Myeloperoxidase-dependent oxidative metabolism of nitric oxide in the cystic fibrosis airway

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Received 30 January 2008; received in revised form 5 August 2009; accepted 1 October 2009
Available online 15 January 2010

Abstract

Background: Decreased expired nitric oxide (eNO) is commonly observed in cystic fibrosis (CF) patients and is usually explained by dysregulation of NO synthase (NOS) isoforms in respiratory tract epithelium. Later stages of this disease are accompanied by intense airway infiltration of phagocytes with high NOS activity, abundant levels of the hemoprotein myeloperoxidase (MPO) and significant production of significant reactive oxygen species.

Methods: This study characterizes the contribution of the high airway levels of MPO to decreased eNO levels in adult CF patients. NO metabolites (NOx) and MPO levels in fresh sputum of control and adult CF patients were determined and related to measurements of eNO and to in vitro consumption of NO in CF sputum.

Results: Despite essentially equal levels of NOx in sputum, eNO was 2- to 3-fold lower in CF patients compared to healthy controls. In CF patients, eNO levels were negatively associated with sputum peroxidase activity. In vivo correlations were confirmed by ex vivo studies of NO consumption by MPO in CF sputum. Immunodepletion studies confirmed MPO as the major heme peroxidase in CF sputum contributing to the hydrogen peroxide (H2O2)-dependent consumption of NO.

Conclusions: In CF airways MPO acts as a phagocyte-derived NO oxidase that diminishes NO bioavailability at airway surfaces, possibly identifying this peroxidase as a potential target for therapeutic intervention.

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Keywords: Myeloperoxidase; Expired nitric oxide; Sputum; Oxidants; Oxidative stress; Neutrophils

1. Introduction

Cystic fibrosis (CF) is a progressive autosomal recessive disease resulting from mutations in the CF transmembrane conductance regulator gene (CFTR) [1], likely modulated by variable expressions of several other genes [2]. The physiological consequences of this disease include a decreased respiratory tract chloride conductance and increased water resorption, resulting in increased mucous viscosity and compromised mucociliary clearance. Due to these abnormalities, patients with CF are highly susceptible to respiratory tract colonization and infection, decreased host defenses and overly exuberant inflammatory responses playing contributory roles [3–5]. The resulting magnitude of the neutrophilic responses and their accompanying proteolytic and oxidative processes are believed to play a major role in the progressive destruction of lung tissues which characterize CF [6,7].

Consistent with heightened inflammatory/immune processes associated with most respiratory tract diseases, elevated levels of expired NO (eNO) are observed in patients with asthma,
bronchiectasis, acute respiratory distress syndrome (ARDS) and unstable chronic obstructive pulmonary disease [8]. However, despite intense inflammation and upregulated immune responses, eNO levels are commonly lower in CF patients compared to those measured in healthy subjects [9–13]. Indeed, eNO levels are even lower in patients with more severe CF-associated lung disease [9]. The mechanisms leading to decreased levels of eNO in CF are presumably multifactorial and include respiratory tract epithelial cell decreased NOS isoform expression [14,15]. The reduction in NO bioavailability at respiratory tract surfaces may contribute to decreased antimicrobial host defenses [16,17] and presumably to the decreased levels of endogenous bronchodilator S-nitrosothiols, including S-nitroso-glutathione (GSNO), reported in CF [18,19].

Although secretory lactoperoxidase, H₂O₂ generating systems and thiocyanate are believed to also play an important role in airway antimicrobial systems [20], and may be defective in CF [5], early bacterial colonization/infec-tion in the CF respiratory tract and the resulting exuberant phagocytic responses result in large amounts of neutrophilic myeloperoxidase (MPO) in airway secretions [21,22]. In the presence of H₂O₂ arising from airway epithelium [23] and neutrophil NADPH oxidases [24] and the bacteria themselves, it can be hypothesized that in later stages of the disease, where infection is presumably most severe, active luminal MPO may be further compromising the expected inflammation-based augmentation of airway NO generation. Herein we provide evidence suggesting that MPO in airway secretions contributes to decreased eNO levels in adult CF patients with respiratory tract infections. These results implicate MPO as a leukocyte-derived airway NO oxidase that limits NO bioavailability in the inflamed CF lung, thus identifying this heme peroxidase as a potential molecular target for therapeutic intervention in CF.

