Upsala J Med Sci 111 (1): 137–153, 2006

Composition of Airway Surface Liquid Determined by X-ray Microanalysis

Inna Kozlova[1], Viengphet Vanthanouvong[1], Marie Johannesson[2], Godfried M. Roomans[1]

[1] *Department of Medical Cell Biology, University of Uppsala, Box 571, Uppsala, Sweden*
[2] *Department of Women's and Children's Health, Uppsala University, Uppsala University Hospital, Uppsala, Sweden*

ABSTRACT

The composition of the airway surface liquid, a thin layer of fluid covering the airway wall, has been debated. Two new techniques to determine the ionic composition of the airway surface liquid are presented. In the first technique, pieces of the airway were shock-frozen and analyzed by X-ray microanalysis in the frozen state in the scanning electron microscope. In the second technique, the airway surface liquid was collected with the help of dextran beads that were allowed to absorb the fluid. The beads were collected in silicon oil, cleaned, dried, and analyzed. Airway surface liquid from pig airways was isotonic to lightly hypertonic, whereas airway surface liquid from mouse and rat airways was hypotonic. The ionic composition of airway surface liquid from rodent airways could be changed by pharmacological stimulation of fluid transport. Transgenic mice with cystic fibrosis (CF) had significantly higher Na and Cl concentrations in the airway surface liquid than normal mice. Nasal fluid was also collected from humans. In CF patients, CF heterozygotes, and rhinitis patients, the levels of Na and Cl in the nasal fluid were signifi-

Received 12 October 2005
Accepted 27 October 2005

cantly higher than in healthy controls. In CF patients K levels were also significantly higher than in healthy controls. The ionic concentrations in fluid collected from patients with primary ciliary dyskinesia (PCD) were not different from normal. Females with CF had significantly higher concentrations of Na, Cl and K in their nasal fluid compared to male patients. The dextran bead technique was also used to determine the ionic composition of the apical fluid in cultures of respiratory epithelial cells from healthy controls and CF patients. In the healthy controls, the fluid was hypotonic. In the CF cell cultures, the apical fluid had a higher Na and Cl concentration than in the controls.

INTRODUCTION

The airway surface liquid (ASL) is a thin layer of fluid covering the airways. The ASL secreted by airway epithelium and submucosal glands is the first line of defense of the airway epithelium and ensures the mucociliary transport of inhaled particles. ASL consists of mucus and an underlying periciliary watery layer. The ionic composition of the ASL plays a crucial role in airway host defense by controlling the ciliary activity, mucin release and antimicrobial activity in the airways. Studies of patients with cystic fibrosis (CF) have strongly suggested that the volume and/or composition of the liquids that line the airway surface are important components of the lung defense.

There are two opposing views regarding what happens with the ASL in CF. Some have claimed that the ASL normally is hypotonic, which provides 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, while still hypotonic, would have a higher salt content than normal, and therefore a reduced activity of defensins [1, 2]. Another group, on the other hand, claims that the ASL is normally isotonic, and that it is isotonic in CF patients, but has a reduced volume, leading to the formation of viscous mucus that facilitates bacterial colonization [3, 4].

However, it has been difficult to determine the exact composition of the ASL. Published data on the composition of the ASL are very divergent and vary from very hypotonic to hypertonic. Tracheal and/or bronchial ASL has been collected both from humans, and from other species, mainly from mouse. According to a review by Verkman et al. [5], published values for the ionic composition of human ASL are fairly closely together and range from 82-91 mM for Na^+ and 82-108 mM for Cl^- [6-9]. For the mouse, however, the variation is large and values range from 6-105 mM for Na^+ and 1-115 mM for Cl^- [10-12]. For monkey and rabbit, values for Cl^- of 112 and 114 mM have been found [13]. High values were found for dogs, where Na+ concentrations ranged from 153-173 mM and the Cl^- concentration was 134 mM [14], and in ferret where Na+ was 167 mM and Cl^- 121 mM [15]. Clearly hypotonic values were found for the rat, where Na^+ was 41 mM and Cl^- 45 mM [16]. A number of different techniques for sampling and analysis have been used, and it would appear that, apart from possible species differences, one source of variation between the results is the sampling technique. Furthermore, studies

have been carried out on cell cultures of airway epithelial cells. According to Matsui et al. [17] values for Na+ and Cl^- were nearly isotonic, whereas Zabner et al. [2] found values for Na^+ and Cl- around 50 mM, and McCray et al. [18] found a value for Cl^- of 18 mM.
We have carried out a study of the composition of ASL using X-ray microanalysis in humans and several animal species. We started with pig airways, which share many structural and physiological similarities with human airways [19, 20]. We continued with rodent (mouse, rat) airways, which gave us the possibility to investigate CF mice [21-23]. We also carried out a study on human nasal fluid, where we investigated both normal humans, CF patients and patients with rhinitis (Vanthanouvong et al., submitted for publication), and we are investigating cell cultures of airway epithelial cells from CF patients and healthy controls (Kozlova et al., submitted for publication)..
We have used two different techniques: X-ray microanalysis[1] of frozen-hydrated specimens [19], and X-ray microanalysis of ASL absorbed into small ion exchange beads [21].

MATERIALS AND METHODS

Pigs

For the pig experiments, pigs of mixed breed with a body weight of 20-30 kg were used [20].

