Analysis of Sequential Aliquots of Hypertonic Saline Solution-Induced Sputum From Clinically Stable Patients With Cystic Fibrosis*

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Study objectives: Sputum induction (SI) is a noninvasive tool for sampling inflamed airways. The purpose of this study was to determine the optimal duration of collection in patients with cystic fibrosis (CF). The hypothesis was that the duration of SI collection would quantitatively and qualitatively alter the content of the induced sputum.

Methods: In 10 clinically stable patients with CF (mean ± SD age, 28 ± 7 years; mean FEV₁, 2.6 ± 0.7 L), SI was performed with 3% hypertonic saline solution at five time points over 20 min. 

Results: SI was well tolerated, with an average maximum fall in FEV₁ of 7% ± 7%. The sample volumes, urea concentrations, interleukin-8 concentrations, total cell counts, and nonsquamous cell counts remained constant (p > 0.05). The percentage of neutrophils decreased from 89% ± 5% to 86% ± 4% (p = 0.03), and the percentage of alveolar macrophages increased 5% ± 2% to 8% ± 4% (p < 0.01). The mean quantitative microbiological counts of nonmucoid Pseudomonas aeruginosa and Staphylococcus aureus decreased over the 20-min time period each by half a log (p = 0.05 and p < 0.01, respectively). Surfactant protein-A concentration increased from 1.6 ± 0.3 to 2.4 ± 0.4 ng/mL (log₁₀; p < 0.001).

Conclusions: We conclude that aliquots of induced sputum are similar in clinically stable patients with CF during 4-min intervals, although there is more alveolar sampling after 20 min. When induced-sputum samples are fractionated for research monitoring of inflammatory or microbiologic indexes, power calculations accounting for these variations over time are required.

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Key words: cystic fibrosis; hypertonic saline; sputum

Abbreviations: CF = cystic fibrosis; CI = confidence interval; ES = expectorated sputum; IL = interleukin; PMN = polymorphonuclear leukocyte; SI = sputum induction; SP = surfactant protein

Hypertonic saline has recently been used as a form of airway clearance in patients with cystic fibrosis (CF),¹ ² and as a means to obtain a lower airway sample for clinical use from patients who are unable to expectorate spontaneously.³ However, in high concentration (10%), hypertonic saline solution has been shown to cause bronchospasm in some patients with CF.⁴ Although sputum induction (SI) has been recognized as a research tool for over a decade with other airway disease such as asthma⁵–¹⁰ and COPD,¹¹–¹³ only very recently has SI been introduced as an outcome measure in CF.¹⁴–¹⁶

BAL has been held as the “gold standard” for airway sampling in patients with CF.¹⁷,¹⁸ BAL has been used to follow serial microbiology cultures,¹⁸–²² in therapeutic trials,²³–²⁵ and to monitor inflammatory mediators and other markers of the underlying pathophysiologic mechanisms of CF.²⁶,²⁷ However, there are problems with the BAL sampling technique in patients with CF. Foremost is the safety issue: young children may require general anesthesia for bronchoscopy and adults require conscious seda-
tion. In addition, the CF lung compartments are not uniform. Inflammation is higher in the upper lobes than in the lower lobes. Some investigators compromise by sampling the lingula. Thirdly, the volume of saline solution instilled during BAL and whether to separate the first aliquot from later samples have not been standardized. The first aliquot from a CF BAL has a much higher percentage neutrophil count than subsequent samples. Finally, the BAL itself may cause an inflammatory response even in normal subjects. Thus, repeated BAL sampling to examine an airway inflammation outcome measure may lead to information reflecting adverse events from the bronchoscopy with BAL procedure rather than adverse events from the study drug.

We have previously shown that SI may provide a more representative airway sample than BAL in patients with CF. When SI is compared with BAL and expectorated sputum (ES), SI had a lower percentage of squamous cells than ES; all three techniques had a similar nonsquamous cell differential count, quantitative microbiology correlated best between ES and SI, and cytokine measurements by all three techniques were similar once corrected for the dilution of BAL.

