Developmental Changes of Ferret Tracheal Mucin Composition and Biosynthesis†

Margaret W. Leigh,*,‡ Pi-Wan Cheng,‡,§ and Thomas F. Boat‡

Departments of Pediatrics and Biochemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Received April 10, 1989; Revised Manuscript Received July 17, 1989

ABSTRACT: We characterized the chemical composition of mucins secreted by ferret tracheal explants and the activities of key mucin glycosyltransferases in ferret tracheal epithelium during a period of rapid postnatal maturation of the mucin-secreting structures. Ferret tracheal explants secrete three major groups of high molecular weight glycoconjugates: (1) those susceptible to bovine testicular hyaluronidase; (2) those resistant to hyaluronidase and exhibiting high density ($\rho \geq 1.60$ g/mL); and (3) those resistant to hyaluronidase and exhibiting low density ($1.45 \leq \rho < 1.60$ g/mL). The hyaluronidase-resistant, low-density glycoconjugates have typical mucin properties and constitute 36% of total glycoconjugates released in newborns but only 8% in adult ferrets. Mucin secretory rate per unit surface area of trachea progressively decreases with age. Mucin amino acid and total carbohydrate contents do not vary; however, the sialic acid content increases, and fucose content as well as blood group A activity of the mucins decreases with age. Four glycosyltransferases involved in mucin biosynthesis [Galβ3GalNAc:(GlcNAc-GalNAc)β6 *N*-acetylglucosaminyl-, GalNAc:β3 galactosyl-, Gal:α2 fucosyl-, and GalNAcα2→6 neuraminyltransferase] are present in tracheal epithelium of ferrets at all ages. Activities of all but the neuraminyltransferase decrease with age. The relatively greater neuraminyltransferase activity is consistent with increased incorporation of sialic acid into secreted mucins over the same age span. Conversely, diminution of fucosyltransferase relative to galactosyltransferase activity may contribute to the lower fucose content and lower blood group A activity of mucins secreted by mature ferret tracheas. We speculate that these age-related differences in biosynthetic enzymes, mucin properties, and mucin secretory rates are likely to be reflected in properties of airway surface liquids and their protective functions.

Tracheobronchial mucins are large glycoproteins secreted by goblet cells in the surface epithelium and submucosal glands. These glycoproteins are major contributors to the rheologic properties of airway mucus and thereby influence the clearance of inhaled particles by mucociliary mechanisms. Tracheobronchial mucins contain 10–20% peptide, 70–80% carbohydrate, and 1–8% sulfate by weight (Boat & Cheng, 1978). Serine and threonine account for 30–50% of the amino acids (Boat & Cheng, 1978). Numerous oligosaccharide chains are attached to a polypeptide core by O-glycosidic *N*-acetylgalactosamine to serine or threonine linkages. These chains contain varying amounts of five sugars: L-fucose, D-galactose, D-*N*-acetylglucosamine, D-*N*-acetylgalactosamine, and sialic acid. Oligosaccharides are synthesized by a stepwise transfer of sugars from respective sugar nucleotides to growing chains by specific glycosyltransferases (Schachter, 1978). The final structure of each oligosaccharide chain is determined by the sequence of addition and specific linkage of sugars. Changes in the relative activities of the various glycosyltransferases can alter oligosaccharide composition and structure (Schachter, 1978). Very little information is available concerning developmental changes in the composition and biosynthesis of mammalian tracheobronchial mucins.

Recently, we found that ferret tracheal epithelium and submucosal glands undergo dramatic growth and development over the first 28 postnatal days (Leigh et al., 1986a,b). Submucosal glands are rudimentary at birth and develop into complex tubuloacinar structures with abundant secretory granules by 28 days of age (Leigh et al., 1986b); therefore, glands are not important secretory structures during the early maturation period. At birth, secretory cells are the major cell type in the surface epithelium (Leigh et al., 1986b) but differ from goblet cells in that secretory granules are sparse. During the first 28 days of life, the secretory cells become less prevalent, gradually assume a typical goblet cell morphology, store secretory products with increasingly acidic-staining properties, and secrete more highly sulfated glycoconjugates (Leigh et al., 1986a,b). The purpose of this study was to determine if developmental sequences also include changes in the carbohydrate composition of mucins secreted by the postnatal ferret trachea and in the activities of relevant glycosyltransferases in this tissue.

Experimental Methods

Animals. Pregnant ferrets were obtained from Marshall Research Animals (North Rose, NY) at 30–34 days of gestation (normal gestation is 41 days), housed in steel cages, and fed dry cat food ad libitum. Newborn ferrets were studied within 12 h of delivery. Ferrets studied at 14 and 28 days of age were allowed to suckle undisturbed. Adult female ferrets, at least 8 months of age, were obtained directly from the supplier.

Tracheal Organ Culture. Tracheas were removed from ferrets under pentobarbital anesthesia, cut into strips, and placed in Medium 199 (GIBCO, Grand Island, NY) containing 0.2 μg/mL amphotericin B and 100 μg/mL gentamicin (Leigh et al., 1986a). Four microcuries of D-[6-^{3}H]glucosamine (specific activity 25–40 Ci/mmol) and 50 μCi of carrier-free [^{35}S]sulfate (ICN Biomedicals, Inc., Irvine, CA) were

† This work was supported by Grants HL 01519 and HL 19171 from the National Institutes of Health. In addition, M.W.L. was a recipient of the Edward Livingston Trudeau Scholar Award from the American Lung Association.

* Address correspondence to this author at the Departmant of Pediatrics, CB 7220, 635 Burnett-Womack Bldg., University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7220.

‡ Department of Pediatrics.

§ Department of Biochemistry.

