Electrophysiological Properties of the Airway

Epithelium in the Murine, Ovalbumin Model of Allergic Airway Disease

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The electrophysiological properties of cultured tracheal cells (CTCs) were examined in a murine (C57BL/6J), ovalbumin (OVA)-induced model of allergic airway disease (AAD) at early (3-day OVA-aerosol) and peak (10-day OVA-aerosol) periods of inflammation. Transepithelial potential difference, short-circuit current (I_{sc}), and resistance (R_T) were lower in CTCs from 10-day OVA-aerosol animals compared to CTCs from naïve mice. In cells cultured for 5 weeks, R_T was greater in naïve CTCs than in 10-day OVA-aerosol CTCs at all times ($P < 0.01$). The I_{sc} response to mucosal amiloride (10^{-4} mol/L) was increased in CTCs from 10-day OVA-aerosol mice compared to naïve mice ($6.0 \pm 0.37 \mu\text{A}/\text{cm}^2$ versus $1.8 \pm 0.56 \mu\text{A}/\text{cm}^2$; $P < 0.001$) with intermediate values for CTCs from 3-day OVA-aerosol mice. The cAMP-induced increase in I_{sc} was blunted in 10-day OVA-aerosol animals compared to CTCs from naïve mice ($9 \pm 12\%$ versus $39 \pm 7\%$; $P < 0.01$) with intermediate values for CTCs from 3-day OVA-aerosol mice. There was no difference in mannitol flux in naïve compared to 10-day OVA-aerosol CTCs. Similar results were found using intact tracheas mounted in a perfusion chamber. These data demonstrate changes in airway epithelial cell function in the OVA-induced model of AAD that may contribute to the pathogenesis of airway inflammation. (*Am J Pathol* 2004, 164:1849–1856)

Airway epithelial cells regulate the depth of periciliary fluid by the active transport of sodium and chloride ions and play a major role in mucociliary transport.^{1,2} Airway epithelial cells are also bombarded daily by airborne, foreign antigens (Ag), noxious agents, and a myriad of microorganisms. In response to a number of these stimuli, the airway epithelium has been shown to release inflammatory and proinflammatory cytokines and chemokines.^{3–11} In response to Ag exposure, the inflammatory cytokines and growth factors released by airway epithelial cells contribute to the repair process and remodeling

observed in asthma.^{7,12,13} Cytokines released by resident and recruited cells in the airways such as interleukin-4, interleukin-13, and interferon- γ produce changes in airway epithelial function including alterations in epithelial barrier function and electrolyte secretion.^{14,15} Airway epithelial cells, thus, play a major role not only in homeostasis but also in the generation and maintenance of airway inflammation.

Our understanding of the role of the airway epithelium in diseases such as asthma and allergy has been greatly enhanced by the development of techniques for primary culture of airway cells from a variety of species.^{1,16–21} The recent development of a successful method to culture mouse airway epithelial cells has provided the opportunity to investigate *in vitro* the electrophysiological properties of the airway epithelium in the ovalbumin (OVA)-induced murine model of allergic airway disease (AAD).²¹

In this study, we characterized the electrophysiological and ion transport properties of isolated, cultured tracheal epithelial cells from naïve mice and from mice with OVA-induced AAD during the development of airway inflammation. We also examined the electrophysiological properties of intact tracheas from naïve mice and from mice with OVA-induced AAD. Compared to tracheal epithelial cells from naïve mice, the airway epithelial cells from mice with AAD demonstrated a lower transepithelial potential difference, short-circuit current, and tissue resistance (without change in the paracellular pathway). In addition, cultured tracheal epithelial cells from mice with AAD demonstrated enhanced inhibition of sodium absorption in response to amiloride and a blunted response to increases in intracellular cAMP. Similar results were found using intact tracheas mounted in a perfusion chamber.

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Materials and Methods

OVA-Induced Model of AAD

The OVA-induced murine model of AAD has previously been described and is characterized by airway eosinophilia, histological evidence of peribronchial and perivascular airway inflammation, clusters of lymphocytes, increased serum IgE levels, and airway responsiveness to methacholine.²² Briefly, female, C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were immunized with three weekly intraperitoneal injections of 25 μ g of OVA in alum. One week after the last injection, the mice were exposed to 1% aerosolized OVA in physiological saline, 1 hour/day for 3 or 10 consecutive days (estimated inhaled daily dose = 80 μ g/mouse). The aerosols were generated by a BANG nebulizer into a 7.6-L inhalation exposure chamber with a chamber airflow of 10 L/minute. Aged-matched female, C57BL/6J animals were used as control (naïve) animals.

