Epithelial lining fluid solute concentrations in chronic obstructive lung disease patients and normal subjects

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Effros, Richard M., Barry Peterson, Richard Casaburi, Jennifer Su, Marshall Dunning, John Torday, Julie Biller, and Reza Shaker. Epithelial lining fluid solute concentrations in chronic obstructive lung disease patients and normal subjects. J Appl Physiol 99: 1286–1292, 2005. First published May 26, 2005; doi:10.1152/japplphysiol.00362.2005.—The exhaled breath condensate (EBC) method represents a new, noninvasive way to detect inflammatory and metabolic markers in the fluid that covers the airways [epithelial lining fluid (ELF)]. However, respiratory droplets represent only a very small and variable fraction of the EBC, most (~99.99%) of which is water vapor. Our objective was to show that ELF concentrations could be calculated from EBC values by using any of three dilutional indicators (urea, total cations, and conductivity) in nine normal and nine chronic obstructive lung disease (COPD) subjects. ELF concentrations of Na+, K+, Ca2+, Mg2+, total cations, urea, and conductivity varied over a 10-fold range among individuals, but concentrations of these constituents (except Ca2+) remained well correlated (r2 = 0.44–0.83, P < 0.001). Dilution (D) of respiratory droplets in water vapor was calculated by dividing plasma concentrations of the dilutional indicators by EBC concentrations. Estimates of D were not significantly different among these indicators, and urea D averaged 10,800 ± 2,100 (SE) in normal and 12,600 ± 3,300 in COPD subjects. Although calculated Na+ concentrations in the ELF were less than one-half those in plasma, and concentrations of K+, Ca2+, and Mg2+ exceeded those in plasma, total cation concentrations in ELF were not significantly different from those in plasma, indicating that ELF is isotonic in both normal and COPD subjects. ELF amylase concentrations (measured with an ultrasensitive procedure) indicated that saliva represented <10% of the respiratory (ELF) droplets in all but three samples. Dilutional and salivary markers are essential for interpretation of EBC studies.

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Most inflammatory diseases of the lung are associated with increases in tissue concentrations of a variety of inflammatory mediators, which can be detected in sputum, bronchoalveolar lavage (BAL), and tissue samples. However, many patients are unable to produce sputum unless exposed to irritating solutions. Both lavage and biopsy approaches involve some risk to patients, and they cannot be used on a routine basis for monitoring the course of lung diseases or the response of these disorders to therapy. It is, therefore, understandable that considerable interest has grown in the exhaled breath condensate (EBC) approach, which may provide a noninvasive method for collecting and analyzing respiratory fluid.

A large number of publications have reported increases in EBC concentrations of inflammatory mediators in a variety of diseases that are known to be associated with tissue inflammation (12, 16, 20). However, it cannot be concluded that increased mediator concentrations in the EBC reflect comparable increases in the respiratory fluid [epithelial lining fluid (ELF)]. Most of the water collected in the EBC represents water vapor, a gas, which is formed by evaporation from respiratory surfaces and is then converted to droplets outside the lungs with cooling (4, 5, 7). The presence of nonvolatile mediators and other molecules in the ELF indicates that droplets of ELF that contain these solutes represent a very small fraction (~0.01%) of the EBC. Reported increases in EBC mediator concentrations could reflect increases in the relative number and volume of true droplets, rather than increases in mediator concentrations in ELF.

We found that electrolyte and urea concentrations in the EBC varied considerably among normal subjects and even vary when serial samples are collected from the same individuals at 30-min intervals (4, 5, 7). However, concentrations of these nonvolatile solutes remained proportional. This observation was consistent with the hypothesis that much of the variation in EBC concentrations was due to changes in the relative volumes of respiratory droplets that were collected in the EBC. We have endeavored to solve this fundamental deficiency of EBC studies by identifying a variety of indicators that can be used to calculate the dilution (D) of respiratory droplets by water vapor. We reasoned that the best indicators for this purpose would be markers that are present in the same concentrations in the plasma and ELF. We selected three “dilutional” indicators for this purpose: urea, total cation concentration, and the conductivity of lyophilized samples of EBC (4, 5, 7). Average D of these indicators were about the same, indicating that 1) any of these indicators could be used to calculate D, 2) the respiratory droplets are approximately isotonic in normal subjects, and 3) true respiratory droplets represent a very small fraction of the total EBC. These observations have been confirmed by Dwyer (3), who reported that urea concentrations are similarly very low and variable among normal subjects.

