Ocular Mucins: Purification, Metabolism and Functions

Anthony P. Corfield[1], Stephen D. Carrington[2], Sally J. Hicks[2], Monica Berry[3] and Roger Ellingham[3]

[1]*Department of Medicine Laboratories, Bristol Royal Infirmary, Bristol BS2 8HW, UK,* [2]*Department of Anatomy, School of Veterinary Science, University of Bristol, Bristol BS2 8EJ, UK and* [3]*Department of Ophthalmology, University of Bristol, Bristol Eye Hospital, Bristol BS1 2LX, UK*

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Corresponding author: Dr Steve Carrington, Department of Anatomy, School of Veterinary Science, Southwell St, Bristol BS2 8EJ, UK. Tel.: 0117 928 8358 or 0117 928 9000, ext. 7401, fax: 0117 925 4794, e-mail: S.Carrington@bristol.ac.uk.

Abstract—Mucins are present at the ocular surface in both secreted and membrane-bound forms. Mucins are produced in part by the conjunctival goblet cells, and are complemented by non-goblet secretions. This review focuses on secreted ocular mucins. They are present in the tear film, probably both in gel and soluble form, and play a role in lubrication and ocular defence. It is apparent that mucins are highly adapted to their functions. State of the art techniques for mucin purification and analysis are presented. Density gradient centrifugation, gel filtration, ion-exchange chromatography and agarose gel electrophoresis are discussed, together with methods of oligosaccharide analysis. Reagents for the detection of mucin are considered in conjunction with these methods, which we have employed in the analysis of human and canine ocular mucins. The general structure of mucins is reviewed. The biosynthesis and glycosylation of ocular mucins are not yet fully understood, and are discussed in relation to currently established concepts. The impact of disease on the nature and secretion of mucins is considered, as well as the physiological and pathological significance of mucus degradation. © 1997 Elsevier Science Ltd

1. INTRODUCTION—MUCINS IN THE TEAR FILM

The tear film is vital for the normal function of the ocular surface, influencing transparency, optical quality and defence against the external environment. A functional understanding of the tear film depends on a knowledge of its components and their interactions. The classical description of the tear film (Wolff, 1954) encompassed three layers: an outer lipid layer comprising meibomian secretions, an intermediate aqueous layer derived from the lacrimal and other accessory glands, and an inner mucus layer. The model put forward by Holly and Lemp (Holly and Lemp, 1971; Holly, 1973) proposed that the surface of the cornea was hydrophobic, and required the presence of a mucus layer in order to protect and hydrate it. This has been revised in the light of data showing that the corneal surface possesses an extracellular glycocalyx, now considered to be an integral part of the tear film. This structure is rich in carbohydrates, at least 1 μm thick, essentially hydrophilic and inherently wettable (Nichols *et al.*, 1985; Tiffany, 1990, 1994a; Dilly, 1994).

In recent work, laser interferometry and confocal microscopy were used to accurately measure the thickness of the tear film and its component layers. Earlier estimates of overall tear film thickness were around 7 μm. The recent data indicate a figure between 35 and 40 μm, the majority (≈ 30 μm) contributed by a mucous gel situated adjacent to the epithelial surface (Prydal *et al.*, 1992, 1993; Dilly, 1994) (Fig. 1). The existence of such an extended gel layer has focused attention on the contribution of mucins to the physiology of the tear film: specialized defensive, surfactant and rheological properties of the tear film may be reflected in the structure of its mucins (Holly and Lemp, 1971, 1977; Holly, 1973; Dilly, 1994).

Ocular mucins were initially believed to be entirely derived from conjunctival goblet cells. More recently, an alternative synthetic pathway has been defined in the stratified epithelial cells of the ocular surface (Dilly, 1985; Greiner *et al.*, 1985; Gipson *et al.*, 1992; Dilly, 1994; Gipson *et al.*, 1995; Gipson and Inatomi, 1997). Goblet cells probably secrete the most mucin, but shedding of membrane-bound mucin from conjunctival and corneal cells may also contribute to secreted mucus.

This chapter is concerned mainly with the biology, biochemistry and functions of secreted ocular mucins. The ocular surface glycocalyx is considered only briefly.