0006-2960/89/0428-9440$01.50/0

added to each milliliter of culture medium to metabolically label the glycoconjugates and facilitate purification of mucins. Tissues were incubated at 35 °C in a water-saturated environment containing 5% CO_2 and 45% O_2. Culture media were changed at 24-h intervals for up to 6 days. Harvested media collected in several experiments were pooled according to age and frozen at −20 °C.

Isolation and Purification of Mucins. Pooled media were treated with 2-mercaptoethanol (125 mM, Sigma Chemical Co., St. Louis) and centrifuged at 500*g* for 5 min to remove cellular debris. Each sample was concentrated 10–20-fold on a Amicon Diaflo membrane with a molecular weight cutoff of 20 000 (Amicon Corp., Danvers, MA). The concentrated samples were dialyzed in deionized 8 M urea containing 5 mM dithiothreitol followed by alkylation with 15 mM iodoacetamide in 0.05 M Tris-HCl, pH 8.5 (Boat et al., 1976). Urea was removed by dialysis, and samples were then incubated for 5 h at 37 °C with 500 units/mL bovine testicular hyaluronidase (type VI-S, Sigma) and 400 U/mL bovine pancreatic deoxyribonuclease I (Sigma) in 0.1 M sodium acetate, pH 5.0, containing 3 mM $MgCl_2$ and 2 mM phenylmethanesulfonyl fluoride (Sigma). Samples were centrifuged at 20000*g* for 15 min to remove insoluble material prior to column chromatography on Sepharose CL-6B.

Fractions in the Sepharose CL-6B void volume, containing radiolabeled high molecular weight glycoconjugates, were pooled and concentrated (Amicon Diaflo membrane). Further purification of mucins was accomplished by cesium chloride density-gradient centrifugation (Starkey et al., 1974). Crystalline cesium chloride (Gallard-Schlesinger Industries) was added to each sample to attain a starting density of 1.58 g/mL. The samples were centrifuged at 130000*g* and 5 °C for 38–40 h. Fractions were removed sequentially from the top of each tube by a Densiflow pump (Hooke Buchler AutoDensi Flow IIC). The density of each fraction was determined from the refractive index. ^3H and ^{35}S were quantitated by scintillation counting with correction for quenching. For each sample, the fractions with densities less than 1.60 g/mL were combined and recentrifuged at a starting density of 1.48 g/mL by using the conditions described above. Fractions containing ^3H- and ^{35}S-labeled material in the density range 1.45–1.59 g/mL were pooled and extensively dialyzed against distilled water.

Compositional Analyses. Sialic acid was determined by the modified thiobarbituric acid assay (Aminoff, 1961). Neutral sugar content was determined by gas–liquid chromatography of the trimethylsilyl derivatives following methanolysis as described previously (Boat et al., 1974). Hexosamines were determined by high-performance liquid chromatography of the phenylisothiocarbamyl derivatives after hydrolysis in a 4 N HCl at 100 °C for 4 h (Cheng, 1987). Amino acid content was determined by high-pressure liquid chromatography of phenylisothiocarbamyl derivatives according to a procedure modified from that of Bidlingmeyer et al. (1984). Specific modifications include addition of 200 mM boric acid to sodium acetate buffer and the utilization of an internal standard, 6-aminohexanoic acid, which is eluted 1–2 min before tyrosine. Inclusion of boric acid improves separation of hexosamines from glutamic acid and serine. Between 200 and 300 μg of mucin are required for the compositional analyses described above.

β Elimination. The purified samples from newborn and adult ferret tracheas were treated with 0.05 M NaOH–1 M $NaBH_4$ at 45 °C for 18 h (Carlson, 1968). The solution was then acidified with 4 N acetic acid and evaporated under vacuum. Borate was removed by repeated additions of methanol followed by evaporation. Galactosaminitol in the treated samples was analyzed by HPLC (Cheng, 1987).

Blood Group Titers. Blood group titers (Boat et al., 1976) were measured with 2 hemagglutinating units of anti A serum (Hyland), anti B serum (Hyland), or H(O)-specific lectin from *Ulex europaeus* and a 1% suspension of human A, B, or O red blood cells. Serial 2-fold dilutions of the glycoconjugate samples were prepared from an initial concentration of 100 μg/mL.

Assay of Glycosyltransferase Activities. For each age group, tracheal epithelial scrapings were homogenized in ice-cold 0.25 M sucrose by successive forceful passage through 18-, 25-, and 27-gauge needles. The activities of each of the four glycosyltransferases described as follows were assayed by using a single homogenate pool for each age. (1) UDP-Gal:GalNAcαSer/Thrβ3 galactosyltransferase activity was assayed by using asialo ovine submaxillary mucin as acceptor (Cheng & Bona, 1982). The radiolabeled disaccharide removed from the galactosylated acceptor by alkaline borohydride treatment coeluted with Galβ1→3GalNAc-ol[1] by HPLC on an amino column without derivatization and on a C-8 column after perbenzoylation (Cheng & Bona, 1982; Cheng et al., 1985). β-Galactosidic linkage was confirmed by susceptibility of the product to bovine testicular β-galactosidase (Cheng & Bona, 1982). (2) UDP-GlcNAc: Galβ3GalNAcαSer/Thr(GlcNAc-GalNAc)β6 *N*-acetylglucosaminyltransferase activity was assayed by employing freezing-point depression glycoprotein as acceptor (Cheng et al., 1985). The radiolabeled trisaccharide eliminated from the N-acetylglucosaminylated acceptor coeluted with Galβ1–3-(GlcNAcβ6)GalNAc-ol on HPLC employing both an amino column without derivatization and a C-8 reverse-phase column after perbenzoylation (Cheng et al., 1985). The [^{14}C]GlcNAc was completely removed from the product by jack bean β-hexosaminidase, indicating a β-glycosidic linkage (Cheng et al., 1985). (3)GDP-Fuc:Galα2 fucosyltransferase was assayed by using freezing-point depression glycoprotein as acceptor (Cheng & DeVries, 1986). The radiolabeled trisaccharide released from the fucosylated acceptor coeluted with Fucα2Galβ3GalNAc-ol on HPLC using both an amino column without derivatization and a C-8 column after perbenzoylation (Cheng et al., 1985). The α-fucosyl linkage was established by the complete cleavage of [^{14}C]fucose from the trisaccharide with *Turbo cornutus* α-fucosidase (Miles Laboratory). (4) The procedure for measurement of CMP-NeuAc:GalNAcαSer/Thrα2→6 neuraminyltransferase activity was assayed by employing asialo ovine submaxillary mucin as acceptor (Cheng et al., 1980). The disaccharide removed from the acceptor coeluted with NeuAcα2→6GalNAc-ol on HPLC with an amino column under the conditions described by Bergh and van den Eijnden (1983). The [^{14}C]NeuAc was removed completely from the disaccharide by *Clostridium perfringens* neuraminidase, indicating an α-glycosidic linkage of neuraminic acid to GalNAc (Cheng et al., 1980).