The current study sought to determine whether a sputum sample obtained after a short time period (4 min) may reflect that of a longer time period, to examine whether 20 min of SI collection would sample the alveolar space as manifest by a change in WBC differential and surfactant concentration, and to determine if aliquots of induced-sputum samples showed changes in microbiologic and inflammatory indexes over time.

**Materials and Methods**

**Subjects**

Subjects were recruited from the CF clinic at the University of Washington, Seattle, WA. All subjects had a proven diagnosis of CF. All subjects were ≥ 18 years old and gave written informed consent for this study, which was approved by the Human Subjects Committee of the University of Washington. All subjects had an initial FEV₁ > 40% predicted and were in clinically stable condition. Subjects remained receiving all long-term medications, including antibiotics.

**Study Design**

On the day of SI, spirometry was performed at baseline, 10 min after inhalation of 180 µg of albuterol via metered-dose inhaler prior to SI, and 5 min following the completion of SI. Peak flow measurements were obtained every 4 min of the SI procedure to ensure that significant bronchospasm (20% fall) was not occurring. Spirometry was measured in all patients with a Cybermedic spirometer (Cybermedic; Boulder, CO) according to American Thoracic Society guidelines.
observations. The regression model for each outcome measure included indicator variables for the effects of sampling time, and a test that the time variables were jointly equal to zero was performed. The null hypothesis for these tests was that sampling times, taken together, had no effect on the outcome of interest. A test for linear time trend was also performed for each outcome measure by fitting a regression in which time was modeled as a continuous variable. Finally, the mean change calculated as the 20-min value minus the 4-min value with 95% confidence intervals (CIs) was calculated.

Sample Size Calculations

This was a pilot study. There was no prior information from which a sample size calculation could be made. However, we performed a post hoc power analysis to determine what size effect could have been missed given the sample and results of our pilot study.

Results

Demographic and Safety Profile

Ten patients with CF (eight male) entered and completed the study. Three patients were homozygous for DF508, six patients were heterozygous for DF508, and one patient's alleles were G551D/N1303K. Mean age was 28 ± 7 years (range, 18 to 38 years). Three subjects were prescribed inhaled tobramycin and one patient was prescribed oral ciprofloxacin at the time of the collection. Four adverse events occurred in three subjects, including throat irritation in three subjects and headache in one subject. The baseline prealbuterol FEV1 was 3.0

Sample Volumes

The sample volumes determined by weight were 3.0 ± 1.8 mL, 2.8 ± 1.5 mL, 4.6 ± 6.6 mL, 4.8 ± 5.1 mL, and 3.8 ± 2.1 mL, collected at 4, 8, 12, 16, and 20 min, respectively. The mean change from the 0- to 4-min sample to the 16- to 20-min sample was 0.81 mL (95% CI, 0.43 to 2.05). There was no significant difference in volume of induced-sputum aliquots over time (p = 0.12).

Urea

Urea was detected in all subjects and in all samples. The mean urea concentration was 4 ± 1 mg/dL, 3 ± 1 mg/dL, 3 ± 1 mg/dL, 4 ± 1 mg/dL, and 3 ± 1 mg/dL at the five time points. The mean change from the 0- to 4-min sample to the 16- to 20-min sample was 0.20 mg/dL (95% CI, -0.65 to 0.25). There was no linear trend in urea concentration with time (p = 0.99).

Total Cell Counts and Cell Differentials

The total cell counts of samples collected at 4, 8, 12, 16, and 20 min (mean ± SEM) were 1.8 ± 1.6 ¥ 107/mL, 1.8 ± 1.8 ¥ 107/mL, 1.8 ± 1.7 ¥ 107/mL, 1.6 ± 1.7 ¥ 107/mL, and 1.8 ± 1.8 ¥ 107/mL, respectively. There was no sample at the 20-min time point in one patient. There was no difference in nonsquamous cell count over time (p = 0.99). The percentage of squamous cells were 5.9 ± 6.6%, 6.8 ± 10.1%, 4.7 ± 5.2%, 5.1 ± 4.7%, and 3.4 ± 2.6% for each time point (p = 0.83). The percentage of neutrophils (mean ± SD) were 89 ± 5%, 88 ± 4%, 87 ± 4%, 84 ± 6%, and 86 ± 4%, sequentially (Fig 1), thus slightly decreasing over time (p = 0.03). The percentage macrophage count was 5 ± 2%, 7 ± 3%, 7 ± 3%, 10 ± 5%, and 8 ± 4%, thus mildly increasing over time (p < 0.01; Fig 1).

