X-ray microanalysis of airway surface liquid in the mouse

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Kozlova, Inna, Harriet Nilsson, Mia Phillipson, Brigitte Riederer, Ursula Seidler, William H. Colledge, and Godfried M. Roomans. X-ray microanalysis of airway surface liquid in the mouse. Am J Physiol Lung Cell Mol Physiol 288: L874–L878, 2005. First published December 23, 2004; doi:10.1152/ajplung.00303.2004.—The ionic composition of airway surface liquid (ASL) has been debated, and, in particular for the mouse, a wide range of values has been published. Two techniques were developed to measure the elemental composition of the ASL. X-ray microanalysis of ASL was carried out at low temperature on trachea removed from isolurane-anesthetized animals and shock-frozen. In the second technique, dextran beads were placed on top of the epithelium of the trachea removed from pentobarbital-anesthetized animals, left to equilibrate with the ASL, dried, and subjected to X-ray microanalysis. Both techniques showed that mouse tracheal ASL has significantly lower concentrations of Na and Cl (60–80 mM) than serum. Differences between the two techniques were due to different sampling of mucus. CFTR(−/−) mice had significantly higher concentrations of Na and Cl in their ASL than age-matched controls. Pilocarpine or isoproterenol stimulation significantly reduced the ion concentrations in tracheal ASL. ASL was also collected with the dextran bead method from the nasal cavity in situ in pentobarbital-anesthetized animals. In control animals, the elemental composition of nasal fluid was similar to that of tracheal ASL. Pilocarpine stimulation caused a significant increase in Na, Cl, and K; stimulation with isoproterenol or phenylephrine caused a significant increase only in K. It is concluded that mouse ASL under unstimulated conditions is hypotonic, which may be related to the relative paucity of submucosal glands in the mouse trachea.

Cystic fibrosis; ion transport

The airway epithelium is covered by a thin layer of liquid, the airway surface liquid (ASL), which is mainly secreted by the submucosal glands. ASL consists of mucus and an underlying periciliary watery layer that enables the cilia to clear the mucus. From studies of patients with cystic fibrosis (CF), as well as studies of patients with exercise-induced asthma, it has become clear that the volume and/or ionic composition of the ASL are important for lung function (1, 3, 4, 23). In its turn, volume and/or ionic composition are affected by ion transport mechanisms in the airway epithelium (11, 22). With regard to the airways in CF patients, several different theories have been proposed (reviewed in Ref. 22). Already early on, it was suggested that the ASL in CF patients would have higher concentrations of Na and Cl than normal (13). One of the current theories (17, 23) claims that the ASL normally is hypotonic. This would provide an optimal environment for the defensins, proteins that play a role in the defense against bacteria. According to this view, the ASL in CF patients, although still hypotonic, would have a higher salt content than normal and, therefore, a reduced activity of defensins. Another theory (3, 4) claims that the ASL is normally isotonic and that it is isotonic in CF patients but has a reduced volume, which would lead to the formation of viscous mucus that facilitates bacterial colonization.

It has been difficult to settle this dispute by determining the exact composition of the ASL. Published data on the composition of the ASL vary considerably (reviewed in Ref. 22). This may, in part, be due to species differences. For the ASL in humans, most reported data for Na and Cl concentrations are in the range of ~80–100 mM. For the mouse, however, values for Na varying from 6 to 105 mM and for Cl varying from 1 to ~120 mM have been reported (2, 7, 18, 19, 21). It is of interest to have data for the mouse, since several variants of transgenic mice with CF are available.

In the present paper, we have used X-ray microanalysis to determine the elemental composition of the ASL in normal and CF mice. Two methods were used: analysis of frozen-hydrated mouse trachea and analysis of the watery fraction of the ASL collected by dextran beads. Both methods show that the ASL in the mouse is somewhat hypotonic.

Materials and Methods

Animals. For the physiological experiments, ~50 NMRI mice (B&K, Sollentuna, Sweden) of both sexes, ~1 mo old, were used. In addition, five NMRI mice of ~15–18 mo of age were used as controls for comparison with CFTR(−/−) mice of similar age (see below). The animals were anesthetized with pentobarbital. In some experiments, the anesthetized animals were injected intraperitoneally with pilocarpine (50 mg/kg body wt) or isoproterenol (10 mg/kg body wt), and the trachea was removed 15 min after the injection. In experiments where nasal fluid was collected, the animals received an intraperitoneal injection with pilocarpine, isoproterenol (dose as above), or phenylephrine (10 mg/kg body wt).

