Polydispersity of Normal Human Conjunctival Mucins

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Purpose. To isolate all constituent mucins from human conjunctival mucus.

Methods. Mucins were extracted from human conjunctiva in guanidine hydrochloride and protease inhibitors. The mucins were isolated by density gradient centrifugation, gel filtration, and ion exchange chromatography. Throughout purification, the mucin profile was monitored by agarose electrophoresis and vacuum blotting. Blots were probed for peptide and carbohydrate epitopes. The latter included 1E3 and TKH2 specific for Tn and sialyl-Tn, respectively, considered tumor-related antigens. In vivo impression cytology specimens of normal conjunctival goblet cells also were probed with the same reagents. Oligosaccharides were released from isolated mucins by alkaline β-elimination and then size fractionated.

Results. Human conjunctival mucins consist of at least three size populations; the largest is excluded on Sepharose CL2B. The two largest populations are polydisperse. Their overall electrophoretic pattern is conserved between individuals. Similar charge distributions were detected in different buoyant density ranges from the density gradient centrifugation: a less charged population containing three components and a highly charged population with two components on agarose electrophoresis. Cross-reaction with 1E3 and TKH2 was detected throughout purification in the largest mucins, which were presumably mature, and in impression cytology. Oligosaccharides from mucins in each buoyant density were largely in the monosaccharide and disaccharide range, consistent with Tn and sialyl-Tn standards.

Conclusions. Secreted human conjunctival mucins are polydisperse, with discrete components appearing consistently in pooled and individual samples. They have a unique oligosaccharide pattern containing Tn and sialyl-Tn. This indicates normal roles in normal human ocular mucins for these antigens, which are disease markers in other tissues. Invest Ophthalmol Vis Sci. 1996; 37:2559-2571.

Mucins constitute an important part of the precorneal tear film. Secreted and membrane-bound mucins are produced by epithelial squamous cells of the conjunctiva and cornea and by goblet cells in the conjunctiva.1-4 The mucous gel laid down by these cells functions to lubricate and cushion the ocular surface and to protect against and trap particles, microorganisms, and toxins.5,6 The structural features specific to ocular mucins that impart these properties have not been elucidated. Recent evidence suggests that the precorneal tear film is 35 to 45 μm thick and that the majority of its thickness is made up by the mucin-containing gel.7 This has revived interest in the nature and function of ocular surface mucins.

Secreted mucins from other mucosal surfaces show a number of characteristic properties: very high molecular weights in the 2,000 to 40,000 kDa range, subunits of 300 to 2,000 kDa8 linked through disulfide bridges, and gel formation at 0.5% to 1% concentrations.9 Oligosaccharide side chains typically comprise 60% to 80% of the dry weight of these molecules. These side chains vary in length and branching, and their charges range from neutral to highly acidic. Glycosylation patterns are tissue specific and disease specific.11 Colonic mucins have predominantly extended and branched oligosaccharide chains with more than eight sugar residues.12 In contrast, saliva contains a large mucin with long oligosaccharide chains and a small mucin with as many as five sugar residues in its oligosaccharide side chains.13 Among the cancer-re-
TABLE 1. Mucin Detection Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Specification</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Periodic acid-Schiff</td>
<td>Vicinal glycol groups</td>
<td>General reagent for glycosylated material</td>
</tr>
<tr>
<td>Wheat germ agglutinin</td>
<td>Sialic acids, GlcNAc</td>
<td>Not ascribed to any mucin gene</td>
</tr>
<tr>
<td>Antibody aM1</td>
<td>Peptide core of gastric mucin</td>
<td>Epitopes have common identity with other mucins</td>
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<tr>
<td>1E3</td>
<td>Tn epitope</td>
<td>Ser/Thr-GalNAc</td>
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<tr>
<td>TKH2</td>
<td>Sialyl Tn epitope</td>
<td>Sialyl-α2-6 GalNAc-Ser/Thr</td>
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MUCIN REAGENTS

