invited review

Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease

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Verkman, A. S., Yuanlin Song, and Jay R. Thiagarajah. Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. Am J Physiol Cell Physiol 284: C2–C15, 2003; 10.1152/ajpcell.00417.2002.—Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) protein, an epithelial chloride channel expressed in the airways, pancreas, testis, and other tissues. A central question is how defective CFTR function in CF leads to chronic lung infection and deterioration of lung function. Several mechanisms have been proposed to explain lung disease in CF, including abnormal airway surface liquid (ASL) properties, defective airway submucosal gland function, altered inflammatory response, defective organelar acidification, loss of CFTR regulation of plasma membrane ion transporters, and others. This review focuses on the physiology of the ASL and submucosal glands with regard to their proposed role in CF lung disease. Experimental evidence for defective ASL properties and gland function in CF is reviewed, and deficiencies in understanding ASL/gland physiology are identified as areas for further investigation. New model systems and measurement technologies are being developed to make progress in establishing lung disease mechanisms in CF, which should facilitate mechanism-based design of therapies for CF.

cystic fibrosis transmembrane conductance regulator; epithelium; fluorescent indicators; Pseudomonas aeruginosa

Cystic fibrosis (CF) is the most common inherited lethal disease in Caucasians. Chronic lung infection and deterioration of lung function are the major causes of morbidity and death in CF. Although the genetic defect in CF was discovered in 1989—mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFTR)—the mechanisms by which CFTR mutations cause lung disease remain uncertain. A number of mechanisms have been proposed to link the CF genotype to clinical disease, some of which include abnormal airway surface liquid (ASL) composition, defective airway submucosal gland secretion, defective intracellular vesicle function, loss of CFTR regulation of other transporting proteins, defective intrinsic antimicrobial function, hyperabsorption of airway fluid, excessive inflammatory responses, and others (reviewed in Refs. 7, 13, 62, 63, 83). Convincing evidence is lacking that these or other mechanisms are responsible for airway disease in CF, and there is no consensus in the field. Determination of the mechanism linking genotype to disease is of critical importance in developing therapies to treat CF, because therapies indicated by some mechanisms are clearly contraindicated by others.

Considerable attention has focused on possible abnormalities in the properties of the ASL, the thin layer of liquid that coats the upper and lower airways and provides a unique interface between inspired/expired air and the airway epithelium. The composition, volume, and physical properties of the ASL depend on secretions from airway submucosal glands, the transporting properties of surface epithelial cells, and convective movement of fluid up the airways. Abnormal composition and physical properties of the ASL and glandular secretions is proposed to promote chronic bacterial colonization of the airways by impairing mucociliary clearance and the activities of endogenous
antimicrobials and by providing an environment conducive to bacterial growth and Pseudomonas aeruginosa biofilm formation. This review focuses on the physiology of the ASL and airway submucosal glands with regard to their proposed role in CF lung disease.

AIRWAY DISEASE IN CF: THE HYPOTHESES

Figure 1 summarizes the principal hypotheses relating abnormalities in ASL and glandular physiology in CF to chronic bacterial infection and progressive deterioration in lung function. Three hypotheses involving abnormal ASL composition attempt to link defective CFTR function to chronic bacterial infection of the airways by primary changes in the ionic content, pH, or oxygenation of the ASL. The “high salt hypothesis” postulates that the normally low ASL NaCl concentration becomes high in CF, inhibiting the activity of endogenous antimicrobials such as defensins (24, 73). Defective CFTR chloride transport in CF is proposed to prevent chloride absorption by the airways. This model predicts that [NaCl] should be low (<60 mM) in normal subjects and high (>100 mM) in CF. The “low pH hypothesis” postulates that the ASL is abnormally acidic in CF, inhibiting mucociliary clearance mechanisms (13). Defective CFTR-dependent bicarbonate transport in CF is proposed to acidify the ASL. The “low oxygenation hypothesis” postulates that ASL oxygen content is low in CF because of increased oxygen consumption in CF airway epithelial cells and possibly slowed oxygen diffusion in the ASL, resulting in enhanced P. aeruginosa growth and biofilm formation and impaired clearance (85). Defective CFTR-epithelial sodium channel (ENaC) interaction is proposed to increase epithelial cell sodium absorption and cellular oxygen consumption in CF, producing in the steady state an oxygen gradient in the ASL with reduced averaged oxygen concentration. In contrast to these mechanisms involving abnormal ASL composition, the “low ASL volume hypothesis” postulates that ENaC hyperactivity and consequent sodium hyperabsorption in CF results in a viscous, dehydrated ASL that impairs mucociliary function and facilitates bacterial adherence (7).

The “defective gland function hypothesis” postulates that the primary defect in CF is reduced fluid secretion by airway submucosal glands and possibly altered secretion of mucous glycoproteins (34, 59, 80). One motivation for this hypothesis is the much greater expression of CFTR in serous epithelial cells lining glandular acini than in other tissues in the airways and lung (19). Defective CFTR chloride transport in CF glands would impair salt and water secretion, resulting in reduced secreted fluid volume, increased protein concentration, and increased viscosity. Progressive deterioration in gland function from mucus plugging would further reduce fluid secretion, leading to a dehydrated ASL as well as impairment of antimicrobial secretion by serous cells. The defective gland function hypothesis predicts reduced volume and increased protein concentration and viscosity in gland fluid secretions in CF, and possibly altered gland fluid ionic content, pH, and protein composition.