Results

After reductive carboxymethylation and treatment with bovine testicular hyaluronidase, ^3H-labeled glycoconjugates in ferret tracheal secretions separate into three distinct peaks on Sepharose CL-6B: I, a void volume peak; II, an included

[1] Abbreviations: GalNAc-ol, *N*-acetylgalactosaminitol; TF, transferase.

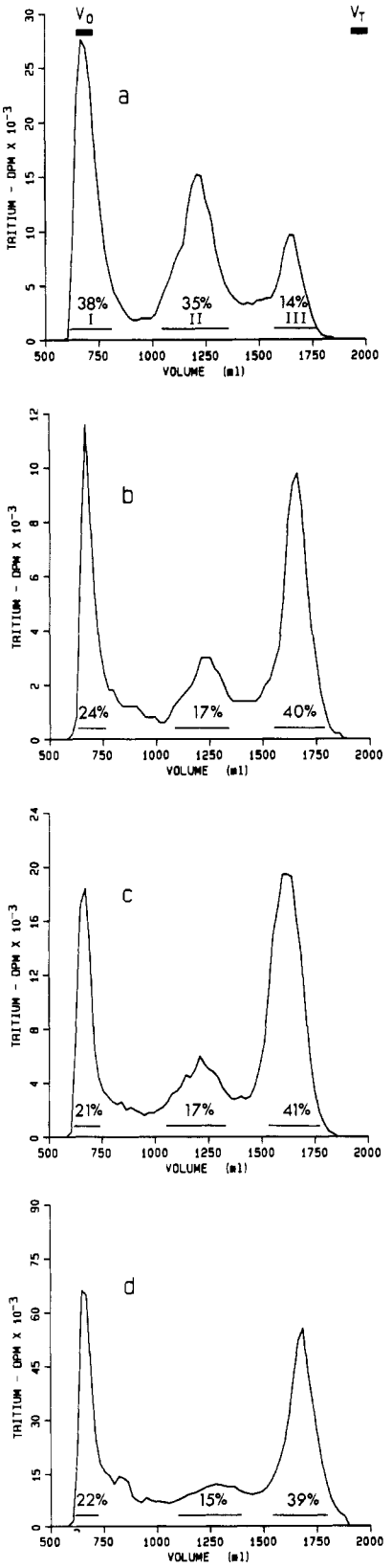

FIGURE 1: Sepharose CL-6B chromatography of ^{3}H-labeled tracheal glycoconjugate after treatment with bovine testicular hyaluronidase. The chromatograms for ^{3}H-labeled glycoconjugates from newborn, 14-day old, 28-day old, and adult ferrets are shown in parts a, b, c, and d, respectively. The column (5 × 98 cm) was eluted at a flow rate of 1 mL/min with 50 mM Tris-HCl, pH 7.0, containing 100 mM NaCl and 8 mM 2-mercaptoethanol. Fractions of 10.8 mL were collected and ^{3}H counts were measured in a 0.1-mL aliquot of every other fraction. V_0 (void volume) and V_T (total volume) were estimated by the elution of native bovine tracheal mucin and phenol red, respectively. The bars indicate the fractions combined for peaks I–III. Percentages refer to proportion of total ^{3}H counts in each peak.

Table I: Distribution (%) by Density of High Molecular Weight ^{3}H-Labeled Glycoconjugates Secreted by Tracheal Explants of Postnatal Ferrets

	buoyant density[a] (g/mL)	
age	<1.60	≥1.60
newborn	94.8	5.2
14 days	81.2	18.8
28 days	63.9	36.1
adult	41.1	58.9

[a] Cesium chloride density gradient centrifugation was performed with a starting density of 1.58 g/mL.

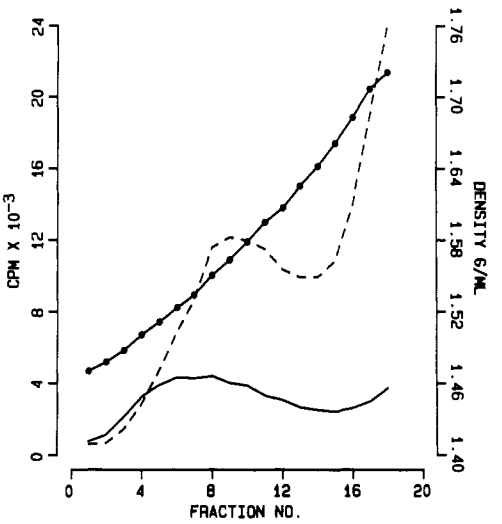

FIGURE 2: First CsCl density gradient centrifugation of glycoconjugates released by 28-day-old ferret tracheas in vitro. Glycoconjugates in peak I (Figure 1) were subjected to CsCl density gradient centrifugation with a starting density of 1.58 g/mL. ^{3}H counts (—), ^{35}S counts (---), and buoyant density (●) are shown for each fraction. The fractions with densities less than 1.60 g/mL were combined for further purification.