Reagents used to identify mucin-containing fractions at every stage of isolation and purification and mucins in impression cytology are described in Table 1. They were periodic acid-Schiff (Sigma), 0.4 mg/ml horseradish peroxidase-linked wheat germ agglutinin (WGA; Vector Labs, Peterborough, UK), or antibody against the peptide core of human gastric mucin (aM1, a gift from Dr. J. Bara, Paris, France), against Tn or against sialyl-Tn epitopes (1E3 and TKH2, a gift from S-i Hakomori, Seattle, WA). Antibodies were used at a dilution of 1:100 to 1:1000 tissue culture supernatant. Cross-reaction with these reagents was followed in dot blots or vacuum blots on polyvinylidene difluoride membrane (Millipore, Watford, UK). Horseradish peroxidase conjugates were visualized using diaminobenzidine (Sigma).

Mucin Purification

CsCl Density Gradient Centrifugation. Buoyant density is a result of molecular composition and architecture and can be used to isolate lipid-containing compounds, proteins, mucins, or nucleic acids. Carlstedt et al21 have shown good separation of mucins from other glycoproteins and nucleic acids after centrifugation on CsCl gradients. Homogenates were brought to a density between 1.3 and 1.4 g/ml with cesium chloride and centrifuged at 150,000 g in 13.5 ml sealed tubes on a Beckman (Palo Alto, CA) 70 Ti rotor for 24 hours at 10°C. The density of 0.5 ml fractions was established by weighing. Fractions were pooled in the isopycnic density ranges 1.3 to 1.35, 1.35 to 1.4, 1.4 to 1.45, 1.45 to 1.5, and 1.5 to 1.55 g/ml and then were desalted by either dialysis against Tris–HCl buffer or through a Sephadex G10 (Sigma) 20 cm × 2 cm column.

Size Fractionation–Gel Filtration. Density gradient centrifugation does not separate by molecular mass. Mature mucins and their subunits or degradation products may have the same buoyant density. To separate different molecular sizes, each isopycnic pool was fractionated further by gel filtration on a Sepharose CL2B (Phar-
FIGURE 1. Mucin-like material in CsCl gradients. Dot blots of 20 μl aliquots from each density gradient were probed with (A) WGA; (B) aM1; (C) 1E3; (D) TKH2 as described in Methods. The blots were scanned with a flatbed scanner. Density plots were obtained with Scan Analysis 2.50 (Biosoft, Cambridge, UK). Horizontal axes represent the buoyant density of the fractions (g/ml) from calculated regression lines for each individual gradient (StatView 4.5; Abacus Concepts, Berkeley, CA). Vertical axes represent the intensity of labeling in arbitrary units, normalized to 100% for each individual. Nine individual gradients are shown for both sexes, age range 17 to 78 years.

Preparation of Mucin Subunits. Most mature mucin molecules are built of disulfide-linked subunits. Analysis of these subunits provides information on mucin species diversity and modes of aggregation. Subunits were prepared from multidonor samples of known buoyant density and hydrodynamic volume. Aliquots were suspended in 6 M guanidine HCl in 0.1 M Tris-acetate, pH 8, reduced with 10 mM dithiothreitol (Sigma) for 5 hours at 37°C and alkylated with 25 mM iodoacetamide (Sigma) at room temperature for 14 hours in the dark.