These hypotheses for airway disease in CF are not mutually exclusive, so that multiple mechanisms may operate in parallel. For example, defective gland fluid secretion and fluid hyperabsorption by airway surface epithelia could synergistically produce a viscous dehydrated ASL, which could reduce oxygen diffusion and create a relatively hypoxic environment, impairing P. aeruginosa clearance and enhancing biofilm transfor-

![Diagram](image-url)

Fig. 1. Hypotheses linking defective CFTR function to airway disease in CF. See text for explanations. ASL, airway surface liquid.
On the other hand, the plausibility of some of the proposed hypotheses is uncertain. For example, a conceptual difficulty with the high salt hypothesis is that the low ASL salt concentration in normal airways predicted by this hypothesis would require either a water-impermeable airway epithelium, which is not the case (22, 51), the presence of non-salt osmolytes, or the action of a surface phenomenon capable of maintaining an osmotic imbalance. The low oxygenation hypothesis requires increased oxygen consumption by CF epithelia in vivo, which has not yet been proven, as well as slow oxygen diffusion in the thin ASL layer.

The testing of these hypotheses has presented a formidable challenge because of difficulties in establishing suitable model systems and in measuring the physical parameters of the ASL and glandular secretions in intact airways. Well-differentiated airway epithelial cells grown on a porous support at an air-liquid interface have been used as a cell culture model to study ASL properties. The airway cell cultures recapitulate many native airway functions such as ion transport and ciliary beating. However, cell culture models have been highly variable from laboratory to laboratory; they cannot recapitulate the complex in vivo airway anatomy, hormonal regulation, and cellular heterogeneity, and they are not subject to time-varying air composition (moisture/PCO2/PO2) and convective fluid transport as they are in vivo. Also, the ASL depth in airway cell culture models is measured as <25 μm, whereas that in intact mammalian airways may be >50 μm, raising concerns that the determinants of ASL composition and volume in cell culture models may differ from those in intact airways in vivo (37, 65). Transgenic mouse models of targeted CFTR deletion or mutation are a potentially useful alternative; however, CF mice develop little or no lung disease (26). There are also a number of potentially important human vs. mouse species differences in airway physiology: submucosal glands are infrequent in mouse airways below the larynx, and the mouse airway epithelium appears to express an alternative chloride channel that may substitute functionally for the defective CFTR. Large animal models of CF do not yet exist. Measurements in intact normal vs. CF human airways are probably the most relevant to study ASL/gland physiology, recognizing the caveat that human airway anatomy and function are altered in response to chronic infection and inflammation. It is thus difficult to determine whether differences in normal vs. CF human airway physiology are related to the primary CFTR defect or to secondary consequences of the disease process.

**PHYSIOLOGY OF THE AIRWAY SURFACE LIQUID**

As depicted in Fig. 2, the large airways (trachea, bronchi) contain numerous submucosal glands and are lined by ciliated pseudostratified columnar epithelial cells with relatively few goblet and brush cells. The epithelium in bronchioles is more columnar, with Clara cells scattered among ciliated cells (10). Light and electron microscopy define two ASL layers: the periciliary liquid or sol layer adjacent to the airway epithelium covering the cilia, and the overlying viscous gel layer (48, 93). The cilia are bathed in the periciliary liquid, whose pH, ionic composition, and physical properties are thought to be important in mucociliary clearance. The ASL is an aqueous solution containing ions, glycoproteins such as mucins, and other proteins including lactoferrin, defensins, lysozyme, IgA, antimicrobial surfactant proteins, secretory leukoprotease inhibitor, human salivary histatin, and cathelicidin. The ASL is thought to play an important role in airway hydration, innate immunity, and antimicrobial defense. In principle, the ASL could be hyposmolar, isosmolar, or hyperosmolar (compared with blood osmolality) depending on the relative influence of epithelia transport, surface tension effects, and convective/evaporative fluid losses. For example, a hypertonic ASL

**Fig. 2.** Schematic of ASL in large and small airways, showing gel and periciliary liquid layers and convective fluid transport up the airways. *Inset:* scale drawing of the airway surface epithelium and ASL along with several tools used to sample ASL. See text for explanations.
might result from evaporative water losses across a relatively water-impermeable barrier, whereas a hypotonic ASL might result from avid ion absorption or possibly surface tension phenomena (94).

**ASL Ionic Composition**

Over the past 20 years several quite different experimental approaches have been applied to measure ASL ionic composition (8, 25, 37). As summarized in Table 1, there is remarkable variability in reported data from laboratory to laboratory with the use of different methods. For example, ASL [Na+] has been reported in the range from <5 to >150 mM. Much of the information has been obtained from analysis of fluid collected by filter paper and capillary methods. In the filter paper method as first introduced by Boucher et al. (8), ASL is collected by using predried strips of filter paper strip that make contact with the surface of an airway or cell culture. By introducing a filter paper strip through a fiber-optic bronchoscope, Boucher et al. (8) reported [Na+] and [Cl] to be near isotonic in canine tracheal ASL. However, using the same approach to sample ASL from human airways, Joris et al. (41) reported a hypotonic ASL under normal conditions that became near isotonic after airway irritation or acute infection. ASL composition was not different in normal vs. CF humans. Using a similar filter paper collection method, Knowles et al. (43) reported approximately isosmolar nasal and bronchial fluids, with no differences in ionic composition between normal and CF subjects. In collections using capillaries (outer diameter 600 μm) or PE-10 tubing in rat and mouse trachea, Govindaraju et al. (25) and Cowley et al. (16) reported much lower [Na+], [Cl–], and [K+] (see Table 1). Micropipettes have also been used to aspirate ASL directly from xenografts and cell cultures (2).

Filter paper, capillary sampling, and micropipette methods are invasive ASL sampling methods that require direct contact with the ASL (see Fig. 2, inset, lower left). These methods have been criticized because the sampled volumes are often substantially greater than the expected fluid volume of the thin ASL film (5 μl per cm2 of airway surface for 50-μm-thick ASL) (42). The sampled ASL fluid may thus be contaminated by cellular and interstitial fluids induced by capillary suction forces, and fluid composition may be altered by mechanical stimulation of the airway surface and submucosal glands. Indeed, the high ASL [K+] reported in several studies (8, 41, 43) may be related to perturbation or damage to surface epithelial cells.

