$ by chi-square analysis). Osteoporosis, another potential complication of long term steroid therapy, was not considered in these studies. In summary, alternate day steroids appear to have a modest beneficial effect on pulmonary function, but the long term risks are substantial.

An obvious solution to the systemic side effects of steroids is the direct delivery of drug to the airways by the inhaled route. However the available data on this approach is limited. The three peer reviewed publications addressing this issue are summarized in the Table below.

None of these studies demonstrated a significant benefit of inhaled steroids on pulmonary function. Nor were they of sufficient duration to address the pressing safety concerns. The body of evidence on the use of inhaled steroids in childhood asthma demonstrates that high doses of inhaled steroids may adversely affect growth and markers of bone mineralization. The systemic absorption of these drugs from the CF airways is not known. That coupled with the greater vulnerability of CF patients to growth retardation, diabetes, osteoporosis and pseudomonal infection make the use of inhaled steroids a matter of serious concern.

Cost is another issue that argues against the routine use of inhaled steroids in CF. For example, the cost of beclamethasone diproprionate at the dosage level used by Nikolaizik et al is substantial (retail cost at our pharmacy- approximately $120 to $172 per month depending on which preparation is prescribed). In the current health care environment, can we justify this cost for a therapy that is not well validated? Increasing the complexity of the medical regimen is an additional concern. Data suggests that compliance with inhaled medications is already lower than for oral medications. Bronchodilators, mucolytics and inhaled antibiotics are also prescribed for many patients. Additional therapies such as inhaled steroids may decrease the compliance rate for these better validated drugs.

In summary, despite widespread use, there is no convincing evidence that inhaled corticosteroids are efficacious in CF. Significant concerns about safety remain unresolved, particularly for the use of high doses of these drugs in children. Furthermore the issues of cost and compliance with a complex regimen weigh in against the

Trial	Drug/dose	Design	Subjects	Results
Schiotz et al	Beclomethasone 100 ug QID	Double-blind, placebo controlled	13-steroid 13-placebo	-No improvement in inflammation, FEV1 or FVC
van Haren et al	Budesonide 800 ug BID	Double-blind, crossover	12-total	-Improvement in sx's, no difference in FEV1 or FVC
Nikolaizik et al	Beclomethasone 800 ug BID	Randomized, non-blinded	25-steroid 24-placebo	-No difference in FEV1 or FVC

routine use of this form of therapy. Additional randomized clinical trials addressing this issue are needed. In the meantime this therapy should be reserved for patients with significant bronchospasm that is not adequately controlled by bronchodilators. Patients selected for a therapeutic trial of inhaled steroids should be carefully monitored for subjective and objective response. In the absence of clearcut improvement, the drug should be discontinued.

References

1. Koch C, McKenzie SG, Kaplowitz H, Hodson ME, Harms HK, Navarro J, Mastella G. Interantaional practice patterns by age and severity of lung disease in cystic fibrosis: data from the ERCF. Ped-Pulmonol 1997; 24(2):147-54.
2. Greally P, Hussain MJ, Vergani D, Price JF. Interleukin-1 alpha, soluble interleukin-2 receptor, and IgG concentrations in cystic fibrosis treated with prednisolone. Arch-Dis-Child 1994; 71(1):35-9.
3. Eigen H, Rosenstein BJ, FitzSimmons S, Schidlow DV. A multicenter study of alternate-day prednisone therapy in patients with cystic fibrosis. CF Foundation Prednisone Trial Group. J-Peiatr 1995; 126(4):515-23.
4. Donati MA, Haver K, Gerson W, Klein M, McLaughlin FJ, Wohl MEB. Long-term alternate day prednisone in cystic fibrosis. Ped Pulmonol 1990; Supp 5:277.
5. Schiotz PO, Jorgensen M, Flensborg EW, Faero O, Husby S, Hoiby N, Jacobsen SV, Nielsen H, Svehag SE. Chronic *Pseudomonaa aeruginosa* lung infection in cystic fibrosis. A longitudinal study of immune complex activity and inflammatory response in sputum sol-phase of cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infections: influence of local steroid treatment. Acta-Paediatr-Scand 1983; 72(2):283-7.
6. van Haren EHJ, Lammers JWJ, Heijerman HGM, Groot CAR, van Herwaarden CLA. The effects of the inhaled corticosteroid budesonide on lung function and broncial hyperresponsiveness in adult patients with cystic fibrosis. Respiratory Medicine 1995; 89:209-214.
7. Nikolaizik WH, Schoni MH. Pilot study to assess the effect of inhaled coricosteroids on lung function in patients with cystic fibrosis. J-Pediatr 1996; 128(2)271-4.
8. Barnes PJ, Pedersen S, Busse WW. Efficacy ad safety of inhaled corticosteroids on lung function in patients with cystic fibrosis. New developments. Am-J-Respir-Crit-Care-Med 1998; 157(3 Pt 2):S1-S53.
9. Tashkin DP. Multiple dose regimens. Impact on compliance. Chest 1995; 107(5 Suppl):176S-182S.
10. Conway SP, Pond MN, Hamnett T, Watson A. Compliance wiht treatment in adult patients with cystic fibrosis. Thorax 1996; 51(1):29-33.