Radiolabeled subunits were obtained from aliquots of individual V₀ fractions by reduction as above. After a 4-hour incubation in the dark at room temperature with ¹⁴C-iodoacetamide (1.85 MBq, 2.22 GBq/mmol; Amersham, Little Chalfont, UK), the alkylation was brought to completion with 25 mM iodoacetamide for an additional 10 hours. Equivalent aliquots were alkylated with ¹⁴C-iodoacetamide without prior reduction to assess background incorporation. Mucins were precipitated with 95% ethanol with 1% sodium acetate at −70°C and then dialyzed against a buffer containing 10 mM piperazine (Sigma), 6 M urea (Sigma), and 1% CHAPS, pH 8.
Analysis of Purified Native Mucins and Subunits

Ion Exchange Chromatography. Mucins or subunits with different charges can be separated using ion exchange chromatography. Charge is conferred largely by the carbohydrate side chains. Sample size dictated that we analyze radioalkylated subunits of individual mucins. The fractionation of mucins by ion-exchange chromatography on mono-Q resin has been optimized for separation and recovery using lithium perchlorate gradients. A 2 ml column of MonoQ ion exchanger (Pharmacia) was equilibrated in a buffer containing 10 mM piperazine, 6 M urea, and 1% CHAPS. Dialyzed radiolabeled mucin samples were applied to the column, and the eluting volume was reapplied until its radioactive counts were at background levels. The column was washed with buffer, and a linear gradient of 0 to 0.4 M lithium perchlorate (Sigma) was applied and collected in sixty 250 µl fractions. A final wash with buffer containing 0.8 M lithium perchlorate was collected in an additional twenty 250 µl fractions. The lithium perchlorate gradient was monitored using the electrical conductivity of the eluant with and without sample loading.

Agarose Gel Electrophoresis. Migration on agarose is a result of mass and charge relationship. After each chromatographic separation, agarose gel electrophoresis provides a measure of purity of the separated material or of dispersity resulting from other factors. Agarose gel electrophoresis was performed on native mucins, reduced and alkylated mucin subunits, and (nonreduced) alkylated samples. Electrophoresis was performed on 1% agarose gels in 40 mM Tris acetate, 1 mM ethylenediaminetetraacetic acid, pH 8, containing 0.1% (wt/vol) sodium dodecyl sulfate, at room temperature for 18 hours at 20 V. Transfer onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) was achieved under vacuum (40 cm H₂O for 120 minutes) with 3 M sodium chloride, 300 mM sodium citrate, pH 7 (20 × standard sodium citrate buffer). After incubation of the blot with 30% metha-
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FIGURE 4. Vacuum blots of electrophoretic profiles on 1% agarose gels: native and reduced and alkylated mucins. Samples were separated by agarose gel electrophoresis and vacuum blotted onto polyvinylidene difluoride membranes, as described in Methods, and were visualized with WGA and aM1 as indicated. Polydisperse material is clearly seen in both V4 and V5. Note the similarity between individual and multidonor samples, as well as the characteristic pattern in each buoyant density range. (a) Multidonor sample V4; lanes labeled with sample buoyant density. (b) Multidonor sample V5; lanes labeled as in a. (c) Individual sample V4; individuals labeled il-il4, buoyant density 1.3 to 1.4 g/ml. (d) Individual sample V5; labeling and density as in c. N = native; R = reduced and alkylated.

Analysis of Oligosaccharide Chains on Purified Mucins. Glycosylation of mucin is tissue specific. It is expected that carbohydrate side chains, which occupy most of the volume of the mucin molecule, contain physiologically important epitopes. Oligosaccharide chains were released from an individual sample of conjunctival mucin, in three buoyant density ranges, by β-elimination as described previously.27 Samples were dissolved in 1 ml of 50 mM sodium hydroxide. They were incubated for 6 hours at 45°C with sodium boro-[3H]-hydride (37 GBq, 307 GBq/mmol; Amersham) before adding cold borohydride for 12 hours to complete the β-elimination. Unreacted sodium boro-[3H]-hydride was removed (released as tritium) in a radioactive fume hood by adding glacial acetic acid. The solution was passed through Dowex 50 H+ (50 to 100 mesh) before rotary evaporation to dryness at 35°C. To re-
FIGURE 5. Vacuum blots of electrophoretic profiles on 1% agarose gels: cross-reaction with WGA, 1E3, TKH2, and aM1. Sepharose CL2B V1 and V2 fractions from the buoyant density range from 1.35 to 1.45 g/ml were compared on agarose gel electrophoresis. Vacuum blots were probed with WGA, 1E3, TKH2, and aM1, as indicated. Both fractions were polydisperse, with characteristic electrophoretic profiles. Glycosylated material (WGA positive) had a larger range of mobilities than mucins cross-reacting with any antibody. Reduction and alkylation results in loss of antibody cross-reactivity. N = native; R = reduced and alkylated.