2. Methods

2.1. Materials

3,3′,5,5′-Tetramethylbenzidine (TMB) and N,N-dimethylformamide (DMF) were from Sigma-Aldrich (St Louis, Missouri, USA). DNase (Pulmozyme™) was a gift from Genentech (South San Francisco, CA). Mouse monoclonal antibodies against human MPO were from The Binding Site (Birmingham, England). Rabbit polyclonal antibodies against human MPO and purified human MPO were from Calbiochem (San Diego, California, USA). Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase were from ZyMax™ (San Francisco, California, USA). All other chemicals were of the highest grade from Fisher Scientific (Houston, Texas, USA) or Sigma-Aldrich.

2.2. Patients and sputum preparation

The studies were performed with approval from the Office of Human Research Protection/Institutional Review Board (IRB) of the University of California, Davis. All subjects gave informed consent. The patients in this study were typical confirmed adult CF outpatients attending the UC Davis Adult CF Center clinic. They are characterized by chronic airway sepsis (infection) and varying degrees of lung impairment. The patients all had a CF diagnosis based on the CF Foundation published diagnosis criteria. Patients were aged 19–30, 16% of which were female. FEV₁’s ranged from 25 to 80% of predicted. CFTR genotypes were as typical of our adult CF patients with ΔF508 prominently represented. No patients were on steroids (an exclusion criterion). Most cultured positive for Pseudomonas and were receiving standard CF management, except that none were actively receiving antibiotics including tobi or azithromycin at the time of the study. Controls were aged 19–44, 28% of which were female. All were free of respiratory disease and on no medications. All had FEV₁’s in the normal range (>80% of predicted). Spontaneously expectorated sputa from adult CF patients (>18 years of age) were collected, frozen immediately and stored at −80 °C until assay. Both controls and CF patients were asked to extensively void saliva from the mouth immediately prior to expectorating sputum, thus minimizing the extent to which saliva contributes to MPO quantitation. It should be recognized that CF sputum peroxidase activity is orders of magnitude higher than saliva or control sputum. Nonetheless, although minimized, we cannot exclude contributions of small amounts of saliva to the reported measurements. Subsequently, sputum was thawed and incubated in an approximately equal volume of 50 mM phosphate buffer (pH 7.4) containing 0.4 mg/ml DNase (0.2 mg/ml final concentration). Samples were vortexed for 30 s, incubated at room temperature for 10 min and centrifuged at 2500 g for 20 min at 4 °C [25]. It should be noted that this methodological approach probably causes lysis of neutrophils with release of their contained MPO. As both NO and H₂O₂ are diffusible, NO oxidation by MPO can occur in both intracellular and extracellular compartments [26]. Supernatants were removed for analysis of protein content, heme peroxidase activity and NO consumption after addition of quantitative amounts of NO. Expectorated sputa from healthy controls were induced by inhalation of hypertonic sterile saline (3%) generated by an Ultraneb 99 ultrasonic nebulizer (De Vilbiss Health Care, Sommerset, PA) [27]. During 12–15 min of inhalation, subjects were encouraged to first eliminate saliva, followed by mouth rinse with water, and then instructed to cough for sputum collection. The sputum was frozen and processed as described above. In a second set of CF patients (where expired NO was measured), sputum was processed by sonication to lyse sputum-associated leukocytes followed by centrifugation. The resulting pellet and supernatant were treated with DNase as described above. The supernatants from both the centrifugation step and DNase treatment were assayed for heme peroxidase activity and NO consumption after addition of exogenous NO.

2.3. Expired NO (eNO) measurements

A chemiluminescence-based NO analyzer (Sievers, Boulder, CO) was used to measure eNO of healthy subjects and CF
patients. Calibrations were performed using authentic NO gas standards. On-line orally-expired NO measurements were obtained using the restricted breath technique in accordance with ATS guidelines [28]. Patients performed serial expiratory vital capacity maneuvers at various expiration rates (40 and 100 ml/s). This method measures lower respiratory tract NO and minimizes nasal contributions, but does not rigorously partition NO production between alveolar/small airways and large airway compartments [29–31], nor take into account salivary contributions [13,32].