Our laboratory’s original reports were confined to normal subjects (4, 5, 7). We have extended these observations in this study to a comparison of normal subjects with patients who...
have chronic obstructive lung disease (COPD). To improve the reliability of the calculations of D, concentrations of the dilutional indicators were also made in the plasma rather than assuming average normal values for these indicators.

METHODS

Clinical information. Eleven normal subjects and 10 subjects with COPD were initially selected, but two normal subjects and one COPD subject were subsequently deleted from the study because EBC urea concentrations were too low to measure reliably for calculating D of respiratory droplets by water vapor (see below). The normal population was composed of three men and six women (average age: 60 ± 6 yr old (SD)) who did not smoke in recent past and had no history of lung disease. The COPD population was composed of five men and four women (average age: 65 ± 4 yr old) who were not recent smokers and had chronic obstructive lung function [forced expiratory volume in 1 s (FEV₁) < 75% predicted, FEV₁/forced vital capacity (FVC) < 75%]. To avoid any effect of recent bronchodilator therapy on EBC data, a bronchodilator was not administered to assess reversibility of obstruction. On the average, they had a history of 57 pack-yr of smoking, and all but one were on maintenance bronchodilators, which were not used during the 1 h before collection of condensates.

Spirometry was performed in all subjects (Sensormedics, Yorba Linda, CA), and measurements of FEV₁ and FVC were compared of smoking, and all but one were on maintenance bronchodilators, and race (1); n, number of subjects. COPD, chronic obstructive lung disease; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; FEV₁/FVC, ratio of FEV₁ to FVC.

| n  | FVC, % predicted | FEV₁, % predicted | FEV₁/FVC, × 100%
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Values (means ± SD) are based on standard values corrected for age, height, and race (1); n, number of subjects. COPD, chronic obstructive lung disease; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; FEV₁/FVC, ratio of FEV₁ to FVC.

Pulmonary function studies

Table 1. Pulmonary function studies

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Values (means ± SD) are based on standard values corrected for age, height, and race (1); n, number of subjects. COPD, chronic obstructive lung disease; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; FEV₁/FVC, ratio of FEV₁ to FVC.

Chemical analysis. The conductivity of the condensate samples was measured before and after lyophilization (YSI model 3200 conductivity meter, Yellow Springs, OH). Conductivity of the plasma was measured in unlyophilized, diluted samples. One-milliliter samples were placed in an inverted YSI 3252 conductivity cell, as described by the manufacturer. Cation concentrations were measured with an ion chromatograph (Metrohm model 761 compact IC, 0.5-ml sample loop, Metrosep C 2 100 column). The eluent contained 4 mM tartaric acid and 0.75 mM dipicolinic acid for analyses of K⁺ and Ca²⁺. Crown ether (0.5 mM) was added for Na⁺ and NH₄⁺ and Mg²⁺ analyses. (The crown ether was used to increase the distance between the Na⁺ and NH₄⁺ peaks.) Condensate urea concentrations were determined by measuring the concentration of NH₄⁺ released from incubating 1 ml of the lyophilized condensates with purified urease (Sigma, St. Louis, MO). Urea concentrations in the diluted saliva were measured with a sensitive, nonenzymatic procedure that is not influenced by the presence of NH₄⁺ in the samples (12, 13). Amylase concentrations in the EBC and saliva were measured with an EnzChek amylase assay kit (E-11954) from Molecular Probes (Eugene, OR). This ultrasensitive procedure is based on the release of a fluorescent dye from a starch substrate.

The coefficient of variance of repeated (5–10) measurements of electrolyte concentrations in the same sample at 5 μmol/l was <2%, and the coefficient of variance of urea at 1 μmol/l was ~20%. The lower limits of detection were 0.5 μM for each of the ions, 0.25 μM for urea, 2.5 μM NaCl for conductivity, and 0.1 ml/ml for amylase.

