Winters, Scot L., and Donovan B. Yeates. Interaction between ion transporters and the mucociliary transport system in dog and baboon. J. Appl. Physiol. 83(4): 1348–1359, 1997.—To gain insight into the role of epithelial ion channels, pumps, and cotransporters in regulating airway water and mucociliary transport, we administered inhibitors of the Na⁺ channel (amiloride), 3Na-2K-adenosinetriphosphatase (acetylstrophanthinidin), and Na-K-2Cl cotransporter (furosemide) to anesthetized dogs and/or baboons. Tracheal ciliary beat frequency was measured by using heterodyne laser light scattering. Tracheal mucus velocity (TMV) and bronchial mucociliary clearance (BMC) or lung mucociliary clearance were measured by using radioaerosols and nuclear imaging. Respiratory tract fluid output was collected by using a secretion-collecting endotracheal tube. In six dogs, amiloride aerosol (lung deposition, 96 ± 11 µg (means ± SE)) had minimal effect, whereas acetylstrophanthinidin aerosol (lung deposition, 71 ± 9 µg) increased BMC, and furosemide (40 mg iv) markedly increased TMV. In five baboons, TMV increased after iv furosemide administration (2 mg/kg) as well as by aerosol (lung deposition, 20 ± 3 µg), coincident with increases in ciliary-mucus coupling from 11.5 ± 0.1 to 29.5 ± 0.4 and 46.5 ± 0.7 µm/beat, respectively. Furosemide also increased lung mucociliary clearance in baboons. In dogs, respiratory tract fluid output increased after intravenous furosemide from 2.2 ± 0.5 to 6.8 ± 1.7 mg/min. When combined with dry-air inhalation, furosemide failed to stimulate TMV and reversed the inhibition of BMC by dry air. Thus pharmacological manipulation of the Na-K-2Cl cotransporter and the 3Na-2K-adenosinetriphosphatase pump may provide increases of clinical relevance in airway hydration and mucociliary transport.

Interaction between ion transporters and the mucociliary transport system in dog and baboon

We hypothesized that the unperturbed tracheobronchial airways favor absorption of water into the mucus, leading to a low basal level of mucociliary transport, which would be further reduced during acute inspiration of dry air. On the basis of in vitro studies, inhibition of Na⁺ channels or 3Na-2K-ATPase pumps can be predicted to inhibit flux of ions and water from the lumen into the mucosa, increasing airway hydration and leading to an increase in mucociliary transport. In the unperturbed airways, Cl⁻ secretion is low (35). If this low Cl⁻ flux is associated with water flux from the mucosa into the lumen, then inhibition of the Na-K-2Cl cotransporter may slightly reduce the already low basal level of mucociliary transport. However, if administration of the Na-K-2Cl cotransporter inhibitor furosemide induces a decrease in intracellular chloride and cellular volume associated with an increase in epithelial permeability to water, an increase in airway fluid depth and mucociliary transport will result. If, as in 1, inhibition of the cotransporter inhibits water flux into the airways, administration of furosemide before inspiration of dry air will further reduce the dry-air-induced decrease in mucociliary transport. On the other hand, if, as in 2, furosemide causes an increase in water flux from the mucosa into the lumen, the decrease in mucus transport due to dry air will be negated. We addressed the above hypotheses and questions regarding the joint regulation of airway ion/water flux and mucociliary transport by investigating responses of the canine (and in specific cases the baboon) mucociliary transport system in vivo to amiloride (an inhibitor of mucosal Na⁺ channels), acetylstrophanthinidin (an inhibitor of the basolateral 3Na₂K⁻ATPase pump), furosemide (an inhibitor of the basolateral Na-K-2Cl cotransporter), inhalation of dry air, and the combination of furosemide and inhalation of dry air.

The bronchi of both dogs and humans absorb Na⁺ (2, 15), and the tracheae of dogs and humans can both secrete Cl⁻ and absorb Na⁺ in vitro (35, 36). However, the balance between these effects under short-circuit conditions, with the canine trachea being predomi-
nantly chloride secreting and the primate trachea being predominantly sodium absorbing. Thus canine and primate airways may be considered to have different basal "set points," suggesting that basal levels of ion and water fluxes differ. We hypothesized that furosemide would produce similar effects on the mucociliary transport system in both canine and primate airways, assuming both airways possess similar mechanisms to respond to perturbation. Furthermore, although intravenous (iv) administration of furosemide may inhibit the basolateral Na-K-2Cl cotransporter in the airway epithelium, mucociliary transport responses might be attributed to systemic effects of this agent. On the basis of the expectation that mucociliary responses to furosemide would be due to its action on the airway epithelia, we postulated that aerosolized and iv administration of furosemide would produce similar effects on mucociliary transport. To investigate the above hypotheses, we also studied the baboon tracheobronchial mucociliary transport system and its response to iv and aerosolized furosemide.

Parameters monitored individually or in combination included tracheal diuretic beat frequency (CBF), tracheal mucus velocity (TMV), mucociliary clearance of the bronchi (BMC) or entire lung (LMC), and volumetric respiratory tract fluid output at the larynx (RTFO) in complementary experiments.

**METHODS**

The experimental procedures and protocols for these studies on dogs and baboons were approved by the Animal Care Committee of the Biological Resources Laboratory at the University of Illinois at Chicago. The Biological Resources Laboratory is sanctioned by the American Association for Accreditation for Laboratory Animal Care.