The CFTR mutant (−/−) mice (14) (female, ages 15–18 mo) were raised in Hannover (Germany), transported by air to Uppsala (Sweden), and kept for 1 wk before the start of the experiment on a crude fiber-deficient diet with addition of a laxating salt solution (Oralav; B. Braun, Melsungen, Germany). The mice were anesthetized by spontaneous inhalation of isoflurane (Forene; Abbott Scandinavia, Kista, Sweden). The inhalation gas was administered continuously through a breathing mask (Simtec Engineering, Therwill, Switzerland) and contained a mixture of 40% oxygen, 60% nitrogen, and 2.2% isoflurane. Before harvesting of the trachea, the mice were terminated by spinal translocation. These mice were compared with control NMRI mice of...
approximately the same age. The experimental protocol was approved by the Regional Committee on Animal Experimentation for Uppsala County.

**Frozen-hydrated specimens.** The trachea was removed and immediately frozen in liquid propane cooled by liquid nitrogen to avoid compression during dissection. The trachea was then dissected into tracheal rings under liquid nitrogen. The pieces were stored in liquid nitrogen until analysis. For analysis, the tracheal rings were placed onto a specially designed holder and transferred to a Philips 525 scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) equipped with a Bio-Rad (Hemel Hempstead, UK) Polaron T7400E cold stage. The samples were coated with a thin carbon layer in the cold stage, at a temperature of $-190^\circ$C, and kept at this temperature throughout analysis (Fig. 1).

After preliminary experiments, an accelerating voltage of 10 kV was chosen to minimize overpenetration of the beam. The samples were analyzed by a LINK AN 10000 energy-dispersive spectrometer system (Oxford Instruments, Oxford, UK). Analysis was carried out for 500 s with a beam size of 200 nm, a beam current of $-15 \mu A$, a count rate of 230–235 counts/s, and a detector dead time of 5%.

Typically, eight to ten analyses were carried out per sample. For quantitative analysis, the data were compared with the results obtained on a standard consisting of a salt solution of known composition to which 5% albumin had been added. The standard solution was spread out in a thin layer over an aluminum planchet, shock-frozen, transferred to the cold stage of the scanning electron microscope, and analyzed under the same conditions as the specimen. Quantitative analysis was carried out using the ratio of characteristic to continuum intensity and by comparing this ratio with that obtained by analysis of the standard salt solution (16).

**Ion-exchange beads.** Dextran (Sephadex G-25) beads (diameter 20–40 µm; Pharmacia, Uppsala, Sweden) were equilibrated for 10 min with the ASL in the trachea in the following way. A small amount of beads was placed in the opening at the base of a Microlance 3 needle (0.8 × 40 mm; Becton Dickinson, Dublin, Ireland). The needle was connected to a syringe previously filled with air, and the beads were sprayed evenly over the tracheal surface by pressure on the syringe. Earlier experiments have shown that saturation of the beads with a salt solution is obtained after 5 min (20). After absorption of the ASL, the beads were recovered by being flushed with hydrophobic volatile silicone oil (Dow Corning 200/1cS; BDH, Poole, UK) and collected in a watch glass (12). Under a preparation microscope, all adhering fluid and debris were removed from the beads, and single beads were transferred onto nylon electron microscopy grids (Agar Scientific, Stansted, UK), which had been submerged into the oil. The grid with beads was slowly lifted out of the oil bath and mounted onto an aluminum holder covered with carbon adhesive tape and left at room temperature for evaporation of the oil.

Nasal fluid was collected in Sephadex beads as follows. The Sephadex G-25 beads were applied to double-sided tape (3M, Minneapolis, MN) attached to a filter paper (width 1–2 mm, length 5 mm; Schleicher and Schuell, Dassel, Germany) (20). The filter paper with the beads was inserted into one or both nostrils of the mouse, with beads facing the nasal septum, and were kept there for 10 min. Then, the filter paper with saturated beads was removed from the nostril and carefully washed in silicon oil to ascertain that no fluid was left on the outside of the beads. In some cases, the beads were separated and each bead was individually moved to a nylon electron microscopy grid until the grid contained 10–15 beads. The grid was then carefully removed from the oil, dried by evaporation of the oil at room temperature, and mounted on a specimen holder. In other cases, when it was difficult to separate the beads from the tape, the filter paper with the beads was carefully washed in the silicon oil and mounted on a specimen holder with the beads facing upwards.

Venous and arterial blood was collected from the mice, and both plasma and serum (after clotting for 24 h at 4°C) were collected. Sephadex beads were equilibrated with serum or plasma for 10 min. After that, the beads were recovered by being flushed with silicon oil and transferred to grids as described above.

Grids with Sephadex beads were carbon coated before analysis. X-ray microanalysis of the beads was carried out at room temperature with the instrumentation described above at 20 kV for 100 s with a beam size of 100 nm. Typically, 10–12 beads were analyzed from each sample. For quantitative analysis, the data were compared with the results obtained on beads soaked in salt solutions of different concentrations (50–250 mM) (20).

