Heterogeneity of the composition and thickness of tracheal mucus in rats

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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.

ultrastructure; epiphase; microscopy; lipid; glycoprotein

UNDERSTANDING OF THE respiratory mucociliary apparatus has been limited by the inability to reliably preserve mucus in situ. Epithelial surfaces of the upper and middle respiratory tract contain many ciliated cells. Their cilia are immersed in a watery serous layer (the hypophase) that facilitates the ciliary return stroke. During their power stroke, cilia stiffen, extend to a mucous layer (the epiphase), and propel mucus toward the pharynx. The hypophase and epiphase cleanse, moisten, and warm inspired air, protecting underlying epithelial cells from direct insult. However, the thicknesses of these supracellular layers have only been estimated, not quantified.

The reported rate of movement of mucus appears to have considerable species (and experimental) variation and may be as slow as 2 mm/min or as fast as 35 mm/min (4, 17, 21). Modeling studies suggest that mucus does not flow evenly but preferentially concentrates along troughs or grooves (1). Variable thickness of mucin coats, if real, may be significant. If mucins are unevenly layered, there may be thin or bare regions that are more susceptible to injury; thus generalized estimates of mucus thickness may be misleading. Passage of viruses and bacteria through the epiphase and hypophase is not well understood due to an inability to preserve microbes within an intact mucous coat. Peripheral domains of glycoprotein mucins are able to interact with bacterial adhesins (12); therefore, bacteria should be retained in tracheal epiphase in a resolvable manner. In addition, there is considerable interest and controversy as to the possible contributions of lung- and airway-derived lipid to the mucous layer (2, 3, 5, 9, 15). Lipids, if present in appreciable amounts, could form an osmotic barrier, thus protecting the underlying epithelial cells from noxious water-soluble agents such as sulfur dioxide gas. If present as a surface layer, lipids would complicate our perception of the sticky mucous lining of the airways. However, the current method for most biochemical studies of normal mucus, airway lavage, dilutes and mixes mucus with cellular debris, lower airway secretions, and lavage fluid (usually saline) to such an extent that the composition of tracheal epiphase can only be estimated.

The purpose of this study was to test the hypothesis that mucus is unevenly distributed in trachea, based on previous observations of heterogeneous flow rates of mucus in dogs (8). A recently developed nonaqueous fixative is reported, and the fixative appears to be ideal for preserving airway mucus in situ. The fixative consists of osmium tetroxide dissolved in a water-immiscible perfluorocarbon. Because osmium tetroxide is an effective fixative of lipids, there was also an opportunity to estimate the lipid content of mucus.

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 cetylpyridinium chloride containing safranin O (n = 3), aqueous buffered glutaraldehyde containing ruthenium red (n = 3), aqueous buffered glutaraldehyde containing alcin 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.

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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 and then were postfixed in 1% buffered osmium tetroxide for 60 min (with the exception of the 1 trachea in the aqueous glutaraldehyde section that was not postfixed to determine if postfixation affected mucus retention). After postfixation, they were dehydrated in ascending concentrations of ethanol followed by propylene oxide, infiltrated, and embedded in epon/araldite. Tracheas fixed in nonaqueous conditions were rinsed in pure FC-72 to remove any unbound osmium tetroxide and were then immersed in 100% ethanol. Vials containing tracheas in ethanol were placed under mild vacuum to remove residual FC-72, which is more volatile than ethanol, for 3 h. Once in pure ethanol, the “nonaqueous” specimens were processed in the same manner as the other tissues.

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 organelar 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 fixative (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 $6 \pm 9 \mu m (n = 66)$ if sites without apparent mucus were excluded and was $5 \pm 8 \mu m (n = 82)$ 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 $5 \pm 8 \mu m$, 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, $11 \pm 15 \mu m$; ventral, $2 \pm 3 \mu m$; and left and right lateral, $4 \pm 6$ and $3 \pm 4 \mu m$, 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-

Fig. 8. Within the nonaqueous fixation samples, sites determined to have no epiphase by light microscopy were observed by transmission electron microscopy to have a thin (~0.2 µm) coat (arrowheads) with a clear hypophase region beneath. Magnification, ×14,000; bar, 1 µm.

Fig. 9. In regions with thicker epiphase, cells and cellular debris (arrowheads) were commonly observed within the mucous coat. Magnification, ×3,500; bar, 1 µm.

Fig. 10. Thin epiphase includes a stack of lipid sheets (arrow) but little or no lipid on the surface and bottom of the mucus. Magnification, ×26,000; bar, 1 µm.
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
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 randomness 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, could have been some displacement of the trachealis muscle region. Heterogeneity of the epiphase along the length of the trachea was not examined in this study, likely contributed to the reported heterogeneity. Heterogeneity of epiphase 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 epiphase thickness by light and electron microscopy being comparable. Thinner regions of epiphase, particularly along the ventral aspect of the trachea, may indeed be more susceptible to insult. Grooving or roughing of mucus adjacent to the trachealis muscle region appears to occur. Another variable of physiological significance is the extent of hydration of the epiphase. 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 surfactant are observed in all parts of the epiphase and need to be considered in modeling studies of gas diffusion and particle penetration of the epiphase. 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.

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REFERENCES