peak; and III, a near salt peak (Figure 1). The proportion of ^{3}H counts in peak I is largest in the newborn (38%) and is constant at other ages (21–24%). Conversely, the relative proportion of ^{3}H counts in peak III is low in the newborn (14%) but is nearly 3 times larger for the other ages (39–41%) (Figure 1). Because peak III is absent in samples not treated with hyaluronidase, this peak probably contains oligosaccharides from degradation of chondroitin sulfates A and C, dermatan sulfate, and/or hyaluronic acid (Meyer, 1971). The percentage of ^{3}H label in peak II remains relatively constant (15–17%) except in the newborn ferret (35%). The amino acid composition of peak II is not characteristic of mucins.[2] These age-related changes in the distribution of ^{3}H label occur largely in the first 2 weeks of life and suggest that the harvested secretions from tracheal explants of newborn ferrets contain a larger proportion of hyaluronidase-resistant high molecular weight glycoconjugates and a smaller proportion of hyaluronidase-susceptible glycoconjugates than those from older ferrets.

The fractions from the void peak at each age were pooled, concentrated, and subjected to successive cesium chloride density gradient centrifugations. By use of a starting density of 1.58 g/mL, high molecular weight glycoconjugates were separated into two fractions: one with buoyant densities less than 1.60 g/mL and the other with densities greater than or equal to 1.60 g/mL (Table I, Figure 2). The proportion of

[2] Manuscript in preparation.

Table II: Amino Acid and Carbohydrate Compositions of Hyaluronidase-Resistant, Low-Density Glycoconjugates Secreted by Ferret Tracheal Explants

	residues per 1000 amino acids			
	newborn	14 days	28 days	adult
amino acids				
Ser	215	217	246	240
Thr	190	172	192	183
Gly	129	136	115	112
Pro	95	67	91	85
Glu	89	88	86	84
Ala	50	46	51	47
Arg	37	36	28	27
Val	36	32	30	28
Met	19	37	24	29
Asp	19	21	37	45
His	24	32	26	26
Ile	31	33	19	19
Leu	21	20	17	17
Phe	13	21	11	15
Lys	13	18	12	11
Cys-cm[a]	13	14	7	16
Tyr	7	11	10	14
sugars				
galactose	1344	1249	1374	1076
GalNAc	730	649	713	782
GlcNAc	591	536	566	582
fucose	250	222	224	171
sialic acid[b]	138	167	310	314
mannose	≤6	<1[c]	<1	<1
glucuronic acid	<1[c]	<1	<1	<1
total sugars	3053	2823	3187	2925
sugar content (%) by wt (excluding sulfate)	85.0	83.9	85.9	84.9

[a] Cys-cm: (carboxymethyl)cysteine. [b] Sialic acid is calculated as *N*-acetylneuraminic acid. [c] The limit of sensitivity of gas–liquid chromatography.

^{3}H-labeled glycoconjugates with densities less than 1.60 g/mL decreased with age from 95% at birth to 41% in the adult. A similar distribution for ^{35}S-labeled glycoconjugates was observed (data not shown). All fractions with a buoyant density less than 1.60 g/mL were combined and subjected to a second CsCl density gradient run at a starting density of 1.48 g/mL. The profiles of this second CsCl density gradient centrifugation are shown in Figure 3. A single peak of radiolabeled glycoconjugates was observed at the densities between 1.45 and 1.58 g/mL at all ages except adult when this preparation contained an additional small amount (7% of the ^{3}H-labeled glycoconjugates in this density gradient profile) at a buoyant density of 1.37 g/mL. [^{35}S]Sulfate-labeled glycoconjugates were recovered at a slightly higher buoyant density than tritium-labeled glycoconjugates at each age. The density of the glycoconjugates from newborn ferrets (^{3}H peak density 1.49 g/mL and ^{35}S peak density 1.50 g/mL) is less than that from the more mature ferrets (^{3}H peak densities 1.51, 1.53, and 1.51 g/mL and ^{35}S peak densities 1.54, 1.56, and 1.54 g/mL for 14 days, 28 days, and adult, respectively). Even though quantitative comparisons are not possible (see Figure 3 legend), it is apparent that the relative [^{35}S]sulfate incorporation is less for glycoconjugates from newborns than for those from ferrets at older ages.

The glycoconjugates isolated within the buoyant density range 1.45–1.59 g/mL have similar amino acid compositions at all ages (Table II). Serine and threonine make up 39–44% of the total amino acids. The hydroxy amino acids plus glycine, proline, glutamic acid, and alanine constitute 73–78% of the total amino acids. The purified glycoconjugates contain galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose,