Ion-sensitive microelectrodes have been used to measure ASL ion composition in situ in cell culture models and intact trachea in vivo. A microelectrode is advanced into the ASL by micromanipulation or fiber-optic bronchoscopy, and the electrode remains in contact with the epithelial surface (generally for >10 s) to obtain a stable measurement. Using solid-state Na+-sensitive microelectrodes introduced in vivo by bron-

### Table 1. ASL ionic content in intact airways and in epithelial cultures grown at air-liquid interface

<table>
<thead>
<tr>
<th>Experimental Approach</th>
<th>Assay</th>
<th>System</th>
<th>[Na+]</th>
<th>[Cl–]</th>
<th>[K+]</th>
<th>Year</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Intact airways</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter paper, bronchoscopy</td>
<td>Flame photometry</td>
<td>Dog trachea</td>
<td>158±7</td>
<td>134±8</td>
<td>29±2</td>
<td>1981</td>
<td>7</td>
</tr>
<tr>
<td>Filter paper, bronchoscopy</td>
<td>Amperometric titration</td>
<td>Human trachea/bronchus</td>
<td>82±6</td>
<td>84±9</td>
<td>29±5</td>
<td>1993</td>
<td>41</td>
</tr>
<tr>
<td>Filter paper, nose clip</td>
<td>Chloridometry</td>
<td>Human nasal cavity</td>
<td>132±3</td>
<td></td>
<td></td>
<td>1996</td>
<td>73</td>
</tr>
<tr>
<td>Filter paper, bronchoscopy</td>
<td>Fluorescence indicators</td>
<td>Human lower airway</td>
<td>80±5</td>
<td>88±10</td>
<td>21±4</td>
<td>1997</td>
<td>43</td>
</tr>
<tr>
<td>Na+-selective microelectrode</td>
<td>Electrical conductivity</td>
<td>Canine trachea</td>
<td>141±17</td>
<td></td>
<td></td>
<td>1997</td>
<td>43</td>
</tr>
<tr>
<td>Cold probe collection</td>
<td>X-ray microanalysis</td>
<td>Mouse trachea</td>
<td>5.5±0.3</td>
<td>1.3±0.3</td>
<td>1.1±0.2</td>
<td>1998</td>
<td>3</td>
</tr>
<tr>
<td>Microcapillary collection</td>
<td>X-ray microanalysis</td>
<td>Fetal tracheal xenograft</td>
<td>64±8</td>
<td>65±13</td>
<td>2.4±0.5</td>
<td>1999</td>
<td>2</td>
</tr>
<tr>
<td>Capillary collection</td>
<td>Conductivity/UV</td>
<td>Mouse trachea</td>
<td>87±3</td>
<td>57±3</td>
<td>4.7±0.4</td>
<td>2000</td>
<td>16</td>
</tr>
<tr>
<td>Fluorescence indicators</td>
<td>Ratio imaging</td>
<td>Mouse trachea</td>
<td>115±4</td>
<td>140±5</td>
<td></td>
<td>2001</td>
<td>37</td>
</tr>
<tr>
<td>Cl–selective microelectrode</td>
<td>Electrical conductivity</td>
<td>Human nasal cavity</td>
<td>107±4</td>
<td>120±6</td>
<td>8.7±0.4</td>
<td>2002</td>
<td>9</td>
</tr>
<tr>
<td>Microdialysis</td>
<td>Flame photometry</td>
<td>Mouse nasal cavity</td>
<td>117±11</td>
<td>117±11</td>
<td></td>
<td>2002</td>
<td>27</td>
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<tr>
<td><em>Epithelial cell cultures grown at air-liquid interface</em></td>
<td></td>
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<td></td>
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<tr>
<td>Filter paper</td>
<td>Chloridometry</td>
<td>Canine trachea</td>
<td>125±11</td>
<td>104±6</td>
<td>6±2</td>
<td>1997</td>
<td>43</td>
</tr>
<tr>
<td>Micropipette aspiration</td>
<td>Chloridometry</td>
<td>Canine trachea</td>
<td>124±3</td>
<td>125±7</td>
<td>11±5</td>
<td>1997</td>
<td>43</td>
</tr>
<tr>
<td>Cl–selective electrode</td>
<td>Electrical conductivity</td>
<td>Human airway</td>
<td>50±4</td>
<td>37±6</td>
<td>11±5</td>
<td>1998</td>
<td>52</td>
</tr>
<tr>
<td>Radiotracer/dilution</td>
<td>Radioactivity</td>
<td>Human airway</td>
<td>0±4</td>
<td>37±6</td>
<td>11±5</td>
<td>1998</td>
<td>94</td>
</tr>
<tr>
<td>Radiotracer/dilution</td>
<td>Radioactivity</td>
<td>Mouse trachea</td>
<td>18±6</td>
<td>18±6</td>
<td></td>
<td>1999</td>
<td>53</td>
</tr>
<tr>
<td>Microcapillary collection</td>
<td>鼠 fluorscence indicators</td>
<td>Mouse airway</td>
<td>~140</td>
<td>~125</td>
<td></td>
<td>2001</td>
<td>77</td>
</tr>
<tr>
<td>Cl–selective microelectrode</td>
<td>Electrical conductivity</td>
<td>Bovine trachea</td>
<td>97±5</td>
<td>118±3</td>
<td></td>
<td>2001</td>
<td>37</td>
</tr>
</tbody>
</table>

ASL, airway surface liquid.
choscopy, Knowles et al. (43) reported near-isotonic \([\text{Na}^+]\) in human and canine airways. Using Cl\textsuperscript{-}-sensitive microelectrodes, Tarran et al. (77) measured ASL \([\text{Cl}^-]\) of \(\sim 130 \text{ mM}\) in human bronchial cell cultures. In situ measurement of ASL properties has considerable advantages over fluid sampling methods; however, a major concern in the use of the microelectrodes is that direct contact is required between the relatively large microelectrode tip (generally \(>100 \mu\text{m}\) in diameter) and thin ASL (\(<100 \mu\text{m}\)). Surface tension and mechanical effects might perturb native ASL properties and introduce uncertainties in reproducibility in contact area between the ASL and microelectrode surface.