move borate, the evaporation was repeated five times, redissolving each time in methanol, and finally dissolving in 1 ml of 0.1 M pyridine acetate, pH 5.

A Biogel P4 (200 to 400 mesh; Biorad, Hemel Hempstead, UK) column 90 cm × 1 cm equilibrated in 0.1 M pyridine acetate buffer, pH 5, was used to separate the oligosaccharide chains. Eighty 1 ml samples were collected, and the radioactive profile of elution was established. For calibration lactose (Sigma), stachyose (Merck, Poole, UK) N-acetylglactosaminitol (Sigma), and sialyl-N-acetylglactosaminitol prepared from ovine submaxillary mucin were eluted from the column in conditions identical to the mucin samples. Detection of standard oligosaccharides was achieved by phenol sulfuic acid reaction for hexoses and orcinol-iron (III) chloride–hydrochloric acid assay of total sialic acid.

Impression Cytology

To eliminate spurious results caused by biases during sampling or sample concentration, we sought in vivo confirmation of each biochemical finding during mucin isolation and purification. Investigations on healthy volunteers followed the tenets of the Declaration of Helsinki and were approved by the local ethics committee. The volunteers, staff members at the Bristol Eye Hospital, are men and women ranging in age from 22 to 55 years. The bulbar conjunctiva was imprinted using pure nitrocellulose filters (0.45 μm, TransBlot; BioRad) and minimal pressure. Each membrane was removed from the conjunctiva after several seconds and was pressed, cell side down, onto a poly-L-lysine-coated glass slide. Membranes were later dissolved in acetone, endogenous peroxidase inhibited with 90% methanol 1% H2O2, and nonspecific binding blocked with 1% bovine serum albumin in phosphate-buffered saline overnight, before they underwent immunohistochemistry.

RESULTS

Mucin Purification

Membrane-attached mucins were removed from our samples during CsCl density gradient centrifugation. The samples include mucin synthesized and stored in the conjunctiva before secretion.

CsCl Density Gradient Centrifugation. CsCl density gradients of multidonor and individual samples contained mucinlike material in the isopycnic range, 1.3 to 1.55 g/ml, as shown by cross-reaction with carbohydrate reagents and anti-mucin antibodies (Fig. 1). This range of buoyant densities is characteristic of mucins. Material cross-reacting with specific reagents is glycosylated, as demonstrated by binding WGA; Tn epitopes (labeled by 1E3) are predominant in the higher density range, 1.4 to 1.5 g/ml, whereas sialyl-Tn (labeled by TKH2) is present (in three less well-defined peaks) across the whole range.

Size Fractionation—Gel Filtration. Mucinlike material from the density gradients was pooled for further analysis. Size fractionation of a multidonor sample is shown in Figure 2. The material eluted in the excluded fraction (MWt > 106, V0) is WGA, aM1, TKH2, and 1E3 positive in each buoyant density range. A second peak (V1) also positive for all the above reagents, occurs early in the included volume, and a
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FIGURE 6. Charge-fractionated $V_o$ mucins: radioactive profiles of MonoQ elution. Reduced and $^{14}$C-radioalkylated subunits from two individual samples were analyzed for charge distribution by ion-exchange chromatography on MonoQ. Mature mucins were obtained by density gradient centrifugation and separation of $V_o$ by Sepharose CL2B gel filtration. Radioalkylation permits sensitive detection of mucins in the eluent and on vacuum blots. Lithium perchlorate concentration (thin lines) was calculated from eluent conductivity in a sample-free run. Specific radioactivity is shown in thick lines, (a) 95-year-old woman, buoyant density 1.3 to 1.35 g/ml, reduced and alkylated (b) 95-year-old woman, buoyant density 1.3 to 1.35 g/ml, (native) alkylated (c) 67-year-old man, buoyant density 1.35 to 1.4 g/ml, reduced and alkylated (d) 67-year-old man, buoyant density 1.35 to 1.4 g/ml, (native) alkylated.