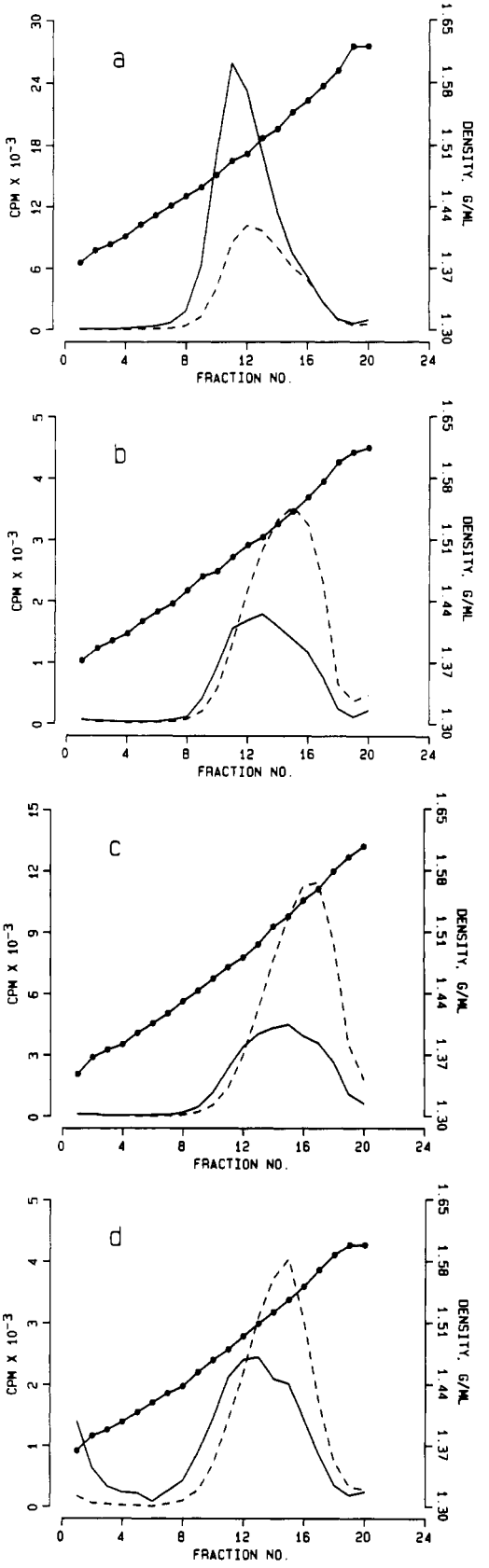

FIGURE 3: Second CsCl density gradient centrifugation of glycoconjugates released by newborn (a), 14-day-old (b), 28-day-old (c), and adult (d) ferret tracheal explants. Fractions with densities less than 1.60 g/mL from the first CsCl density gradient centrifugation (Figure 2) were pooled and subjected to a second centrifugation at a starting density of 1.48 g/mL. ^{3}H counts (—), ^{35}S counts (---), and buoyant density (●) are shown for each age. Relative amounts of ^{35}S and ^{3}H incorporation are not strictly comparable between age groups because secreted material from each age was pooled from several experiments performed at different times, precluding correction for ^{35}S decay.

Table III: Rate of Tracheal Mucin Secretion by Ferrets at Different Ages

age	secretory rate (μg/whole trachea·day)	tracheal surface area[a] (cm^2)	secretory rate ($\mu g/cm^2$ SA·day)
newborn	22.6	0.70 ± 0.02	32.2
14 days	33.4	2.0 ± 0.06	16.7
28 days	39.2	5.6 ± 0.13	7.0
adult	32.0	16.7 ± 0.15	1.9

[a] Tracheal surface area (SA) was measured in previous studies (Leigh, 1986a) and is presented as mean ± standard error of the mean.

and sialic acid, which account for 84–86% of the glycoconjugates by weight (excluding sulfate). The amounts of mannose and uronic acid are negligible. The distribution of individual sugars does vary with age. Galactose and fucose contents are relatively constant for the first 28 days and then decrease by 20% and 32%, respectively, by adulthood. Sialic acid content increases progressively until 28 days when its content is similar to that in the adult and more than twice that in the newborn. The hexosamine content does not vary appreciably with age.

After alkaline borohydride treatment, 47–48% of *N*-acetylgalactosamine in the hyaluronidase-resistant, low-density glycoconjugates is converted to *N*-acetylgalactosaminitol for both newborn and adult ferret tracheas. No glucosaminitol could be detected at either age after this treatment. Purified ferret tracheal hyaluronidase-resistant, low-density glycoconjugates inhibit blood group A hemagglutination but have no B or O (H) activity. The blood group A activity for the tracheal mucins decreases significantly with age: at 0, 14, and 28 days, the titer of a 100 μg/mL solution is 4096, but by adulthood, the titer is 1024. These characteristics of the hyaluronidase-resistant, low-density glycoconjugates, together with their compositional analysis, are consistent with the properties of mucins. SDS–polyacrylamide gel electrophoresis (3–12% gradient) of the isolated mucins was performed to further assess their purity. No contaminating proteins stainable with Coomassie blue or silver staining were observed for the mucins isolated from adult or 28- or 14-day-old ferret tracheas. In the newborn preparation, 2–3 faint protein bands with molecular weight less than 200 000 were detected with silver but not Coomassie blue staining. These contaminating proteins represented less than 0.2% of the material applied (100 μg).

Mucin secretory rates per whole trachea and per square centimeter of tracheal surface area for each age are shown in Table III. The secretory rates per whole trachea for all ages except newborn are relatively constant despite the large change in size of the tracheas. Mucin secretion per unit surface area of trachea is 17 times higher in the newborn then in the adult and progressively decreases with age.

The activities of the four glycosyltransferases measured in ferret tracheal epithelium as a function of postnatal ages are shown in Table IV. Except for CMP-NeuAc:GalNAc-Thr/Serα2→6 neuraminyltransferase, which maintains a relatively constant specific activity throughout maturation, the specific activities of the other three glycosyltransferases are highest in the newborn and decrease as the ferret tracheas mature. Both fucosyltransferase and *N*-acetylglucosaminyltransferase activities decrease more rapidly with age than galactosyltransferase activity. The relative enzyme activities for neuraminyl- to galactosyltransferase and for neuraminyl- to *N*-acetylglucosaminyltransferase demonstrate the carbohydrate chain termination enzyme (neuraminyltransferase) versus the chain elongation enzyme activities (Cheng & Bona,

Table IV: Developmental Changes of Glycosyltransferase Activities[a] in Tracheal Scrapings of Postnatal Ferrets

enzymes[b]	nmol of sugar transferred h^{-1} (mg of protein)$^{-1}$			
	newborn	14 days	28 days	adult
Gal TF	214.5	128.5	124.4	127.0
GlcNAcTF	20.3	6.3	7.3	2.5
Fuc TF	40.3	22.4	14.3	7.4
NeuAc TF	2.3	2.2	2.4	1.8
relative enzyme activities				
NeuAc TF/Gal TF	0.01	0.02	0.02	0.01
NeuAc TF/GlcNAc TF	0.11	0.35	0.33	0.72
Fuc TF/Gal TF	0.19	0.17	0.12	0.06