Animals sensitized to OVA by intraperitoneal injection and challenged with 3 days of aerosolized OVA are referred to as 3-day OVA-aerosol animals whereas animals sensitized to OVA by intraperitoneal injection and challenged with 10 days of aerosolized OVA are referred to as 10-day OVA-aerosol animals. These two time points were chosen because eosinophilia begins at 3 days and peaks at 10 days.²²

Epithelial Cell Cultures

The isolation and culture of tracheal epithelial cells was performed as described by Davidson and colleagues.²¹ Briefly, the trachea was removed from sacrificed, anesthetized mice, split lengthwise, and placed in dissociation media consisting of Ca^{2+} , Mg^{2+} -free minimal essential medium with Pronase (0.14%) and DNase (0.01%) for 60 minutes at 37°C. The tracheal husks were removed and the cells were centrifuged, washed, and suspended in airway media [Dulbecco's modified Eagle's medium-F12 with 5% fetal calf serum, 1% penicillin and streptomycin, and insulin (12 U/100 ml)]. After a 2-hour incubation at 37°C to remove fibroblasts, the cells in suspension from two tracheas were placed in precoated 6.5-mm transwells (Costar, Cambridge, MA) and incubated at 37°C in 95% O_2 -5% CO_2 . On day 4 in culture, when the cells were 80 to 90% confluent, an air-liquid interface was created by removing the medium from the apical surface and replacing the basolateral media with 600 μ l of Ultrosor G medium (1:1 mixture of Dulbecco's modified Eagle's medium and Hams F-12 containing 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2% Ultrosor G serum substitute) (Biosepra, Paris, France). Within 24 hours of creating an air-liquid interface, a dry mucosal surface was maintained and the cells developed a trans-epithelial resistance. The maintenance of a dry mucosal surface coupled with the development of a resistance across the cultured cells is indicative of polarization.

Histological Studies

Light microscopy with hematoxylin and eosin (H&E) staining of cells was performed on isolated tracheal cell suspensions that were used for the cell cultures. In addition, isolated cells were stained with antibodies to pancytokeratin and α -smooth muscle actin to distinguish epithelial cells from smooth muscle cells and with periodic acid-Schiff staining to determine the presence of goblet cells.

For the pancytokeratin and α -smooth muscle actin studies, we used the following antibodies: mouse monoclonal anti-human pancytokeratin (1:100 dilution; Sigma, St. Louis, MO) and mouse monoclonal anti-human α -smooth muscle actin (1:400 dilution, Sigma). Negative controls were performed by omitting the primary antibodies. In these studies, tracheal cells were grown in culture for a total of 10 days. Cells were washed in phosphate-buffered saline (PBS), fixed in 1:1 acetone:methanol for 5 minutes, washed, and cut from the culture insert. The specimens were blocked with PBS containing 2% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA), 0.2% Tween 20, 2% bovine serum albumin, 7% glycerol, and 1% anti-mouse immunoglobulin for 20 minutes at room temperature. After washing, the specimens were blocked again with blocking solution to which anti-mouse immunoglobulin had been omitted, incubated for 1 hour at room temperature with the primary antibody diluted in blocking solution, and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) in blocking solution. After washing, the membrane was mounted cell side up onto a slide, covered with a coverslip, and sealed and stored at 4°C in the dark until read in a confocal microscope.

Static histomorphometric measurements were made on primary epithelial cell cultures on days 7 to 9 after seeding using the BioQuant computerized image analysis system (Bio-Quant; R & M Biometrics, Nashville, TN) interfaced with a Nikon E400 microscope (Nikon Inc., Melville, NY). Confluence was measured by determining cell density from edge to edge through the center of the transwell. Cell size was determined in a minimum of 125 cells located throughout each of four quadrants in each transwell.

Electrophysiological Studies

Cultured Cells

The resistance of the cultured tracheal cells (CTCs) on transwells (R_T) was monitored weekly for 5 weeks using a Millicell-ERS (Millipore Corp., Bedford, MA) after the addition of 200 μ l of USG medium at 37°C to the apical surface. Recordings were performed in tracheal cells that were isolated and cultured from naïve and 10-day OVA-aerosol animals. The resistance of the transwell alone (200 $\Omega \cdot \text{cm}^2$) was subtracted from the resistance measurements of the CTCs.