Mice

For the physiological experiments on control mice, NMRI mice of both sexes, about 1 month old, were used. In some experiments, the anesthetized animals were injected intraperitoneally with pilocarpine (50 mg/kg body weight), or isoproterenol (10 mg/kg body weight), 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 weight) [22].

The CFTR (-/-) mice (female, age 15-18 months) [22, 23] were transported by air to Uppsala and kept for one week before the start of the experiment, on a crude fiber deficient diet, and with addition of a laxating salt solution. These mice were compared with control NMRI mice of approximately the same age.

---

[1] X-ray microanalysis is a technique of elemental analysis, which does not distinguish between elements occurring in an ionized form (ions) and elements occurring in a non-ionized form. Therefore, in this paper, when reference is made to results obtained by X-ray microanalysis, this reference will be made to the non-ionized form of the element (Na, Cl, etc.), even though the element may be ionized when it occurs in a cell or in the extracellular fluid. Likewise, reference will be made to P, S, etc., regardless of the chemical state (phosphate, protein-bound sulfur etc.), in which these elements occur in reality.

Rats

In the rat experiments, adult Sprague-Dawley rats (male and female, 7- 8 weeks old, 250-300 g), were used [24].

Cultured cells

16HBE14o$^-$ (16HBE) and CFBE41o$^-$ (CFBE) (homozygous for the DF508 mutation) cells [25] were grown in Eagle's Minimal Essential Medium (EMEM) to confluence in tissue culture flasks. The confluent cells were trypsinized, split, and seeded on Costar inserts on a tissue culture 24-well plate. The inserts had been coated with 100 µl of a solution containing 0.01% collagen type I from calf skin. The coated surface was sterilized by ultraviolet light for 2-3 h.

Some membranes with cells were chosen for analysis as soon as confluence was reached in liquid-liquid interface cultures, other cultures were allowed to differentiate in air-liquid interface on the membranes for 3-4 weeks. Liquid-liquid cultures of confluent 16HBE and CFBE cells were allowed to equilibrate with isotonic PBS for 24 h prior to the experiment. 7 µl of PBS was added to the apical surface of the cells, which gave a layer with a depth of about 200 µm. After 24 h the membrane was frozen in liquid propane cooled by liquid nitrogen to -190°C. The membrane was placed onto a specially designed holder and transferred to a Philips 525 scanning electron microscope equipped with a Bio-Rad Polaron 7400E cold stage (Kozlova et al., submitted for publication).

Humans

Nasal fluid was collected from several different groups of subjects (Vanthanouvong et al., submitted for publication). The control group consisted of healthy volunteers (non-smokers). In addition, CF patients, CF heterozygotes, patients with primary cilia dyskinesia (PCD), and allergy/rhinitis patients were included in the study.

All CF and PCD patients were treated at Uppsala Cystic Fibrosis Center, Uppsala University Hospital, and the CF heterozygotes were mothers of these patients. The CF patients were classified according to the severity of their disease (taking into account lung function and the need for intravenous antibiotics) in "mild", "medium" and "severe". All but one of the rhinitis patients were children or teenagers on regular control at the Allergy Clinic, Children's Hospital, Uppsala University Hospital, and one of them was an adult staff member at the Clinic. All subjects in the group were allergic as judged by positive skin tests, IgE-antibody tests and/or clinical history. All had a perennial allergy to fur-coated animals or a seasonal allergy with birch-timothy pollen sensitivity, most of them both. Sampling was performed during or immediately after the pollen season in May-June, i.e., during relevant exposure for the vast majority of the patients.

Analysis of animal trachea in situ

The trachea or bronchi were removed from the anesthetized animals and dissected in a specially designed chamber, in which the humidity could be kept constant at close to 100% [19, 20, 22]. The chamber consisted of a Perspex box, with a retractable plastic sheath on one side, which could be opened for handling of the specimen. A water reser-

voir at 37°C was placed at the bottom of the chamber, and the specimen was placed on a perforated shelf. Humidity was monitored with a hygrometer.

Tissue pieces were frozen in liquid propane cooled by liquid nitrogen. The pieces were stored in liquid nitrogen until analysis. For analysis, the tissue pieces were placed with the mucosal side up onto a specially designed holder and transferred to a Philips 525 scanning electron microscope equipped with a Bio-Rad Polaron 7400E cold stage. The samples were coated with a thin carbon layer in the cold stage, at a temperature of -190°C, and kept at this temperature throughout analysis. After preliminary experiments, an accelerating voltage of 9 or 10 kV was chosen to minimize overpenetration of the beam.

The samples were analyzed by a LINK AN 10000 energy-dispersive spectrometer system. For quantitative analysis, the data were compared to the results obtained on a drop of frozen salt solution of known composition analyzed under the same conditions. 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.

Analyis of ASL collected by beads

In the animal studies, Sephadex G-25 beads (diameter 20-40 µm) were spread evenly on the surface of the (dissected pieces of the) trachea and left during 30 min in the humidity chamber described above. Saturation of the beads with a salt solution was obtained after 5 min [21]. After absorption of the ASL, the beads were recovered by flushing with hydrophobic volatile silicone oil and collecting the beads in a watch-glass. Under a preparation microscope, all adhering fluid and debris were removed from the beads, and single beads were transferred onto specimen grids, which had been submerged into the oil. The specimen grids used were nylon grids covered with a thin Formvar film. The grid with beads was slowly lifted out of the oil bath and mounted onto an aluminum holder covered with round carbon adhesive tape and left at room temperature for evaporation of the oil. Grids with Sephadex beads were carbon coated prior to analysis [21].