2. WHAT ARE MUCINS?

Mucins are large glycoconjugates with molecular weights in the range 5×10^2 to over 4×10^4 kDa. Sugar residues contributing up to 85% of dry weight are mainly O-linked to one of the two domains of a polypeptide backbone (Fig. 2). The
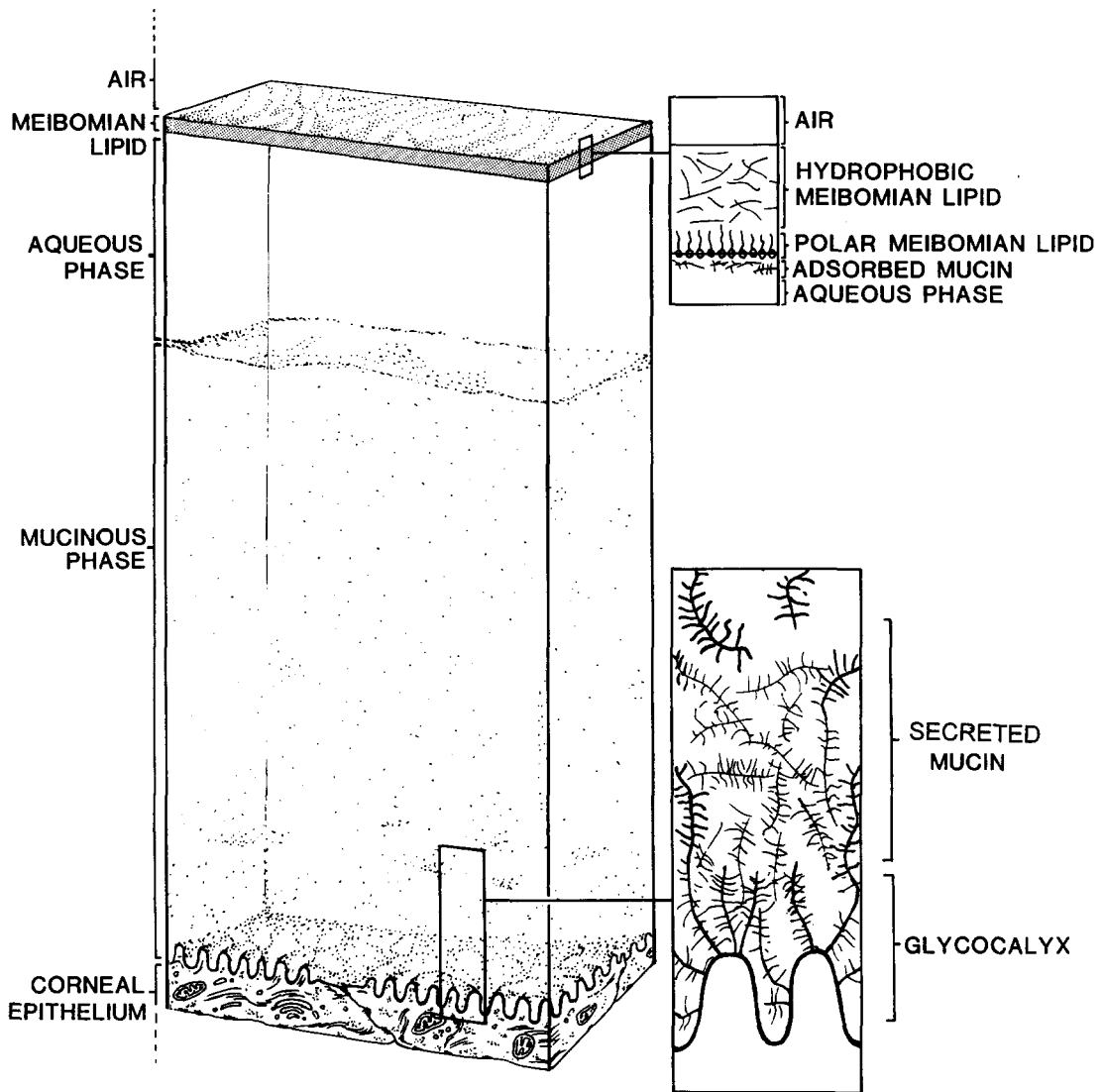
Fig. 1. Diagram showing the composition of the preocular tear film. Insets show interactions occurring at the glycocalyx, mucinous phase and meibomian lipid/aqueous phase.

multiple functions exhibited by mucins relate to their unique chemical structure (Carlstedt *et al.*, 1985; Strous and Dekker, 1992; Allen and Pearson, 1993; Forstner and Forstner, 1994; Van Klinken *et al.*, 1995).