third peak, in the retarded volume ($V_r$) elutes beyond the lower resolution limit of the column. The retarded fraction has not been analyzed further. Thus, in every buoyant density range, there is a mixture of molecules of different hydrodynamic volume. Individual samples produce similar patterns (results not shown).

Reduction and alkylation with $^{14}$C-iodoacetamide of the largest mucins permitted tracing of radioactive subunits on a second gel filtration on Sepharose CL2B. The size of $V_o$ subunits was not decreased enough to allow separation by this gel. $V_o$ subunits eluted in $V_o$ (Fig. 3A) and are distinct from the mucins that eluted in $V_i$. The second peak observed in the radioactive profile did not bind WGA (Fig. 3B); therefore, it does not contain glycosylated material resulting from the reduction of disulfide bonds. It is likely to contain unreacted $^{14}$C-iodoacetamide.

The electrophoretic patterns of size-fractionated mucinlike material indicate polydispersity. Various mobilities were observed in native and (nonradioactive) reduced-alkylated mucins belonging to either $V_o$ (Fig. 4a) or $V_i$ (Fig. 4b). All samples migrated more slowly than immunoglobulin M, which was included to assist comparison between blots. Individual and multidonor sample patterns were similar to each other (Figs. 4c, 4d). The mobilities of $V_o$ fractions were similar across the whole buoyant density range analyzed and were not altered significantly by reduction–alkylation. Native $V_i$ fractions showed a consistent pattern across all densities, but reduction and alkylation resulted in increased mobility of a slow component and, thus, a more banded pattern. Mucins in $V_i$ have higher mobilities than the $V_o$ mucins in the same isopycnic range, consistent with elution position on gel filtration.

The anti-mucin core antibody, aM1, recognized discrete bands within the WGA-positive regions. In $V_i$, we observed a faster migrating WGA and an aM1-positive band, which did not appear in $V_o$. The strongest labeling was observed in the density range 1.35 to 1.45 g/ml for both $V_{o}$ and $V_{i}$ mucins. Cross-reaction with aM1 was lost after reduction and alkylation. This loss
FIGURE 7. Charge-fractionated mucins: vacuum blots of electrophoretic profiles on 1% agarose. Charge-fractionated mucins from a 95-year-old woman, density 1.3 to 1.35 g/ml, were separated by agarose gel electrophoresis, blotted onto a polyvinylidene difluoride membrane, and detected with WGA. Odd-numbered lanes represent (native) alkylated samples, whereas even-numbered lanes represent their reduced and alkylated counterparts. Lanes 3 to 10 = first peak (see Fig. 6a and refer to Ion-Exchange Chromatography in the Results section). Lanes 13 to 14 = second peak (see Fig. 6a and refer to Ion-Exchange Chromatography in the Results section). Rainbow markers (RB, maximal molecular weight 200 kDa) and immunoglobulin M are included for comparison between blots.

FIGURE 8. Charge-fractionated V0 mucins: vacuum blots of electrophoretic profiles on 1% agarose. Charge-fractionated mucins from a 67-year-old man, density 1.35 to 1.4 g/ml, were detected with WGA. Odd-numbered lanes represent (native) alkylated samples, whereas even-numbered lanes represent their reduced and alkylated counterparts. Lanes 3 to 10 = first peak (see Fig. 6c and refer to Ion-Exchange Chromatography in the Results section). Lanes 13 to 14 = second peak (see Fig. 6c and refer to Ion-Exchange Chromatography in the Results section). Rainbow markers (RB, maximal molecular weight 200 kDa) and immunoglobulin M are included for comparison between blots.

of labeling is a characteristic of the conformational epitope detected by this antibody. Blots of agarose electrophoresis gels of this density range also cross-reacted with 1E3 and TKH2 (Fig. 5). The mobility of this material is similar to aM1-positive mucins.