Nasal fluid of mouse and rat

Nasal fluid of mouse and rat was collected in Sephadex beads in the following way [22, 24]: The Sephadex G-25 beads were applied to double-sided tape attached to a filter paper (width 1-2 mm, length 5 mm). The filter paper with the Sephadex beads was inserted into either one or both nostrils of the mouse, beads facing the nasal septum, and kept there for 10 min. After this period, the filter paper with saturated beads was removed from the nostril and carefully washed in hydrophobic volatile silicon oil to ascertain that no fluid was left on the outside of the beads. 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.

Human nasal fluid

Analysis of human nasal fluid was carried out in the following way [26]. The subjects were asked to close their nose with a nose clip for 10 min, and subsequently, nasal fluid was collected by inserting in the vestibule of the nose ion exchange beads mounted on double-sided tape on filter paper. The nostrils were kept closed for another 10 min. Normally, this is sufficient to saturate the filter paper and the beads with nasal fluid. In some CF patients, the nostrils were kept closed for a longer time (15-20 min), because of their reduced secretion. In some of the allergy/rhinitis patients also this longer time was necessary, if use of a nasal decongestant had dried out the mucous membranes of the nose. At the end of the period, the filter papers were removed, while the subjects held their breath, and the filter papers were transferred and carefully washed in hydrophobic volatile silicon oil.

X-ray microanalysis of the beads was carried out with the instrumentation described previously. Typically 10-12 beads were analyzed from each sample. For quantitative analysis, the data were compared to the results obtained on beads soaked in salt solutions of different concentrations (50mM – 250mM).

RESULTS

Pig

The results of the X-ray microanalysis of frozen-hydrated pig trachea, placed with the mucosal side up, are shown in Figure 1. The results show that [Na] is around 120 mmol/kg wet weight (about the same in mM) and [Cl] and [K] are around 50 mmol/kg wet weight.

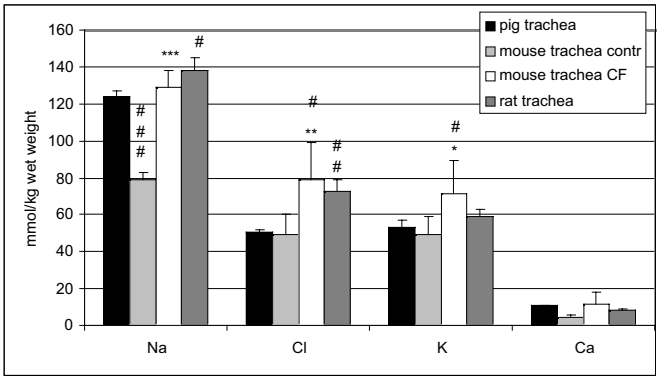

Figure 1. X-ray microanalysis of frozen-hydrated trachea in the pig, control mice, transgenic CF mice, and rats. Concentrations are given in mmol/kg wet weight. Statistically significant differences between pig and control mice and rats are indicated by # ($p<0.05$), ## ($p<0.01$), and ### ($p<0.001$), and statistically significant differences between control mice and cystic fibrosis mice are indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Note that the levels of Na are much lower in the mouse trachea than in the pig trachea.

Figure 2 shows the results of the X-ray microanalysis of Sephadex G-25 beads that had absorbed ASL for 30 min. The Na and Cl concentrations in the ASL were slightly lower than in plasma. The K concentration was higher than in plasma, but lower than in the measurements on frozen-hydrated ASL.

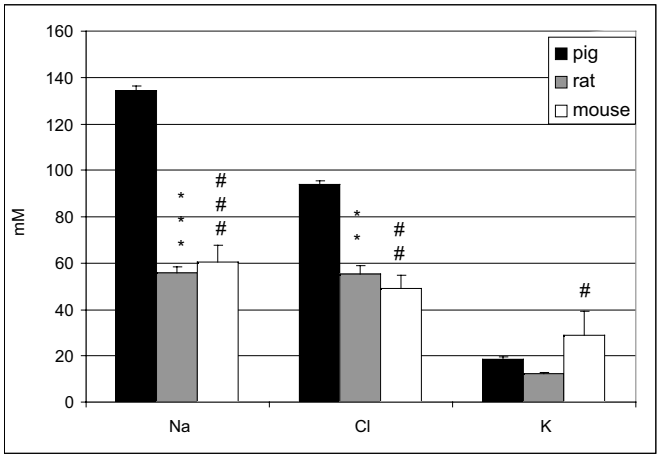

Figure 2. X-ray microanalysis of tracheal ASL collected by Sephadex beads in the pig, rat, and mouse (control). Concentrations are given in mM. Statistically significant differences between pig and rat are indicated by asterisks (** p<0.01, *** p<0.001) and between pig and mouse by # (# p<0.05, ## p<0.01, ### p<0.001). Note that the levels of Na are much lower in the mouse and rat trachea than in the pig trachea.

