Heterogeneity of the composition and thickness of tracheal mucus in rats

DAVID E. SIMS AND MARGARET M. HORNE
Department of Anatomy and Physiology, Atlantic Veterinary College,
University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada C1A 4P3

Sims, David E., and Margaret M. Horne. Heterogeneity of the composition and thickness of tracheal mucus in rats. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1036–L1041, 1997.—Inability to preserve airway mucus in situ has limited our understanding of its structure and function. This light- and transmission electron-microscopic study of rat tracheal mucus used a nonaqueous fixative that retains mucus (epiphase) over a lucent layer (hypophase). The fixative is a 1% solution of osmium tetroxide dissolved in a perfluorocarbon. The mean thickness of rat tracheal epiphase was 5 µm, with significant variation (0.1–50 µm) around the tracheal circumference. Tracheal mucus was thickest at the trachealis muscle region and contained cells, cellular debris, and a variable amount of surfactant and lipid, estimated at 4–16% of the total epiphase in five rats, with a mean composition of 9%. Lipid was observed on the surface of the epiphase, embedded within mucus, and at the epiphase-hypophase interface. Refined study of developmental, physiological, and pathological alterations to the airway coat may benefit from this approach.

MATERIALS AND METHODS

A thin mucous layer situated over a serous layer and attached to epithelial cells by just the tips of kinocilia is intrinsically fragile. Regardless of the method of preservation, great care has to be taken to avoid disrupting the mucus. In this study, tracheas were dissected from rats immediately after death by an overdose of pentobarbital sodium. Approximately 10-mm lengths from the pharyngeal swelling to the thoracic inlet were removed and were gently immersed in one of the following fixatives: aqueous cacodylate-buffered glutaraldehyde (n = 3), aqueous buffered glutaraldehyde containing safranin O (n = 1), aqueous buffered glutaraldehyde containing ruthenium red (n = 3), aqueous buffered glutaraldehyde containing alcian blue (n = 3), buffered neutral Formalin (BNF; n = 3), aqueous buffered osmium tetroxide (n = 3), or osmium tetroxide dissolved in a perfluorocarbon (n = 7).

After postfixation in buffered aqueous osmium tetroxide, tracheas were dehydrated and were embedded in plastic resin. Blocks were trimmed to expose tracheas at approximately the middle of the samples or ~5 mm from the pharyngeal swelling. Thicker sections for light microscopy (0.8 µm) and thinner sections for transmission electron microscopy (silver-gold, ~90 nm) were cut and stained. Mucous thickness was measured with a ×40 objective lens (final magnification ×400), using an ocular reticule. As indicated in Fig. 1, 12 sites of measurement per trachea were selected based on placement of site 2 in the center of the trachealis muscle. Thickness of the mucous coat was further quantified by electron microscopy. Randomly obtained electron micrographs were used for volume-fraction estimation of lipid content of the mucous (epiphase) layer.
Methods. Sprague-Dawley and Long-Evans rats, weighing 250–300 g, were obtained, cared for, and treated in compliance with guidelines established by the Canadian Council on Animal Care. The two strains and both sexes were randomly used. Reagents used were 10% BNF (Fisher Scientific, Orangeburg, NJ); osmium tetroxide (Pelco International, Redding, CA) used as a 1% solution in either aqueous cacodylate buffer or FC-72 perfluorocarbon (3-M, London, ON, Canada); glutaraldehyde (Pelco International) prepared at a concentration of 2.5% in 0.5 M sodium cacodylate buffer (JBEM, Dorval, PQ, Canada) and adjusted to pH 7.3 with 1.0 N HCl; ruthenium red (Polysciences, Warrington, PA) used at 0.1% (wt/vol) concentration; safranin O (Fisher Scientific) used at 0.1% (wt/vol) concentration; and alcian blue (BDH, Toronto, ON, Canada) used at 0.5% concentration. The protocols for using ruthenium red, alcian blue, and safranin O were obtained from Hayat (7).

Tracheas were dissected immediately after euthanasia by an overdose of pentobarbital sodium. They were dabbed with cotton gauze to remove any blood on the cut ends and were gently immersed in fixative. After 90 min of fixation in the primary fixative at room temperature, including changes of the fixation solutions at 10 and 60 min, tracheas in aqueous solutions were briefly rinsed in 0.05 M cacodylate buffer (JBEM, Dorval, PQ, Canada) and adjusted to pH 7.3 with 1.0 N HCl; ruthenium red (Polysciences, Warrington, PA) used at 0.1% (wt/vol) concentration; safranin O (Fisher Scientific) used at 0.1% (wt/vol) concentration; and alcian blue (BDH, Toronto, ON, Canada) used at 0.5% concentration. The protocols for using ruthenium red, alcian blue, and safranin O were obtained from Hayat (7).