2.4. Heme peroxidase activity measurements

Heme peroxidase activity was determined by measuring the rate of H$_2$O$_2$-dependent oxidation of TMB. Aliquots of each sample, containing 5 µg of total protein, were mixed with sodium acetate buffer (300 mM, pH 5.4, containing 1.2 mM TMB prepared in DMF) and H$_2$O$_2$ (300 µM) in a total of 200 µl in microtiter plate wells. The rate of TMB oxidation was monitored at 630 nm over 2 min on a PowerWave$_{XI}$ UV–Vis plate reader (Bio-Tek Instruments, Inc). Rates are expressed as ΔOD$_{630}$/min/mg protein. In some experiments dapsone (1 mM) was included in the assay buffer.

2.5. NO consumption measurements

Measurements of NO catabolism were made using a NO-specific electrode (World Precision Instruments) and data recorded using a Macintosh-based PowerLab data acquisition software package (ADInstruments, Inc.). Aliquots of the solubilized sputum samples were incubated in PBS (containing 100 µM DTPA) in a glass chamber equilibrated at 37 °C. When a stable baseline was achieved, NO (800 nM) was injected as a bolus from a saturated anaerobic stock NO solution (~1.7 mM). Once NO had begun to decay at a slow basal rate, hydrogen peroxide (10 µM) was added. The difference in the rates of NO decay before and after the addition of H$_2$O$_2$ was used to determine the MPO-dependent rate of NO consumption in the soluble sputum samples.

2.6. ELISA for MPO

High-binding Costar brand 96 well plates were coated with a mouse monoclonal antibody to MPO (The Binding Site, Inc.; prepared in 50 mM sodium bicarbonate buffer, pH 9.6; 0.7 µg/well) overnight at 4 °C. Wells were then washed three times with Tris/HCl buffer (50 mM, pH 7.8, containing 150 mM NaCl and 0.1% Tween-20) and then blocked for 1 h at room temperature. The secondary and tertiary antibodies were extensively and alkaline phosphatase substrate solution added (100 µl/well of a 1.5 mg/ml 4-nitrophenylphosphate solution prepared in 1 M diethanolamine, pH 9.8, containing 0.5 mM MgCl$_2$). The reaction was stopped by the addition of NaOH (1 M, 50 µl/well, containing 1 mM EDTA) and the plate read at 405 nm.

2.7. Western blot analysis of MPO

Aliquots of sputum samples containing low, medium and high amounts of MPO protein (as determined by ELISA) were separated by SDS-PAGE on 12% gels under reducing conditions. Proteins were transferred to nitrocellulose membranes and incubated overnight in PBS containing 0.05% Tween-20 and 5% non-fat dry milk. Membranes were incubated with MPO antibody (rabbit polyclonal antibody against human MPO from Calbiochem) at a dilution of 1:2500. A horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody (diluted 1:10,000), followed by detection using enhanced chemiluminescence.

2.8. Immunodepletion of MPO

Sputa from CF patients processed as noted above were subjected to immunodepletion of MPO using a polyclonal antibody against human MPO. Briefly, sputum supernatants were pre-cleared by incubation with protein-G sepharose (25 µl), and the resultant supernatant incubated with 10 µg of polyclonal MPO antibody for 2 h at 37 °C. Protein-G sepharose (50 µl) was then added and incubation continued for 1 h. Complexes were then removed by centrifugation, and rinsed three times with PBS. Supernatants were then assessed for MPO protein by ELISA and NO consumption activity.

2.9. Quantitation of NO metabolites (NO$_x$) in sputum

Nitrite (NO$_2$) and nitrate (NO$_3$) in soluble supernatants of DNase-treated CF sputum and induced sputum from healthy subjects were detected as NO following reduction with vanadium III under acidic conditions at 95 °C, on an Antek 7020 Chemiluminescence NO detector (Antek Instruments, Houston, TX) as previously described [22].

2.10. Statistics

All data are expressed as the average±SD. Details of individual statistical tests are noted in the figure legends.
3. Results

3.1. Heme peroxidase activity and eNO

Orally-expired NO (eNO) was quantified in adult CF patients and the levels compared to the heme peroxidase activity in their respective sputum samples. There was no significant relationship between eNO levels and MPO activity in the supernatant of sputum solubilized by centrifugation alone (data not shown). However, when the pellet remaining after centrifugation was subjected to DNase treatment (which liberates extensive quantities of MPO from the insoluble mucus), low levels of expired NO correlated with high levels of heme peroxidase activity in the solubilized sputum of CF patients (Fig. 1A,C). Compared to normal controls, CF patients had 2- to 3-fold lower eNO levels. As shown for two expiratory flow rates (40 and 100 ml/s), a dramatically low eNO to heme peroxidase ratio characterizes adult CF patients. These data support our hypothesis that the previously described NO oxidase activity of MPO [26,33] may contribute to decreased eNO in CF patients with significant respiratory tract infections.