Electrophysiological measurements were made on tracheal cells that has been grown in culture for 7 to 14 days

after seeding. These epithelial cells grown on transwells were mounted in a modified Ussing chamber (surface area = 0.32 cm²) apparatus. Each chamber half was connected to a jacketed reservoir containing 4 ml of Ringer's solution (pH 7.4) maintained at 37°C and aerated with 95% O₂-5% CO₂. A bubble lift apparatus recirculated the bathing solution through the half chambers. Ussing chamber experiments were not performed with cultured cells that had an R_T less than 200 Ω · cm² because these cultured cells had not established a monolayer and were unable to maintain a dry mucosal surface in the presence of an air-liquid interface. The composition of the Ringer's solution was (mmol/L): 140 Na, 124 Cl, 21 HCO₃, 5.4 K, 2.4 HPO₄, 1.2 Mg, 1.2 Ca, and 10 glucose.

Transepithelial potential difference (Ψ_{ms}) was measured between two KCl-agar bridges connected by calomel half-cells to an automatic voltage clamp electrometer (model 616C; Bioengineering Div., University of Iowa, Ames, IA). Short-circuit current (I_{sc}) was determined by passing sufficient current through the tissue to nullify Ψ_{ms} using two Ag/AgCl electrodes immersed in saturated KCl solution and connected to the tissue bath by KCl-agar bridges. R_T was determined by passing short duration, biphasic current pulses through the tissue (via the voltage-clamp electrometer) and recording the voltage response.^{23,24} R_T was calculated using Ohm's law by dividing the voltage response by the known current pulse; chamber fluid resistance was automatically subtracted by the electrometer. Experiments were performed under short-circuit conditions, interrupted every 10 minutes for 30 seconds to record Ψ_{ms} and R_T.

Intact Tissue

The basic perfusion system for measuring the electrophysiological properties of intact tracheas has been described previously.²⁵ Briefly, tracheas were removed *in toto* and placed in a small Plexiglas chamber filled with Ringers solution and the ends were tied to two plastic cannulas 3 to 5 mm apart. The tracheal lumen was perfused with an identical solution using gravity at a rate of 30 μl/minute. Both solutions were maintained at 37°C and bubbled with a 95% O₂-5% CO₂ gas mixture. Ψ_{ms} was measured by two KCl-agar bridges (one in the tracheal lumen and the other in the chamber bath) as described above. I_{sc} was measured by passing a current through a fine silver wire threaded in the tracheal lumen and a spiral silver wire surrounding the submucosa of the trachea according to the method of Spring and Paganelli.²⁶ Tissue resistance (R_T) was measured using Ohm's law as described above.

Measurement of Electrophysiological Responses

The electrophysiological responses to the sequential addition of amiloride (10⁻⁴ mol/L, Sigma) to the mucosal bathing solution and to the addition of a cAMP cocktail [2.5 μmol/L forskolin, 250 μM 8-bromoadenosine-cAMP,

and 250 μmol/L 8-(4-chlorophenylthio)-cAMP] (Sigma) to both the mucosal and submucosal bathing solution were measured. Drugs were tested on CTCs from naïve, 3-day OVA-aerosol and 10-day OVA-aerosol animals and on whole tissue from naïve and 10-day OVA-aerosol animals.

Chloride and Mannitol Fluxes

Chloride fluxes were measured in CTCs on transwells that has been pretreated with amiloride during a control period and for 60 minutes after addition of the cAMP cocktail. ³⁶Cl (4 μCi; ICN, Irvine, CA) was added to the mucosal bathing solution for mucosal-to-submucosal flux (J_{ms}) and to the submucosal bathing solution for submucosal-to-mucosal flux (J_{sm}). After 20 minutes, samples (0.5 ml) were taken from the cold side every 10 minutes for 60 minutes and an equal volume of an identical solution, but containing no isotope was added. Samples were counted in a liquid scintillation counter (Beckman LS 1801) and the flux in μEq/cm² · hour was calculated.