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The ideal stain for light-microscopic examination of plastic sections proved to be an azure II-methylene blue-safranin O combination originally described by Laczkó and Lévai (11). Conventional toluidine blue staining enabled resolution of mucus, but contrast and clarity were inferior, and considerably more time was required for staining. For electron microscopy, thin sections were mounted on copper grids and were stained with 5% uranyl acetate in 50% ethanol for 30 min followed by triple lead salts (18) for 2 min in the absence of carbon dioxide.

Volume fraction of lipid in the mucus epiphase was determined by taking random micrographs of the epiphase at an initial magnification of ×20,000. At that magnification, the operator of the electron microscope cannot easily distinguish subcomponents of the mucus layer and, hence, is not likely to introduce a bias to the sampling. Ten micrographs were taken from each of five tracheas. Micrographs were printed at a final magnification of ×52,000. Regions containing cells or cellular debris were excluded. A dot-grid transparency was placed over the micrograph, and dots were counted that lay over surfactant/lipid vs. other, presumably glycoprotein, matrices. Mucus presented itself in varying degrees of hydration. Care was taken to count only those dots directly over electron-dense particles and not the lucent spaces between the particles. Surfactant was counted with lipid in this study, but membranes that appeared to be of organellar origin were excluded.

Statistical analysis was performed with MiniTab and SAS software. Mucous thickness was compared for intra- and interanimal variance by the general linear model. Volume fraction of lipid within the epiphase was subjected to F-test
comparison between animals. Values are presented as means ± SD, with significance indicated by P < 0.05.

RESULTS

Light microscopy. Aqueous glutaraldehyde, with or without safranin O, aqueous osmium tetroxide, and BNF yielded no mucous coat (Fig. 2), with one exception in the trachealis muscle region of a BNF-fixed trachea. Ruthenium red- and alcian blue-fixed tracheas had sites showing material overlying the hypophase (3 of 36, and 20 of 36, respectively), but with both fixatives there was considerable swelling and rupture of mucous cells and preservation of a flocculant material that was not consistent with a mucous coat (Fig. 3). In contrast, there was a discernable mucous epiphase over a clear hypophase in tracheas fixed in nonaqueous fixatives (Figs. 4–7). Therefore, only the nonaqueous fixed tracheas were subjected to further analysis. When heterogeneity of mucus thickness and composition became evident, additional samples were analyzed to increase statistical confidence. Within seven cross-sectioned tracheas analyzed at 12 sites each, 66 of 84 possible sites had a discernable mucous epiphase over a clear hypophase. Sixteen sites had no resolvable mucus (Fig. 4), several sites had much thicker mucus (Figs. 5–7), and two sites had a flocculant material indicative of disrupted epiphase (Fig. 6).

A heterogeneous distribution of mucus was observed, with a tendency for thicker mucus in the region of the trachealis muscle. Entire cells could be resolved within the mucus, most often along the sides of the trachealis muscle (sites 1 and 3), where a troughing or grooving of the epithelium occurred. Overall mean thickness of the mucus was 6 ± 9 µm (n = 66) if sites without apparent mucus were excluded and was 5 ± 8 µm (n = 82) if they were included as zero values. There was significant variation between animals, indicating a lack of uniformity in the thickness of mucous epiphase.

Electron microscopy. When sites of tracheas determined above to have no discernable mucus were examined by electron microscopy, they were found to have a layer of mucus that was too thin for resolution by light microscopy (Fig. 8). The mucus at those sites was between 0.1 and 0.3 µm thick. The surface layer of mucus was smooth between thicker and thinner regions, suggesting that variations were physiological, not artifactual (Fig. 4).

Adjacent to the trachealis muscle, epiphase was observed to be thicker, often containing cellular debris (Fig. 9), whereas epiphase on epithelia opposite the trachealis muscle usually did not (Figs. 8 and 10–12). Based on this observation, the mean thickness of mucus as determined by light microscopy was recalculated, with 0.2 µm replacing zero values for nonresolvable mucus. Then 82 of 84 possible test sites had mucus with a mean thickness of 5 ± 8 µm, the same as...
determined by light microscopy. Interanimal differences of mucous thickness remained significant.

Noting a tendency for thicker mucus in the region of the trachealis muscle, the observation points were reconfigured such that the circumference of the trachea was divided into 4 regions instead of 12. These were named the dorsal (adjacent to the trachealis muscle), ventral, and left and right lateral regions. Sampling points 1, 2, and 3 were merged into the dorsal region, points 4, 5, and 6 were merged into right lateral, etc. The thickness of mucus in these regions based on 21 or 22 sample sites per region was as follows: dorsal, \(11 \pm 15\) µm; ventral, \(2 \pm 3\) µm; and left and right lateral, \(4 \pm 6\) and \(3 \pm 4\) µm, respectively. Mucous was significantly thicker in the dorsal region.