Analysis of Purified Mucins

Analysis of charge distribution and oligosaccharide side chains will define the tissue specificity of ocular mucins.

Ion Exchange Chromatography. Reduced and radioalkylated V0 mucins from two individual samples subjected to MonoQ ion exchange chromatography eluted with similar profiles across a range of isopycnic densities (Figs. 6a, 6c). There is a major peak of lower charge (fractions 24 to 38, further referred to as the first peak) and a minor peak (the second peak) of higher charge (fractions 54 to 62). As expected, there was little label incorporation in native mucins (Fig. 6b, 6d).

To obtain sufficient sample for loading onto agarose gel electrophoresis, fractions spanning the leading edge, peak maximum, and trailing edge, respectively, of the first mucin peak were combined. All the material under the second peak was combined for agarose electrophoresis. A WGA-probed blot from a 95-year-old female donor, 1.3 to 1.35 g/ml, is shown in Figure 7. The leading edge of the first peak (lane 4) contains...
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FIGURE 9. Charge-fractionated V0 mucins: vacuum blots of electrophoretic profiles on 1% agarose. Odd-numbered lanes represent (native) alkylated samples, whereas even-numbered lanes represent their reduced and alkylated counterparts detected with WGA. Lanes 3 to 10 = 95-year-old woman, 1.35 to 1.4 g/ml. Lanes 3 and 4 = leading edge of first peak; lanes 5 and 6 = first peak maximum; lanes 7 and 8 = trailing edge; lanes 9 and 10 = second peak. Lanes 11 to 16 = 67-year-old man, 1.4 to 1.45 g/ml. Lanes 11 and 12 = first half of first peak; lanes 13 and 14 = second half of first peak; lanes 15 and 16 = second peak. Rainbow markers (RB, maximal molecular weight 200 kDa) and immunoglobulin M are included for comparison between blots.

the slowest running mucin species (band a) and a small quantity of mucin with higher mobility (band b). The first half of the peak maximum (lane 6) contains most of mucin b and an additional species (band c) migrating slightly faster than band a. The second half of the peak maximum (lane 8) contains less labeled material, in a pattern similar to the first half. Lane 10, the trailing edge of the first peak, contains band c and a more strongly labeled band b. The fractions between the two peaks (lane 12) contain a shadow of the material found in the more charged peak. This latter peak (lane 14) contains a faint, slowly migrating band (band d, not visible in lane 12) and a faster migrating species (band e). All the bands are remarkably tight for combined fractions labeled with a general reagent. In summary, the less charged peak contains two mucin species of low mobility and one species with high electrophoretic mobility. The higher charged peak contains one low mobility and one high mobility negatively charged species. Charge separation and agarose electrophoresis thus distinguish five mucin species in the 1.3 to 1.35 g/ml isopycnic range.

The electrophoretic mobilities of charge separated mucins with buoyant density between 1.35 and 1.4 g/ml (67-year-old male donor; Fig. 8) are remarkably similar to the more buoyant range described above, except for an extra band (band f) of high mobility in the first peak (lanes 6 and 8). This sixth band is demonstrated clearly in mucins of the same density extracted from the 95-year-old female donor (Fig. 9, lanes 4 and 6), despite the combination of more fractions in each lane on the agarose gel. There is insufficient resolution to identify species in the 1.4 to 1.45 g/ml isopycnic range (Fig. 9, lanes 11 to 16). All other bands, however, are remarkably tight for combined fractions labeled with a general reagent.