**Morphological studies.** For morphological studies, tissue was removed from the anesthetized animal and immediately fixed in 2.5% glutaraldehyde in water or different concentrations of sodium cacodylate buffer (0.025, 0.05, 0.1, or 0.15 mM). The tissues were kept in fixative for 24 h at 4°C and then postfixed with osmium tetroxide, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections were cut for electron microscopy, contrasted with uranyl acetate and lead citrate, and viewed at 75 kV in a Hitachi 7100 transmission electron microscope.

**Statistics.** Data are presented as means ± SE. Differences between more than two groups were determined by ANOVA, and differences between two groups were determined using Student’s t-test.

**RESULTS**

X-ray microanalysis of the ASL layer in frozen-hydrated mouse trachea (Fig. 1) showed a Na concentration of $\sim 80$ mM and a Cl concentration of $\sim 50$ mM (Fig. 2). The concentration of K was considerably higher than expected for an extracellular fluid. The fluid also contained large amounts of P and S. There was no significant difference in elemental composition between the young (1 mo) control animals and the old (15–18 mo) control animals (Fig. 2). The ASL in CF mice had significantly higher concentrations of Na and Cl compared with their age-matched controls (Fig. 2). Data for Mg, P, S, and Ca were not significantly different among any of the groups (Mg, P, S not shown).

Transmission electron microscopy of CF mouse trachea showed concretions in what appears to be a gland duct (Fig. 3) but is otherwise a normal ultrastructure of the surface epithelial cells.

Whereas X-ray microanalysis of ASL in frozen-hydrated trachea mainly samples the upper mucous layer of the ASL, the

![Fig. 1. Scanning electron micrograph of frozen-hydrated mouse trachea (at $-190^\circ$C) as mounted for X-ray microanalysis. The airway lumen (black) is to the left. The thin layer of airway surface liquid (ASL) is seen as a dark gray layer. The outer layer of the tracheal wall consists of cartilage (C). Bar = 100 µm.](http://ajplung.physiology.org/)
Sephadex beads sample the aqueous component. The data on the ionic composition of the aqueous component of the tracheal ASL also showed concentrations of Na and Cl, each ~60 mM (Fig. 4). As a comparison, data on mouse serum or plasma prepared for analysis in a way similar to the ASL are given (Fig. 4). The concentration of K in tracheal ASL was much higher than that in serum.

Cholinergic stimulation of the animal by pilocarpine resulted in a significant decrease of all elemental concentrations in the tracheal ASL (Fig. 4), with the exception of Ca, of which the concentration in tracheal ASL from normal mice is at the limit of the detection method. Adrenergic stimulation with isoproterenol had a similar, but less pronounced, effect (Fig. 4).

Transmission electron microscopy of the surface epithelium of mouse trachea fixed in a buffer with a strength of 200 mosmol/kgH2O (equivalent to 100 mM NaCl) did not show any damage to the cells, compared with tissue fixed in a buffer with a strength of 300 mosmol/kgH2O (Fig. 5). In surface epithelium fixed in a buffer with a strength of 100 mosmol/kgH2O, minor damage was observed in the form of small vacuoles in the apical part of some of the cells (not shown).

The elemental composition of nasal fluid from mice was not significantly different from that of ASL from the trachea. Stimulation with pilocarpine caused a significant increase in Na, Cl, and K in the nasal fluid, whereas stimulation with isoproterenol and phenylephrine only caused a significant increase in the K concentration (Fig. 6).

**DISCUSSION**

X-ray microanalysis of the ASL in frozen-hydrated mouse trachea indicates a hypotonic composition of the ASL. This finding is confirmed by the analysis of the watery fraction of the ASL, absorbed by the Sephadex beads. The concentration of Na plus K is ~90 mM in the watery fraction. The ionic composition of the ASL can be affected by pharmacological treatment. Analysis of frozen-hydrated trachea indicates that the ASL in CF mice has a higher content of NaCl than that in control mice.
There is a significant difference between the data from the frozen-hydrated tracheae and the data from the Sephadex beads. The data on the frozen-hydrated ASL show higher levels of, e.g., P and K, elements associated with cellular contents rather than with extracellular fluid. Because the ASL in the mouse is only ~10 μm thin, measurements in the frozen-hydrated state are technically difficult. It could be proposed that the electron beam would penetrate the thin ASL layer and excite the underlying cells, including their cilia. Theoretically, the penetration of the electron beam in ice at an accelerating voltage of 10 kV can be calculated to 2–3 μm (15). This is not sufficient for overpenetration of the ASL layer. Although it cannot be completely excluded that the tips of the cilia could be excited by the electron beam, it is not likely that the high K and P content of the ASL is due to excitation of the underlying cells. If the Na in the X-ray spectra were (mainly) due to the fluid, and K (mainly) to the underlying cells, one would expect a negative correlation between Na and K, with the Na:K ratio depending on the extent of overpenetration. It was, however, found that there was no negative correlation between Na and K in the individual measurements (Fig. 7).