3.2. NO metabolites in sputum

As shown in Fig. 2, and despite the rather large spread of data, the levels of total NO metabolites ($\text{NO}_x$) were not significantly different between normal controls ($112\pm25 \mu\text{M}$) and adult CF patients ($132\pm30 \mu\text{M}$). This suggests that the decreased eNO observed in CF patients with airway infections, compared to controls, is not due to an overall compromised NO synthesis within the respiratory tract. In contrast, these results suggest that NO catabolism within the airway prior to exhalation in the gas phase is responsible for the decline in eNO in CF patients with airway infections. When NO is oxidized by MPO, $\text{NO}_2^-$ is generated [26,33]. Continued oxidation of $\text{NO}_2^-$ results in the formation of $\text{NO}_3^-$ by MPO and other oxidative processes. Thus, the relative quantities of $\text{NO}$, $\text{NO}_2^-$ and $\text{NO}_3^-$ provide mechanistic insight into potential routes of NO metabolism in the CF lung. In contrast to total $\text{NO}_x$ levels which did not differ between controls and CF patients, the ratio of $\text{NO}_2^-$ to $\text{NO}_3^-$ was lower in CF sputum ($0.26\pm0.1$) compared to that observed in induced sputum from healthy subjects ($0.46\pm0.1$). This suggests that more $\text{NO}_3^-$ was generated relative to $\text{NO}_2^-$ in CF compared to control subjects.
further confirmation of a highly oxidizing milieu in the CF airway [7].

3.3. Relationship between peroxidase activity, MPO protein and NO consumption

Heme peroxidase activity was measured in the DNase-treated CF sputum by determining the rate of TMB oxidation. The rate of TMB oxidation (which reflects heme peroxidase activity) ranged from ~1 to 23 OD$_{650}$/min/mg protein in CF sputum ($n=16$). An association was observed between the level of sputum heme peroxidase activity and H$_2$O$_2$-dependent NO consumption rates (Fig. 3), as quantified with an NO-specific electrode. This trend, however, did not reach statistical significance ($p=0.163$). Of note, the NO oxidizing activity of CF sputum (and purified MPO) remains quantitatively and kinetically essentially the same over pH values between 6.0 and 8.0 (data not shown).

To determine whether the peroxidase responsible for TMB oxidation and NO consumption was MPO, the amount of MPO protein in the samples was determined by a MPO-specific ELISA. Higher rates of H$_2$O$_2$-dependent NO consumption were observed in sputum specimens containing larger quantities of MPO protein (Fig. 4A). This association did reach statistical significance ($p=0.004$). In addition, sputum samples shown to contain low, medium and high amounts of MPO determined by ELISA were separated by SDS-PAGE and a western blot was probed for the MPO heavy chain. The intensity of staining on the blot agreed qualitatively with the results obtained by ELISA (Fig. 4B).

Lactoperoxidase (LPO) has been identified as a major heme peroxidase in normal disease-free respiratory tract secretions [34] and is also able to consume NO [33] and be modulated itself by NO [35]. While our data suggest that the peroxidase responsible for NO consumption in CF sputum is MPO, it is possible that LPO may contribute. To define the contribution of LPO, peroxidase activity and NO consumption measurements were performed in the presence of 4,4′-diaminodiphenylsulfone (dapsone, 1 mM), as lactoperoxidase is more sensitive to inhibition by dapsone than MPO [36]. Dapsone did not inhibit peroxidase activity nor did it significantly alter NO consumption in CF sputum samples (data not shown), yet the peroxidase activity and NO consumption activity of purified LPO are inhibited by dapsone. These data further support a role for neutrophil-derived MPO as the predominant peroxidase at respiratory tract surfaces in CF patients.

Fig. 2. Expectorated sputum NO$_x$ (NO$_2^-+NO_3^-$) levels are not different between normal and CF patients. A scatter plot is shown for 6 normal controls and 12 CF patients. The average±SEM for controls and CF patients was 133±30 µM and 132±30 µM, respectively. A two-sided Wilcoxon–Mann–Whitney test revealed that there was no statistically significant difference in sputum NO$_x$ between the two groups ($p=1.0$).