To determine whether changes in R_T were because of changes in the transcellular or paracellular pathway, mannitol fluxes were performed in cultured cells on transwells from naïve and 10-day OVA-aerosol animals. ¹⁴C-mannitol (4 μCi, ICN) was added to either the mucosal or submucosal bathing solution. Sufficient nonradiolabeled mannitol was added to both bathing solutions to result in a final mannitol concentration of 5 mmol/L. Samples were taken every 20 minutes from the cold side for 100 minutes and an equal volume of an identical solution, but containing no isotope was added. Samples were counted in a liquid scintillation counter (Beckman LS 1801) and the flux in nm/cm² · hour was calculated.

Statistical Analysis

All results are expressed as means ± SEM of "n" number of observations. Sets of data were compared using analysis of variance and paired and unpaired Student's *t*-test as appropriate. A *P* value less than 0.05 was considered statistically significant.

Results

Airway Epithelial Cell Cultures

Each trachea generated ~1.5 to 4.0 × 10⁵ cells of which >95% were viable by trypan blue exclusion. More than 95% of the cells were epithelial in origin as demonstrated by H&E staining of cell suspensions, the presence of beating cilia, the typical cobblestone appearance of the cells in culture, and the localized pancytokeratin staining (Figure 1). Staining with antibodies to α-smooth muscle actin was negative (<1%). An occasional goblet cell was observed in cultures from naïve animals by periodic acid-Schiff staining but was always less than 1% of the total cells. Even in animals with AAD, less than 1% of the total cells was periodic acid-Schiff-positive. Within 24 hours of creating an air-liquid interface (day 5 in culture), more

Figure 1. Representative fluorescent immunohistochemical photo of mouse tracheal cells in culture with pancytokeratin staining demonstrating the typical cobblestone appearance. **A:** Cells in culture from naïve animals 10 days after creating an air-liquid interface. **B:** Cells in culture from 10-day OVA-aerosol animals 10 days after creating an air-liquid interface. Scale bars, 10 μ m.

than 90% of the transwells had developed a resistance greater than background and the cells were noted to be confluent by light microscopy. These cultured cells maintained a dry mucosal surface. Phase contrast microscopy demonstrated that primary cultures of murine tracheal epithelium had a cobblestone appearance typical of epithelia. Confocal microscopy in the *z* plane revealed a monolayer with pancytokeratin staining at the apical surface and basally located nuclei compatible with a polarized epithelium. Consistent with this finding was the presence of a transcellular resistance. There was no difference in the viability, staining characteristics, or appearance of the tracheal cells from the OVA-induced AAD animals compared to naïve animals (Figure 1).

Histomorphometric measurements at 7 to 9 days in culture after seeding demonstrated no difference in confluence between naïve ($n = 5$) and 10-day AAD ($n = 5$) cultured cells ($97.5 \pm 0.03\%$ versus $99.7 \pm 0.002\%$), cell number ($5.5 \pm 0.2 \times 10^5$ versus $5.7 \pm 0.3 \times 10^5$ cells/transwell; $P = 0.55$) or cell size (59.4 ± 2.6 versus 57.0 ± 3.1 μ m; $P = 0.56$). The resistance of the naïve epithelial cells was significantly higher than the resistance of the 10 day AAD cells in culture (2892 ± 369 versus 364 ± 27 $\Omega \cdot \text{cm}^2$; $P < 0.0001$).

Resistance

More than 90% of the naïve and 10-day OVA-aerosol airway epithelial cell cultures developed a monolayer with a dry mucosal surface and a resistance greater than background. In cells cultured on transwells in which the resistance was measured weekly throughout 5 weeks, at every time point, resistance was lower in cultured cells from 10-day OVA-aerosol animals than in tracheal cell cultures from naïve animals (Figure 2). R_T continued to increase throughout time in the tracheal cell cultures from naïve animals. In tracheal cell cultures from 10-day OVA-aerosol animals, R_T increased for 14 days and then remained stable for up to 35 days.

Electrophysiological Measurements

Baseline electrophysiological properties for naïve tracheal cells grown in culture on transwells for 7 to 14 days and mounted in Ussing chambers are shown in Table 1. The airway epithelial cells in culture generated a negative transepithelial potential difference (mucosa negative rel-

Figure 2. Tissue resistance in tracheal cells from naïve animals (squares) and 10-day OVA-aerosol animals (diamonds) cultured on transwell filters with an air-liquid interface. The air-liquid interface was established on day 4 in culture. Each point represents the mean $\pm$ SEM of a minimum of 10 observations. The resistance of the transwell filter has been subtracted from the reported values. At every time point, the resistance of the 10-day OVA-aerosol tracheal cell cultures was lower than the resistance of the naïve tracheal cell cultures.

ative to submucosa). Compared to cultured cells from naïve animals, cultured cells from 10-day OVA-aerosol animals had a significantly lower baseline transepithelial potential difference, short-circuit current, and resistance. Changes in Ψ_{ms} , I_{sc} , and R_T were intermediate for cultured cells from 3-day OVA-aerosol animals but did not reach statistical significance.