Mouse

Figure 3 shows a scanning electron micrograph of frozen-hydrated mouse trachea, which allows the thickness of the ASL layer to be estimated at around 10 µm. Fig-

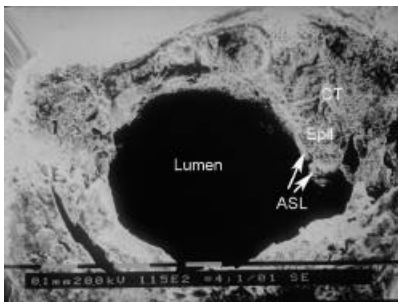

Figure 3. Scanning electron micrograph of frozen-hydrated mouse trachea, showing the lumen, the airway-surface liquid (ASL), the epithelium (Epit) and the connective tissue (CT). The magnification is indicated by bars (bar = 100 µm).

ure 1 shows the results of X-ray microanalysis of the ASL-layer in frozen hydrated mouse trachea which gave a Na concentration of about 80 mmol/kg wet weight and a Cl concentration of about 50 mmol/kg wet weight. The concentration of K was considerably higher than expected in an extracellular fluid. The fluid also contained large amounts of P and S. There was no significant difference in elemental composition between the young control animals and the old control animals (not shown). The ASL in CF mice had significantly higher concentrations of Na and Cl compared to their age-matched controls (Fig. 1). Data for Mg, P, S and Ca were not significantly different between any of the groups.

When Sephadex beads are placed onto the trachea and left there for 30 min, the

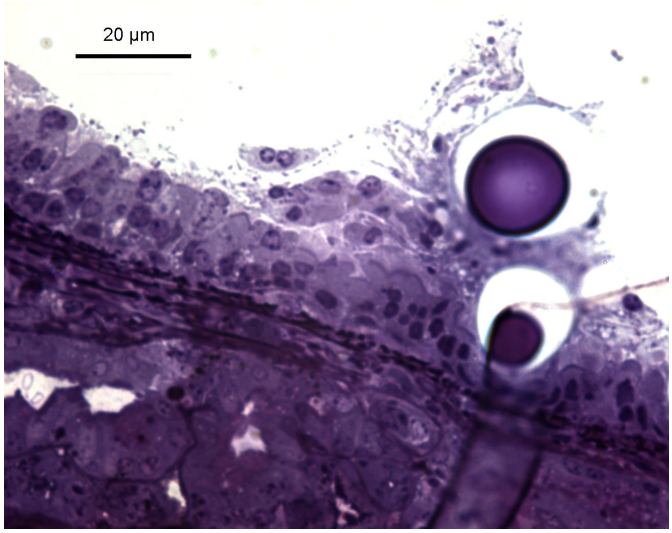

Figure 4. Light microscopical image of Sephadex G-25 beads embedded in the mucus layer on mouse tracheal epithelium. The mucus layer contains cell and cell debris. The magnification is indicated by a bar of 20 µm.

beads sink through the ASL layer and reach the tips of the cilia as shown in Figure 4. It can be seen in this figure that the mucus layer contains cells or cell debris.

While X-ray microanalysis of ASL in frozen-hydrated trachea mainly samples the upper, mucous layer of the ASL, the 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 of each around 60 mM (Fig. 5a), much lower than in the pig.

Cholinergic stimulation of the animal by an intraperitoneal injection of pilocarpine resulted in a significant decrease of the elemental concentrations in the tra-

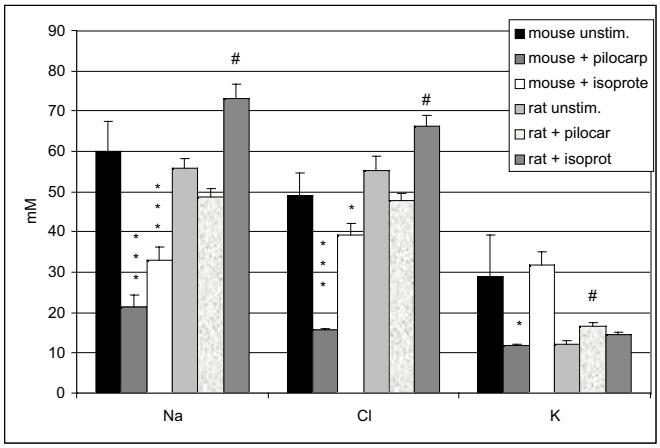

figure 5a

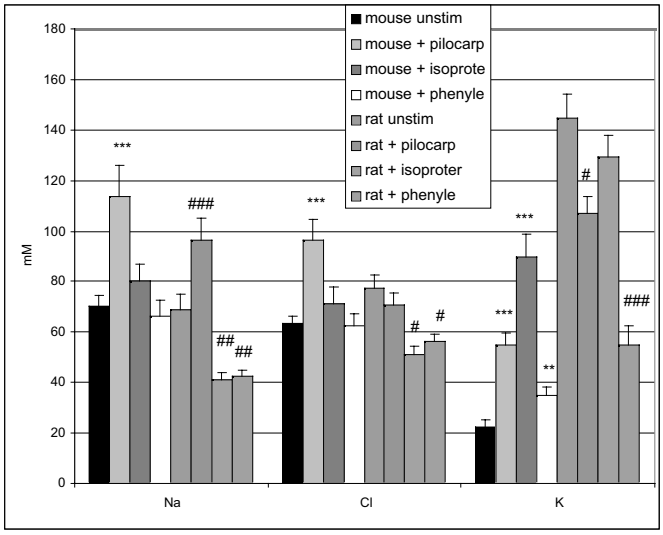

figure 5b

Figure 5. X-ray microanalysis of (a) tracheal fluid, and (b) nasal fluid in the mouse and rat. The composition of the fluid after cholinergic stimulation (pilocarpine), β-adrenergic stimulation (isoproterenol) and α-adrenergic stimulation (phenylephrine) is compared to the composition of fluid in unstimulated control animals (* $p<0.05$, ** $p<0.01$, ***$p<0.001$for the mouse, and #$p<0.05$, ##$p<0.01$, and ### $p<0.001$ for the rat).

cheal ASL (Fig. 5a), 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 (data 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. 5b).