Fig. 3. Relationship between ex vivo NO consumption rates and sputum peroxidase activity in CF patients. Data represent sputum from 16 individual adult CF patients. Whereas there was a trend toward a direct relationship, the two-sided $p$-value using Spearman’s rank correlation coefficient ($p=0.163$; ASE=0.222) to assess the association between NO consumption rate and peroxidase activity was 0.163, indicating no statistically significant association.

Fig. 4. Ex vivo NO consumption rates are directly proportional to sputum MPO protein levels in CF patients. MPO protein levels in CF sputum, determined by ELISA (A), were generally confirmed by Western blotting (B). Low, medium, and high in panel B refer to representative CF patients who had MPO levels determined by ELISA in the lowest third, the middle third, and the highest third, respectively (two independent patients per category). The two-sided $p$-value using Spearman’s rank correlation coefficient ($p=0.004$; ASE=0.164) to assess the association between NO consumption rate and MPO protein was 0.004, which indicates that there was a statistically significant association between NO consumption rate and MPO protein.
3.4. Effects of immunodepleting MPO on NO consumption

To further define the role of MPO as a catalyst of NO catabolism in CF sputum, experiments were performed using specific antibodies to deplete MPO. Immunodepletion of MPO resulted in substantially reduced NO consumption rates in CF sputum (Fig. 5A), that were paralleled by a decrease in MPO protein (Fig. 5B). Collectively, these results provide strong evidence supporting a role for MPO as the major heme peroxidase responsible for catalyzing H$_2$O$_2$-dependent NO consumption in CF sputum. The data may illuminate a mechanism contributing to decreased exhaled NO in adult CF patients with on-going progressive respiratory tract infections.

3.5. Bidirectional modulation of peroxidase/H$_2$O$_2$-dependent NO consumption in CF sputum

MPO can act as a NO oxidase by generating substrate radicals (such as tyrosyl radicals) that oxidize NO [26,37]. Thus, we determined the effect added tyrosine would have on NO consumption in CF sputum. Real-time traces indicating NO oxidation are shown for the reaction with CF sputum in the absence and presence of tyrosine (Fig. 6A,B) or Trolox (Fig. 6D,E). Addition of tyrosine to sputum increased H$_2$O$_2$-dependent NO consumption in a concentration-dependent manner (C). Also shown are peroxidase/H$_2$O$_2$-dependent NO consumption rates in CF sputum (D) and CF sputum supplemented with 500 µM Trolox (E). Trolox decreases peroxidase/H$_2$O$_2$-dependent consumption of NO in a concentration-dependent manner (F). Values in C and F represent the average±SD.
manner (Fig. 6C). In contrast, addition of Trolox decreased NO consumption in a concentration-dependent manner (Fig. 6F). These data provide a means of achieving either enhanced or diminished NO oxidation in CF respiratory tract secretions as measured in expectorated sputum.

4. Discussion

CF airways are infiltrated by extraordinarily high numbers of neutrophils resulting in the release of high concentrations of MPO into the respiratory tract fluids [21,22]. The levels of expired NO (eNO) are consistently low in CF patients [9–12] compared to patients with other inflammatory lung diseases or healthy subjects. The complex pathophysiology of this disease has made it difficult to characterize all of the mechanisms contributing to this finding. Evidence is conflicting, with levels of NO metabolites measured in some studies being elevated [12,38] while other studies have shown variably reduced expression of inducible NO synthase [14,39]. Importantly, pharmacologic manipulation of NO in patients with CF has been proposed as a therapeutic approach to modulating disease progression [19,40,41]. In the present study, we have shown that the levels of expired NO negatively correlate with MPO activity in CF sputum, supported by in vitro studies showing that CF sputum with high levels of MPO oxidize NO at high rates.