Microbiology

The number of organisms in study subjects ranged from two to seven. In addition to the presence, the quantitation of a specific set of organisms was examined. Mucoid and nonmucoid Pseudomonas aeruginosa (mucoid n = 9, nonmucoid n = 5), Staphylococcus aureus (n = 6), Burkholderia cepacia (n = 1), Stenotrophomonas maltophilia (n = 4), Acinetobacter (formerly Alcaligenes) xylosidans (n = 2), Haemophilus influenzae (n = 0), Enterococcus (n = 1), Acinobacter (n = 1) andRalstonia pickettii (n = 6) were selected. Quantitation of each organism in each sample was also examined (Fig 2). There was a mean half-log decrease in both nonmucoid P aeruginosa and S aureus with time (p = 0.05 and p < 0.01, respectively). The mean changes in nonmucoid P aeruginosa density and S aureus were 0.54 log10 cfu/mL (95% CI, -1.60 to 0.52) and -0.55 log10 cfu/mL (95% CI, -1.42 to 0.32), respectively. There was no change in bacterial density of mucoid P aeruginosa (p = 0.12). R pickettii was seen in 60% of subjects but at low colony counts up to 7,600 colonies per milliliter.
Inflammatory Mediators

IL-8 was detected in all subjects and in all samples. The mean ± SD concentration (log$_{10}$) was 1.8 ± 1.4 ng/mL, 1.8 ± 1.5 ng/mL, 1.9 ± 1.6 ng/mL, 1.8 ± 1.6 ng/mL, and 1.8 ± 1.5 ng/mL at the five time points, respectively. The mean change in IL-8 (log$_{10}$) was -0.026 ng/mL (95% CI, -0.103 to 0.051). There was no linear time trend in IL-8 concentrations (p = 0.99; Fig 3).

Interpretation of Results in Light of Multiple Comparisons

As 10 different statistical outcome measures were performed, a value of p ≤ 0.005 is required to maintain the overall risk of a type I error equal to 0.05. We can reasonably conclude that only SP-A is statistically remarkable (p < 0.001).

SPs

SP-D was not found in any sample. SP-A was detected in all subjects and in all samples. The mean ± SD concentration (log$_{10}$) was 1.62 ± 0.29 ng/mL, 1.95 ± 0.62 ng/mL, 2.23 ± 0.58 ng/mL, 2.30 ± 0.59 ng/mL, and 2.40 ± 0.44 ng/mL at the five time points, respectively. The mean change in SP-A (log$_{10}$) was 0.79 ng/mL (95% CI, 0.46 to 1.11). There was a linear time trend in SP-A concentrations (p < 0.001; Fig 4).

Discussion

Serial aliquots of induced sputum in clinically stable adult CF patients with mild-to-moderate obstructive lung disease show significant changes over time in SP-A concentration and a 3% increase in alveolar macrophages. This provides evidence for increased distal airway sampling over time with the SI procedure in patients with CF. In addition, there was a 3% decrease in polymorphonuclear leukocytes (PMNs), and a half-log decrease in P. aeruginosa and S. aureus density between the 0- to 4-min and 16- to 20-min collections.
SI was relatively well tolerated by all subjects. However, two subjects had a >15% fall in FEV₁ with this procedure even when premedicated with albuterol. However, the adverse effects associated with the sedation and the procedure of bronchoscopy with BAL on pulmonary function and oxygenation in patients with CF is not well reported but would appear greater than those of SI. Throat irritation was felt by three subjects.