Rat

X-ray microanalysis of the layer of ASL in frozen-hydrated rat trachea showed a concentration of about 140 mmol/kg wet weight (roughly equivalent to 140 mM) for Na, and about 60 mM for K. In contrast, the Cl concentration was only about 80 mM (Fig. 1). The concentrations of P and S were quite high, around 110 mM and 75 mM, respectively (not shown).

X-ray microanalysis of tracheal fluid and nasal fluid (Fig. 5) collected under unstimulated conditions showed that the elemental composition of these fluids was markedly different from that of plasma. Concentrations of Na and Cl were significantly lower in both tracheal fluid and nasal fluid compared to plasma. In tracheal fluid, K was slightly, but significantly higher than in plasma. In nasal fluid extremely high K values were found (Fig. 5b). The very high K concentration was the main difference between tracheal and nasal fluid in the rat.

Pharmacological stimulation resulted in relatively minor changes in the composition of the ASL lining the trachea (Fig. 5a). Isoproterenol significantly increased the Na content of the fluid, and pilocarpine and phenylephrine the K content. Pilocarpine stimulation also increased the Mg concentration. The effects of stimulation on nasal fluid were somewhat different: pilocarpine significantly increased Na and Cl, isoproterenol significantly increased K and phenylephrine significantly decreased K but all the stimulations significantly increased the Ca concentration (Fig. 5b).

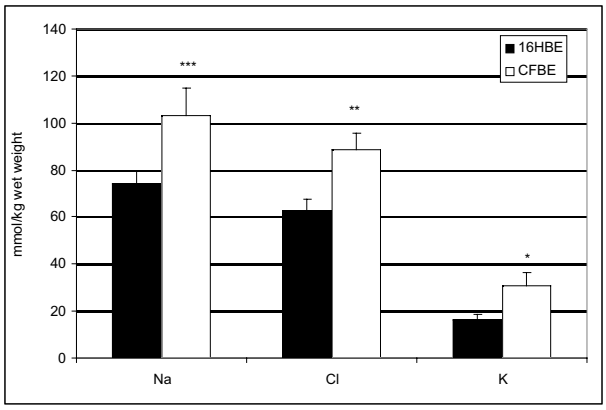

Figure 6. X-ray microanalysis of apical fluid covering cultures of normal (16HBE) and CF (CFBE) airway epithelial cells. Statistically significant differences between CF cells and controls are indicated by asterisks (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

Cultured respiratory epithelial cells

Analysis of the apical fluid in frozen-hydrated specimens of liquid-liquid interface cultures showed that after equilibration for 24 h, the Na and Cl concentrations in the apical fluid had decreased, both in CF cells and in control cells, compared to the original PBS, in which the Na^+ concentration is 150 mM, and the Cl^- concentration 140 mM. The decrease was less in the CF cultures, and the Na and Cl concentrations were significantly higher in the apical fluid of CF cells compared to controls (Fig. 6). Both in control cells and CF cells, the apical fluid was clearly hypotonic.

Human nasal fluid

The analysis of beads with nasal fluid showed that in CF patients, CF heterozygotes, and rhinitis patients, the levels of Na and Cl in the nasal fluid were significantly higher than in healthy controls. In CF patients also the levels of K were significantly higher than in healthy controls (Fig. 7).

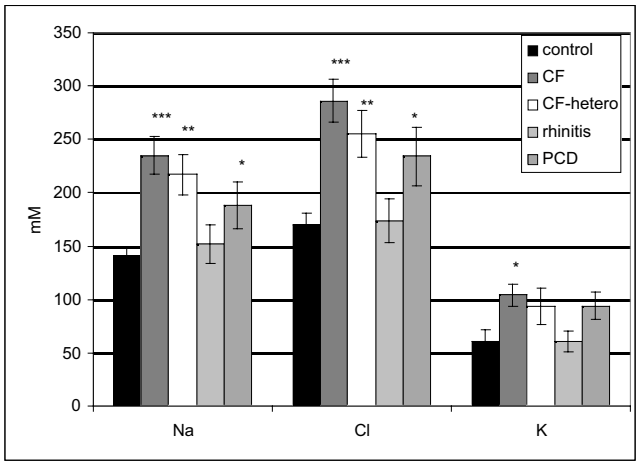

Figure 7. X-ray microanalysis of nasal fluid from patients with different airway diseases and healthy controls. The fluid was collected by the Sephadex bead method. Statistically significant differences between healthy controls and diseased patients are indicated by asterisks (* $p<0.05$, ** $p<0.01$, ***$p<0.001$).

The elemental composition of the fluid collected from PCD patients was not different from that of the healthy controls. No significant difference in Na, Cl or K was observed between healthy males and females. However, females with CF had significantly higher concentrations of Na, Cl and K in their nasal fluid compared to CF-males (Fig. 8).