After addition of amiloride (10^{-4} mol/L) to the mucosal bathing solution, there was a small decrease in Ψ_{ms} and I_{sc} in naïve tracheal cells in culture. The I_{sc} -response to mucosal amiloride (10^{-4} mol/L) was increased in CTCs from 10-day OVA-aerosol mice compared to naïve mice ($P < 0.001$) with intermediate values for CTCs from 3-day OVA-aerosol mice. (Table 1, Figure 3). Both the absolute change (Figure 3) and the magnitude of the response to amiloride (Table 1) were increased in tracheal cell cultures from 3-day and 10-day OVA-aerosol mice. Submucosal addition of amiloride had no effect on electrophysiological parameters.

Naïve tracheal cells grown in culture on transwells responded to the cAMP cocktail with an increase in I_{sc} and a decrease in R_T ($P < 0.05$). The cAMP-induced increase in I_{sc} was blunted in cultured cells from 10-day OVA-aerosol animals compared to cultured cells from naïve mice ($9 \pm 12\%$ versus $39 \pm 7\%$; $P < 0.01$) with intermediate values for cultured cells from 3-day OVA-aerosol mice (Table 1). Submucosal addition of ouabain (10^{-3} mol/L), an inhibitor of the Na-K ATPase pump, reduced the I_{sc} to 0 suggesting that sodium and chloride are the only actively transported ions in murine airways.

Chloride and Mannitol Fluxes

Unidirectional chloride fluxes were performed in a separate group of tracheal cell cultures (Table 2). The elec-

Table 1. Electrophysiologic Properties of Cultured Tracheal Epithelial Cells from Naïve, 3-Day OVA-Aerosol and 10-Day OVA-Aerosol Mice*

	Ψ_{ms} (mV)	Isc ($\mu A/cm^2$)	R_T (Ωcm^2)
Naïve ($n = 28$)			
Baseline	-5.72 ± 1.64	11.3 ± 0.49	498 ± 16
Amiloride	-4.60 ± 0.63	9.5 ± 1.03	480 ± 25
cAMP	-6.54 ± 1.04	$16.1 \pm 1.0^{\dagger}$	$409 \pm 33^{\ddagger}$
3-Day OVA-aerosol ($n = 10$)			
Baseline	-4.98 ± 1.85	11.0 ± 1.4	448 ± 62
Amiloride	-3.0 ± 0.65	$6.3 \pm 0.6^{\S}$	469 ± 10
cAMP	-4.54 ± 2.28	$10.8 \pm 1.7^{\P}$	398 ± 33
10-Day OVA-aerosol ($n = 16$)			
Baseline	$-3.43 \pm 0.65^{\dagger}$	$9.4 \pm 1.3^{\ddagger}$	$383 \pm 31^{\ddagger}$
Amiloride	$-1.30 \pm 0.36^{\dagger\S}$	$3.4 \pm 0.55^{\dagger\S}$	$392 \pm 22^{\dagger\S}$
cAMP	$-2.0 \pm 0.50^{\P}$	$7.3 \pm 0.66^{\P}$	$273 \pm 51^{\P\P}$

*Electrophysiologic measurements were performed in the following sequence: baseline, amiloride, and cAMP.

$^{\dagger}P < 0.05$ compared to baseline.

$^{\ddagger}P < 0.05$ compared to naïve animals at baseline.

$^{\S}P < 0.01$ compared to naïve animals after amiloride.

$^{\P}P < 0.001$ compared to naïve animals after cAMP.