Analysis of Oligosaccharide Chains on Purified Mucins.

The size of oligosaccharide side chains released by β-elimination from V0 mucins of a 67-year-old female donor were analyzed by chromatography on Biogel P4 (Fig. 10). The three isopycnic ranges—1.3 to 1.35, 1.35 to 1.4, and 1.4 to 1.45 g/ml—have similar, but not identical, distributions of side chain sizes. All oligosaccharide chains are short, eluting in the region between sialyl N-acetylgalactosaminol and lactose. No radionuclide-labeled chains are excluded on Biogel P4.

Impression Cytology

Impression cytology specimens from normal human volunteers stained with periodic acid-Schiff and labeled with aM1, 1E3, and TKH2. Staining and cross-reaction were strongest in goblet cells, where a distinctly granular pattern of labeling was seen, suggestive of mucin-containing granules (Fig. 11). The aM1-labeled impression showed streaks of mucin (Fig. 11B) pulled from broken goblet cells by a previous impression at the same site.

DISCUSSION

Polydispersity is found in many mucus secre-
tions19,21,30,32 and has contributed to difficulties in mucin purification. We have applied recent methods of mucin purification, including extraction in guani-
dine hydrochloride with protease inhibitor cocktail and isopycnic density gradient centrifugation in cesium chloride, to prepare native (undegraded) mucins.9,21,30 These methods improve separation of mu-
FIGURE 10. Biogel P4 chromatography of total oligosaccharides released by alkaline $\beta$ elimination from $V_o$ mucins of a 69-year-old woman. Oligosaccharides were released from mucin fractions by alkaline $\beta$ elimination and reduction with tritiated borohydride. After removal of excess radioactive borohydride and salts, the oligosaccharides were applied to a column of Biogel P4, and the radioactivity in individual fractions was measured. Standard oligosaccharides were fractionated under the same conditions. (A) Mucin oligosaccharide elution patterns. Each trace represents a different buoyant density range, as indicated. (B) Elution of standard oligosaccharides. Note that there are no oligosaccharides eluting in the $V_o$ fraction of the Biogel P4 column.

cins from other components and guarantee the isolation of the whole mucin complement from a tissue preparation. Combined with ion exchange chromatography and agarose gel electrophoresis, they optimize the fractionation of mucin subpopulations.

Initial attempts to isolate secreted ocular mucins were made in humans$^{12,34,35}$ and rabbit.$^{36}$ In these studies, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to analyze and detect mucins. Some glycosylated material failed to enter or migrate beyond the stacking gel and could not be optimally analyzed. In the absence of chaotropic solvents, the presence of an insoluble residue was observed. Different components were identified that may represent mucin aggregates, fragments, or subunits.$^{34,35}$ The molecular weights of these components were estimated to range between $2 \times 10^3$ and $2 \times 10^6$ kDa. By first separating by density gradient centrifugation, followed by size fractionation, and then refining this fractionation by ion exchange chromatography and agarose electrophoresis, we resolved the whole complement of mucins that either are ready or are being prepared for secretion. This approach overcomes the limitations of the previous methods.

Across the whole buoyant density range typical of mucins, size fractionation indicated the presence of a large molecular weight fraction that was unlikely to represent immature mucins. A small molecular weight fraction, also present at all buoyant densities, is unusual for mucosae analyzed this way.$^{31,38}$ This fraction may be equivalent to the small molecular weight mucins previously described on ocular epithelia.$^{3,34,35}$ The presence of mucins in $V_o$, $V_i$, and possibly $V_o$ of Sepharose CL2B columns, regardless of the method of detection, suggests a variety of mucins in conjunctival mucus. It is unclear whether different reagents always identify the same mucin.

We have limited our analysis to mucins not linked or anchored to membranes. Our samples probably contained surface-secreted mucin and breakdown products, but tissue homogenates by their nature will enrich stored or immature mucins prepared for secretion. Degraded and immature mucins have not been studied yet.