Although the sex difference in ion concentrations was not significant for PCD and rhinitis patients, there was a significant difference between male and female

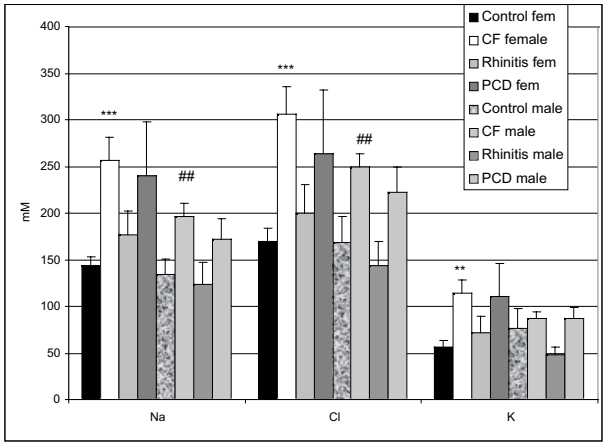

Figure 8. Differences in elemental composition of the nasal fluid between male and female patients with different airway diseases or healthy controls. Statistically significant differences between males and females are indicated by symbols (** p<0.01, ***p<0.001 for females and ##p<0.01 for males).

patients when all patients with airway diseases (CF, PCD, and rhinitis) were pooled. The CF patients classified as ”severe” had significantly higher K concentrations in their nasal fluid than the patients classified as ”mild” or ´”medium” (Fig. 9). Within the group of cystic fibrosis patients, there was no significant correlation with age, FEV1 or FVC%. Within the other groups, there was no significant correlation between age and elemental content of the nasal fluid.

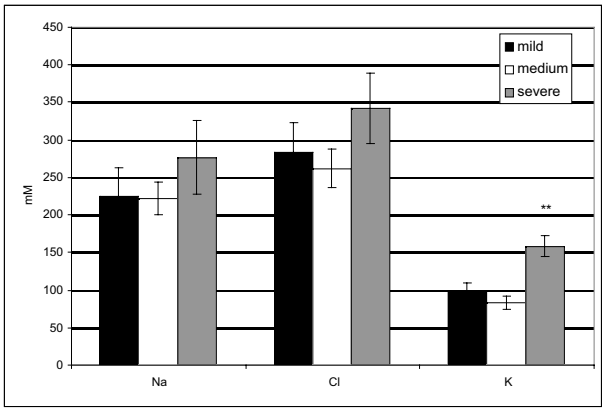

Figure 9. Differences in elemental composition between CF-patients (males and females pooled) with a mild, medium, or severe disease (taking into account lung function and the need for intravenous antibiotics). Statistically significant differences between groups are indicated by asterisks (** p<0.01).

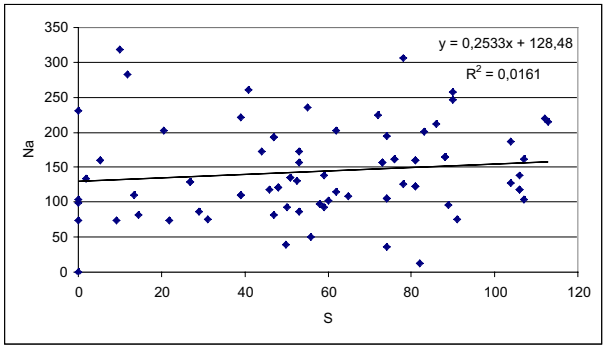

Figure 10. Correlation between Na and S concentrations in frozen-hydrated pig trachea. The line through the points shows a weakly positive correlation between Na and S.

DISCUSSION

The results indicate that in the pig, which is close to the human with respect to fluid transport in the airways, the Na and Cl concentrations of the ASL are close to isoosmolar. There are, however, major differences in the results of the ASL measured *in situ* in frozen-hydrated specimens, and the results obtained on the Sephadex beads. The ASL *in situ* contains substantially higher concentrations of P, S, and K than the ASL in the Sephadex beads. The elements P, S and K are "cellular", rather than "extracellular" elements. One could suspect that the measurements on the ASL in the frozen-hydrated state, underlying tissue, or at least cilia, are excited by the electron beam, and thus contribute to the spectrum. There are, however, several arguments against this notion.

First, the spatial resolution of analysis at 9 or 10 kV in a frozen-hydrated specimen can be calculated to be in the order of 2-3 µm, which is much less than the actual thickness of the ASL than can be measured from micrographs where the trachea is mounted sideways. Finally, if the results were due to overpenetration of the electron beam and excitation of the underlying epithelium, one would expect a negative correlation between e.g., Na and P or S, or Na and K, since a measurement with little or no overpenetration would sample the overlying ASL, and show high Na and low P, S, and K, whereas a measurement with much overpenetration would mainly excite the epithelium and show high P, S, and K and lower Na. Analysis of the data in Figure 10, however, shows that the correlation between Na and S is not negative, and the same result is obtained for the relation between Na and P or K. Similar results were obtained for the rat and the mouse.

The difference, then, between the measurements of ASL *in situ* in frozen hydrated specimens and in the beads must be due to the fact that actually different things are measured. The measurements of the ASL in the frozen-hydrated specimens mea-

sure the upper (mucous) layer of the fluid, whereas the beads absorb the watery component of the layer. The upper part of the ASL likely contains glycoproteins and exfoliated cells and cell debris that could add "cellular" elements such as P, S and K to the layer. If mucus and debris is left on the beads, higher values for P, S and K are obtained.