Equations and statistics. The D of respiratory droplets (ELF) by water was calculated from the equations:

\[
D_{area} = \frac{\text{Volume}_{EBC}}{\text{Volume}_{ELF}} \times \frac{[\text{Urea}]_{plasma}}{[\text{Urea}]_{ELF}}
\]

\[
D_{cations} = \frac{\text{Volume}_{EBC}}{\text{Volume}_{ELF}} \times \frac{[\text{Na}^+] + [\text{K}^+] + [\text{Ca}^{2+}] + [\text{Mg}^{2+}]}{[\text{Na}^+] + [\text{K}^+] + [\text{Ca}^{2+}] + [\text{Mg}^{2+}]}_{plasma}
\]

\[
D_{conductivity} = \frac{\text{Volume}_{EBC}}{\text{Volume}_{ELF}} \times \frac{[\text{Conductivity}]_{plasma}}{[\text{Conductivity}]_{ELF}}
\]

where the asterisks indicate that the conductivity or urea measurement must be made on lyophilized samples; brackets denote concentration; \(D_{area}, D_{cations}, \text{ and } D_{conductivity}\) are area, cations, and conductivity D, respectively; and \(\text{Volume}_{EBC}\) and \(\text{Volume}_{ELF}\) are BCF and ELF volumes, respectively. Lyophilization removes most of the volatile constituents (including NH₄⁺ and HCO₃⁻). The concentrations of ions and amylase in the ELF were calculated from EBC concentrations with the equation:

\[
[Solute]_{ELF} = [Solute]_{EBC} \times D_{area}
\]

Urea was used in these calculations rather than conductivity or cation concentrations to minimize the effect of shared variables on correlation coefficients of electrolyte concentrations.

The volumetric fraction (F) of the ELF that represented saliva (Fsaliva) was calculated from the equation:

\[
F_{saliva} = \frac{V_{saliva}}{V_{ELF}} = \frac{[\text{Amylase}]_{ELF}}{[\text{Amylase}]_{saliva}}
\]

where \(V_{saliva}\) and \(V_{ELF}\) are saliva and ELF volume, respectively. Statistical analyses were conducted with SigmaStat version 2 software (Jandel, San Rafael, CA). A Kruskal-Wallis one-way analysis of variance on ranks with a Student-Newman-Keuls pairwise multiple-comparison procedure was used to compare plasma and ELF concentrations. Two-way analysis of variance was used to compare mean values of \(D_{area}, D_{cations}, \text{ and } D_{conductivity}\) and to compare total cation concentrations with conductivity measurements in the plasma of the...
normal and COPD populations. Solute concentration and D data were compared by linear regression and correlation analysis. A t-test was used to compare conductivity before and after lyophilization and to compare salivary and EBC concentrations of amylase. With the exception of average subject age, mean values are indicated with SEs of the means in the text and Figs. 1, 3, and 5–7, and a probability that means are different at a \( P < 0.05 \) level was considered significant. The correlations were analyzed between all of the ions and the urea concentration. These studies were approved by the Human Research Review Committees, and consent was obtained from each subject before each study.

RESULTS

Removal of volatile constituents. Lyophilization of the EBCs reduced the conductivity of these solutions by 95% (see Fig. 1) in both the normal and COPD populations. This observation is consistent with that of an earlier study in normal subjects, in which it was found that \( \text{NH}_4^+ \) represented 95% of the total cations in the EBC in normal subjects, and that nearly all of this cation (and other volatile ions) can be readily removed from the samples by lyophilization (4). Residual concentrations of \( \text{NH}_4^+ \) in the EBC samples averaged 1.5 ± 0.5 μmol/l in these normal subjects and 2.8 ± 1.4 μmol/l in the COPD subjects.