The measurements of the ASL in frozen-hydrated trachea do, however, measure something different from the measurements of the Sephadex beads. The latter collect the watery component of the ASL without mucus. In the frozen-hydrated trachea, the entire ASL is sampled, with probably a predominance of the upper mucous layer. Airway mucus has a composition that is different from that of the watery fraction (20). Apart from mucus macromolecules contributing S, this layer also contains cell debris that would contribute elements such as P and K. Leakage from dead cells could partially explain the relatively high K concentration in the watery fraction. Negatively charged macromolecules (phosphates and mucins) could also attract and bind cations, resulting in higher cation concentrations in the mucus than in the fluid component.

Both techniques agree, however, on the fact that the ASL in mouse is hypotonic. The data also show that neither age nor the method of anesthesia affect the composition of the ASL. Our results agree reasonably well with data obtained by Cowley et al. (7), who found 87 mM for Na and 57 for Cl. However, our concentrations are lower than those published by Caldwell et al. (5), Song et al. (18), Tarran et al. (19), and Verkman (21), who found chloride concentrations of ~115–120 mM. On the other hand, our values are much higher than those given by Baco et al. (2), who found concentrations <10 mM. It may seem unreasonable that airway surface epithelial cells could be continuously exposed to a hypotonic fluid, but ultrastructural investigations appear to confirm that the cells are not noticeably damaged by a fluid with a salt concentration of ~100 mM (200 mosmol/kgH2O), which is close to the 90 mM Na plus K measured.

The relatively low ionic concentrations in mouse ASL are in contrast with our findings in the human (20) and the pig (10). It is well known that the mouse airway has much fewer submucosal glands than pig and human airways (6). Light microscopy of the control mouse tracheae used in the present study failed to find a noticeable number of submucosal glands. It could be hypothesized that much of the ASL is produced by glands as an isotonic fluid and that the surface epithelium absorbs ions from the ASL (3). In animals with few glands relative to the extent of surface epithelium, this would result in lower ionic concentrations in the ASL compared with airways with many glands, such as pig and human airways.

CF mice have significantly higher concentrations of Na and Cl in their ASL than the controls. This appears to disagree with findings of Zahm et al. (24), who found no significant difference between ASL in control and CF mice, but since those data are presented as mmol/kg dry weight, whereas our data are in mmol/kg wet weight, these studies are difficult to compare. One should be careful, however, in extending data from the mouse to the human, because of the differences in airway architecture.

In one of the CFTR(−/−) mice, a concretion in a duct of a submucosal gland was observed. In general, transgenic mice with CF have been reported to show no evident signals of airway disease that would be comparable to the human airway with dilated gland ducts and inpsissated mucus (9), but a recent report found that in long-living CFTR(−/−) mice, airway pathology developed that resembled that in CF patients (8). Our findings can thus be related to the age of the mice.

The ionic composition of the ASL can be changed by pharmacological treatment. The effect of stimulation on the ionic composition of tracheal and nasal fluid, respectively, is markedly different. The effects on the nasal fluid can be explained by assuming secretion by glands in the nose, where the cholinergic stimulation gives rise to secretion of a NaCl-rich fluid, whereas the β-adrenergic agonist isoproterenol mainly causes secretion of protein-rich secretory granules, to

![Fig. 6. X-ray microanalysis of nasal liquid collected with the Sephadex bead method. Data (in mM, means ± SE) are given for control (5 animals), pilocarpine-stimulated (4 animals), isoproterenol-stimulated (4 animals), and phenylephrine-stimulated mice (4 animals). Significant differences between stimulated and control trachea are indicated, ***P < 0.01, ****P < 0.001.](http://ajplung.physiology.org/)

![Fig. 7. Correlation between Na and K in measurements of frozen-hydrated trachea, showing that the Na concentration is independent of the K concentration.](http://ajplung.physiology.org/)
which, apparently, K is bound. The α-adrenergic agonist phenylephrine has only small effects. In the trachea, however, both pilocarpine and isoproterenol cause a significant decrease in ionic concentrations. This remarkable effect can only be explained by assuming that the ASL is diluted due to secretion of water (fluid secretion can be observed macroscopically). The origin of this water (the tracheal wall or the distal airways or alveoli) remains to be elucidated. In the trachea, different from the situation in the nose, submucosal glands would not contribute significantly to the fluid under stimulated conditions. Admittedly, the doses of the agonists used in the present study are very high and some unspecific effects may be present. However, the data provide “proof of principle” that the ionic concentrations in the ASL can be manipulated by pharmacological treatment. The experimental system used in the present study offers the possibility to directly test the effect of drugs on the ionic composition and water content of the ASL, which may be helpful for research on diseases where one wishes to increase the hydration of the fluid lining the airway wall.

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