S18.3
CLINICAL CONTROVERSIES IN CF NUTRITION SUPPLEMENTATION: WHY AND FOR WHOM? THE PRO SUPPLEMENTATION DISCUSSION

Virginia A Stallings, MD
Division of GI and Nutrition, Children's Hospital of Philadelphia,
Department of Pediatrics School of Medicine, University of Pennsylvania, Philadelphia, PA/USA

Malnutrition, growth failure, abnormal body composition, delayed sexual and skeletal maturation are all frequently and well documented in the population of infants, children, adolescents and adults with cystic fibrosis (CF) and associated pancreatic insufficiency. There is no indication that the various CF gene mutations directly code for these abnormalities of growth, development and nutritional status.

Growth failure and malnutrition are caused by a state of chronic negative energy balance. The negative energy balance is because many people with CF can not, day in and day out over a lifetime eat enough food, particularly higher fat food, to meet their energy requirements. The energy requirements are higher for people with CF than for unaffected, well nourished children and adults due to the consequences of CF, including: (1) increased resting energy expenditure (ie, chronic inflammation, infection, altered protein and carbohydrate metabolism, altered cellular water and electrolyte metabolism); (2) unusual losses (ie, pancreatic insufficiency-related malabsorption, hepatobiliary-related malabsorption, renal losses with untreated diabetes); and, (3) abnormal body composition (loss of body fat in proportion to fat-free mass) resulting in a higher metabolically active body mass.

So, the population of people with CF is at-risk for abnormalities of growth and nutritional status. The consequences of poor growth and malnutrition on the course of CF in an individual are not fully understood. Yet, most care providers acknowledge that malnutrition is associated with depressed immune function, decreased wound healing, decreased cognitive function, suboptimal muscle and bone mass, poorer quality of life and loss of growth potential, compared to family/genetic potential. These are not good associations for people with a life threatening, complex, chronic disease.

The next issue is how do we care providers diagnosis growth failure, pubertal delay and malnutrition in a clinical setting. This varies tremendously from physician to

physician and nutritionist to nutritionist. It should incorporate accurate clinical data (longitudinal information/ velocity/percentiles/z score calculations) for weight, height, head circumference (up to 3 or 5y), triceps skinfolds (fat stores), arm muscle (muscle/ fat free mass stores), and pubertal stage (Tanner) assessment. The documentation of biological parental and sibling height and pubertal history are also needed. Most laboratory assessments are normal and do not contribute to the process of the diagnosis of malnutrition and growth failure (except in critical illness or end stage disease) in this setting. The issues of diagnosis, care provider bias, and patterns of practice will be discussed.

Based upon the simplest measures of growth of children with CF in the US (weight and height records from the CF Patient Registry) growth failure is common, and these data will be reviewed. This is in contrast to better patterns of growth reported in Canada and Australia.

If abnormalities of growth, body composition, pubertal development or other categories of malnutrition are documented, then a treatment plan to improve energy balance is the next step. Ideally, one would work to increase energy intake (more food, more fat, more kcal) and decrease malabsorption (enzymes, bile acids, etc) and causes of increased expenditure (treat infections, etc).