An alternative method to collect ASL is to freeze the ASL onto a cold probe at liquid nitrogen temperature. After surgical exposure of the tracheal mucosa through a window, a cold metal probe is manipulated down onto the mucosal surface to freeze a portion of the ASL, and a window, a cold metal probe is manipulated down onto the mucosal surface to freeze a portion of the ASL, and introduce uncertainties in reproducibility in contact area between the ASL and microelectrode surface. An alternative method to collect ASL is to freeze the ASL onto a cold probe at liquid nitrogen temperature. After surgical exposure of the tracheal mucosa through a window, a cold metal probe is manipulated down onto the mucosal surface to freeze a portion of the ASL, which adheres to the cold probe for assay by X-ray probe microanalysis. Using this method, Baconnais et al. (3) reported very low \([\text{Na}^+]\), \([\text{Cl}^-]\), and \([\text{K}^+]\) (<10 mM) in mouse trachea. Using essentially the same technique, Zahm et al. (95) reported that the ASL salt “content” of normal and CF mice did not differ, although absolute ionic concentrations were not determined. However, potential concerns with the cold-probe approach include perturbation of the ASL by surgical exposure of the tracheal mucosa and probe contact as well as uncertainties in the depth of the sampled frozen fluid.

A radiotracer-dilution method has been applied to measure ASL \([\text{Na}^+]\) and \([\text{Cl}^-]\) using \(^{22}\text{Na}\) and \(^{36}\text{Cl}\), with \(^{3}H_2\text{O}\) as an ASL volume marker. Cell cultures are equilibrated with \(^{22}\text{Na}\), \(^{36}\text{Cl}\), and \(^{3}H_2\text{O}\), and the ASL is aspirated rapidly after nonradioactive aqueous buffer is added to the apical surface of the cultures. Using this method, Zabner et al. (94) reported ASL \([\text{Na}^+]\) of 50 mM and \([\text{Cl}^-]\) of 37 mM in cultured bronchial cells from normal humans and greater ASL \([\text{Na}^+]\) of \(\sim 100 \text{ mM}\) and \([\text{Cl}^-]\) of \(\sim 90 \text{ mM}\) in human CF cells. Using a similar dilutional method in cultured airway cells from wild-type and CF mice, McCray et al. (53) reported a much lower ASL \([\text{Cl}^-]\) of \(\sim 15 \text{ mM}\). However, a concern with these studies is the rapid diffusional exchange of \(^{3}H_2\text{O}\) across the cell layer, resulting in an underestimate in ionic concentrations (because more \(^{3}H_2\text{O}\) was extracted than was contained in the ASL). In addition, there are concerns about the completeness of ASL washout and filter edge effects, where fluid can accumulate at the interface between the flat filter surface and the curved wall due to surface tension.

Our laboratory recently developed an alternative strategy to assay ASL ionic composition and osmolality that overcomes some but not all of the concerns mentioned above. Ion-sensitive fluorescence indicators are introduced into the ASL by addition of a small volume of low-boiling point perfluorocarbon into which the fluorescent dyes are physically dispersed by sonication. The perfluorocarbon evaporates rapidly, leaving only solid residue to dissolve in the aqueous ASL (37). For measurements in airway epithelial cells grown on a porous support at an air-liquid interface, the cell insert is mounted in an incubation chamber at 37°C in a 5% CO\(_2\) atmosphere with basolateral-side perfusion (Fig. 3A). Fluorescent dyes for \([\text{Na}^+]\) and \([\text{Cl}^-]\) were developed that remain confined to the ASL compartment and give two-color fluorescence in which one color is ion sensitive and the other is not. For example, the Cl\textsuperscript{-}-sensitive indicator consisted of a blue-fluorescing Cl\textsuperscript{-}-sensitive quinolinium fluorophore conjugated to dextran together with a red-fluorescing Cl\textsuperscript{-}-insensitive tetramethylrhodamine fluorophore. The red-to-blue fluorescence ratio provided a quantitative measure of ASL \([\text{Cl}^-]\). The dyes were calibrated in situ in the ASL using ionophores, with data for the Cl\textsuperscript{-} dye shown in Fig. 3B. With this approach, ASL \([\text{Na}^+]\) was 97 mM and \([\text{Cl}^-]\) was 118 mM in the bovine cell cultures. A different method was applied to measure ASL osmolality, because it is not possible to design an osmolality-sensitive fluorescent dye. Osmotically sensitive 400-nm-diameter liposomes were generated in which calcine (green fluorescing) was encapsulated in the liposomes at self-quenching concentrations as a volume-sensitive marker, together with sulforhodamine 101 (red fluorescing) as a volume-insensitive reference (35). As determined from green-to-red fluorescence ratios, ASL osmolality in bovine epithelial cell cultures was 325 mosmol/kgH\(_2\)O.

The fluorescent indicator and microscopy methods were also applied to measure ASL properties in mouse trachea in vivo and in excised human bronchi. In anesthetized mice, a window was created in the trachea, the ASL was stained with fluorescent indicators (dispersed in perfluorocarbon), and the window was covered with transparent plastic to permit normal respiration and visualization of the stained ASL at the posterior mucosal surface (Fig. 3C, left). Alternatively, for ratio imaging of the brighter \([\text{Na}^+]\) and pH indicators, the perfluorocarbon suspension was introduced into the trachea using a blunt feeding needle, and the trachea was exposed (but not cut) for fluorescence microscopy (Fig. 3C, right). In mouse trachea, \([\text{Na}^+]\) was 115 mM, \([\text{Cl}^-]\) was 140 mM, and osmolality was 330 mosmol/kgH\(_2\)O. Similar values were found in CFTR null mice. To study ASL properties in human bronchi, bronchial fragments were incubated for 30–60 min at 37°C in 5% CO\(_2\) and mounted in a chamber as in Fig. 3A. ASL \([\text{Na}^+]\) was 103 mM and \([\text{Cl}^-]\) was 92 mM in normal human bronchi. Although the fluorescence indicator approach is less invasive than methods that require ASL sampling or direct contact, there remain concerns about surgical exposure of airways as well as the authenticity of the ASL formed by an excised human bronchus.