Figure 3. The short circuit current (Isc) response to amiloride and cAMP in CTCs from naïve (white bars), 3-day OVA-aerosol (gray bars), and 10-day OVA-aerosol (black bars) animals grown on transwells and mounted in Ussing chambers. Data are presented as the mean $\pm$ SEM of 10 to 28 transwells/condition.

trophysiological responses to amiloride and to the cAMP cocktail were similar in these cells in culture compared to the entire group. Net chloride absorption (J_{net}) was present after addition of amiloride (baseline) in all tracheal epithelial cells mounted in Ussing chambers. After addition of cAMP, there was a significant increase in Isc and a significant decrease in R_T in tracheal cell cultures from naïve animals. This was associated with a significant increase in both unidirectional chloride secretion and absorption. Net chloride absorption decreased but was still present. All of the change in short-circuit current, however, was accounted for by the change in net chloride transport. Net chloride absorption was decreased in 3-day and 10-day OVA-aerosol tracheal cell cultures at baseline (after amiloride) compared to naïve animals. After addition of the cAMP cocktail, there was no significant change in J_{net} .

There was no difference in mannitol flux between 10-day OVA-aerosol tracheal cell cultures (9.76 ± 0.38 nm/cm² · hour, $n = 5$) and naïve (9.85 ± 1.58 nm/cm² · hour, $n = 5$) tracheal cell cultures suggesting that the change in resistance is because of a change in transcellular

Table 2. Chloride Fluxes in Cultured Tracheal Epithelial Cells from Naïve, 3-Day OVA-Aerosol, and 10-Day OVA-Aerosol Mice*

	Ψ_{ms} (mV)	Isc ($\mu A/cm^2$)	R_t (Ωcm^2)	J_{sm} ($\mu Eq/cm^2 \cdot hour$)	J_{ms} ($\mu Eq/cm^2 \cdot hour$)	J_{net} ($J_{sm} - J_{ms}$)
Naïve ($n = 10$)						
Baseline	-5.0 ± 0.7	8.6 ± 1.4	$556 \pm 167^{\dagger}$	1.70 ± 0.33	3.06 ± 0.02	-1.36 ± 0.34
cAMP	-6.8 ± 1.4	$14.8 \pm 1.9^{\ddagger}$	$453 \pm 133^{\ddagger}$	$2.82 \pm 0.26^{\ddagger}$	$3.98 \pm 0.24^{\ddagger}$	-1.16 ± 0.10
3-Day OVA-aerosol ($n = 6$)						
Baseline	$-2.3 \pm 1.0^{\ddagger}$	6.1 ± 2.0	463 ± 102	1.71 ± 0.91	1.78 ± 0.90	$-0.07 \pm 0.57^{\ddagger}$
cAMP	-4.0 ± 2.0	10.8 ± 2.1	395 ± 189	1.87 ± 0.44	$2.05 \pm 0.47^{\P}$	$-0.18 \pm 0.38^{\P}$
10-Day OVA-aerosol ($n = 8$)						
Baseline	$-1.1 \pm 2.4^{\ddagger}$	$2.6 \pm 1.0^{\ddagger}$	409 ± 95	2.36 ± 0.54	2.47 ± 0.65	$-0.11 \pm 0.82^{\ddagger}$
cAMP	$-2.3 \pm 1.4^{\P}$	$6.5 \pm 1.8^{\P}$	$302 \pm 54^{\S}$	2.48 ± 0.18	$2.68 \pm 0.62^{\P}$	$-0.20 \pm 0.26^{\P\P}$

*Tissues were treated with mucosal amiloride (10^{-4} mol/L).

$^{\dagger}R_{ism} = 616 \pm 46 \Omega cm^2$ $R_{tms} = 501 \pm 147 \Omega cm^2$.

$^{\ddagger}P < 0.05$ compared to naïve animals at baseline.

$^{\S}P < 0.01$ compared to naïve animals after cAMP.

$^{\P}P < 0.001$ compared to naïve animals after cAMP.

Table 3. Electrophysiologic Properties of Intact Tracheas Mounted in Perfusion Chamber

	Ψ_{ms} (mV)	Isc $\mu A/cm^2$	Rt (Ωcm^2)
Naïve ($n = 5$)			
Baseline	-3.60 ± 0.32	14.4 ± 1.6	250 ± 54
Amiloride	$-2.92 \pm 0.58^*$	$9.4 \pm 1.2^*$	313 ± 75
cAMP	-1.50 ± 0.67	27.1 ± 1.7	$215 \pm 52^\dagger$
10-Day OVA-aerosol ($n = 5$)			
Baseline	-2.06 ± 0.62	15.7 ± 0.90	135 ± 28
Amiloride	$-1.23 \pm 0.56^*$	$7.5 \pm 3.6^*$	174 ± 58
cAMP	-1.42 ± 0.46	8.7 ± 4.2	163 ± 52

Electrophysiologic measurements were performed in the following sequence: baseline, amiloride, cAMP.