Working in the field of CF is fairly unique, as families and patients are given significant information about nutrition and growth as part of standard care. As care providers, we are (almost) always committed to treatment in steps of the least invasive intervention to the more invasive. In nutrition and growth, that means significant attention to: nutrition education, behavioral education (child, family, food-related and medication-related parenting skills), normal child development education, and solving social/financial/child care problems. Mediation use and compliance are also evaluated and modified when needed and when possible. Using these approaches, we would aim to improve energy balance by increasing intake and the absorption of real food, then use real food supplement strategies (put more butter or cream in products, etc) and then turn to supplements.

When appropriate and standard approaches to improve growth and nutrition status are taken and fail, the care team must consider the next set of interventions to treat the important diagnosis of growth or pubertal delay or other forms of malnutrition. These nutritional supplementation interventions must be presented fairly to the family and, when chosen, must be implemented with the skill and attention to follow up care accorded to all forms of therapy provided to any patient. Nutritional supplementation is in the required continuum of care to provide optimal nutritional care for people with CF. To tolerate malnutrition or growth failure without nutritional intervention (all modes and products considered) represents suboptimal care. Enough food and good growth are usually highly desirable to the family and patient. We must improve our skills at diagnosis and treatment to match the needs of the patient with the product and technology available.

An approach to the follow-up protocol and setting and achieving nutritional rehabilitation goals will be discussed.

S18.4
CLINICAL CONTROVERSIES IN CF NUTRITIONAL SUPPLEMENTATION: WHY AND FOR WHOM? – CON

Ross Shepherd MD, FRACP
Department of Gastroenterology and Nutrition, Royal Children's Hospital,
Department of Paediatrics, University of Queensland, Brisbane Q4029 Australia

Malnutrition and growth retardation have been common complications of CF, known to adversely affect prognosis. The observation that raised energy expenditure contributes to energy imbalance is now well documented, probably related to both increased cellular metabolic activity and pulmonary dysfunction. However it can be argued that with appropriate attention to optimising absorbed nutrient intake and aggressive pulmonary therapy from early (pre-symptomatic) CF life, normal growth and nutritional status is achievable in the majority of CF sufferers. Possible exceptions include those with short bowel syndrome and those who develop diabetes or liver disease. The benefits of a dietary policy which encourages high nutrient intakes via a high fat diet were well documented in the seminal outcome studies of survival, growth and pulmonary function comparing the better nourished Toronto Clinic and the less well nourished Boston Clinic over 10 years ago (Corey et al, 1988). Similarly, studies from Australia (Richardson et al 1998), comparing data from an adult CF clinic in 1983 (prior to changing dietary policy) with similar data obtained in 1996, have shown that malnutrition has decreased from 62 to 9%. These CF adults are now of normal height and their mean dietary intake now exceeds the CF RDI of 120% normal RDI (a significant increase over 1983). These results have been achieved in the main by consistent attention to achieving an appropriate absorbed dietary intake and the use of nutritional supplementation in only few selected cases.

The goal of achieving normal nutrition and growth in CF requires an individualised and age related approach to achieving an adequate oral energy intake. With mod-

ern pancreatic enzyme therapy, energy losses of less than 10% of intake can generally be achieved (Thomson et al 1993). In CF infants, despite an inherent increase in energy expenditure (Thomson et al 1995), most will thrive on human milk or standard formula (Holliday and Allen 1991). In older children and adolescents appropriate energy intakes can be achieved by the oral route using a variety of nutritional and educational approaches (Bentur et al 1996). Eating behavior strategies may be necessary for those consuming less than appropriate intakes (Stark et al, 1997). The key to achieving a consistent high-energy diet in these age groups is a moderate to high palatable fat intake. Fat has both practical and biological advantages. First, it is energy-rich and occurs in foods highly palatable at all ages. Secondly, it is known that for the maintenance of normal body composition, the fuel mix oxidised must match the nutrient distribution in the diet, and is influenced by circulating substrate and hormone concentrations, which among other things, reflect the degree of replenishment of body fuel reserves. The amount of body fat is the key factor on which an understanding of body weight regulation should be based (Flatt 1995). If energy requirements are not met, depletion of body fat and more seriously,cell mass,occurs (Shepherd et al 1989). In addition, with prolonged aerobic activity there is normally an increase in the proportion of fat and a decrease in the proportion of carbohydrate oxidised as fuel. (Flatt 1987), but in undernourished CF patients, high RQ values are found, reflecting an increase in the proportion of carbohydrate used as a metabolic substrate, because fat stores are depleted (Bowler et al 1993, Shepherd et al 1997). Veterinary scientists have known for many years that "fat adaptation" (combination of a high fat diet and physical conditioning) favours endurance in racehorses, and recent studies have shown that it even helps sprinting, apparently through enhanced regulation of glycolysis, generating more power when needed, with lower CO_2 production per unit of work (Kronfeld 1997). With these arguments, it is of no surprise that the major difference between outcomes of the Toronto and Boston Clinics revealed in 1988 was assumed related to the high fat dietary policy in Toronto.