Mucins secreted on to epithelial surfaces throughout the body form viscoelastic gels and constitute a physical barrier with functions specific to their mucosal location. Native secreted (synthetically complete and undegraded) mucins share a common structural organization, displaying subunits linked end to end through disulphide bridges (Carlstedt *et al.*, 1985; Strous and Dekker, 1992; Forstner and Forstner, 1994) (Fig. 2). A different group of mucins is integrated into cell membranes. These are generally smaller than secreted mucins and do not form extracellular gels. Normally, they lack disulphide-linked subunits and are not secreted via classical goblet cell vesicles (Hilkens *et al.*, 1992; Shimizu and Shaw, 1993; Van Klinken *et al.*, 1995). Mucin metabolism is described in Section 7.
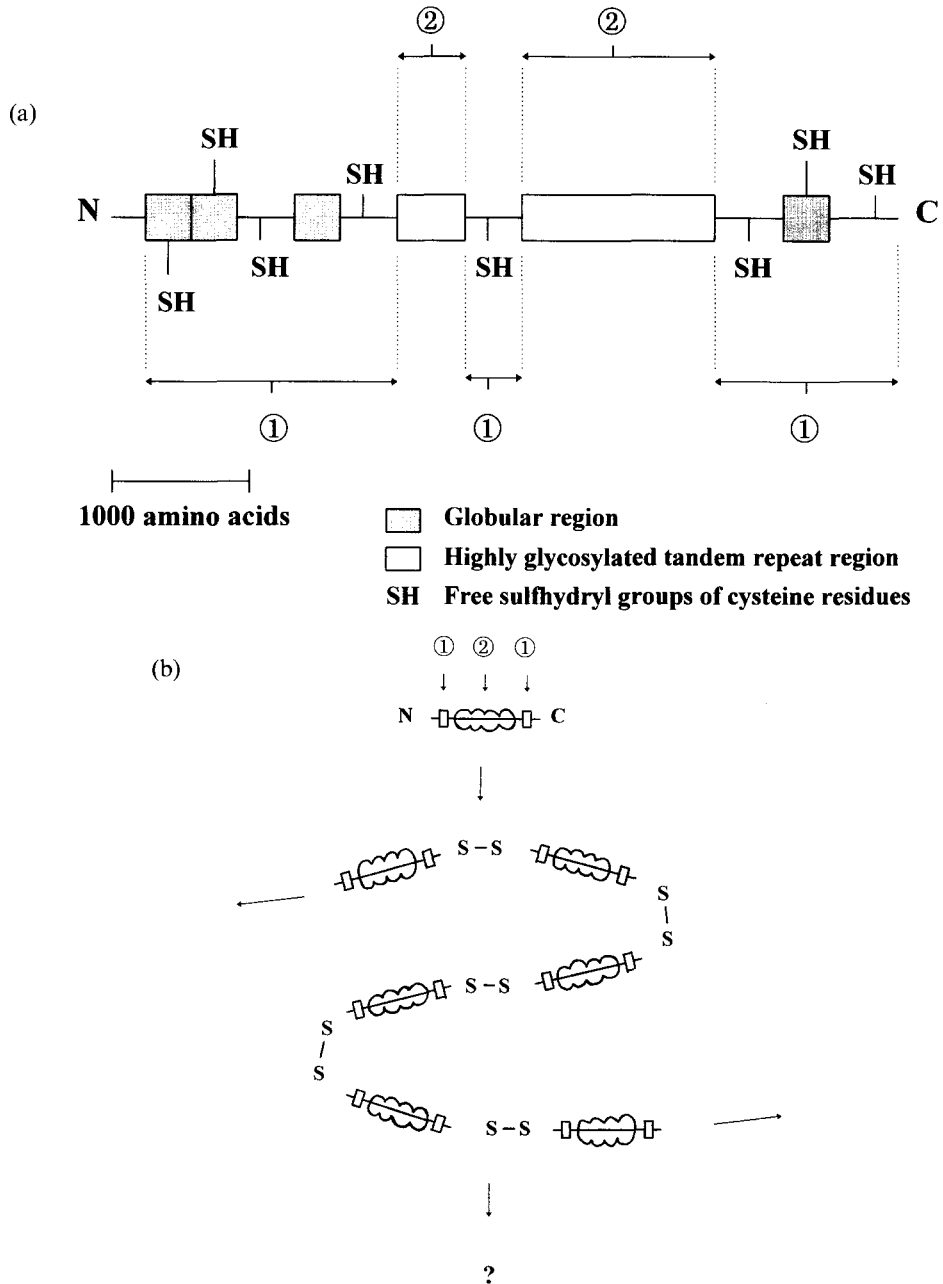
Fig. 2. Schematic representation of a typical secreted mucin, based on information known for MUC2 (Gum, 1995). (a) The monomeric subunit contains two main polypeptide regions: (1) protease-susceptible region: low glycosylation, some O-linked and N-linked oligosaccharide chains; high cysteine content; presence of von Willebrand factor motifs, containing intramolecular disulphide bridges. (2) Protease-resistant region: high glycosylation, mostly O-linked through GalNAc to serine and threonine; a small number of N-linked chains are present; variable number tandem repeat (VNTR) sequences, rich in serine, threonine and proline; low cysteine content. (b) Simplified representation of the proposed multimerization of subunits into native mucins. Linear (head to head or tail to tail) covalent linkage through disulphide bridges is shown. The number of S–S bonds and the precise linkage nature are not known. (1) and (2) indicate protease-susceptible and protease-resistant regions as in (a).