Dog preparation. Six healthy adult mongrel dogs (Canis familiaris; 1–3 yr old; 5 male, 1 female; and 20–30 kg in weight) were studied. Each dog was fasted 12 h before each study but was allowed free access to water. The dogs were anesthetized with thiamylal sodium (Surital, 0.025 mg/ml, to effect; Parke-Davis, Morris Plains, NJ) and placed in the supine position. The head and neck of the dog were extended, and the maxilla was stabilized with gauze across the premaxillary teeth. For control and pharmacological perturbation studies, airway patency was maintained by using a size 10 endotracheal tube, through which the dog was allowed to spontaneously breathe room air that was humidified and conditioned by intermixing and passage through the tube. For dry-air inhalation studies, airway patency was maintained using a 37-Fr double-lumen endobronchial tube (Malinckrodt Medical, St. Louis, MO), with the endobronchial portion distal to the tracheal cuff trimmed. Thus spontaneous inspiration was achieved through one lumen and expiration was through the other, with no intermixing or humidification. The endotracheal tube cuff in every experiment was positioned within or just distal to the larynx. The resulting eucapnic ventilation was confirmed by monitoring end-expired carbon dioxide concentration (airway gas monitor 252; Datex Instrumentarium, Helsinki, Finland), which remained stable near 4.5% throughout all experiments. Arterial blood samples were also drawn at 30-min intervals throughout the experiment from an indwelling femoral artery catheter and analyzed for pH, oxygen, and carbon dioxide content (IL-1312; Instrumentation Laboratories, Lexington, MA) to rule out the occurrence of inadequate blood oxygenation or acid-base homeostasis. Mean values (±SE) for pH were 7.35 ± 0.00, and the partial pressures of carbon dioxide and oxygen were 40.9 ± 0.4 and 84.0 ± 0.8 Torr, respectively. Electrocardiogram (850, Datascop, Paramus, NJ) and femoral arterial blood pressure were monitored (P23 ID Gould Statham, Gould, Cleveland, OH) to verify normal circulation hemodynamics. Rectal temperature was monitored (5800; Omega, Stanford, CT), and warm-water blankets and electrical heating pads were used to maintain body temperature as necessary (36–38°C). Supporting fluid [0.9% NaCl injection, US Pharmacopoeia (USP)] at a nominal rate of 0.5 ml/min and maintenance doses of thiamylal sodium were delivered through a cephelic iv catheter. Doses of anesthetic were titrated to approach extinction of the palpebral reflex and jaw muscle tone and yet maintain minimal effect on the other monitored physiological parameters. Each dog was allowed to stabilize for at least 30 min after intubation before data collection.

Baboon preparation. Five healthy adult baboons (Papio cynocephalus anubis; 10–12 yr old; 2 male, 3 female; 15–40 kg in weight) were studied. The baboon preparation was identical to that of the dog with the following exceptions. The baboons were preanesthetized with ketamine hydrochloride (Ketaset, 100 mg/ml, 10 mg/kg im; Aveco, Fort Dodge, IA) to allow safe handling, transport to the laboratory, and insertion of an iv catheter. Anesthesia with thiamylal sodium began ~40 min after ketamine administration, followed by intubation with the use of a size 5.5 endotracheal tube. Mean values (±SE) for arterial blood pH were 7.350 ± 0.003, and the partial pressures of carbon dioxide and oxygen were 43.7 ± 0.7 and 75.3 ± 1.5 Torr, respectively.

The estimation of aerosol deposition in the lung during perturbation. The dose of agents deposited within the lungs was determined by radioaerosol techniques. The volume and dose of aerosolized furosemide, acetylstrophanthin, and amiloride delivered to the lungs were estimated by aerosolization of a solution of human serum albumin (MediPhysics) labeled with 99mTc. The radioactively labeled albumin was dispersed in the particular solution to be aerosolized at ~70 µg/ml albumin and 7.4 MBq/ml radioactivity. Aerosol delivery conditions to the dog or baboon mimicked the specific perturbation. The duration of aerosol delivery during these characterization studies varied between 1 and 4 min. Agents were assumed to deposit similarly to the solution of human serum albumin. Both animal models were calibrated for radioactivity count sensitivity to lung geometry and tissue absorption by iv injection of macroaggregated albumin (MediPhysics, Arlington Heights, IL). The macroaggregated albumin was labeled with ~3.7 MBq of 99mTc, an isotope that has a 6-h half-life, emits gamma rays, and was assumed to be totally trapped within the pulmonary alveolar capillary bed within a few minutes of the injection. The known injection activity was correlated with the activity of a point source (typically 50 MBq) placed 50 cm above the gamma camera. The gamma-camera sensitivity was recalibrated to a similar reference point source each day of experimentation and was in general ~4,000 counts·min⁻¹·MBq⁻¹ in the lung. Radioactive deposition within the dog lung demonstrated that the pulsed aerosol ventilation system deposited 0.36 ± 0.04 ml during the 2-min delivery with the use of isotonic saline as the carrier. Characterization of the aerosolized furosemide delivery in the baboon, using furosemide solution as the carrier, indicated deposition of 2.0 ± 0.3 ml within the lungs for the 6-min delivery. This provides the basis for the calculated deposition of the mass of agents delivered to the lungs.
Measurement of CBF. CBF within the trachea was measured by nonstationary time-frequency analysis of laser-light scattering described elsewhere in detail (3). In this system, a helium-neon laser beam was transmitted down the axis of a hollow stainless steel probe (30 cm long, 8 mm OD). The beam exited perpendicular to the probe so that the 7-µm focal spot, 5 mm from the probe surface, was coincident with the surface of the ciliated epithelium in the midtrachea of the anesthetized animal. The endotracheal tube was positioned cephalad and dorsal to the probe. Mixing the Doppler-shifted back-scattered photons from the beating cilia with non-Doppler-shifted back-scattered photons produced constructive and destructive interference. These photons were collected by a photomultiplier tube (R649; Hamamatsu, Bridgewater, NJ), and the temporal output was analyzed by a computer with the use of nonstationary time-frequency analysis. Collection time for the 512 samples analyzed was 3 ms each. CBF was defined as the predominant frequency in each collection period. Time-frequency analysis required ∼1.5 s, so CBF values were determined approximately every 3 s. Data collection continued for at least 60 min after beginning the perturbation.