Induction of sputum with 3% hypertonic saline solution has been shown to be relatively safe in patients with moderate-to-severe asthma, and was associated with no serious adverse events in our 10 subjects with CF. The majority of CF patients have reactive airway disease, but the degree of airway reactivity to the challenge of hypertonic saline solution in the limited number of CF subjects studied to date appears to be less than that observed among asthmatic subjects. Microbiology results suggested SI may be a useful tool in the multicentered research setting. Aliquots of induced sputum appear to provide a representative sample as quantitative bacterial cultures only varied by approximately a half-log count over time. In asthmatic subjects, fractional analysis of sequential induced sputum at 4-min intervals over a 20-min interval showed a decrease in percentage of PMNs and an increase in percentage of macrophages after the 0- to 4-min aliquot. In the CF population, the total cell counts are higher than those seen in asthmatics, reflecting the chronic infected airways of CF patients. The change in aliquots appeared to be less pronounced than those of asthmatics, so that a 4-min collection may be more representative of a longer time sample in CF subjects. Thus, shorter sputum collection times may be adequate for CF subjects.

Microbiology results suggested SI may be a useful tool in the multicentered research setting. Aliquots of induced sputum appear to provide a representative sample as quantitative bacterial cultures only varied by approximately a half-log count over time. In standard induced-sputum sample processing, dithiothreitol has to be added to the sputum sample to homogenize it. However, the addition of dithiothreitol decreases microbiological counts after 2 h. Thus, for multicenter studies, when microbiological samples may be sent to a centralized laboratory, it appears that a sample from the first 4-min aliquot may vary from the last aliquot by a half-log count.

Inflammatory mediators were found in concentra-
tions equivalent to or greater than those previously reported in patients with CF. The presence of hypertonic saline solution may increase the amount of Na+ and Cl− in airway surface liquid and rehydrate the periciliary fluid. Hyperosmolarity has been shown to stimulate IL-8 production.

**Figure 3.** Concentrations of IL-8 from induced-sputum aliquots. IL-8 was detected in all subjects and in all samples. There was no linear time trend in IL-8 concentrations (p = 0.99).

**Figure 4.** Concentrations of SP-A from induced-sputum aliquots. SP-A was detected in all subjects and in all samples. There was a linear time trend in SP-A concentrations (p < 0.001). HSPA = human surfactant protein-A.
in human bronchial epithelial cells in vitro, but we doubt that a 20-min exposure to hypertonic saline solution in vitro would allow adequate time to increase intracellular IL-8 production.

Type II alveolar epithelial cells are responsible for producing and secreting surfactant and most of the surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) into the alveolar lining fluid. Deficiencies in SP-A and SP-D in lavage fluid have been documented in various human inflammatory lung diseases, including pneumonia, ARDS, and CF. Greene et al demonstrated that reduced levels of SP-D in BAL fluid from patients with ARDS are associated with a worse outcome. Greene et al hypothesize that the low surfactant levels are not just a biomarker of acute lung injury, but that their absence exacerbates the dysfunctional inflammation characteristic of acute lung injury. As anticipated, the concentration of SP-A increased in the later time samples in our study, reflecting that the content of these later samples contained more alveolar fluid.

Sputum induction has become a well-validated research tool for evaluating a variety of indexes of inflammation, both cellular and humoral, in non-CF diseases of the airways such as asthma. This study demonstrates that SI is well tolerated and provides valid information regarding the markers of inflammation and the microbiology of the airways in subjects with CF. If patients or research subjects are able to expectorate at least 1 mL of sample using hypertonic saline solution, then this can provide an adequate sample for clinical information. However, further studies are needed to examine modest, but significant changes in CF pathogen density and PMN count for use of serial aliquots as outcome measurements in clinical trials.

In summary, fractional analysis of induced sputum from clinically stable CF subjects shows that aliquots of induced sputum provide representative samples for microbiologic and inflammatory indexes, and this suggests it should be possible to apply the varied processing methodologies necessary for different outcome measurements in multicenter studies.

REFERENCES