2.1. Mucin Genes and Polypeptides

The identification of nine mucin (MUC) genes has provided important data that could not be obtained by classical biochemical studies (for a review, see Gipson and Inatomi, 1977). Full sequence data are only available for MUC1, MUC2 and MUC 7. One of these mucin genes, MUC1, encodes a membrane-bound mucin possessing the characteristics typical of such molecules, including a transmembrane sequence and a cytoplasmic tail (Gum, 1992; Gendler and Spicer, 1995). Other MUC genes are candidates for the major secreted mucins found in the tear film. However, complete gene sequence data are required to confirm whether their products are secreted or membrane-inserted.

Each MUC gene contains a variable number of characteristic tandem repeats (VNTR), flanked by non-repeating sequences. The VNTR sequences code for peptide domains rich in serine, threonine and proline. Allelic variation of the number of tandem repeats contributes to mucin polymorphism. Serine and threonine are linkage sites for oligosaccharide side-chains. The flanking sequences, which have not been fully sequenced in all cases, code for poorly glycosylated globular peptide domains containing numerous cysteine residues. These residues form disulphide bridges that are involved in the aggregation of mucin subunits and the folding of the peptide chain. In MUC2 and MUC5AC, the globular domains also contain motifs similar to those found in von Willebrand factor. These are implicated in storage and secretion phenomena (Gum, 1992, 1995; Gendler and Spicer, 1995). In MUC3, a disulphide-bond linked motif analogous to the epidermal growth factor receptor has been identified (Kim et al., 1995). The positioning of the disulphide bonds in these peptide sequences is significant for the identification of such motifs. Analysis of the nucleotide sequence alone does not predict their presence (Gum, 1995).

2.2. Mucin Glycosylation

The glycosylation of MUC gene polypeptides varies from tissue to tissue and is a feature of mucin structural complexity (Gum, 1992; Gendler and Spicer, 1995). The tissue-specific nature of mucin glycosylation is believed to be related to the functional requirements at each mucosal site (Roussel et al., 1988; Corfield et al., 1995; Montreuil et al., 1996).

Each serine and threonine residue in the mucin polypeptide backbone is a potential site for O-linked N-acetyl-galactosamine (GalNAc), which is the first sugar in all oligosaccharide chains. The VNTR regions contain the bulk of the oligosaccharide side chains in a mucin molecule. Repetition of these regions amplifies the overall proportion of carbohydrate within the molecule. The oligosaccharide side chains contain characteristic monosaccharides: galactose, fucose, N-acetylhexosamines and sialic acids, but no uronic acids and little mannose (Carlstedt et al., 1985; Strous and Dekker, 1992; Forstner and Forstner, 1994). Chains vary in length and branching, with charges ranging from neutral to highly negative, depending on the presence of sialic acid and sulphate (Roussel et al., 1988; Montreuil et al., 1996) (Fig. 3).