EBC solute concentrations. As indicated in Fig. 2, considerable differences were observed among subjects in the concentrations of various solutes in EBCs. However, correlation \( (r^2) \) of each pair of variables exceeded 0.44 \((P < 0.001)\) with the exception of \( \text{Ca}^{2+} \). Normal subjects; ○, COPD patients.
centrations of each of the constituents in the EBC. However, correlations between each of these parameters were significant \( (r^2 > 0.44) \), with the exception of Ca\(^{2+}\). No differences were observed in mean solute concentrations between normal and COPD subjects (Fig. 3). Furthermore, mean values calculated for total cation concentrations in the EBC were not significantly different from the mean value of conductivity of lyophilized samples (expressed in terms of molar NaCl concentration). The absence of a significant difference could be related to the small sample size of the populations. However, cation concentrations estimated from the conductivities of the plasma samples were slightly greater than the total cations measured by ion chromatography \( (151 \pm 4 \text{ vs. } 145 \pm 2 \text{ mmol/l in normal subjects and } 154 \pm 4 \text{ vs. } 140 \pm 2 \text{ mmol/l in COPD subjects}) \). \( (P < 0.05) \). Urea concentrations in the condensate averaged \( 0.73 \pm 0.13 \mu\text{mol/l} \) in the normal subjects and \( 0.97 \pm 0.31 \mu\text{mol/l} \) in the COPD patients (means not significantly different). Plasma concentrations of urea averaged \( 6.07 \pm 0.43 \text{ mmol/l in normal subjects and } 6.01 \pm 0.18 \text{ mmol/l in the COPD subjects.} \) The plasma urea concentrations were significantly higher than those found in the saliva \( (1.66 \pm 0.83 \text{ mmol/l in normal subjects and } 2.05 \pm 0.80 \text{ mmol/l in COPD}) \). \( (P < 0.05) \). Na\(^{+} \), K\(^{+} \), and Ca\(^{2+} \) represented the predominant cations in the lyophilized samples collected from both the normal subjects and COPD patients (Fig. 3).

**D of respiratory droplets by water vapor.** Because EBC concentrations of the measured solutes were very variable among subjects, much variability was also present in the values calculated for D, which ranged from 4,000 to \( > 50,000 \) (Fig. 4). Three EBC samples were excluded from the analysis because concentrations of urea were too low to be measured reliably (when \( D_{\text{urea}} > 100,000 \)). However, \( D_{\text{cations}}, D_{\text{urea}}, \) and \( D_{\text{conductivity}} \) were significantly correlated with one another (Fig. 4), and there were no significant differences between mean values of these estimates of D (Fig. 5).

**ELF solute concentrations.** As indicated in Fig. 6, the concentrations of individual nonvolatile solutes estimated in the ELF from the EBC data using \( D_{\text{urea}} \) were distinctly different from those found in the plasma. Concentrations of Na\(^{+} \) in the ELF were much lower than those found in the plasma of both normal subjects and those with COPD, whereas concentrations of K\(^{+} \), Ca\(^{2+} \), and Mg\(^{2+} \) were significantly higher in the ELF than in the plasma of these populations. Nevertheless, the total concentrations of cations in the ELF were not significantly different from plasma concentrations in both normal subjects and those with COPD. No differences were detected between the ELF or plasma concentrations of cations in the normal and COPD populations.

**Contribution of saliva to ELF.** EBC amylase concentrations averaged \( 0.36 \pm 0.08 \text{ mU/ml in the normal subjects and } 0.60 \pm 0.15 \text{ mU/ml in the COPD subjects} \) \( (P \text{ not significant}) \). Salivary amylase concentrations averaged \( 111,000 \pm 48,000 \text{ mU/ml in normal subjects and } 254,000 \pm 75,000 \text{ mU/ml in the COPD subjects} \) \( (P \text{ not significant}) \). Estimated concentrations of amylase in the ELF \( (\text{Eq. 4}) \) were \( < 10\% \) of those in saliva in all but three samples. The cationic concentrations in the saliva also differed significantly from those estimated in the ELF: salivary Na\(^{+} \) concentrations were significantly lower and salivary K\(^{+} \) concentrations were significantly higher than those calculated for the ELF \( (\text{Fig. 7}) \), and the total cation concentration of the saliva was less than that observed in plasma or ELF. Concentrations of NH\(_4\)\(^{+} \) in the saliva averaged \( 18.6 \pm 4.7 \text{ mmol/l in the normal subjects and } 15.9 \pm 2.0 \text{ mmol/l in the COPD patients} \) \( (P \text{ not significant}) \).