Aerosol deposition for mucociliary transport studies. An iron oxide colloid was prepared and labeled with 59mTc (34) to produce 20 ml of a 1.1% sol with an activity of ∼22 GBq. The system to generate the aerosol has been described (9, 19, 20). Briefly, the aerosol was produced by a syringe pump at 2 ml/min to a jet nebulizer operating at an airflow rate of 8 l/min. The aerosol was dried by heated dilution air and then again concentrated by virtual impaction. By this mechanism, aerosol particles were preferentially propelled through holes in a perforated plate with a small fraction of air (16 l/min) because of their high inertial energy relative to air. The majority of air (100 l/min) was redirected perpendicular to the perforated plate and exhausted through a filter. The radioaerosol delivery system was maintained at 2 kPa positive pressure, and aerosol was delivered to the animal through a solenoid-controlled valve that opened 1 s every 10 s. A second valve allowed exhalation for 3 s immediately after inhalation. The radioactivity deposited was monitored throughout the 2-min inhalation period by a gamma camera (PhyGamma III; Nuclear-Chicago, Des Plaines, IL) positioned 80 cm from the baboon’s head, with the beam being collimated to 15 cm. The gamma camera collimation system was a line of CsI(Na) phoswich-scintillation sandwiches arranged in a line (each crystal being 8-mm thick) that were positioned over the anterior midline of the trachea exterior to the neck. With the use of active (pulse height and shape) and passive (tungsten plates) collimation and shielding, sensitive measurements of temporal radioactivity were made at six positions. From time-response curves, the time at which a bolus was in direct apposition to a given detector was estimated by subtracting the background level from the time-response peak and visually bisecting the peak into equal areas. The relation of this time to detector separation distance enabled the calculation of a TMV for each bolus by linear regression. Only peaks that were evident in at least three channels were considered. Data collection continued up to 90 min after beginning challenge (up to 120 min after radioaerosol deposition).

Measurement of RTFO. RTFO was collected by using a modified endotracheal tube, further described in a companion paper (40). Briefly, a V-shaped retention foot that conformed to the interarytenoid groove (or posterior commissure) was molded in epoxy near the caudal cuff end, both to assist placement and to stabilize the device by preventing both caudal movement and rotation. A stainless steel tube, 2 mm ID, was also affixed axially across the deflated cuff and secured circumferentially at both ends. A mucus-collection catheter (polytetrafluoroethylene; 1.29 mm ID, 1.90 mm OD) was passed inside this tube so that its tip just protruded from the caudal end of the steel tube and the retention foot. The
The catheter was free to move within this pathway and was adjusted independently of the endotracheal tube so that the catheter was close enough to the mucosa to allow fluid collection without obstruction of the catheter tip. The other end of the catheter was connected to a test tube maintained in an incubator at 37°C. Gentle intermittent suction applied to the test tube caused any airway lining fluid at the posterior commissure to be transported within the catheter and collected in the test tube.

The cuff was inflated in the larynx immediately distal to the vocal cords. Vacuum of up to 15 kPa was applied to the collection test tubes (and the RTFO-collection catheter) during three sampling periods: 0–15, 30–45, and 60–75 min. Collected samples sealed within the tubes were allowed to stand in the incubator up to 1 h after the end of the experiment to allow for foam coalescence. The samples were then withdrawn into a graduated syringe to estimate RTFO volume. RTFO rates were estimated only from the volumetric sums of the last two samples (30–45 and 60–75 min) to avoid the influence of animal preparation before collection. These sums were divided by the time spanning from the end of the first collection to the end of the third collection, usually 1 h.

The canine tracheal circumference was used in calculations of an RTFO transport depth (see Data analysis and statistical significance). It was estimated by scaling of the radiographic image and was confirmed in postmortem study in one of the dogs considered representative of the cohort.

Agents. Furosemide for both injection and aerosolization was standard injection grade (furosemide injection USP, 10 mg/ml or ~0.030 M in water; sufficient NaCl to render the solution isotonic). Amiloride solution was prepared from amiloride hydrochloride (Sigma Chemical, St. Louis, MO), which was first dissolved in dimethylsulfoxide (Sigma) to enhance solvation. This solution was then diluted with isotonic NaCl to attain 0.15 M NaCl to attain a 1.0 mM amiloride solution that also contained ~60 mM (0.5%) dimethylsulfoxide. Acetylstrophanthidin (Sigma) was also first dissolved in dimethylsulfoxide, followed by a similar dilution with isotonic saline to 0.45 mM acetylstrophanthidin. Dry air consisted of unconditioned breathing air (USP: ~50 parts water per million parts air).

Perturbations of airway lining fluid. Furosemide (an inhibitor of serosal Na-K-2Cl cotransport) was delivered through a cuffed iv catheter over a period of 2 min. Each dog received 40 mg of furosemide, equivalent to ~1.6 mg/kg, and each baboon received 2.0 mg/kg. In studies of the dog in which furosemide only was administered (not furosemide and dry air), urine output was collected from a urinary catheter; 500 ml were usually collected by the end of the experiment, and the iv delivery rate of isotonic NaCl was adjusted to compensate for the approximate urine output. In studies of the baboon in which furosemide was administered, no urinary catheter was used, and supporting fluid delivery was not adjusted. Aerosolized furosemide was generated during the 6-min delivery by a Fisoneb ultrasonic nebulizer (Fisons, Rochester, NY) equipped with a one-way inspiratory valve connected to the endotracheal tube through a T piece with a one-way valve for exhalation (Hans Rudolph). Particle-size distribution for the aerosolized furosemide was not measured in these studies, but, under similar conditions, this nebulizer is reported to produce mass median diameters of 5.8–6.9 µm (11).