[a] Number of tracheas used to prepare the epithelial homogenates for these studies: 50, newborn; 30, 14 days; 15, 28 days; 10, adult. [b] The full names of the four glycosyltransferases are as follows: Gal TF, UDP-Gal: GalNAcαSer/Thrβ3 galactosyltransferase; GlcNAc TF, UDP-GlcNAc:Galβ3GalNAc(GlcNAc-GalNAc)β6 *N*-acetylglucosaminyltransferase; Fuc TF, GDP-Fuc:Galα2 fucosyltransferase; NeuAc TF, CMP-NeuAc:GalNAcαSer/Thrα2→6 neuraminyltransferase.

1982; Cheng et al., 1985) in the tracheal epithelium. Neuraminyltransferase activity relative to galactosyltransferase activity does not change appreciably with age; but, the ratio of neuraminyl- to *N*-acetylglucosaminyltransferase activity increases with age and is 6-fold greater in adult than in newborn epithelium. The ratio of fucosyl- to galactosyltransferase is indicative of how readily fucose is transferred to the newly formed β-galactoside to generate the blood group H determinant (Cheng & DeVries, 1986). Fucosyltransferase activity relative to galactosyltransferase activity is highest in newborns.

Discussion

The ferret is a particularly useful model for studying secretion of macromolecules by tracheobronchial epithelium. Like human airways but unlike the airways of most small laboratory animals, the ferret airways contain mucus-secreting cells in both the surface epithelium and the submucosal glands. We have previously reported (Leigh et al., 1986a) that ferret tracheal explants secreted high molecular weight glycoconjugates that may be classified by their susceptibility or resistance to bovine testicular hyaluronidase degradation. In this study, the high molecular weight glycoconjugates are further classified on the basis of buoyant density in CsCl: (1) hyaluronidase-susceptible; (2) hyaluronidase-resistant, high-density ($\geq$1.60 g/mL); and (3) hyaluronidase-resistant, low-density (1.45–1.58 g/mL). The hyaluronidase-resistant, low-density glycoconjugates secreted by explants of ferrets at all ages (newborn to adult) are mucins on the basis of their compositional analysis, blood group activity, and presence of O-glycosidic linkage of oligosaccharide to peptide core. The peak buoyant density and the composition of ferret tracheal mucins are similar, with minor exceptions, to those reported for tracheobronchial mucins from other sources (Boat et al., 1976; Creeth et al., 1977; Bhaskar & Reid, 1981; Woodward et al., 1982; Ringler et al., 1987; Rose et al., 1979, 1987; Feldhoff et al., 1979; Sachdev et al., 1980; Lamblin et al., 1979). For example, serine and threonine are the two most abundant amino acids in respiratory mucins, accounting for 21–46% of total amino acids in the studies cited above and 39–44% for the ferret mucins in this study. For some mucins, the serine content is greater than or closely approximates the threonine content (Ringler et al., 1987) as we observed in the ferret, but in most other airway mucins, threonine content exceeds that of serine.

The proportion of secreted high molecular weight glycoconjugates that are mucins decreases with age. Of the total

^{3}H-labeled glycoconjugates in the harvested media of newborn ferret tracheas, 38% are in the Sepharose CL-6B peak I, of which 95% have a buoyant density of 1.45–1.58 g/mL; therefore, 36% of the total newborn ^{3}H-labeled glycoconjugates are mucins. For adult ferret tracheas, 22% of ^{3}H-labeled glycoconjugates are in peak I, and 34% of these have a buoyant density of 1.45–1.58 g/mL (correcting for the 7% with a density less than 1.45 g/mL); therefore, only 7.5% of total adult ^{3}H-labeled glycoconjugates are typical mucins. In a previous study, we showed that the rate of release of radiolabeled hyaluronidase-resistant glycoconjugates per square centimeter of tracheal surface area is 6-fold greater for newborn than for adult ferret tracheas (Leigh et al., 1986a). On the basis of the age-related differences in mucin yield after cesium chloride density gradient centrifugation, the rate of mucin secretion in that experiment would be 17-fold greater for the newborn than for the adult ferret tracheas, consistent with the results of this study. From these two studies, we conclude that tracheas from newborn ferrets are more active as organs of mucin secretion and that mucins are the predominant secretory product early in life. Because newborn ferret tracheas have only primordial submucosal glands without secretory granules (Leigh et al., 1986b), their mucins must be released by the secretory cells in the surface epithelium. Tracheas from older ferrets release smaller amounts of glycoconjugates per surface area, and these glycoconjugates are largely glycosaminoglycans and, as yet uncharacterized, hyaluronidase-resistant, high-density ($\rho \geq 1.60$ g/mL) glycoconjugates. Canine and normal human tracheal explants also release a large proportion of high-density glycoconjugates (Bhaskar et al., 1986). Because the mucins secreted by tracheal explants of mature ferrets constitute such a small proportion of the total high molecular weight glycoconjugates, studies of modulation of airway secretions may require a density gradient centrifugation step following gel filtration to identify a mucin-specific effect.