**DISCUSSION**

**D of ELF droplets in the EBC.** The principal attraction of the EBC approach has been the potential of noninvasively detect-
ing and measuring concentrations of cytokines and other indicators of interest in the ELF. The discomfort, risks, inconvenience, and expense of alternative approaches, such as BAL, sampling of tracheal fluids, and induced sputum collections, can thereby be avoided. However, there are two major problems with the EBC approach, which must be surmounted before it can become a useful research or clinical tool. These are related to the D of respiratory droplets in exhaled water vapor and uncertainty regarding the sites of generation of the droplets of "respiratory" fluid (ELF) that are present in the EBC. These problems can only be addressed by the development of appropriate dilutional and locational indicators.

Most (~99.9%) of the EBC is composed of water vapor, which is generated by evaporation from the pulmonary epithelium. Because the exhaled air is virtually saturated with water at body temperature, the amount of water vapor exhaled each minute is set by the minute ventilation. Full saturation of the exhaled air provides an essential function of keeping the exchange surfaces and airways moist, thereby facilitating gas diffusion and reducing the possibility that drying will result in increases in the concentrations of inflammatory mediators and other injurious solutes in the ELF. It must be emphasized that water vapor is a gas until it is cooled in the condenser, and solutes cannot be delivered to the condenser by the water vapor.

The presence of nonvolatile solutes in the EBC samples must indicate the release of droplets from the respiratory surface and their subsequent deposition on the condenser walls. Unlike water vapor, these droplets are formed by convective rather than diffusional processes, and it is likely that most are derived from the airways, where turbulence of the exhaled air is more likely to occur. Also, unlike the formation of water vapor, there is no reason to believe that the formation of respiratory droplets in the lungs is regulated, provides an important function, or is related to the rate of water vapor production. Considerable variation was found in EBC solute concentrations of normal subjects, even when these were collected sequentially (4, 5, 7). The observation of increased concentrations of inflammatory mediators in the exhaled air could be due to either increased concentrations in the ELF or simply to an increase in the number and size of respiratory droplets that were generated.

**Urea concentrations in the ELF.** There are several reasons for assuming that concentrations of urea are the same in the epithelial lining (ELF) and plasma. There is no evidence for production, active transport, or catabolism of urea in the lungs, and urea is not volatile. Equilibration of urea in fluid-filled lungs requires ~3 h, but should be much more rapid in air-filled lungs, which contain much less water in the air spaces (6). These properties of urea have been responsible for its adoption as a dilutional indicator in studies of BAL over the past 20 yr (19). Because of the rapidity with which urea diffuses from the blood to the air spaces, lavage must be completed very expeditiously to avoid additional urea entering the instilled fluid during the procedure. This is not a problem in EBC studies, which do not involve instillation of fluid into the lungs.

**Electrolyte concentrations in the ELF.** Controversy persists regarding the osmolality of the fluid that lines the respiratory surfaces (ELF). The extremely shallow depth of the ELF layer on respiratory surfaces complicates sampling of ELF. Quinton (18) reported that the ELF was hypotonic in normal subjects and isotonic in patients with cystic fibrosis. If true, the lungs would resemble the sweat glands in this respect, as the sweat in normal subjects is more hypotonic than in cystic fibrosis. Furthermore, there is evidence that antimicrobial peptides (defensins), which are released by pulmonary epithelial cells, are only active in hypotonic solutions (9). Recurrent infections in cystic fibrosis lungs might, consequently, be related to abnormally high electrolyte concentrations in the ELF of these subjects.
The observations of Quinton (18) were challenged by other investigators, who were unable to reproduce these findings (21). Quinton collected ELF by applying filters to the airway surfaces. It was argued that the volume of fluid collected in this fashion was greater than that which would be expected on the airway surfaces that were sampled, and some of the fluid may have been derived from the tissues as well as the ELF. A variety of studies appeared to indicate that the ELF is isotonic in both normal subjects and those with cystic fibrosis, in whom the physiological disorder may be related to a proportionate decrease in both water and solutes, with increases in the viscosity rather than the osmolality of the ELF (21).