MPO is an NO oxidase which utilizes substrate radicals to oxidize NO to NO₂ [26]. Thus, a plausible explanation for the lowered expired NO in CF with intense respiratory tract inflammation is the catalytic consumption of NO by MPO. We have shown that DNase-treated sputum consumes NO and that the sputum with the highest rates of NO consumption also had the highest levels of MPO (Fig. 4). NO oxidase activity is not restricted to MPO. Lactoperoxidase and eosinophil peroxidase can also consume NO [33]. While eosinophils are prevalent during hypersensitive responses such as allergen induced asthma [42], their numbers are not elevated in CF [43,44]. While it is clear that most inflammatory lung diseases are associated with increased expired NO and increased heme peroxidase activity, it should be pointed out that the levels of heme peroxidases in asthma is orders of magnitude lower as compared to levels reported in CF. It seems possible that expired NO levels could be even higher in asthma if the heme peroxidases (ie. eosinophil peroxidase) were not expressed by the infiltrating phagocytic cells. Respiratory tract lactoperoxidase is involved in respiratory tract antimicrobial activities [23]. However, in the present studies selective inhibition of lactoperoxidase by dapsone did not inhibit the peroxidase-dependent consumption of NO or the oxidation of TMB. Using an ELISA method for MPO protein and immunoblotting protein in the samples with an antibody to the heavy chain of MPO, we have demonstrated that the peroxidase consuming NO in CF sputum is highly associated with MPO. Lastly, immunodepletion studies have revealed that MPO is the primary NO consuming heme peroxidase in CF sputum.

The bidirectional modulation of MPO-dependent NO consumption by Trolox or tyrosine offers a novel theoretical construct for altering NO bioavailability in CF airways (Fig. 7). For example, administration of Trolox into the CF airway would increase bioavailable NO and increase its pleotropic airway effects [45]. In contrast, administration of tyrosine would accelerate the oxidative consumption of NO and further decrease airway NO bioavailability. Interestingly, ascorbic acid (vitamin C), a reductant that has been suggested for airway administration as an ‘antioxidant’ and a possible CFTR activator [46], would act like tyrosine and accelerate MPO-dependent consumption of NO [26]. Of relevance, recent studies have suggested that exogenous NO₂ at the mildly acidic pH of airway secretions can be transformed into NO that could potentially facilitate the killing of mucoid bacteria in anaerobic biofilms [40]. Not only would this provide for increased bioavailable NO, therapeutic concentrations of NO₂ (~15 mM) could also serve to inhibit MPO and its NO consuming activity [47].

Some limitations of the current methodology should be mentioned. In order to mimic H₂O₂ generation systems by phagocytes, bacteria and/or respiratory tract cells, small amounts of H₂O₂ (10 μM) were added to activate MPO which may or may not reflect the scenario in the infected and inflamed CF lung. Although our data suggest that MPO is the primary catalyst of H₂O₂ and NO consumption, we cannot exclude the contribution of other heme peroxidases or redox-active metals within CF sputum [48]. Because of the wide variation of sputum NOx values and great variability of sputum volume production by individual CF patients, no attempts were made to present stoichiometric correlations among eNO, sputum MPO and sputum NOx (as would be ideal). Finally, the present data make no attempt to quantitate the number of other potential mechanisms for NO oxidative metabolism in the CF airway as might be expected to occur via reactions with other reactive oxygen species (ie. superoxide) or even the colonized bacteria themselves.

![Fig. 7. Schematic illustrating potential pathways for modulating MPO-dependent NO metabolism in airway secretions of CF patients. Second order rate constants are given with units of M⁻¹ s⁻¹ for reactions. Tyrosine enhances MPO-dependent consumption of NO, whereas Trolox inhibits MPO-dependent consumption of NO.](image-url)
In conclusion, the consumption of NO by MPO in CF airways may contribute, at least in part, to the reduction in expired NO measured in this patient population. Pharmacologic inhibition of MPO may provide a useful therapeutic approach in CF by decreasing oxidant burden and preserving the functions of NO as a bronchodilator, bacteriostatic agent, and anti-inflammatory mediator.

Acknowledgements

We wish to thank Genentech, Inc. for kindly providing DNase (Pulmozyme™). This work was supported by the Cystic Fibrosis Foundation (BMM and CEC), a Fellowship from the Cystic Fibrosis Research, Inc. (VTV), and a Training Grant in Comparative Respiratory Biology and Medicine (NHLBI #HL07013; JSH) and a NIH grant (NHLBI #HL2506; JPE). We wish to recognize the generous support of Pam Fair and Glen Sullivan. Statistical support was made possible by Grant Number UL1 RR024146 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. Information on Reengineering the Clinical Research Enterprise can be obtained from http://nihroadmap.nih.gov/clinicalresearch/overview-translational.asp.

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