Solutions of amiloride (an inhibitor of mucosal Na⁺ transport) and acetylstrophanthidin (an inhibitor of serosal Na⁺/K⁺ exchange) were delivered by using a pulsed aerosol ventilation system with a Microstat ultrasonic nebulizer (Mountain Medical Equipment, Denver, CO) initially charged with 5 ml of solution. The nebulizer was slightly modified to increase the delivered aerosol dose. Spiral grooves on the baffle were removed, and two mouthpiece tubes were joined vertically to increase the aerosol chamber volume. Particle-size distributions for the aerosolized amiloride and acetylstrophanthidin were not measured in these studies, but, under similar conditions, this nebulizer is reported to produce typical mass median diameters of 2.6–3.4 µm (19). Breathing air (USP) entered the system at 60 l/min and was humidified (3210/3211 Bird Products, St. Paul, MN). The humidified air was vented to exhaust through a bypass valve (Automatic Switch, Florham Park, NJ) that was open except during aerosol delivery to the dog. During delivery, a solenoid valve controller (University of Illinois Bioinstrumentation) directed airflow through a pneumotachograph (Fleisch, Richmond, VA) coupled to a pressure transducer (MP45–14 Valdyne, Northridge, CA). This signal from the carrier demodulator (CD-12, Valdyne), which represented airflow, was integrated for precise control of aerosol ventilation volume. After 600 ml were delivered, inspiration was terminated. Preset controller times governing exhalation and delay between breaths resulted in 16 inspirations/min during the 2-min delivery. The piping and fittings (Swagelok, Solon, OH) were stainless steel. To suppress condensation, they were warmed by an electrical heating tape (Barnstead/Thermolyne, Dubuque, IA) that surrounded the piping.

Dry-air perturbation consisted of spontaneous inspiration of dry air for a duration of 90 min through one lumen of the double-lumen endotracheal tube with exhalation being through the other, as assured by one-way valves (Hans Rudolph) on the inlet and outlet sides of the endotracheal tube and by continuous dry-air flow at 15 l/min across a T piece attached to the inlet valve. The perturbation combining dry air and furosemide in the dog was identical to the dry-air perturbation study except for the administration of 40 mg furosemide iv as dry air delivery was begun.

Dog protocol. Each dog underwent a control study and five studies in which the airway lining fluid was perturbed. First, to evaluate the role of Na⁺ channels, 3Na₂K-ATPase pumps, and Na-K-2Cl cotransporters in regulating mucociliary transport, three studies were conducted with aerosolized amiloride, aerosolized acetylstrophanthidin, and iv furosemide. To evaluate the response of the airway to dehydration, a fourth study was conducted with inspiration of dry air. To evaluate whether furosemide might ameliorate the effects of dehydration, a fifth study was conducted with iv furosemide and inspiration of dry air combined. The control study for each dog included 2 min of ventilation by the pulsed-aerosol ventilation system but with no aerosol delivered to the lungs. Although vehicle control studies were not conducted as part of this work, other studies (3, 40) suggest the diluents (dimethylsulfoxide, water, and isotonic saline) would have a minor effect, if any, on the monitored parameters when deposited in these small quantities over a short duration. Each perturbation was repeated in complementary experiments up to three times while one or more of the following assays were recorded: measurement of CBF, measurement of TMV and BMC, and measurement of RTFO. CBF was always recorded alone as a separate set of experiments to eliminate any interference on the other assays caused by the presence of the stainless steel CBF probe within the trachea. RTFO was also recorded during separate experiments because this assay was fully developed only after some studies were already complete. The control study and the study in which furosemide was administered consisted of three sets of experiments (CBF, TMV-BMC, and RTFO). In the combination perturbation study using both dry air and furosemide, TMV and BMC were recorded simultaneously in one set of experiments, and no complementary experiments recording CBF were conducted. This protocol resulted in a total of 13 experiments for...
each dog, not including gamma-camera calibration and aerosol characterization studies. These experiments were separated by a quasi-random period of weeks to months. Animals were not studied more than once per week.

Baboon protocol. Each baboon underwent three studies (a control and two perturbation studies), each consisting of two sets of experiments. Experiment A measured CBF, and experiment B measured TMV and LMC. During the control experiments, the baboons did not receive 6 min of isotonic saline aerosol (vehicle control). On the basis of other results (40), this was not expected to be a confounding factor. The two perturbations included iv furosemide and spontaneous inhalation of aerosolized furosemide.

Data analysis and statistical significance. The same cohorts of dogs and baboons were used in the control and perturbation studies. Statistical analysis was performed by using commercially available software (SigmaStat 1.0; Jandel Scientific Software, San Rafael, CA). R24% was examined for differences among the treatment groups with the use of the Friedman repeated-measures analysis of variance on ranks. Blood-gas results, monitored indexes (CBF, TMV, BMC, LMC, and RTFO), and the derived indexes described below were all analyzed for the effects of varying treatments and the effects over time by using the Kruskal-Wallis analysis of variance by ranks. When significant differences in the median values among the treatment groups were detected, Dunn’s method was used to isolate the group or groups that differed from the control. A P value of <0.05 was considered significant. The indexes were evaluated in time periods of 15 min with time 0 coincident with the start of each perturbation. The time span of 15 min was chosen arbitrarily. For ease of notation, subscripts separated by commas refer to the period in minutes from which the values were derived (e.g., BMC0,15). The results, which did not satisfy testing for distribution normality, are nevertheless related in text and figures as means ± SE.