Mucins from many sources have been examined for carbohydrate content and oligosaccharide patterns. These data constitute a vast body of structural information (Moore and Tiffany, 1979; Roussel et al., 1988; Schachter and Brockhausen, 1992; Montreuil et al., 1996). Mucin-type oligosaccharide chains show a great variety in length, branching and terminal substitution. Chains consist of three regions: core, backbone and periphery (Fig. 3). Variation exists in each region. Thus, for any mucin molecule, hundreds of O-linked oligosaccharides may occur.

The importance of individual carbohydrate structures is best illustrated by the blood group antigens. These carbohydrate motifs are present on secreted mucins, depending on an individual's secretor status. The specificity of this glycosylation is reflected in the fidelity of blood group antigen expression for each individual. The specificity of glycosylation pathways resulting in this level of fidelity is the same for all oligosaccharides expressed in mucins: each mucin possesses structures typical for its site of expression. It is important to identify these structures in order to correlate them with tissue-specific function.

(a)   **PERIPHERAL UNITS**

blood group B

sialic acid
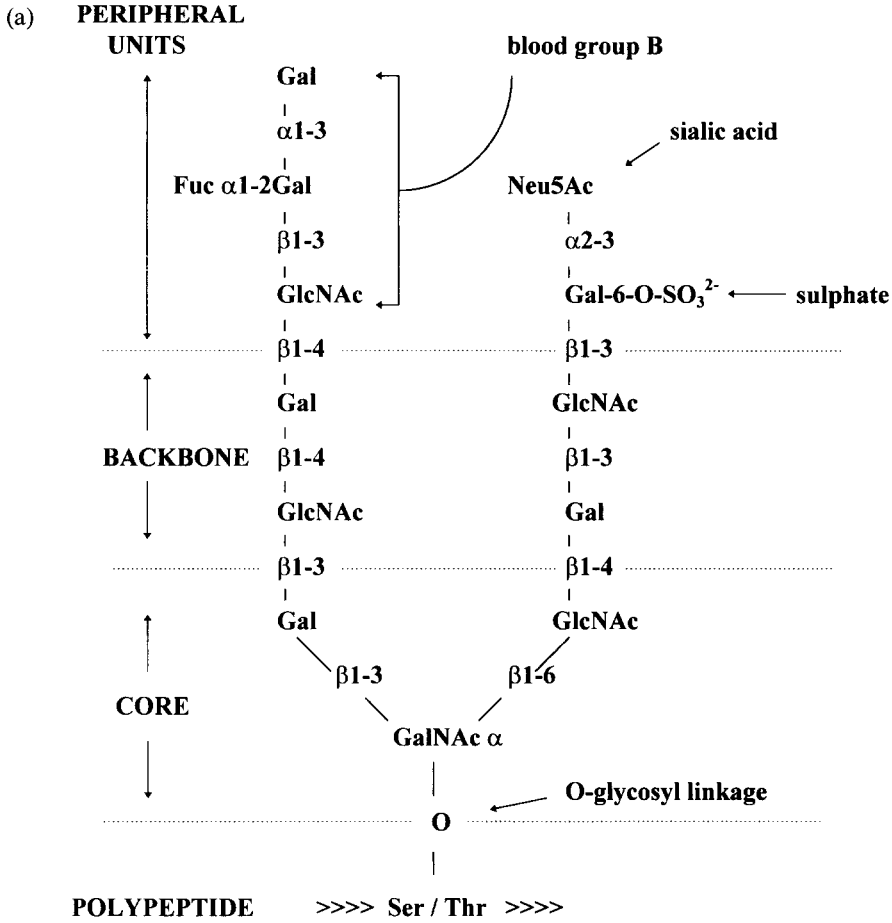
$$\text{Gal}$$
$$\mid$$
$$\alpha\text{1-3}$$
$$\mid$$
Fuc α1-2**Gal**   **Neu5Ac**
$$\mid$$
$$\beta\text{1-3} \qquad\qquad \alpha\text{2-3}$$
$$\mid$$
GlcNAc   **Gal-6-O-SO$_3$$^{2-}$** ◄—— **sulphate**
$$\mid$$
$$\beta\text{1-4} \cdots\cdots\cdots\cdots \beta\text{1-3}$$
$$\mid$$
Gal   **GlcNAc**