Lipid profiles were seen throughout the epiphase (Figs. 10–12), taking the appearance of mono- and bilayers, sheets and whorls, and surfactant-like com-
plexes. Lipid was observed on the luminal surface of the epiphase (Fig. 11), within the epiphase (Fig. 10), and at its base (Figs. 11 and 12). A volume fraction of surfactant and lipid in the epiphase was calculated based on point counting from 10 randomly selected micrographs from each of five animals. The epiphase was composed of $9 \pm 9\%$ surfactant and lipid. Means of lipid composition for individual animals ranged from 4 to 16%, with interanimal variance indicating significant difference.

**DISCUSSION**

Nonaqueous fixatives offer significant advantages over conventional aqueous fixatives. They may retain more protein (14) and lipid (13). Perfluorocarbons are also desirable for their oxygenating properties (10, 16). Lacking in osmotic or pH-related forces, nonaqueous solvents interfere with cell physiology and structure to a minimal extent (19, 20). As is shown in this report, osmium tetroxide dissolved in a perfluorocarbon preserves more of the components of tracheal mucus in situ than has previously been possible.

Ruthenium red and alcian blue likely impart a labeling effect on carbohydrates (7). As mucus dissolves during an aqueous fixation process, some label is bound to the cell surface and some to mucin, possibly creating a cross-linking effect as well as a flocculant label. However, in this study, neither ruthenium red nor alcian blue preserved a mucous layer consistent in appearance with what might be expected for a predominantly glycoprotein substance, and both caused unacceptable cellular swelling of tracheal epithelium. Osmium tetroxide presented in a nonaqueous manner reliably fixed mucus, whereas aqueous glutaraldehyde (with or without additives), osmium tetroxide, and Formalin did not. Glutaraldehyde and formaldehyde do not dissolve in perfluorocarbon, so the remainder of the possible solvent-fixative combinations are not testable. The relatively small size of osmium atoms and the

Fig. 11. Region of epiphase with extensive coverage by a monolayer of lipid (arrow; note contrast between this epiphase surface and that shown in Fig. 10) along with numerous lipid profiles within the mucus. Magnification, $\times 46,000$; bar, $1 \mu m$.

Fig. 12. Higher magnification of part of Fig. 8. Surfactant is shown with its characteristic lattice-like profile. Lipid bi- and trilayers cover a significant portion of the surface and about one-half of the bottom of the epiphase. Magnification, $\times 46,000$; bar, $1 \mu m$. 
appearance of retained mucus suggest that the micrographs of this report reflect the biological nature of mucus and not a label attached to it.

Two possible caveats that must be considered during interpretation of these data are the position of the trachea immediately before fixation and the random-ness with which the tracheas were sampled. The rats used in this study were placed in sternal recumbency after onset of anesthesia. However, during the surgical removal of tracheas, animals were laid on their backs; hence, there could have been some displacement of epithelial toward the trachealis muscle region that was caused by gravity. Viscous properties of mucus and the natural grooving of tracheal epithelium would tend to limit this effect. Heterogeneity of the epithelium along the length of the trachea was not examined in this study and may play a role in the interanimal variations of thickness and lipid content observed.

Heterogeneity of epithelium in rat trachea applies to both its thickness and composition. The normal mucous coat of rat trachea varied from thin (~0.2 μm) to thick (~50 μm), with estimates of epithelial thickness by light and electron microscopy being comparable. Thinner regions of epithelium, particularly along the ventral aspect of the trachea, may indeed be more susceptible to insult. Grooving or troughing of mucus adjacent to the trachealis muscle region appears to occur. Another variable of physiological significance is the extent of hydration of the epithelium. A given amount of glycoprotein may have increased thickness by addition of water. Hydration states of tracheal mucus, a random event within this study, likely contributed to the reported heterogeneity.

In disease states, the lipid profile of mucus may be altered to include glycolipids (2); their ultrastructural interpretation awaits further study. Lipid and surfac-tant are observed in all parts of the epithelium and need to be considered in modeling studies of gas diffusion and particle penetration of the epithelium. Investigations of developmental, physiological, and pathological processes of the entire mucociliary apparatus, which have previously been compromised (6), will benefit from the use of nonaqueous fixation.

Address for reprint requests: D. E. Sims, Dept. of Anatomy and Physiology, Atlantic Veterinary College, Univ. of Prince Edward Island, Charlottetown, PE, Canada C1A 4P3 (E-mail: sims@upei.ca).

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