To evaluate the effects of the perturbations on the depth of airway lining fluid being transported along the trachea, an estimated depth in micrometers was derived. Assuming that uniform transport occurred along the essentially circular, cylindrical surface of the trachea, individual RTFO (cm²/time) can be factored by the TMV (cm/time) and the estimated circumference of the trachea (in cm) to obtain an estimate of the tracheal fluid depth (in cm) for each RTFO collection period, i.e., 15–45 or 45–75 min. Comparisons were based on the cohort over a period of 15–75 min.

To compare the effects of airway lining fluid perturbations on the interrelationship between airway regions, an index of the coordination was derived in percent bronchial clearance per centimeter of tracheal mucus transport from individual BMC and TMV results during the 30 min after the start of the perturbation. TMV values were assumed indicative of the TMV from either time 0 or any previous TMV value, to the next TMV value or, if no following values existed, to the end of the experiment. Specifically, the BMC over each minute was divided by the TMV at that time. Comparisons were based on the cohort over the 30 min after the beginning of challenge.

To compare the effects of airway lining fluid perturbations on the conversion of tracheal ciliary beat into mucus transport, an index of cilia-mucus interaction was also derived in micrometers transport per ciliary beat from individual TMV and CBF results. By using the same assumptions and TMV time intervals as given above, TMV/CBF could be estimated for each CBF data point. Comparisons were based on the set cohort over the entire experiment.

RESULTS

The responses of the canine mucociliary transport system, as indicated by CBF, TMV, and BMC, to lung deposition of 96 ± 11 µg of amiloride or 71 ± 9 µg of acetylstrophanthidin and to iv administration of 40 mg of furosemide are shown in Fig. 1. For the study in which amiloride was administered, CBF in the 15 min before the perturbation was started, was significantly higher than in the control study. The CBF from the first 15 min after the sham control perturbation was started, was significantly elevated with respect to subsequent time periods in the control study (Fig. 1A). This is possibly caused by mechanical disturbance of the system inherent in moving equipment and the coupling of ventilatory circuits. Considering CBF at all times throughout the study, CBF was increased compared with the control by each of the perturbations. However, the increases that reached statistical significance (many CBF observations) are limited in magnitude and perhaps importance, i.e., particularly those after administration of amiloride. After furosemide injection, TMV increased from the control result of 10.9 ± 0.7 to 14.4 ± 0.5 mm/min (Fig. 1B). The R24% for the control study was 36.9 ± 3.0% compared with 33.9 ± 11.7, 28.5 ± 9.5, and 42.5 ± 6.6% for the study in which amiloride, acetylstrophanthidin, or furosemide was administered, respectively. This indicator of radioaerosol deposition pattern did not vary significantly either between test perturbations or between individual dogs. However, the study in which acetylstrophanthidin was administered had the lowest R24% value. BMC was markedly increased compared with the control study by administration of aerosolized acetylstrophanthidin (17.2 ± 1.3 vs. 9.0 ± 1.3%, BMC15,30 vs. control, respectively; Fig. 1C). The increase in BMC caused by administration of furosemide iv (14.2 ± 1.5 vs. 9.0 ± 1.3%, BMC15,30 vs. control, respectively) did not reach statistical significance.

The response in baboons of CBF, TMV, and LMC to administration of 2 mg/kg iv furosemide or lung deposition of 20 ± 3 mg of furosemide is shown in Fig. 2. In the study in which aerosolized furosemide was inhaled, CBF was significantly lower than the comparable CBF in the control study during the 15 min before the inhalation was started (Fig. 2A). CBF during the 60 min after inhalation of the aerosolized furosemide was remarkably still lower (7.9 ± 0.3 vs. 10.1 ± 0.3 Hz, CBF0.60 vs. control, respectively). TMV markedly increased after furosemide iv (TMV0.90, 6.6 ± 0.4 mm/min; Fig. 2B) compared with the control study (TMV0.90, 2.1 ± 0.1 mm/min). An even more dramatic increase was observed in the 45 min after furosemide aerosol (11.2 ± 1.4 vs. 2.2 ± 0.1 mm/min, TMV0.45). The increase in LMC caused by administration of furosemide iv (8.9 ± 1.3 vs. 4.9 ± 0.4%, LMC15,30 vs. control, respectively; Fig. 2C) did not reach statistical significance. Although mucociliary clearance was slightly faster than in the comparable control study before the inhalation of aerosolized furosemide, the increase in LMC after inhalation of aerosolized furosemide was statistically significant within 15 min of the aerosol delivery (LMC0.15, 2.4 ± 0.4 to 7.1 ± 0.6%).
The responses of CBF, TMV, and BMC in dogs to inhalation of dry air or dry air plus furosemide, compared with the control study and the study where only furosemide was administered, are shown in Fig. 3. Dry-air inspiration decreased CBF compared with the control study during the first 15 min of exposure, but, surprisingly, CBF recovered during subsequent dry-air exposure to approach or exceed control values (Fig. 3A). When TMV from the dry air inhalation was considered

Fig. 1. Ciliary beat frequency (CBF; A), tracheal mucus velocity (TMV; B), and bronchial mucociliary clearance (BMC; C) for 6 dogs; symbols are means ± SE in 15-min time intervals. Time 0 is coincident with start of administration of acetylstrophanthidin, amiloride, furosemide, or sham. *Statistically significant difference from control during that specific 15-min interval, \( P < 0.05 \). †Difference from control when comparing data throughout entire time of study, \( P < 0.05 \).