Is ASL ionic composition abnormal in CF? Although the final answer is not in, the preponderance of evidence in cell culture models (52, 77), CF mice (37, 53), and human CF airways (43) suggests that ASL ionic composition is not different in CF. However, there is little information about ASL composition in lower airways, where airway disease in CF has been postulated to begin. Also, there are few data about the possibility
that ASL ionic composition might be altered in response to various agonists and factors released by inflammatory cells/bacteria or other components of CF airway fluid.

**ASL Depth**

As summarized in Table 2, there is remarkable variability in measured ASL depth. A few measurements of ASL depth have been reported in intact, large airways. By light microscopic examination of monkey nasal mucosa, Lucas and Douglas (48) proposed the two-layer ASL theory, which was supported by subsequent studies in rat and rabbit trachea (66, 93). Seybold et al. (68) visualized the air-liquid and epithelial surfaces of sheep trachea by bright-field and dark-field optics, respectively, and reported an ASL thickness of 35–50 μm. In guinea pig trachea, Rahmoune and Shephard (65) estimated ASL thickness to be 87 μm after creating a small window in the trachea and scanning through the ASL with an electrode. However, low-temperature scanning electron microscopy of rapidly frozen bovine trachea samples gave a substantially lower ASL thickness of 6–20 μm (87). In our laboratory, rapid z-scanning confocal microscopy was used to measure ASL thickness in vivo in mice. In the fluorescently stained ASL preparation shown in Fig. 3C, left, ASL thickness was 45 μm (37). ASL thickness was 55 μm in excised human bronchi.

ASL depth in cell culture models has been measured by fluorescence confocal microscopy. When a dense (nonvolatile) perfluorocarbon was used to deliver the dye, ASL thickness was measured to be 7 μm in human airway epithelium cells (77). However, a concern with these measurements was possible surface tension and mechanical effects of the perfluorocarbon layer overlying the aqueous ASL. When a low-boiling point perfluoro-
rocarbon was used to introduce the fluorescent dye, ASL thickness in cultured bovine airway cells was 21 μm (37). ASL thickness was insensitive to a series of transport agonists and inhibitors but decreased substantially when the cultures were exposed to nonhumidified room air.

There is limited evidence that ASL thickness varies with lung volume, airway diameter, and submucosal gland function. Yager et al. (88) used low-temperature electron microscopy to estimate ASL thickness in large and small airways of guinea pig at different lung volumes. They reported that ASL thickness was approximately proportional to the square root of airway internal perimeter, increasing from 0.9 to 1.9 μm with increasing airway diameter from 250 to 1,800 μm with the lung at functional residual capacity; ASL thickness increased twofold at total lung capacity. These values of ASL thickness are, for unclear reasons, much lower than those reported by other investigators. Submucosal gland secretion may also change the ASL thickness. Using rapid-freeze methods and electron microscopy, Wu et al. (86) reported that ASL thickness in excised bovine trachea increased from 23 to 78 μm after stimulation of gland secretion by methacholine.

The majority of information on ASL composition and thickness comes from cell culture models and large airways. We recently developed a “stripped lung” preparation to measure ASL depth and properties in small airways (75). Here, the ASL throughout the airways of a freshly excised lung is stained with a fluorescent dye dispersed in low-boiling point perfluorocarbon. After pleural stripping and limited lung microdissection, fluorescently stained small airways are visualized by confocal microscopy. Preliminary measurements indicated an ASL thickness of ~15 μm in mouse small airways of ~100 μm in diameter.

Is altered ASL thickness important in the pathophysiology of CF? This is a difficult question to answer because of the great variability in reported ASL thicknesses with little consensus in the field. Given the many concerns with the existing studies (perturbation by invasive monitoring, tissue preparation/fixation, limited resolution of light microscopy, evaporation artifacts), the true ASL depth in vivo remains uncertain. Notwithstanding these concerns, an important study by Matsui et al. (52) concluded that ASL depth is decreased in CF, which was attributed to increased ENaC-mediated Na⁺ absorption. Measurements were done in airway cell cultures from normal vs. CF humans. Clearly, additional work is needed to measure ASL depth accurately in vivo and to test whether ASL depth is abnormal in human CF.

### ASL pH Regulation

In contrast to the wide range of reported values for ASL ionic composition and depth, there is good agreement in data from several groups that the ASL is mildly acidic compared with serum. Acidic luminal pH in airways has been shown to inhibit ciliary beating (12), cause bronchoconstriction (1), and lead to epithelial cell detachment from the basement membrane (28). Using pH microelectrodes in the in vitro ferret trachea, Kyle et al. (44) reported ASL pH of 6.85 when the pH of the serosal solution was 7.4, increasing to 6.92 when serosal pH was 8.0. Microelectrode measurements in human airway cultures gave pH ~6.9 (14). Using a pH-stat titration method, Fischer et al. (21) reported an ASL pH of 6.85. Our laboratory used the ratioable pH-sensitive fluorescence indicator BCECF-dextran to measure ASL pH in cell culture models and intact airways, using the preparations described above for measurement of ion concentrations and osmolality. ASL pH in airway cell cultures was 6.98 in the absence and 6.81 in the presence of HCO₃⁻/CO₂ and was sensitive to changes in serosal pH and to Na⁺ or Cl⁻ replacement (36). ASL pH was 7.14 in mouse trachea and was insensitive to amiloride or glibenclamide but quite sensitive to changes in blood pH created by metabolic (HCl or HCO₃⁻ infusion) or respiratory (hyperventilation, hyperventilation, hyperventilation) mechanisms. The results suggested that ASL pH is primarily determined by basolateral fluid pH and that H⁺/OH⁻ transport between the ASL and serosal fluid involves amiloride-sensitive Na⁺/H⁺ exchange and stilbene-sensitive Cl⁻/HCO₃⁻ exchange. It was proposed that the rapid response of ASL pH to changes in systemic acid-base status may contribute to airway hypersensitivity in asthma and other diseases of the airways.

