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40. Calculated on a per day basis, each pregnant animal received: (in milligrams) choline chloride, 20.0; inositol, 10.0; ascorbic acid, 2.0; calcium pantothenate, 1.0; (in micrograms) *p*-aminobenzoic acid, 2000.0; pyridoxine, 600.0; nicotinic acid, 600.0; thiamin, 600.0; menadione, 500.0; riboflavin, 200.0; folic acid, 12.0; biotin, 5.0; vitamin B_{12}, 0.6; (in international units) retinyl palmitate, 300; cholecalciferol, 30.0; and DL-α-tocopheryl acetate, 2.2
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Lysozyme
mucous glycoprotein
organ culture
tracheobronchial epithelium

Human Tracheobronchial Secretions: Development of Mucous Glycoprotein and Lysozyme-secreting Systems

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Summary

Baseline rates for secretion of mucous glycoprotein were similar (680–830 μg/g tissue/24 hr) for cultured tracheal epithelium from newborns of 26–32 weeks' gestation, full term newborns, and older children. Addition of methacholine to culture medium augmented secretory rates of glycoprotein from all tissue sources 3–5-fold. The overall composition of secreted mucous glycoproteins changed little with increasing age. A trend toward less sulfation and toward increased sialic acid and fucose content was noted in secreted glycoproteins from explants of older subjects. Histochemical observations of stored glycoprotein in tracheal tissue, which was subsequently used for organ culture experiments, confirmed that a modest, but consistent sulfate to sialic acid shift occurs during early life. In contrast, baseline secretory rates for lysozyme from tracheal epithelium of preterm infants were one-half as large as rates from epithelium of full term babies and were refractory to cholinergic stimulation. Stimulation of lysozyme secretion by a cholinergic agonist was achieved in all cases by 40 weeks' gestation. We conclude that basal glycoprotein secretion and the mechanism for glycoprotein response to cholinergic stimulation have developed by the earliest

age of viability, but that basal lysozyme secretion is deficient and is unresponsive to cholinergic stimulation in tracheal tissue from preterm newborns.

Speculation

The lungs of preterm infants may be more susceptible to bacterial invasion than lungs of full term infants and older children as a result of deficient lysozyme secretion by tracheobronchial epithelium.

Development of the human tracheobronchial secretory system begins late in the first trimester of gestation (7). Glandular mucus-secreting cells appear between 14 and 17 weeks (6) but serous cells are not found in glands until late in gestation (7). Mucus-secreting (goblet) cells of the surface epithelium also appear during the second trimester (6).

According to Lamb and Reid (9), secretory material stored in gland cells of newborn infants and fetuses stains uniformly for sulfate but not at all for sialic acids, in contrast to glands of older children and adults which contain cells with staining reactions for both sulfated and sialylated glycoproteins. Little is known about the actual secretory activity of this tissue. Both lysozyme and mucous glycoproteins are synthesized and released by cultured tracheobronchial epithelium of preterm and term newborn infants (5). The composition of tracheobronchial secretions at various stages of development has not been delineated. We have employed organ culture, chemical, and histochemical techniques to determine the lysozyme and mucous glycoprotein content of tracheal secretions and the response of these secretory components to cholinergic stimulation (3) from the earliest age of viability through childhood.

MATERIALS AND METHODS

Tracheal explants were obtained from preterm infants (19) with birth weights of 500–1100 g and estimated gestational ages of 26–32 weeks. These infants had succumbed to pulmonary immaturity between 1 and 6 days of life. All full term infants weighed more than 2500 g and had died within the first month of life of nonpulmonary diseases. Older children also died of nonpulmonary causes. None of the subjects had breathed more than 70% oxygen for more than 36 hr.

Tracheas were removed within 2 hr of death. Newborn tracheas were cut into 2–4-mm^2 pieces and cultured as full thickness explants. The submucosa-mucosa layers of tracheas from older children were microdissected from the underlying cartilage and serosa to improve oxygenation and nutrient diffusion to gland cells during organ culture. These layers were also cultured as 2–4-mm^2 explants. Tissue weights were obtained by weighing culture dishes before and after addition of explants.

Explants were cultured in sufficient 199 (Eagle's base)medium to provide a level even with but not covering the surface of explants as previously described (5). Gentamicin (100 μg/ml) and amphotericin (10 μg/ml) were added to all culture media. In some experiments 6×10^6 dpm/ml $Na_2{}^{35}SO_4$ and 1×10^6 dpm/ml 6-D-[^{3}H]glucosamine were also added to the culture medium. Explants were cultured in a 40 ± 1% oxygen, 5% CO_2, water-saturated environment. Medium was removed and replaced every 24 hr for studies of baseline secretory rates.