Fig. 2. CBF (A), TMV (B), and lung mucociliary clearance (LMC; C) for 5 baboons; symbols are means ± SE in 15-min time intervals. Time 0 is coincident with start of administration of iv furosemide, aerosolized furosemide, or sham. *Statistically significant difference from control during that specific 15-min interval, \( P < 0.05 \). †Difference from control when comparing data throughout entire time of study, \( P < 0.05 \).
as one sample throughout the 90-min delivery period, no difference from the control was evident (Fig. 3B). However, when the dry-air exposure was examined in three periods of 30 min each, a reduction was suggested during the first 30 min (TMV₀,₃₀, 8.6 ± 1.0 vs. 10.9 ± 0.7 mm/min), followed by a return to near baseline during the second 30 min (TMV₃₀,₆₀, 11.3 ± 1.1 mm/min), and a suggested TMV increase between 60 and 90 min of exposure (TMV₆₀,₉₀, 15.1 ± 1.7 mm/min). TMV (Fig. 3B) also showed responses that suggested counterbalancing effects of dry air and furosemide. Dry air eliminated the substantial TMV increase after furosemide administration, and furosemide administration eliminated the suggested TMV increase during prolonged dry-air exposure. In the radioactivity retention studies, the R²₄% values for the studies in which dry air and dry air plus furosemide were administered were 44.9 ± 3.9 and 47.5 ± 4.0%, respectively, and were not significantly different from the control or from the study in which furosemide only was administered. Mucociliary clearance in the 15 min before the start of both dry-air and dry-air plus furosemide perturbations was slightly slower than the control study (Fig. 3C). Beyond 30 min of dry-air exposure, BMC was significantly reduced compared with the control (9.3 ± 0.9 vs. 14.0 ± 1.4%, BMC₃₀,₄₅ vs. control, respectively). BMC for the combined perturbation of dry air and iv furosemide (Fig. 3C) was not significantly different from that of the control study and was approximately midway between BMC curves for dry air and furosemide alone.

RTFO for the control study and for the study in which 40 mg of furosemide were administered iv is compared for each of the sampling periods in Fig. 4. The control RTFO may be artificially large in the first collection period because fluid accumulates at the endotracheal tube cuff from the time of intubation, not from the start of collection. Furthermore, secretion may be transiently stimulated by intubation. On the basis of the latter two sampling periods, RTFO collection rate was significantly larger than the control study rate after administration of furosemide iv (6.8 ± 1.7 vs. 2.2 ± 0.5 mg/min, furosemide vs. control, respectively). On the
basis of postmortem measurement of one dog and radiographic image scaling of another dog, the mean diameter of the trachea for the dogs used in these studies was estimated to be 19 mm. By eliminating the time dimension from RTFO and TMV, and assuming a uniform circular cylinder of transport, the estimated airway lining fluid transport depth in the control study was $4.5 \pm 0.9 \mu m$. This value was increased to $8.3 \pm 2.3 \mu m$ by the administration of iv furosemide.

In the dog, coordination of regional tracheobronchial transport, i.e., between BMC and TMV, was maintained similarly to the control study in the studies in which aerosolized acetylstrophanthidin or both dry air and iv furosemide were used (Fig. 5). Bronchial clearance decreased relative to tracheal transport in the studies after perturbation with aerosolized amiloride, iv furosemide, or dry air. In the baboon, lung clearance of radioactivity also decreased relative to tracheal transport compared with the control study during study of both iv furosemide and aerosolized furosemide.

The interaction between tracheal CBF and TMV, as well as its variation in response to perturbation of airway lining fluid and ion transport, is shown in Fig. 6. It is notable that none of the perturbations in the present study significantly decreased the index of micrometers per beat. These values are presented for the dogs (Fig. 6A) and the baboons (Fig. 6B), considering all results from the start of the perturbation to the end of the study – 60 min later. The conversion of ciliary beat into mucus transport for the control study was notably several times less in the baboon ($13.6 \pm 0.3 \mu m/beat$) relative to the dog ($57.8 \pm 0.7 \mu m/beat$). In the dog, the beat-transport translation was not changed by either aerosolized acetylstrophanthidin or dry-air exposure. A minimal increase above the control study was observed for the study in which amiloride was delivered ($69.8 \pm 0.7$ vs. $57.8 \pm 0.7 \mu m/beat$, amiloride vs. control, respectively). The interaction was consistently improved in studies where iv furosemide was administered ($90.0 \pm 0.9 \mu m/beat$). Furosemide also dramatically increased beat-transport translation interaction in the baboon animal model (Fig. 6B) with respect to the control study ($11.5 \pm 0.1 \mu m/beat$), whether delivered iv or by aerosol ($29.5 \pm 0.4$ vs. $46.5 \pm 0.7 \mu m/beat$, respectively).

**DISCUSSION**

One of the major findings of the present study is the considerable evidence that furosemide causes an increase in the net water flux toward the airway lumen, facilitating an increase of mucus transport. 1) Furosemide increased mucociliary transport when administered iv to both dogs and baboons and when administered (90.0 ± 0.9 µm/beat). Furosemide also dramatically increased beat-transport translation interaction in the baboon animal model (Fig. 6B) with respect to the control study (11.5 ± 0.1 µm/beat), whether delivered iv or by aerosol (29.5 ± 0.4 vs. 46.5 ± 0.7 µm/beat, respectively). It can be seen in Fig. 7 that aerosol administration resulted not only in a larger increase in cilia-mucus interaction but also in an earlier maximal response than did iv administration.
Although inhibition of the apical Na⁺-transport system to amiloride observed herein (Fig. 1) is consistent with the increased CBF in Stentor polymorphus (a sessile protozoan) induced by digitoxin (27), another 3Na-2K-ATPase inhibitor. Such inhibitors also dramatically decrease intracellular Cl⁻ concentrations (L. B. Wong, private communication). Whereas TMV did not increase after administration of acetylstrophanthidin in the present study, TMV in dogs was enhanced after administration of an extremely high (0.025 mg/kg) iv dose of acetyloouabain (17), also a 3Na-2K-ATPase inhibitor. The large increase in BMC, without complementary increase in TMV, may in part be the result of a more proximal iron oxide aerosol-deposition pattern, as suggested but not substantiated in R24% analysis.