* $P < 0.05$ compared to baseline.

$^\dagger P < 0.01$ compared to after cAMP.

conductive pathways and not to a change in paracellular shunt resistance.

Electrophysiological Studies in Intact Tissues

The electrophysiological properties of intact tracheas mounted in the perfusion chambers are shown in Table 3 and a typical recording is shown in Figure 4. Compared to CTCs, intact tracheas mounted in perfusion chambers had a lower resistance (R_t) and a higher Isc. There was no significant difference in baseline electrophysiological properties between the intact tracheas from naïve animals and those from 10-day OVA-aerosol animals. R_t in tracheas from 10-day OVA-aerosol animals was however, lower than the R_t in tracheas from naïve animals and approached significance ($P = 0.053$). Tracheas from naïve animals had a $35 \pm 16\%$ decrease in Isc after addition of amiloride and a $65 \pm 17\%$ increase in Isc after addition of the cAMP cocktail. In contrast, tracheas from 10-day OVA-aerosol animals had a $52 \pm 12\%$ decrease in Isc after addition of amiloride and only a $16 \pm 13\%$ increase in Isc after the cAMP cocktail.

Discussion

This study demonstrates changes in the electrophysiological properties of the murine airway epithelium in the presence of OVA-induced AAD and represents another important characteristic of the acute allergic state in this

model. This study also begins to demonstrate the temporal relationship of these changes.

The isolated tracheal cells were determined to be epithelial in origin by H&E differential stain, by positive pancytokeratin staining, and by negative smooth muscle and mucus staining. They rapidly formed a polarized epithelium with the establishment and maintenance of a tissue resistance and a dry mucosal surface with evidence of polarization by confocal microscopy.¹⁹ R_t was similar to what some have found,²⁷ but less in both the absolute magnitude and in the development of an increasing resistance throughout time compared to others.²¹

Tracheal airway cell cultures from naïve animals demonstrated stable electrophysiological properties and chloride fluxes. Cultured cells responded to increases in intracellular cAMP with an increase in Isc and a decrease in R_t . They also demonstrated a significant decrease in Isc in response to the sodium channel blocker, amiloride. Murine airways have been reported by some investigators to have ENac, the amiloride-sensitive sodium channel, and CFTR, the cAMP-stimulated chloride channel,²⁸ and are thus capable of responding to amiloride and cAMP. Others, however, have been unable to find CFTR and co-transporter mRNA in mice tracheas and have suggested that in this region, the entry step for Cl^- secretion is different.²⁹

Under standard culture conditions, the murine respiratory epithelium has been described as inherently sodium absorptive with approximately two of three of the Isc accounted for by Na absorption.³⁰ Differences in the relative abundance of ion transport molecules in tracheal cells can exist, however, and the portion of total ion transport contributed by amiloride sensitive Na^+ or cAMP-dependent Cl^- flow across the mouse trachea is reportedly variable.²⁹ Although the number and appearance of the airway cells from the naïve and from the OVA-aerosol animals were the same, we do not know if our cultures contained populations of cells with different ion transport molecules. The absolute changes in Isc in response to amiloride and the cAMP cocktail were less than those reported by others, but their responses were measured after longer times in total culture (21 to 28 days) compared to our 7 to 14 days in culture.^{21,31} We were concerned that the asthma phenotype might be lost with increasing time in culture. We therefore, completed

Figure 4. Typical recording from naïve trachea mounted in perfusion chamber. Amiloride (10^{-4} mol/L) was added at the first arrow. A cAMP cocktail was added at the second arrow.

all of our measurements at the earliest time point in which a significant resistance had developed and we repeated experiments using intact tissue. Even at this time point, however, airways from OVA-aerosol mice demonstrated differences in electrophysiological properties and in response to secretagogues.

The pattern of bioelectric properties and chloride ion flows for murine tracheal epithelium is different from the pattern observed in human and canine trachea in the basal state.^{32,33} Under short-circuit conditions in tracheal cell cultures from naïve mice, net chloride absorption was observed whereas in other species, under similar conditions, net chloride secretion is present. In humans, net chloride secretion disappears in the smaller, more distal airways similar in size to the mouse trachea. It is possible that transcellular fluxes are so low in murine airways that we were unable to distinguish transcellular from paracellular fluxes even though we were able to detect changes in chloride fluxes after addition of the cAMP cocktail. Alternatively, the mouse trachea may be different from the tracheas of larger animals. Neither amiloride nor indomethacin completely inhibited *I*_{sc}. The residual *I*_{sc} could reflect nonamiloride sensitive sodium absorption or net flow of an unidentified ion species. In the absence of sodium fluxes, we cannot determine which of these two possibilities exist.