Experiments testing the response to methacholine stimulation utilized an initial 20-hr baseline culture period (P_1) and a second 4-hr culture period (P_2) during which 30 μg/ml of methacholine hydrochloride (Sigma) was added to Petri dishes (3). Data for these experiments are reported as P_2/P_1 ratios of the rate at which nondialyzable material was discharged into the medium. Use of the ratio controls for variability in the secretory capacity of different sets of explants (3).

Harvested media were analyzed for lysozyme by the method of Osserman (14), dialyzed exhaustively against distilled water, subjected to methanolysis, and analyzed further for fucose, galactose, total hexosamine, and sialic acid content by gas liquid chromatography of the trimethylsilyl sugar derivatives (4). Nondialyzable ^{3}H and $^{35}SO_4$ were assayed by double isotope methods (4). Previous studies had shown that 80% or more of these two radioactive precursors incorporated into macromolecules by tracheal explants are, in fact, incorporated into mucous glycoproteins (5). Mucous glycoprotein was determined chemically by calculating the sum of its five sugars in dialyzed medium and multiplying this sum by 1.25 (1) to adjust for the 20% peptide content of human tracheobronchial mucous glycoproteins (2).

Two or three segments of each trachea, taken at different levels, were placed in neutral buffered formalin and processed for histologic and histochemical studies. Stains routinely employed included alcian blue (AB) at pH 1.0 or 2.5 followed by periodic acid-Schiff (PAS) (12), AB (pH 2.5)-acid fuchsin (AF) (16), and high iron diamine (HID)-AB (pH 2.5). The AB (pH 2.5)-PAS staining sequence was performed both before and after overnight treatment with 1:4 diluted *Vibrio cholerae* sialidase as described by Lamb and Reid (8). Control sections were incubated with buffer alone and compared with sialidase-treated sections for all observations. AB (pH 2.5) stains all acidic substances (sulfate and sialic acid) blue whereas at pH 1.0, only sulfated materials are stained blue. The AB-AF staining sequence stains sulfated materials purple and materials containing only carboxyl (sialic) acidic groups blue. HID-AB stains sulfated substances brown-black whereas sialic acid-containing substances are stained blue.

The percentage of total mucous gland cells yielding a characteristic color reaction was estimated by two observers (*JK, TB*) by directly counting each cell type in all gland acini appearing in multiple sections of tracheal explants. The estimates, performed independently on two occasions, were reproducible. The small amounts of tracheal tissue which could be alloted from newborn tracheas for histochemical, rather than culture, studies precluded precise quantitation of cells giving a particular staining reaction. Percentage data were grouped into quartiles between 0 and 100 to emphasize that the numbers are carefully determined estimates.

RESULTS

Baseline rates for secretion of mucous glycoprotein and lysozyme by tracheal explants during the first 72 hr of culture are recorded in Table 1. Mucous glycoprotein was discharged into the culture medium at approximately the same rate by tracheal explants from preterm infants, term infants, and older children. On the other hand, baseline rates for lysozyme secretion by explants of term newborns were double that of explants from preterm infants, and were even greater in explants from older children.

Explants from six preterm infants responded to methacholine stimulation by discharging mucous glycoprotein at a rate which was 3–5 times greater than the rate under nonstimulated culture conditions (Fig. 1*a*). However, the rate of secretion of lysozyme did not change with addition of methacholine to culture medium bathing preterm explants. Explants from term newborns discharged both lysozyme and mucous glycoprotein in response to methacholine at a rate significantly faster than the baseline rate

Table 1. *Baseline secretory rates for cultured tracheal epithelium*

Trachea source	No. of tracheas	Rate, μg/g tissue/24 hr ± SEM: Lysozyme	Rate, μg/g tissue/24 hr ± SEM: Mucous glycoprotein
Premature newborns	8	81 ± 12 ($p < 0.02$)	830 ± 226
Term newborns	8	164 ± 29	746 ± 214
Children (2–8 years)	5	294 ± 81	680 ± 120

(Fig. 1*b*). The increase in rate of mucous glycoprotein secretion was comparable with that of tracheal explants from preterm infants.

The chemical properties of mucous glycoproteins discharged into culture medium by explants from premature newborns, term newborns, and older children were compared with respect to $^{35}SO_4/^3H$, sialic acid/galactose, and fucose/galactose ratios (Table 2). Incorporation of ^3H glucosamine into mucous glycoprotein as well as galactose and hexosamine content of mucous glycoproteins discharged *in vitro* did not change appreciably as a function of the gestational or postnatal age of the tissue donor. Therefore, differences of ratios noted in these studies reflect changes in the $^{35}SO_4$, sialic acid, or fucose content. Although mean $^{35}SO_4/^3H$ ratios decreased from 0.32 in glycoproteins from preterm explants to 0.23 in glycoproteins from explants of older children, these differences did not achieve statistical significance. Similarly, the apparent small increase of sialic acid/galactose ratios of secreted glycoproteins as a function of increasing age was not significant. In contrast, the amount of fucose present in mucous glycoprotein increased significantly between the newborn period and childhood.