The effects of iv furosemide in the baboon (Fig. 2) correlated well with the results in the dog (Fig. 1), indicating that basal electrolyte-transport differences between dog and primate airways determined in vitro (35) are not indicative of different physiological responses of the mucociliary transport system in vivo. That furosemide was more effective when delivered by aerosol (Fig. 2) should not be surprising, because furosemide, which has some luminal action on the Na-K-2Cl cotransporter (36, 37), likely achieved a high concentration in the lining fluid, and, because of its polar nature, was unlikely to diffuse readily into the blood.

In an apparent contradiction of our findings, inhaled furosemide has been reported to have no effect on mucociliary clearance in humans (12). In that study, only 10% of the 40 mg aerosolized was likely deposited within the lungs, compared with the 20-mg deposition confirmed within the lungs in the present study. In addition, the mass of furosemide required to cause the same effect in the standard 70-kg human subjects vs. 15- to 40-kg baboons could be substantially larger. Given the differences in deposition and dosing, the study by Hasani et al. (12) suggests only that furosemide deposition at <10% of that used in our experiments may be ineffective.

The transient suppression of BMC, TMV, and CBF during dry-air inspiration (Fig. 3) is consistent with a previous report of TMV reduction in dogs by dry air (13) as well as with our conjecture that the hydration of airway lining fluid in the unstimulated airway is at a low basal level. The proportionate decrease in CBF and TMV during initial dry-air inspiration may be an external consequence of increased viscous resistance from a dehydrated mucus, opposing ciliary motion. However, there was minimal change in the translation of CBF into TMV (Fig. 6), suggesting no important changes occurred in viscous resistance. The decrease of CBF in response to airway dehydration may result from an inhibitory neural suppression of the mucociliary transport system.

The subsequent reversal of dry-air-induced mucociliary suppression, with proportionate increases in TMV and CBF (Fig. 3), is consistent with studies that showed a stimulation of BMC in patients with asthma after 6- to 8-min hyperventilation with dry air (5). The absence

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**Fig. 7.** Temporal coordination of TMV per CBF in 5 baboons. Data are means ± SE showing mucus movement (in µm per ciliary beat) after administration of iv furosemide, aerosolized furosemide, or sham. *Statistically significant difference from control during that specific 15-min interval, P < 0.05. †Difference from control when comparing data throughout entire time of study, P < 0.05.
of recovery of BMC in our results may derive from the lower bronchial hydration stress of spontaneous dry-air inspiration compared with hyperventilation, as both water transport and heat transport occur deeper in the lung with moderate hyperventilation, or it may derive from the prolonged bronchial hydration stress of continuous dry-air inspiration compared with that after cessation of a short (6–8 min) hyperventilation. The recovery of TMV and CBF implies the existence of a compensatory mechanism to increase water transport to the airway in response to dehydration. The time course of the inhibition and recovery of TMV and CBF is similar to the time course of decrease and subsequent increase in expired humidity during dry-air inhalation (30), as well as the delay suggested to be required for insertion of Na-K-2Cl cotransporters (10) or aquaporins into the epithelial membrane (16). The finding that RTFO and the estimated depth of the tracheal fluid increased after furosemide administration (Fig. 4) is suggestive that net water flux to the mucosa increased.

Since informal presentation of our findings, the effects of furosemide combined with dry air have been studied by others. Specifically, Daviskas et al. (4) combined aerosolized furosemide with dry-air hyperventilation in healthy and asthmatic subjects. In healthy subjects, inhalation of the vehicle control appears to have inhibited the increase in mucociliary transport, previously reported to be induced by dry-air hyperventilation (5). Under such conditions, the ability to observe effects due to the administration of furosemide is compromised. The inhibition of a hyperventilation-induced increase in mucociliary transport by furosemide in persons with asthma is consistent with data in the present study and with our initial findings in dogs (42).

The coordination of regional tracheobronchial transport (Fig. 5) appears to be easily disturbed. However, it is quite reasonable that, when altered, bronchial transport should be reduced relative to tracheal transport (or that tracheal transport should be increased relative to bronchial transport) to avoid the accumulation of secretions at the tracheobronchial junction.

Consistent with the lack of change in rabbit tracheal CBF in vitro on exposure to 10−4 M furosemide (31), CBF in the present study was largely unaffected by iv furosemide in both dogs and baboons (Figs. 1 and 2). Aerosolized furosemide decreased CBF while producing the largest increases in mucociliary transport. Therefore, the increases in mucociliary transport induced by furosemide were due to an increase in the distance mucus was transported per ciliary beat rather than to an increase in beat frequency (Figs. 6 and 7).

In the dog control study, the coordination of mucus movement per ciliary beat (Figs. 6 and 7) indicates a mean transport of mucus of 58 µm per ciliary beat. This distance is several times the length of the cilia and suggests that the mucus layer is disengaged and moving above cilia in the recovery phase of the stroke and/or within the metachronal field. The increase of this transport distance to 90 µm with perturbation implies that the mucus layer is advancing toward an optimal interaction with the tips of the cilia, with mucus velocity approximating ciliary tip velocity. Ciliary tip velocities of 1,000 µm/s at a beat rate of 10 Hz (i.e., 100 µm/beat) have been reported (28). The transport of mucus per ciliary beat in the baboon control study (14 µm) is closer to the length of the cilia and thus suggests that the mucus layer is normally more fully engaged with the cilia in the baboon, consistent with the differing flux “set point” in the trachea between the dog (primarily Cl− secreting) and baboon (primarily Na+ absorbing). It is notable that none of these perturbations decreased the effectiveness of the translation of ciliary beat to mucus transport, indicating the robustness of the mucociliary transport system and its ability to adapt to perturbation.