The amino acid composition of ferret tracheal mucins does not appear to vary appreciably with age, suggesting that the peptide core changes little, if any, during maturation. Some of the amino acid contents vary slightly between age groups, but there are no progressive, age-related trends for these changes. We cannot rule out the possibility that some of the minor differences in amino acid and sugar contents may reflect secretion in different proportions of several tracheal mucin species, as proposed by Podolsky for colonic mucins (Podolsky et al., 1986). We predict that the number of sugar chains arising from the peptide core varies little with age on the basis of the essentially constant number of *N*-acetylgalactosamine and serine plus threonine residues per 1000 amino acids and a similar percentage of total *N*-acetylgalactosamine identified as the link sugar. The age-related increase in sialic acid content is consistent with our previous study showing that the secretory granules of the cells in the surface epithelium acquire more acidic histochemical staining properties with age (Leigh et al., 1986b). Studies of human airways, although not systematic, have suggested a similar age-related change in the histochemical staining properties of secretory tissues and the sialic acid content of secreted mucins (Bucher & Reid, 1961; Lamb & Reid, 1972; Boat et al., 1977). Previously, we reported that the $^{35}SO_4/^3H$ ratios of mucins metabolically labeled and released by ferret tracheal explants increased more than 2-fold from newborn to adult ages (Leigh et al., 1986a). Because the sum of hexosamines and neuraminic acid, the sugars into which ^{3}H is incorporated, is relatively constant with age (1352–1589 residues/1000 amino acid residues), the increase in the $^{35}SO_4/^3H$ ratios should represent increased sulfation of mucins. Therefore, increased sulfation as well as increased sialic acid content provides a greater charge density for the glycosylated region of mucins secreted at the older ages.

The four glycosyltransferases assayed in this study were chosen because they were involved in the key steps for mucin-type oligosaccharide chain synthesis and therefore are likely to be found in greater abundance in secretory cells. The size and prevalence of secretory cells in the surface epithelium of ferret tracheas decrease with age (Leigh et al., 1986b; Carson et al., 1988). Therefore, our observation of age-related decreases in the activities of several glycosyltransferases (Gal TF, GlcNAc TF, and Fuc TF) most likely reflects a diminishing number of secretory cells in the tracheal epithelium. Sialyltransferase activity remains constant during this period, suggesting that the activity of sialytransferase per secretory cell increases with age, consistent with the observed increase of sialic acid incorporation into mucins over this developmental period.

A strict correlation of age-related changes in the activities of glycosyltransferases and the contents of respective sugars in secreted mucin is not expected, in part because only selected glycosyltransferases were examined. For example, at least four different β-galactosyltransferases may be involved in the synthesis of tracheal mucins (Cheng & Bona, 1982; Sheares & Carlson, 1983, 1984), but only GalNAc:β3 galactosyltransferase was assayed. Differences in the relative activities of the specific glycosyltransferases that utilize the same acceptor may influence the structure as well as the composition of the mucin oligosaccharide chains that are generated. We propose that the α2→6 neuraminyltransferase is a key biosynthetic enzyme in the developing ferret tracheal secretory cells. This enzyme catalyzes the formation of NeuAcα2→6GalNAcαSer/Thr and prevents the synthesis of Galβ1→3GalNAcαSer/Thr (Beyer et al., 1979; Carlson et al., 1973), a structure needed for further elongation of oligosaccharide chains. Even after Galβ1→3GalNAc is formed, incorporation of NeuAc to C-6 of GalNAc precludes the synthesis of the Galβ1→3(GlcNAcβ6)GalNAc core structure, which is required for the synthesis of Galβ1→3(Galβ3/4GlcNAcβ6)-GalNAc. Both β-galactosides of this oligosaccharide are potential acceptors for α1→2 fucosyltransferase. Therefore, the diminished fucose content of adult tracheal mucins may be due to a decreased synthesis of fucose acceptor structures as well as lower levels of fucosyltransferase relative to galactosyltransferase activity. Furthermore, when fucosylation is limited, fewer precursors are available for the synthesis of the blood group A determinant [GalNAcα1→3(Fucα1→2)-Gal]. Thus, while the glycosyltransferase activities (Table III) shift in the direction predicted by the observed changes in carbohydrate structures, we suggest that the relatively greater neuraminyltransferase activity in more mature tracheas may be a major factor responsible for age-related changes in mucin carbohydrate structure. Structural analyses of ferret mucins at different ages are needed to support the hypothesis but are beyond the scope of this study. Consistent with the notion that shifts in enzyme activity result in changes in carbohydrate structure, Piller et al. (1988) recently reported that activation of human T-lymphocytes is associated with a change in the O-glycosidic carbohydrate core structure of a sialoglycoprotein from Gal1→3(NeuAcα2→6)GalNAc to Galβ1→3-(GlcNAcβ1→6)GalNAc, apparently caused by a decrease in α2→6 neuraminyltransferase activity and a marked increase in β1→6 *N*-acetylglucosaminyltransferase activity (Piller et al., 1988).

We have demonstrated developmental changes in the rate of tracheal mucin secretion as well as carbohydrate composition of mucins and glycosyltransferase activities from ferret tracheal epithelium. Studies of mucins and glycosyltransferase activities in rat intestinal mucosa (Shub et al., 1983; Biol et al., 1987) suggest different patterns of age-related changes. The specific activities of *N*-acetylgalactosaminyltransferase and fucosyltransferase are lower in intestinal mucosa of newborn rats than in adult rats (Biol et al., 1987), which could explain the lower fucose and *N*-acetylgalactosamine contents of their intestinal mucins (Shub et al., 1983). These studies support the hypothesis that one of the cellular bases for developmental changes in the mucin carbohydrate moiety is a shift of the relative activities of the glycosyltransferases as a function of age. The selective advantages of these developmental changes in the glycosylation of the mucins are not known. Conceivably, these changes influence the protective functions of airway mucus such as its interaction with influenza virus (Boat et al., 1976, 1978) and bacteria (Krivan et al., 1988) and the effectiveness of its clearance by the mucociliary system (Mian et al., 1982).

Acknowledgments

We thank Aruna Kumar and Patricia Ropp for expert technical assistance.

Registry No. Gal TF, 81876-97-3; GlcNAc TF, 96697-64-2; Fuc TF, 39434-10-1; NeuAc TF, 71124-50-0.

References

Aminoff, D. (1961) *Biochem. J. 81*, 384–392.