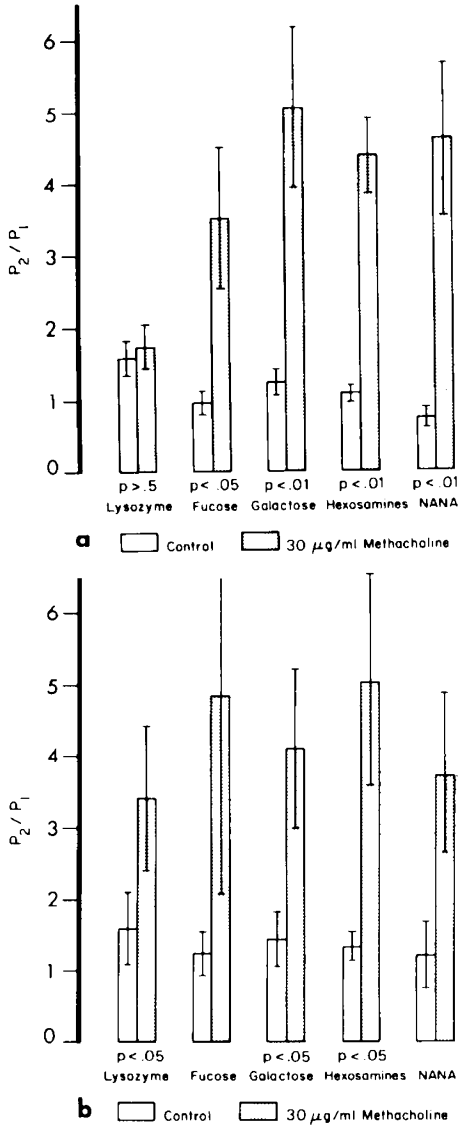

Fig. 1. Methacholine stimulation of secretion by tracheal explants from six preterm newborn infants (*a*) and four term newborns (*b*). P_2/P_1 is the ratio of secretory rates for the second (test) culture period to secretory rates during the first (baseline) secretory period.During the test culture period some sets of explants were incubated without addition of methacholine to the culture medium (controls). Means and standard errors of the means for each secretory product are graphed. Significance of differences between means from control and stimulated explants was assessed by the paired *t*-test.

Histochemical observations of mucous glycoproteins in the preterm and newborn tissues used for culture experiments are recorded in Table 3. Amounts of stored mucous glycoprotein did not vary appreciably with age. Mucus-containing cells characteristically gave an acidic blue or purple staining reaction with the AB (pH 2.5)-PAS sequence at all ages. Less than 10% of the gland cells in tracheas of preterm and full term newborns gave the pink staining reaction of neutral glycoproteins with this stain. Less than 50% of cells in all tracheal tissues stained blue or purple with AB (pH 1.0)-PAS, indicating the presence of sulfated glycoprotein. However, full term tracheal glands generally had less than 25%, whereas preterm tracheas generally had more than 25% of cells which displayed the sulfate staining reaction. This distribution of cells containing sulfated glycoprotein was confirmed by HID-AB and AB-AF staining sequences.

Loss of AB staining at pH 2.5 with prior sialidase treatment was more extensive in full term tracheal glands. However, all but one preterm trachea had at least a small number of gland cells with sialidase-susceptible contents.

Goblet cells of the surface epithelium were sparse in most preterm tracheas, but when present they had acidic staining properties with AB (pH 2.5)-PAS and were predominantly sulfated as demonstrated by AB (pH 1.0)-PAS, HID-AB, or AB-AF stains. Goblet cells were more abundant in full term tracheas but displayed the same histochemical properties as those in preterm tracheas.

DISCUSSION

Our findings suggest that tracheobronchial secretions of infants born before 32 weeks' gestation are relatively deficient in lysozyme for a number of days after birth and that the rate of

Table 2. *Compositional changes of mucous glycoprotein secreted by tracheal explants as function of age*

Trachea source	$^{35}SO_4/^3H$-glucosamine[1]	Sialic acid/galactose	Fucose/galactose
Premature newborns	0.32 ± 0.12	1.08 ± 0.14	0.19 ± 0.03
Term newborns	0.29 ± 0.04	1.30 ± 0.21	0.25 ± 0.03 ($P < 0.01$)
Children (2–8 years)	0.23 ± 0.10	1.35 ± 0.14	0.50 ± 0.03

[1] Labeling expressed as disintegrations per min incorporated into secreted macromolecules per 10^6 dpm of each isotope added to culture medium.