This calculated index of ciliary beat effectiveness, although informative, must be interpreted cautiously because other components of ciliary motion, such as metachronal wave period of the cilia bed (41), amplitude of the ciliary beat, or cilia stroke velocity were not measured and may be regulated independently of the regulation of CBF. Furthermore, this index requires the implicit assumption that the CBF, determined from backscattered photons of a laser beam with a focal diameter of 7 µm on the epithelium in the midtrachea, was representative of ciliary motion throughout the trachea and that measurement of each component of mucociliary transport on separate days of experimentation was representative of the systems response to each perturbation.

In addition to its primary action on the cotransporter, there are other potential mechanisms whereby furosemide may have elicited the observed responses. Furosemide appears to relax smooth muscle constriction by inhibiting prostaglandin synthesis. However, administration of furosemide did not change prostaglandin synthesis in the nasal mucosa (21) or inhibit its production in airway epithelium (18). Diuresis from furosemide might also induce the release of vasopressin, angiotensin, or other mediators that may stimulate mucociliary transport. However, inhalation of furosemide (1 mg/kg) did not cause diuresis either in the present study or in the study of Rastogi et al. (25). Also, furosemide increased mucociliary transport in the dog studies in which urine output was matched with iv volume replacement, as well as in baboon studies where there was no attempt at volume replacement. Furosemide is also reported to inhibit cholinergic and excitatory nonadrenergic, noncholinergic neurotransmission in the airways (6). However, for this effect to cause the observed result, furosemide would have to block inhibitory sensory nerves that regulate secretion so that their inhibition thereby stimulates mucociliary transport. A furosemide-induced increase in microvascular leakage (8) is consistent with our observations.

As stated above, the data strongly imply that furosemide increases the net water balance in the airway lumen. We suggest that it is more likely that furosemide causes this water balance by inhibition of the basolateral Na-K-2Cl cotransporter rather than by other documented or undocumented actions of furosemi-
may seem an antithesis to the generally held paradigm that water flux toward the airway lumen is associated with Cl− transport across the apical epithelial membrane. The data need not be so considered. Rather, the data suggest that an increase in net water content in the airway lumen is not always associated with an increase in net Cl− transport into the airway lumen.

In the case of severe airway perturbation, such as hyperventilation with dry air (4, 5), released mediators may cause a receptor-induced increase of intracellular inositol triphosphate or adenosine 3′,5′-cyclic monophosphate, with the subsequent activation of apical CI− channels and consequent efflux of Cl− from the cell, followed by an obligatory cell shrinkage. Such shrinkage could promote an increase in net water flux from the submucosa to the lumen. Cell shrinkage is also associated with compensatory increased activity of the basolateral Na-K-2Cl cotransporter (10), likely through phosphorylation, in an effort to maintain cellular volume homeostasis. Thus this sequence of events results in an augmentation of basolateral-to-luminal transport of both Cl− and water. Teleologically, such a response would protect a dehydrated epithelial lining.

During mild dry-air perturbation or under homeostatic conditions in which apical Cl− channels are not likely to be receptor activated, augmentation of Cl− transport into the airway lumen is not expected. However, in both mild dry-air and furosemide perturbations (as well as the extreme perturbation above), a predicted decrease occurs in the intracellular Cl− content and cell volume (7, 8, 36). It is possible these decreases act as second messengers (32) to initiate a net water flux from the submucosa to the airway lumen. Such water flux may result from decreased cell volume by increased paracellular permeability to water flux into the airway, analogous to responses in the ophthalmic trabecular network (23). This is consistent with an osmotically induced increase in mannitol permeability in native tracheal epithelia (43), although it is inconsistent with the osmotically induced decrease in mannitol permeability in cultured nasopharyngeal epithelial cells (39). A decrease in intracellular Cl− has also been linked to increased transmembrane permeability to water flux through the increased expression of aquaporins within epithelial membranes (9). Aquaporins permit water flux down an electrochemical gradient (16) that, although it may be minimal during homeostatic perturbations, may still allow ample water movement if the permeability is substantially increased. Finally, active water transport has recently been proposed to rationalize occurrences related to water transport across biological membranes that are not explained by simple flux down an electrochemical gradient (44). Such active water transport may have a role in producing water flux without Cl− transport. Therefore tracheobronchial secretion may not be strictly associated with Cl− flux into the airway but rather with increased net flux of Cl− out of the epithelial cells, resulting from either increased Cl− efflux or decreased Cl− influx.

In summary, the use of aerosolized amiloride to enhance the clearance of secretions from the lungs in normal airways is not supported by the minimal, transient responses observed in mucociliary transport with this Na+−channel inhibitor. The potential stimulation of the mucociliary transport system (BMC and CBF) by aerosolized acetylstrophanthidin-induced inhibition of 3Na−2K−ATPase should be confirmed. The increases in airway fluid transport, mucociliary transport, and mucus transport-ciliary beat when furosemide was administered either to dogs or to baboons, either intravenously or by aerosol, as well as the counterbalancing mucociliary transport responses of dry-air inhalation and furosemide, all suggest an important role of the Na-K-2Cl cotransporter in the regulation of airway hydration and mucociliary transport.

Thus pharmacological manipulation of the periciliary layer may provide an effective mechanism to treat persons with dry, inspissated secretions and to augment an impaired mucociliary clearance system. Aerosolized loop diuretics and cardiac glycosides may be clinically effective in improving the removal of secretions from the airways.

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Address for reprint requests: D. B. Yeates, Dept. of Medicine (M/C 788), Univ. of Illinois at Chicago, 1940 West Taylor St., Rm. 212, Chicago, IL 60612 (E-mail: YEATES-D@UIC.EDU).

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