Thus, both theoretically and practically, the metabolic and nutritional problems seen in CF (ie hyper metabolism, increased lipid utilisation, impaired glucose metabolism, and increased protein turnover) can be overcome by a nutritional policy of advising an energy intake >120% RDI, (individually higher in some cases), via a high fat diet with appropriate pancreatic enzyme therapy, most easily (and metabolically efficiently) achieved by a grazing pattern of food intake (4 meals a day with 3 high energy snacks). Adherence to these recommendations is likely to confer significant advantages in terms of normal growth and nutrition, without the need for nutritional supplementation.

References

1. Corey M, McLaughlin FJ, Williams M, Levison HA. A comparison of survival growth and pulmonary function in patients with cystic fibrosis in Boston and Toronto. *J Clin Epidemiol* 1988: 41 583-591.
2. Richardson I, Nyulasi I, Cameron C, Moore M, Wilson J. (In press) Nutritional status of an adult cystic fibrosis population. *Nutrition* 1998.
3. Thomson M, Clague A, Cleghorn GJ, Shepherd RW. Comparative in vitro and in vivo studies of enteric coated pancrealipase preparations for pancreatic insufficiency. *J Paediatr Gastroenterol & Nutr* 1993, 17, 4, 407-413.
4. Thomson MA, Willmot RW, Wainwright C, Masters B, Francis PJ, Shepherd RW. Resting energy expenditure, pulmonary inflammation and genotype in the early course of cystic fibrosis. *J Pediatr* 1986: 129: 367 – 373.
5. Holliday K, Allen J. Growth of human milk fed and formula fed infants with cystic fibrosis. *J Pediatr* 1991: 118: 77-79.
6. Bentur L, Kalnins V, Levison H, Corey M, Durie PR. Dietary intakes of young children with cystic fibrosis: Is there a difference? *J Pediatr Gastroenterol & Nutr* 1996: 22: 254-258
7. Stark LJ, Mulvihill MM, Jelalian E, Bowen AM, Powers SW, Tao S, Creveling S, Passero,MA,Harwood I,Light M,Lapey A, Hovell MF. Descriptive analysis of eating behavior in school-age children wuth cystic fibrosis and healthy control children. *Pediatrics* 1997; 99:665-671.
8. Flatt JP. Body composition, respiratory quotient, and weight maintenance. *Am J Clin Nutr* 1995: 62: (5 Suppl): 1107S-1117S.
9. Shepherd RW, Holt TL, Greer R, Cleghorn GJ, Thomas BJ. Total body potassium in cystic fibrosis. *J Pediatric Gastroenterol & Nutr* 1989: 9: 200-205.
10. Bowler IM, Green GR, Wolfe SP, Littlewood JM. Resting energy expenditure and substrate oxidation rates in cystic fibrosis. *Arch Dis Child* 1993: 68: 754-759.
11. Shepherd RW, Greer R, Wainwright C, Thomson M. Substrate utilisation in infants with cystic fibrosis. *Pediatr Pulmonol* 1997: Suppl 14; 372; A372.
12. Kronfeld DS. Fat adaptation and exercise: Blood acid-base and lactate responses to sprinting. *American Association of Equine Physiology*—Proceedings 1997; 43:353-354.