Mucins are polymorphic because of their polypeptide tandem repeats$^{8,26}$ and the varying lengths, branchings, and charges of their oligosaccharide side chains.$^{10,26}$ This study shows that human conjunctival mucins are polydisperse, containing mucin species of different buoyant density, hydrodynamic volume, and relative molecular mass whose components have discrete charge characteristics. MonoQ ion exchange chromatography, after density gradient centrifugation and Sepharose CL2B gel filtration, fractionates into tighter bands the species that otherwise appear as long smears on agarose gel electrophoresis.

Density gradient separation of individual samples gave highly reproducible profiles. Mucin core peptide epitope screening (aM1) on density gradient fractions clearly defines the range, 1.3 to 1.5 g/mL, in which mucins are expected. Antibodies to carbohydrate epitopes identified not only material within the mucin
FIGURE 11. Impression cytology of normal human conjunctiva. Impressions were taken from normal volunteers onto nitrocellulose membranes. They were probed with the reagents used to detect mucinlike material in tissue homogenates: (A) periodic acid-Schiff to detect all goblet cells; (B) the antibody aM1 against mucin core peptide; and antibodies against the oligosaccharide chains (C) Tn (IE3) and (D) sialyl-Tn (TKH2). Scale bar = 10 μm.

range but also other glycoconjugates, bearing the same epitopes separated in the lighter fractions. Individual conjunctival mucin samples display overall size-charge distribution patterns, which are highly conserved. Another consistent feature is the presence of large mucin subunits, demonstrated by both electrophoretic profiles (in which reduced subunits comigrate with the native mucins) and gel filtration (in which reduced subunits remain in V0 of a Sepharose CL2B column). Moore and Tiffany35 also found that the largest mucin component of crude human ocular mucus is excluded by Sepharose 4B or 6B, even after reduction.

After reduction and alkylation, we observed loss of labeling with the antibody aM1, as well as with IE3 and TKH2. This did not result from loss of sample because WGA binding was retained. Although loss of aM1 binding is expected because of changes in protein folding,22 IE3 and TKH2 are likely to reflect loss of epitope clustering caused by conformational change.30

The glycosylation pattern demonstrated in this study is unique among human mucins: All oligosaccharide chains are short. Similar results were obtained by β-elimination of crude ocular mucus35 and tear glycoproteins. We did not sequence the oligosaccharide chains. Though several structures are possible, we have evidence for GalNAc-O-Ser/Thr and Neu5Acα2-6GalNAc-O-Ser/Thr in tissue samples and in impression cytology. The mechanism for the production of short oligosaccharides has been described for many tissues,10 but not the eye. It is probably different from the glycosylation inhibition by retinoic acid suggested by Chen and Wolosin41 in corneal epithelium because of the extensive sialylation we observed.

Not all goblet cells cross-reacted with the antiacarbohydrate antibodies IE3 and TKH2. It is known that subgroups of goblet cells can be distinguished by differential staining.42,43

Tn expression has not been demonstrated in mature mucins outside the eye. In other normal human
mucins, sialyl-Tn expression either is restricted to limited areas recognized by TKH2 or is expressed in a cryptic O-acetylated form. Overexpression of Tn and sialyl-Tn has been correlated with progression to adenocarcinoma in the gastrointestinal tract. Circulating Tn and sialyl-Tn epitopes are considered independent predictors of survival. These epitopes are used as a basis for cancer therapy, producing cytotoxic antibodies that may have ocular side effects pertinent to the role of short carbohydrate chains on the ocular surface.

The methods described in this article are sensitive and specific, and we are applying them to study mucins in ocular surface pathology. In healthy human ocular mucus, mechanisms appear to operate that preserve polydispersity within bounds and that limit the length of oligosaccharide side chains.

Key Words
cancer-associated epitopes, conjunctiva, goblet cells, glyco-protein, mucin

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