The mechanisms responsible for generation of a mildly acidic ASL are not known. Fischer et al. (21) detected histamine-stimulated acid secretion from air-
way cell cultures in short-circuit experiments. \( K^+ / H^+ \) or \( H^+ \) pumps may be involved in the apparent active proton secretion process. With regard to CF pathophysiology, it has been postulated that low ASL pH in CF favors bacterial survival and proliferation in the airway lumen by reducing electrostatic repulsion between the bacteria and airway surface, facilitating tighter biofilm formation and hindering access of immune cells (13, 72). Reduced CFTR-dependent \( HCO_3^- \) transport has been proposed to acidify the ASL in CF. One preliminary report suggested acidified nasal fluid from CF patients (13); however, measurements in CF null mice (37) and in CF humans (E. Alton, unpublished data) have failed to show differences in ASL pH compared with appropriate controls.

**PHYSIOLOGY OF AIRWAY SUBMUCOSAL GLANDS**

**Gland Structure**

Submucosal glands can be divided into four distinct regions that have different roles in the production and processing of fluid to be secreted onto the airway surface (54, 55, 59). Glands contain serous tubules and acini that secrete salt, water, and various antimicrobial proteins (Fig. 4). The serous secretions pass through mucous tubules, where high-molecular-weight glycoproteins are added, and then into a collecting duct and a ciliated duct, and finally onto the airway surface. Serous-type epithelial cells lining serous acini and tubules are believed to secrete the majority of gland salt and water, as well as antimicrobial proteins such as lysozyme and defensins (5, 71, 78, 96). Serous epithelial cells express CFTR more strongly than other cell types in the airways (19, 33, 67). Mucous tubules are lined by mucous-type epithelial cells that are packed with secretory granules containing mucins (57). The collecting duct is lined by a nonciliated columnar epithelium. The function of the collecting duct epithelium is largely unknown but has been suggested to modify the ionic composition of secretions from the serous and mucous epithelia (59). Myoepithelial cells lie at the base of many epithelial cells lining the serous and mucous tubules and the collecting duct and may facilitate fluid secretion by mechanical contraction (55, 59). The ciliated duct cells that line gland openings are a continuation of the airway surface epithelium.

Fluid and macromolecule secretion by submucosal glands is regulated by vagal muscarinic nerves, \( \alpha \)- and \( \beta \)-adrenergic nerves, and local mediators including vasoactive intestinal peptide (VIP) (57). Much of the information regarding regulated gland secretion comes from work done two decades ago by Nadel and colleagues. Muscarinic receptor activation, which stimulates both mucous and serous cells, increases gland secretion (81) with little change in overall protein concentration or viscosity (82). In contrast, \( \alpha \)-adrenergic stimulation is relatively selective for serous cells, resulting in secretions with a high fluid content and low viscosity and protein content (56). \( \beta \)-Adrenergic stimulation appears to be relatively selective for mucous cells, resulting in viscous secretions with high protein content (82). Conflicting results were reported for gland stimulation by VIP from measurements of macromolecule content and short-circuit current (15, 61). More recent studies suggest that VIP produces a sustained increase in secretion rate in porcine submucosal glands, as was found for stimulation by the cAMP agonist forskolin (39). Although these studies have addressed the increase in secretion rates by different activating pathways, little is known about compositional differences in agonist-stimulated secreted fluid.

![Fig. 4. Anatomy of submucosal gland fluid secretion in human airways. Schematic of a submucosal gland unit shows serous acini, mucous tubules, and collecting duct, with an expanded view of a serous epithelial cell. See text for explanations. AQP5, aquaporin-5; AE2, anion exchanger 2; NBC, Na\(^+\)-HCO\(_3^-\) cotransporter; NKCC, Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter.](http://ajpcell.physiology.org/)

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Cell Culture Studies of Gland Salt Transport

Most of the information about ion- and fluid-transporting mechanisms across glandular epithelia has been obtained from cell culture models. Primary cell cultures of acinar submucosal gland cells give secretory short-circuit current responses to both calcium- and cAMP-stimulating agonists (90, 92), which are reduced in cells cultured from CF patients (88). Non-CF gland cells generally secrete fluid under basal conditions with a balance maintained between cAMP-dependent Cl⁻ secretion and amiloride-sensitive Na⁺ absorption (38). Transepithelial fluid secretion in the cell cultures is stimulated by 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT-cAMP), methacholine, and UTP. With the use of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and DIDS to block CFTR and calcium-activated chloride channels, respectively, it was concluded that cAMP-stimulated fluid secretion is mediated by CFTR and UTP-stimulated fluid secretion is mediated by calcium-activated chloride channels, whereas methacholine (the major physiological stimulus in airway glands) activates both types of channels. Cells isolated from CF patients showed significant reductions in fluid secretion stimulated by cAMP or methacholine, suggesting a primary defect in gland fluid secretion in CF.