Bergh, M. L. E., & van den Eijnden, D. H. (1983) *Eur. J. Biochem. 136*, 113–118.

Beyer, T. A., Rearick, J. I., Paulson, J. C., Prieels, J. P., Sadler, J. E., & Hill, R. L. (1979) *J. Biol. Chem. 254*, 12531–12541.

Bhaskar, K. R., & Reid, L. (1981) *J. Biol. Chem. 256*, 7583–7589.

Bhasker, K. R., O'Sullivan, D. D., Opaskar-Hincman, H., Reid, L. M., & Coles, S. J. (1986) *Exp. Lung Res. 10*, 401–422.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Biol, M. C., Martin, A., Richard, M., & Louisot, P. (1987) *Pediatr. Res. 22*, 250–256.

Boat, T. F., & Cheng, P.-W. (1978) in *Glycoproteins and Glycolipids in Disease Processes* (Walborg, E. F., Ed.) ACS Symposium Series 80, pp 108–121, American Chemical Society, Washington, DC.

Boat, T. F., Kleinerman, J. I., Carlson, D. M., Maloney, W. H., & Matthews, L. W. (1974) *Am. Rev. Respir. Dis. 110*, 428–441.

Boat, T. F., Cheng, P. W., Iyer, R. N., Carlson, D. M., & Polony, I. (1976) *Arch. Biochem. Biophys. 177*, 95–104.

Boat, T. F., Kleinerman, J. I., Fanaroff, A. A., & Stern, R. C. (1977) *Pediatr. Res. 11*, 977–980.

Boat, T. F., Davis, J., Stern, R. C., & Cheng, P.-W. (1978) *Biochim. Biophys. Acta 540*, 127–133.

Bucher, U., & Reid, L. (1961) *Thorax 16*, 219–225.

Carlson, D. M. (1968) *J. Biol. Chem. 243*, 616–626.

Carlson, D. M., McGuire, E. J., Jourdian, G. W., & Roseman, S. (1973) *J. Biol. Chem. 248*, 5763–5773.

Carson, J. L., Collier, A. M., Gambling, T. M., Leigh, M. W., Boucher, R. C., Hu, S. S., & Boat, T. F. (1988) *Am. Rev. Respir. Dis. 138*, 666–674.

Cheng, P.-W. (1987) *Anal. Biochem. 167*, 265–269.

Cheng, P.-W., & Bona, S. J. (1982) *J. Biol. Chem. 257*, 6251–6258.

Cheng, P.-W., & DeVries, A. (1986) *Carbohydr. Res. 149*, 253–261.

Cheng, P.-W., Moeller, S. L., & Boat, T. F. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2002.

Cheng, P.-W., Wingert, W. E., Little, M. R., & Wei, R. (1985) *Biochem. J. 227*, 405–412.

Creeth, J. M., Bhaskar, K. R., Horton, J. R., Das, I., Lopez-Vidriero, M.-T., & Reid, L. (1977) *Biochem. J. 167*, 557–569.

Feldhoff, P. A., Bhavanandan, V. P., & Davidson, E. A. (1979) *Biochemistry 18*, 2430–2436.

Krivan, H. C., Roberts, D. D., & Ginsburg, V. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6157–6161.

Lamb, D., & Reid, L. (1972) *Br. J. Dis. Chest 66*, 219–225.

Lamblin, G., Lhermitte, M., Degand, P., Roussel, P., & Slayter, H. S. (1979) *Biochimie 61*, 23–43.

Leigh, M. W., Cheng, P.-W., Carson, J. L., & Boat, T. F. (1986a) *Am. Rev. Respir. Dis. 134*, 784–790.

Leigh, M. W., Gambling, T. M., Carson, J. L., Collier, A. M., Wood, R. E., & Boat, T. F. (1986b) *Exp. Lung Res. 10*, 153–169.

Meyer, K. (1971) *Enzymes (3rd Ed.) 5*, 307–320.

Mian, N., Pope, A. J., Anderson, C. E., & Kent, P. W. (1982) *Biochim. Biophys. Acta 717*, 41–48.

Piller, F., Piller, V., Fox, R. I., & Fukuda, M. (1988) *J. Biol. Chem. 263*, 15146–15150.

Podolsky, D. K., Fournier, D. A., & Lynch, K. E. (1986) *J. Clin. Invest. 77*, 1251–1262.

Ringler, N. J., Selvakumar, R., Woodward, H. D., Simet, I. M., Bhavanandan, V. P., & Davidson, E. A. (1987) *Biochemistry 26*, 5322–5328.

Rose, M. C., Lynn, W. S., & Kaufman, B. (1979) *Biochemistry 18*, 4030–4037.

Rose, M. C., Brown, C. F., Jacoby, J. Z., Lynn, W. S., & Kaufman, B. (1987) *Pediatr. Res. 22*, 545–551.

Sachdev, G. P., Myers, F. S., Horton, F. O., Fox, O. F., Wen, G., Rogers, R. M., & Carubelli, R. (1980) *Biochem. Med. 24*, 82–94.

Schacter, H. (1978) in *The Glycoconjugates* (Horowitz, M. I., & Pigman, E., Eds.) Vol. 2, pp 87–181, Academic, New York.

Sheares, B. T., & Carlson, D. M. (1983) *J. Biol. Chem. 258*, 9893–9898.

Sheares, B. T., & Carlson, D. M. (1984) *J. Biol. Chem. 259*, 8045–8047.

Shub, M. D., Pang, K. Y., Swann, D. A., & Walker, W. A. (1983) *Biochem. J. 215*, 405–411.

Starkey, B. J., Snary, D., & Allen, A. (1974) *Biochem. J. 141*, 633–639.

Woodward, H., Horsey, B., Bhavanandan, V. P., & Davidson, E. A. (1982) *Biochemistry 21*, 694–701.