It has also been suggested that solute concentrations are increased in patients with bronchial asthma (2). Hyperventilation could cause excessive evaporative losses of water that could not be replenished from the underlying respiratory mucosa. Airway drying could elevate the concentrations of salts or inflammatory mediators in the respiratory fluid and trigger bronchospasm. Once again, this hypothesis has been disputed by other investigators, who have argued that the respiratory fluid remains isotonic, although mucosal temperature may fall (13).

**Dilutional indicators.** We have selected three candidate indicators for calculating D: urea, total cations, and conductivity of lyophilized samples. As indicated in earlier papers, there is reason to believe that each of these indicators is present in similar concentrations in the ELF and plasma and can, therefore, be used to estimate D (3–7). No significant differences could be found for Durea, Dcations, or Dconductivity in normal or in COPD subjects. This observation is consistent with the hypothesis that the ELF is isotonic.

It is encouraging that mean values of D were not significantly different between normal and COPD subjects in the present study, and differences found in mediator concentrations between these populations could, therefore, be attributed to differences in respiratory fluid concentrations rather than the number of droplets found. However, unless D values are measured and used to correct for D, EBC studies cannot provide assurance that various lung disorders are associated with increased concentrations of mediators in the respiratory fluid, since the large variability of EBC measurements could exceed differences between EBC values measured in normal and abnormal subjects. The failure to measure D is comparable to estimating glomerular filtration rates from urine flows without measuring concentrations of urea, creatinine, or inulin in the urine and plasma.

The discovery that respiratory droplets represent ~0.01% of the EBC presents formidable analytical challenges in any studies of the EBC. Concentrations of cytokines should be ~1% of those in BAL samples and may, therefore, be impossible to measure reliably by conventional ELISA procedures. One advantage of the dilutional approach is that it can provide evidence that dilutional or inflammatory mediator concentrations in specific samples are too low to measure with any degree of reliability.

**Site-specific (locational) indicators.** The principal advantage of the EBC is that it permits collections of samples exhaled from the mouth. Unfortunately, oral collections are associated with another problem: uncertainty regarding the site of droplet formation. Concentrations of amylase were usually very low in the ELF compared with those in the saliva. Furthermore, concentrations of Na⁺ in the EBC were proportionately greater than those found in the saliva. It is, therefore, unlikely that saliva was the source of more than a small fraction of the solutes found in the EBC. However, measurements of amylase must be routinely performed to rule out salivary contamination. Although amylase is also produced in the pancreas, it is unlikely that much of this isozyme would reach the oral cavity. On occasion, amylase is also produced by adenocarcinomas, but it is also unlikely that this was a problem in this group of stable patients. Amylase assays must be able to detect concentrations <1.0 mU/ml. If salivary amylase is 100,000 mU/ml and the D of all droplets by water vapor in the condensate is 10,000, then the presence of 1.0 mU/ml would indicate that 10% of the “respiratory” droplet volume present in EBC is derived from the mouth. The stomach represents another possible source for contamination and may contribute to some of the “acidopnea” described in patients with various lung diseases (8). It may be possible to use pepsin as a “locational” indicator of gastric contamination.

The unexpected observation that ELF Ca²⁺ concentrations are much higher than those in plasma and are not as well correlated with other ELF constituents (Fig. 2) may be related to the release of Ca²⁺ from surfactant components or mucin (10, 14, 17). Surfactant proteins might be used as a marker of the presence of respiratory constituents, which were generated in the distal parts of the lung, where surfactant is produced, and were then transported to the airways, where they were incorporated in droplets released from the airway surfaces.

**Future development of the EBC approach.** It can be anticipated that, like the development of pulmonary function studies of gas exchange over the past several decades, maturation of the EBC approach into a reliable technique will require progressive improvements in the analytical procedures used to analyze extremely low concentrations of dilutional, locational, and diagnostic indicators. Recent progress in protein detection is particularly promising in this regard (8). EBC studies would also be enhanced if the generation of respiratory droplets can be augmented in some manner. Although these are challenging objectives, novel techniques can be expected to emerge in the near future, which will expand the horizon of exhaled markers from a handful of gases to a vast array of nonvolatile inflammatory and metabolic markers.

**ACKNOWLEDGMENTS**

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