The most widely used cell culture system representing serous cells is the lung adenocarcinoma cell line Calu-3. After available established cell lines had been screened, Calu-3 cells were chosen as a model system because they express serous cell markers including a high level of CFTR (69). The mechanism of anion secretion by Calu-3 cells has been actively debated and a number of ion transport models proposed (17, 32, 45, 70). Basal secretion in Calu-3 cells is thought to involve Cl⁻-dependent HCO₃⁻ secretion either directly through CFTR or via a CFTR-regulated apical Cl⁻/HCO₃⁻ exchanger (46). These studies also reported that calcium-elevating agonists such as thapsigargin induced a switch from HCO₃⁻ to Cl⁻ secretion by activating basolateral K⁺ channels and increasing the driving force for Cl⁻ entry (45). Forskolin-stimulated elevation in short-circuit current was generally small and dependent on cell age and growth conditions. However, other short-circuit current and microelectrode studies suggested a robust cAMP-mediated secretion that was postulated to be predominantly Cl⁻-independent HCO₃⁻ secretion involving basolateral Na⁺/HCO₃⁻ cotransport (17) (see Fig. 4). In agreement with previous studies, stimulation by 1-EBIO, which elevates calcium concentration and may also interact directly with CFTR (50), resulted in predominantly Cl⁻ secretion. The proposed mechanism of 1-EBIO activation of Cl⁻ secretion involves basolateral membrane hyperpolarization by K⁺ channels, inhibiting Na⁺/HCO₃⁻ cotransport and stimulating Na⁺/K⁺/2Cl⁻ cotransport. Studies in Calu-3 cells have thus provided some insights into potential mechanisms of secretion across gland serous cells, with the caveat that ion and fluid transport in Calu-3 cells may not mimic that of acinar submucosal gland cells. Similarly, a limitation of primary acinar gland cultures is that they show a mixed serous/mucous cell phenotype (89–91), making conclusions about transport in either cell type difficult. In particular, it remains unclear from the opposing results of Calu-3 cells vs. primary cell cultures whether serous cells in vivo contain an apical calcium-activated chloride pathway.

Studies of Submucosal Gland Function in Intact Airways

There have been relatively few studies of submucosal gland secretion in intact airways due in part to technical difficulties in isolating gland secretions from ASL. Early studies used direct micropipette cannulation of gland openings in feline airways to measure rates of fluid secretion (23, 58, 82). These initial experiments provided basic data on gland secretion rates and neural regulation, although the method employed was technically difficult and could only be used to measure rates from single glands. The importance of Cl⁻ and HCO₃⁻ transport in gland fluid secretion has been demonstrated in several studies by Ballard and coworkers (4, 79) using excised porcine bronchi. Two methods were used to sample gland secretions: direct collection of secreted fluid from the airway surface using forceps, and cannulation of the whole bronchi and aspiration of the luminal liquid. Inhibition of Cl⁻ and HCO₃⁻ transport by different transport inhibitors (bumetanide, DIDS, diphenylamine-2-carboxylic acid, glibenclamide) produced inhibition of gland fluid secretion and alterations in mucus content after acetylcholine stimulation (4, 31), as well as altering the rheological properties (80). However, the sampling methods used in those studies were relatively invasive, and it was not possible to fully isolate gland vs. surface secretions. Using an optical method to follow intact secretions (39, 40), Wine and coworkers in recent studies have characterized gland fluid secretions in porcine bronchi in response to carbachol, VIP, and forskolin. They showed increased gland fluid secretion with both cholinergic and cAMP agonists but found no effect of adrenergic agonists. Removal of HCO₃⁻ or addition of bumetanide inhibited fluid secretion by ~50%, suggesting the involvement of Cl⁻ and HCO₃⁻ in gland fluid secretion. Recently, preliminary studies have begun to address the complex transport properties of different gland regions by using an isolated gland preparation (29). After submucosal gland serous tubules had been digested from porcine bronchi, fluorescence measurements of intracellular pH of serous cells suggested both HCO₃⁻-dependent and -independent pH regulatory mechanisms.

As indicated in Fig. 4, CFTR and other ion transporters provide the route for solute secretion, which creates an osmotic gradient to drive water secretion. Aquaporin-5 (AQP5) is a water-selective transporter expressed at the apical membrane of serous gland cells. On the basis of the finding that AQP5 deletion in mice inhibited fluid secretion by salivary gland, our labora-
ory tested the hypothesis that AQP5 could be rate limiting in fluid secretion in submucosal glands in mouse nasopharynx (76). With the use of fluid collection and imaging methods, fluid secretion was reduced more than twofold in AQP5 null mice, with a reciprocal increase in secreted fluid protein concentration. Thus, when sufficiently reduced, AQP5-mediated water transport can be a rate-limiting step in glandular fluid secretion. It was proposed that upregulation of AQP5 expression and/or function might increase glandular fluid secretion and reduce fluid viscosity in CF.

Tissue studies of submucosal gland function have just started to elucidate the complex physiology of submucosal glands, and though technically difficult, they do not suffer from many of the concerns associated with cell culture systems. The development of robust optical methods to measure gland secretion rate and composition with minimal perturbation of secreted fluid should allow more accurate assessment of the rate, composition, and regulation of gland secretions. Further studies on isolated submucosal glands should provide much needed information on the transport properties of different regions of submucosal glands, including the collection duct epithelium.