The results unequivocally confirm the notion that the Na and Cl concentrations in the watery phase of the ASL of the pig are close to those found in plasma. The elemental composition of the watery layer of the ASL does, however, differ from that of plasma in a number of respects. Concentrations of Mg, P and K are higher than in plasma. For K, where we find a concentration of 20 mM, some literature data are available. In human ASL, Joris et al. [7] found 29 mM and Knowles et al. [8] found 18 mM, which agrees well with our data. Possibly, the source of the K in the fluid component of the ASL is the cell debris in the mucous layer that could leak K+ as well as contain phosphate- and sulfur-containing molecules.

The ion concentrations in human nasal ASL in the healthy controls are slightly hypertonic compared to serum. This does not contradict the notion that an isotonic fluid is produced, but it is likely that some evaporation of water occurs due to the flow of air in the nose, leading to an increase in salt concentration. The sum of [Na]+[K] is larger than [Cl], which may be due to the presence of bicarbonate ions and organic anions (amino acids, glycoproteins) in the mucus. The K concentration is much higher than in serum, which may be due to K leaking out from damaged or dead cells shed from the epithelium. In the model of Boucher [3, 4], it is predicted that the ASL normally is isotonic, and that it is isotonic in CF patients as well, but has a reduced volume (low volume hypothesis"). In contrast, in the model of Welsh et al. [1, 2] the ASL is normally hypotonic, but salt concentrations are increased in CF patients ("high salt hypothesis"). Our finding that in healthy persons nasal ASL is slightly hypertonic would agree best with the "low volume hypothesis". On the other hand, we find that the salt concentration in the nasal ASL from CF patients is significantly higher than normal, which would be more in line with the alternative hypothesis. Also in cultured respiratory epithelial cells from CF patients, the apical fluid contains more Na and Cl than the corresponding fluid in control cultures. The fact that the values for Na and Cl found in CF heterozygotes are higher than normal may be due to the fact that in many CF heterozygotes chloride transport in nasal epithelial cells is abnormal, even though this does not give rise to clinical problems. It is uncertain how much of this increase in Na and Cl is due to a defect in CFTR, and how much is due to epithelial damage caused by chronic inflammation, since patients with rhinitis have ion concentrations that are not significantly different from those found in CF patients.

The finding that CF patients with severe symptoms have higher ion concentrations than patients with mild or moderate symptoms does not contradict the hypothesis that inflammation is an important factor determining the ionic concentrations in the ASL. So far, the composition of the ASL has not been studied in any other diseases besides CF. The hypothesis that epithelial damage caused by inflammation

could cause a leakage of ions into the ASL, resulting in abnormally high Na^+ and Cl^- concentrations, seems reasonable but awaits further testing.

In rodents, however, the situation is different. In the mouse, both techniques agree on the fact that the ASL is hypotonic. Our results agree well with data obtained by Baconnais et al. [27], who found concentrations of around 65 mM for Na and Cl, and reasonably well with data obtained by Cowley et al. [11], who found 87 mM for Na^+ and 57 for Cl^-. However, our concentrations are lower than those published by Verkman [12], Tarran et al. [28] and Caldwell et al. [16], who found chloride ion concentrations of about 115-120 mM. Possibly, results differ between different mouse strains. 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 about 100 mM (200 mOsmol).

The extremely high K^+ concentrations in the rat nasal fluid differ from the situation in the human and even in the mouse, and the source of this K^+ remains to be elucidated. Since the method of collection was the same in the rat as in the mouse and the human, it is unlikely that the high K^+ in the rat fluid is caused by the collection technique. The explanation of this result is made more difficult by the fact that the physiology of the airway submucosal glands has not been well studied. Possibly, the submucosal glands in the nose resemble salivary glands. In these glands, it has been shown that radiation damage or inhibition of the Na^+-K^+ ATPase by ouabain can cause high levels of K^+ in the secreted fluid. Therefore, the high K^+ levels in the nasal secretion may point to the fact that these nasal glands function in a suboptimal way in the rat, and that therefore the K^+ level in the secreted fluid is abnormally high.

Of interest is also that 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 in the mouse, respectively, is markedly different. The effects on the nasal fluid can be explained by assuming secretion by glands in the nose, where the cholinergic agonist pilocarpine would mainly give rise to secretion of a NaCl-rich fluid, whereas the ß-adrenergic agonist isoproterenol would cause mainly secretion of protein-rich secretory granules, to which, apparently, K^+ is bound. The a-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. This notion is supported by the fact that fluid secretion can be observed macroscopically. The origin of this water (the tracheal wall or the distal airways or alveoli) remains to be elucidated. Furthermore, 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 unspecific effects may play a role. However, the data provide “proof of principle” that the ionic concentrations in the ASL can be affected 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.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the collaboration of Harriet Nilsson, Elisabeth Nääs, Dr Birgitta Almgren, Dr Anca Dragomir, Dr Marianne Högman, and Dr Mia Phillipsson (University of Uppsala) in the experiments and of Prof Ursula Seidler and Dr Birgitte Riederer (University of Hannover, Germany) and Prof William H. Colledge (University of Cambridge, U.K.) for the CF mice. The authors gratefully acknowledge the skillful technical assistance of Leif Ljung, Marianne Ljungkvist and Anders Ahlander. The study was supported by the Cystic Fibrosis Foundation, the Swedish Heart-Lung Association, the Swedish Science Research Council, the Swedish Asthma and Allergy Association, and the Swedish Association for Cystic Fibrosis.