Table 3. *Histochemical staining properties of tracheal gland cells*

Source and staining reaction[1]	No. of tracheas with indicated % cells giving staining reaction			
	0–25%	25–50%	50–75%	75–100%
Preterm newborns				
For sulfate (AB (pH 1.0)-PAS)	3	4	0	0
For sialidase-susceptible contents	6	1	0	0
Full term newborns				
For sulfate (AB (pH 1.0)-PAS	5	2	0	0
For sialidase-susceptible contents	1	3	2	1

[1] AB: Alcian blue; PAS: periodic acid-Schiff.

lysozyme secretion is not increased by cholinergic mechanisms in these preterm infants. Studies of serum (18) and amniotic fluid (10) lysozyme as a function of gestational age also show 2–3-fold increases of activity during the third trimester. It appears that mechanisms for synthesis and discharge of lysozyme by several human tissues, including leukocytes which generate serum lysozyme, are not fully functional until relatively late in gestation.

A deficiency of lysozyme in tracheobronchial secretions may reduce the antibacterial capacity of the tracheobronchial tract in very small preterm infants. Lysozyme has a bacteriolytic effect on gram-positive bacteria and along with complement and immunoglobulins may play a role in the destruction of gram-negative bacteria (13).

In contrast to an immaturity of the lysozyme-secreting system, the mechanism for secretion of mucous glycoprotein is well developed by the end of the second trimester of pregnancy. Baseline quantities released *in vitro*, and in response to cholinergic stimulation by preterm explants, are equivalent to those of term newborns and older children. For the first time clearly separable mucous glycoprotein and lysozyme-secreting systems in human tracheobronchial tissue have been demonstrated. Previous work demonstrated parallel secretory rates for these two protein components from unstimulated and stimulated tracheal explants of older children and adults (3), suggesting a common cell of origin. The present results, coupled with the observation that serous cells of tracheobronchial glands appear only near term (7), suggest that serous cells secrete lysozyme whereas mucus cells are the primary source of mucous glycoproteins.

Minor age-related changes in composition of mucous glycoproteins secreted by explants are demonstrated in these studies. The content of galactose, glucosamine, and galactosamine, the three sugar moieties which form the oligosaccharide cores of human and many other mucous glycoproteins (17), is constant from 26–32 weeks of gestational age to childhood. Less sulfation and greater sialic acid and fucose incorporation seem to occur with increasing age, but only a change in fucose content is strongly supported by the present studies. The validity of a small sulfate-sialic acid shift is supported both by histochemical observations and chemical studies of secretions from the same tissues.

Our chemical and histochemical observations differ from the histochemical observations of Lamb and Reid (9) who described sulfated mucous glycoproteins, devoid of sialic acid, in nearly all gland cells of fetal (15–26 weeks' gestation) tracheas and the appearance of sialidase-susceptible secretory material only after several months of extrauterine life. Mucous glycoprotein in tracheal tissue from the youngest subjects in our study did not stain uniformly for sulfate; virtually all preterm tracheal glands and their secreted glycoproteins contained some sialic acid. Differences between our observations and those of Lamb and Reid are not readily explained, but may relate in part to gestational age differences and/or to changes which may have occurred during extrauterine life, which was experienced by all of our trachea donors. The close correlation between results of chemical analyses of newborn tracheal secretions and histochemical analyses of stored secretions strengthens our claim that only modest changes of glycoprotein composition occur after 26 weeks of gestation. Further support for this claim comes from the histochemical studies of Lev (11), which demonstrated staining reactions for both sialic acid and sulfate in human fetal tracheobronchial glands.

An apparently higher secretory rate for mucous glycoproteins from tracheal explants of newborns, especially preterm, can be attributed to an increased gland density at early gestational ages (6). Mucous glycoprotein secretory rate per gland mass *in vitro* probably varies little with age. Since ciliary movement is initiated early in the second trimester of pregnancy (7), the two major components of a functioning mucociliary apparatus are present by 26 weeks of gestation. In fact, we have observed carbon particle transport at a rate approaching 1 cm/min on the surface of explanted tracheal epithelium from newborns of 26–32 weeks' gestation. The effect, if any, of shifts in sulfate, sialic acid, and fucose content of the mucous glycoproteins on mucociliary transport of particles should be the subject of future investigations.

CONCLUSION

Baseline rates for secretion of mucous glycoprotein and secretion of this glycoprotein in response to methacholine are similar, whether from trachea explants of preterm newborns, full term newborns, or older children. A trend toward less sulfation of glycoprotein and toward more sialic acid and fucose incorporation was noted with increasing age. In contrast, tracheal explants from preterm newborns had a low secretory rate for lysozyme and were refractory to cholinergic stimulation. These results suggest that lysozyme secretion is deficient in the tracheobronchial airways of preterm newborns, but that the mucous-secreting system in these airways is functioning adequately by the earliest age of viability.

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19. Postmortem tracheal tissue was obtained with permission of parents as approved by the Human Experimentation Committee of University Hospitals, Cleveland.

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