Submucosal Gland Function and CF

Several lines of evidence suggest an important role for submucosal glands in the progression of airway disease in CF. As mentioned, CFTR is strongly expressed in serous epithelial cells of submucosal glands (19, 33, 67). Autopsy specimens from neonates with CF but that have not yet developed lung disease show distended lumens in submucosal glands, suggesting mucus accumulation (20, 60); however, one report suggested normal CF gland morphology (11). Submucosal glands become massively hypertrophied as CF airway disease progresses, with mucus plugging of airways (6, 74). Cell culture studies have indicated loss of cAMP-induced fluid secretion in gland epithelial cells from CF patients (38). Studies in intact airways have also shown that inhibition of anion secretion reduces gland fluid secretion and increases mucus viscosity (4, 79). The mucus secretions from stimulated submucosal glands are important in determining both the composition and depth (86) of the ASL after airway irritation from inhaled pathogens. Gland secretions are also a key factor for mucociliary clearance from the airway surface and provide all the major antimicrobial proteins involved in airway defense against bacteria. Several groups have thus postulated that the salt content and viscosity of submucosal glandular secretions in CF are abnormal because of the reduction in fluid secretion (34, 59, 79). The resultant hyperviscous secretions are postulated to reduce airway mucociliary clearance and normal airway defenses to bacterial infection. In addition, on the basis of reported functional interactions between CFTR and a Cl⁻/HCO₃⁻ exchanger (46, 64) and transport of HCO₃⁻ by CFTR (30, 47), it has been postulated that the pH of glandular secretions in CF is abnormal. Acidic airway pH has been shown to affect ciliary beating (12) and induce cough (85).

Our laboratory recently developed optical methods to measure the rate, ionic content, and viscosity of fluid secreted from submucosal glands in freshly obtained human bronchi and living mice (34, 75). Fragments of human bronchi or trachea obtained at the time of lung transplantation were mounted in a humidified perfusion chamber, and the mucosal surface was covered with a thin layer of mineral oil (Fig. 5A, top). Individual droplets of secreted fluid were microinjected with fluorescence indicators for measurement of [Na⁺], [Cl⁻], and pH by ratio imaging fluorescence microscopy and of viscosity by fluorescence recovery after photobleaching. After carbachol stimulation, 0.1–0.5 μl of fluid accumulated in spherical droplets at gland orifices in ~3–5 min (Fig. 5A, bottom). In gland fluid from normal human airways, [Na⁺] was 94 mM and pH was 6.97, with no differences in normal vs. CF airways (Fig. 5B). Gland fluid viscosity was measured by fluorescence recovery after photobleaching, in which the translational diffusion of a 10-kDa FITC-dextran was measured from the rates of movement into a cylindrical volume in the fluid droplet that was bleached by a brief intense laser pulse (Fig. 5C, left). Gland fluid viscosity was increased more than twofold in CF gland secretions. Measurements of solute diffusion give linear (zero order) viscosity but do not provide information about nonlinear viscous properties such as adhesivity and thixotropy, which require dynamic measurements such as the movement of magnetic beads in response to time-varying magnetic fields (42). In any case, notwithstanding the caveats mentioned above about studies on chronically diseased human CF airways, these initial observations suggest that salt concentration and pH are not different in gland fluid from normal vs. CF bronchi but that fluid viscosity is significantly elevated in CF. It will be important to determine whether viscosity is elevated in gland fluid before airway disease occurs and with different secretory agonist, and if so, to determine whether the increased viscosity is related to decreased fluid vs. protein secretion and/or altered glycoprotein composition.

RESEARCH DIRECTIONS

Although there is now a considerable body of descriptive information about the properties of the ASL and the function of airway submucosal glands, there is little mechanistic-level information or few definitive data to relate defective CFTR function to CF lung disease. The origins of ASL remain poorly understood in terms of the respective roles of submucosal gland secretions, lower vs. upper airways, and convective flow from distal to large airways. The factors responsible for regulation of ASL depth and composition are not known. There is a paucity of data on ASL volume and composition in vivo and on the properties of the ASL in lower airways. Although recent data suggest that abnormalities in ASL ionic composition and pH do not occur in upper airways in CF, there is a need to

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investigate lower airways, where airway disease in CF is thought to begin, and to reinvestigate upper airways under relevant stresses to which CF airways are exposed such as *P. aeruginosa* toxins and inflammatory mediators. There is limited information on the functioning of intact airway submucosal glands and on mechanisms regulating salt, water, and protein transport in serous vs. mucous vs. ductal epithelial cells. Microdissection and perfusion methods will be useful to resolve acinar vs. ductal function.

Although technical advances have been made to study ASL and gland physiology with improved accuracy and reliability, the systems available for experimentation remain a limiting factor in making progress. There is a need to study ASL and gland properties in intact CF airways prior to the development of severe disease, because the disease process itself causes marked glandular hypertrophy and other chronic changes. Functional evaluation of bronchoscopic biopsy specimens from pediatric CF patients may be useful in this regard. There is a need to develop large animal models of CF that mimic human CF pathophysiology. Although genetic manipulations in large animals remain an attractive approach over the long term, new high-affinity CFTR-selective inhibitors (49) may permit pharmacological creation of CF animal models. Ultimately, it may be concluded that the etiology of lung disease in CF is multifactorial and complex; however, the remarkable predictability of CF lung disease favors a single primary disease mechanism, which may already be listed in Fig. 1 or remains to be identified.

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Fig. 5. Fluorescence measurement of human gland fluid composition and viscosity. **A**, top: schematic of an excised bronchus immobilized with pins on a sponge soaked with HCO₃⁻-containing physiological buffers. **A**, bottom: micrographs of fluorescently stained fluid droplets secreted from submucosal glands shown under bright-field illumination (left) and fluorescence (right). Scale bar = 2 mm. **B**: [Na⁺] (left) and pH (right) in freshly secreted fluid droplets from submucosal glands measured by fluorescence ratio imaging. Each point is the average of measurements done on multiple gland fluid droplets from a different human sample. **C**: gland fluid viscosity measured by fluorescence recovery after photobleaching (left). Photobleaching method shows the bleaching of a vertical cylinder through an FITC-dextran-stained fluid droplet. Fluorescence increases as unbleached dye moves into the bleached area. Curve on the bottom shows fluorescence recovery measured in a secreted fluid droplet from a normal human airway. Half-times (t₁/₂) for fluorescence recovery (proportional to fluid viscosity) from gland fluid droplets of normal (●) and CF (○) human airways are shown at right. [Adapted from Jayaraman et al. (34)].
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