REFERENCES

1. Smith JJ, Travis SM, Greenberg EP, Welsh MJ (1996) Cystic fibrosis airway epithelial fail to kill bacteria because of abnormal airway surface fluid. Cell 85: 229-236.
2. Zabner J, Smith JJ, Karp PH, Widdicombe JH, Welsh MJ (1998) Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro. Mol Cell 2: 397-403.
3. Boucher RC (1999) Molecular insights into the physiology of the 'thin film' of airway surface liquid. J Physiol (Lond) 516: 631-638.
4. Boucher RC (2002) An overview of the pathogenesis of cystic fibrosis lung disease. Adv Drug Deliv Rev 54: 1359-1371.
5. Verkman AS, Song Y, Thiagarajah JR (2003) Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. Am J Physiol Cell Physiol 284: C2-C15.
6. Gilljam H, Ellin A, Strandvik B (1989) Increased bronchial chloride concentration in cystic fibrosis. Scand J Clin Lab Invest 49: 121-124.
7. Joris L, Dab I, Quinton PM (1993) Elemental composition of human airway surface fluid in healthy and diseased airways. Am Rev Resp Dis 148: 1633-1637.
8. Knowles MR, Robinson JM, Wood RE, Pue CA, Mentz WM, Wager GC, Gatzy JT, Boucher RC (1997) Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. J Clin Invest 100: 2588-2595
9. Hull J, Skinner W, Robertson C, Phelan P (1998) Elemental content of airway surface liquid from infant with cystic fibrosis. Am J Respir Crit Care Med 157: 10-14.
10. Baconnais S, Zahm JM, Kilian L, Bonhomme P, Gobillard D, Perchet A, Puchelle E, Balossier G (1998) X-ray microanalysis of native airway surface liquid collected by cryotechnique. J Microsc 191: 311-319.
11. Cowley EA, Govindaraju K, Guilbault C, Radzioch D, Eidelman DH (2000) Airway surface liquid composition in mice. Am J Physiol Lung Cell Mol Physiol 278: L1213-L1220.
12. Verkman AS (2001) Lung disease in cystic fibrosis: is airway surface liquid composition abnormal? Am J Physiol Lung Cell Mol Physiol 281: L306-L308.
13. Caldwell RA, Grubb BR, Tarran R, Boucher RC, Knowles MR, Barker PM (2002) In vivo airway surface liquid Cl- analysis with solid-state electrodes. J Gen Physiol 119: 3-14.
14. Boucher RC, Stutts MJ, Bromberg PA, Gatzy JT (1981) Regional differences in airway surface liquid composition. J Appl Physiol 50: 613-620.

15. Robinson NP, Kyle H, Webber SE, Widdicombe JG (1989) Electrolyte and other chemical concentrations in tracheal airway surface liquid and mucus. J Appl Physiol 66: 2129-2135.
16. Cowley EA, Govindaraju K, Lloyd DK, Eidelman DH (1997) Airway surface fluid composition in the rat determined by capillary electrophoresis. Am J Physiol 273: L895-L899.
17. Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, Boucher RC (1998). Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airway disease. Cell 95: 1005-1015.
18. McCray PB Jr, Zabner J, Jia HP, Welsh MJ, Thorne PS (1999) Efficient killing of inhaled bacteria in DeltaF508 mice: role of airway surface liquid composition. Am J Physiol 277: L183-L190.
19. Kozlova I, Högman M, Roomans GM (2002) X-ray microanalysis of airway surface liquid in frozen-hydrated pig trachea. GIT Imaging & Microscopy 2002(4): 2-3.
20. Kozlova I, Vanthanouvong V, Almgren B, Högman M, Roomans GM (2005) Determination of the elemental composition of airway surface liquid in the pig determined by X-ray microanalysis. Am J Resp Cell Mol Biol 32: 59-64.
21. Nilsson H, Kozlova I, Vanthanouvong V, Roomans GM (2004) Collection and X-ray microanalysis of airway surface liquid in the mouse using ion exchange beads. Micron 35: 701-705.
22. Kozlova I, Nilsson H, Phillipson M, Riederer B, Seidler U, Colledge WH, Roomans GM (2005) X-ray microanalysis of airway surface liquid in the mouse. Am J Physiol Lung Cell Physiol 288: L874-L878.
23. Ratcliff R, Evans MJ, Cuthbert AW, McVinish LJ, Foster D, Andersson JR, Colledge WH (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. Nat Genet 12: 280-287.
24. Vanthanouvong V, Kozlova I, Roomans GM (2005) Ionic composition of rat airway surface liquid determined by X-ray microanalysis. Microsc Res Techn, 68: 6-12
25. Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruenert DC (1994) CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am J Respir Cell Mol Biol 10: 38-47.
26. Vanthanouvong V, Roomans GM (2004) Methods for determining the composition of nasal fluid by X-ray microanalysis. Microsc Res Techn 63: 122-128.
27. Baconnais S, Tirouvanziam R, Zahm JM, de Bentzmann S, Peault B, Balossier G, Puchelle E (1999) Ion composition and rheology of airway liquid from cystic fibrosis fetal tracheal xenografts. Am J Respir Cell Mol Biol 20: 605-611.
27. Tarran R, Grubb BR, Parson D, Picher M, Hirsh AJ, Davis CW, Boucher RC (2001) The CF salt controversy: in vivo observations and therapeutic approaches. Mol Cell 8: 149-150.

Corresponding author: Inna Kozlova
Department of Medical Cell Biology,
University of Uppsala, Box 571,
SE-75123 Uppsala, Sweden