The absorption and secretion of fluid and electrolytes across the airway epithelium involve the movement of ions across the apical and basolateral membranes through ion (Na^+ , K^+ , 2Cl^-) selective channels, the Na-K ATPase pump and the Na-K-2Cl co-transporter. Allergic airway inflammation could affect any of these processes either directly or through secondary mediators such as cytokines or leukotrienes, many of which have been implicated in asthma. Interleukin-4 down-regulates Cl^- secretion and CFTR expression in T84 cells.¹⁵ Amiloride and ouabain have been used to elucidate the nature of sodium absorption across a variety of epithelia. The response to amiloride demonstrates a conductive path of sodium entry across the apical membrane whereas ouabain inhibits the Na-K ATPase pump and sodium extrusion across the basolateral membrane. Our naïve murine airway cells in culture demonstrated both amiloride and ouabain responsiveness. OVA aerosol was associated with an increase in amiloride sensitivity both in absolute magnitude and as a percentage of the basal short-circuit current.

Murine airways were pretreated with amiloride before adding the cAMP cocktail and measuring chloride fluxes. In human, canine, and rabbit bronchi, amiloride has been shown to produce a small but significant chloride secretion secondary to hyperpolarization of the cell interior with a slowing in passive anion entry across the apical barrier.^{32,33} The decrease in net Cl^- absorption in the AAD model that was observed may have been secondary to increased amiloride responsiveness of the tissues because all chloride fluxes were measured after treatment of the tissues with amiloride. It is also possible that the variability in the unidirectional fluxes may have masked small changes in net ion flows, a situation compounded

by the tendency of cAMP-like agents to increase passive flows of both Cl^- and Na^+ .

Similar to murine airway epithelial cell monolayers infected with *Mycoplasma pulmonis*, our AAD monolayers no longer responded to pharmacological stimulators of Cl^- secretion.³¹ In contrast to these infected airways, however, the AAD airways displayed enhanced Na^+ transport with increasing duration of OVA-aerosol exposure. Thus, the murine airway is capable of different responses to different insults.

In vitro characterization of the ion transport properties of murine airway epithelial tissue is challenging with methods only recently being developed. Selection bias could have been a problem with discarding cultures that reflected changes because of allergic airway inflammation. However, only cultured cells that did not generate a resistance sufficient to produce a dry mucosa were discarded and cell cultures from OVA-aerosol mice were no more likely than cell cultures from naïve animals to be discarded.

Similarly edge damage induced by clamping the transwells in the Ussing chamber is no more likely in cultured cells from naïve mice than in cultured cells from OVA-aerosol mice. We used O rings to clamp the transwells in place and to minimize such edge damage. In addition, flow-through edge-damaged areas would be expected to be high (approximate free solution) and this was not observed in the mannitol studies. We cannot, however, rule out edge damage as a potential explanation for the lower resistance in the intact tracheas.

What then could be the pathophysiological consequences of these changes in ion transport? In the presence of net chloride absorption, the increased sodium absorption that was observed in CTCs from 10-day OVA-aerosol animals would result in a decrease in water translocation into the airway lumen and the potential for dehydration and dessication of airway secretions. We have observed in this animal model, the presence of mucus and mucus plugging in the airways that peaks at 14 days of OVA aerosol (unpublished data).³⁴ The changes we have observed in ion transport would facilitate mucus retention as they result in an airway epithelium similar to what has been observed in the cystic fibrosis airway.³⁵

In summary, we have demonstrated that the electrophysiological properties of the airway epithelium have been altered during the acute inflammatory response in the OVA-induced model of AAD. This epithelial response to inflammation in the murine airway is characterized by increased amiloride responsiveness and decreased cAMP responsiveness. This study confirms a role for the airway epithelium in the OVA murine model of AAD. It also offers the opportunity to investigate in a detailed manner the mechanisms for the changes in electrophysiological properties and the time course and the interaction between the airway epithelium and other cells in the lung in the development of airway inflammation.

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