BACKBONE β1-4 β1-3
$$\mid$$
GlcNAc   **Gal**
$$\mid$$
$$\beta\text{1-3} \cdots\cdots\cdots\cdots \beta\text{1-4}$$
$$\mid$$
Gal   **GlcNAc**
$$\searrow \beta\text{1-3} \qquad \beta\text{1-6} \nearrow$$

CORE   **GalNAc** α
$$\mid$$
$$\text{O} \qquad\text{◄—— } \textbf{O-glycosyl linkage}$$
$$\mid$$

POLYPEPTIDE   >>>> **Ser / Thr** >>>>

(b)

Blood group antigens carried on mucin oligosaccharides :-

A   GalNAcα1-3Galβ1-3GlcNAc - R
$$\mid$$
$$\alpha\text{1-2}$$
$$\mid$$
Fuc

Lewis^{a}   Galβ1-3GlcNAc - R
$$\mid$$
$$\alpha\text{1-4}$$
$$\mid$$
Fuc

B   Galα1-3Galβ1-3GlcNAc - R
$$\mid$$
$$\alpha\text{1-2}$$
$$\mid$$
Fuc

Lewis^{x}   Galβ1-4GlcNAc - R
$$\mid$$
$$\alpha\text{1-3}$$
$$\mid$$
Fuc

H (type 1)   Galβ1-3GlcNAc - R
$$\mid$$
$$\alpha\text{1-2}$$
$$\mid$$
Fuc

Sialyl Lewis^{x}   Neu5Acα2-3Galβ1-4GlcNAc - R
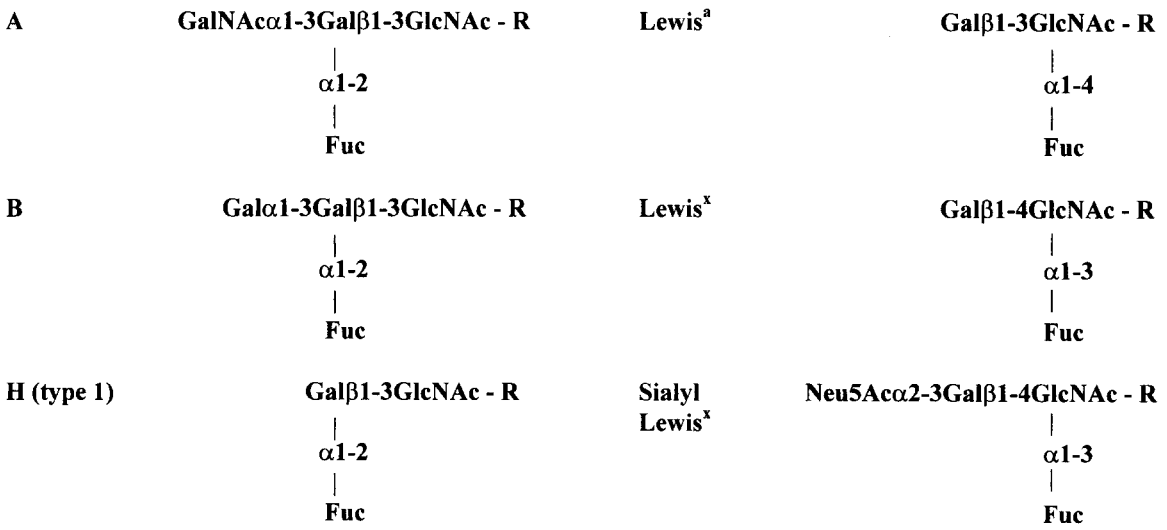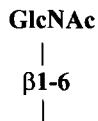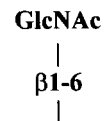$$\mid$$
$$\alpha\text{1-3}$$
$$\mid$$
Fuc

Fig. 3(a, b)

(b) contd.

STRUCTURE OF OLIGOSACCHARIDE ANTIGENS IN MUCINS

O-glycosyl link :-   GalNAcα - O - Ser.Thr

Examples of oligosaccharide core structures :-

core 1   Galβ1-3GalNAcα - O - Ser/Thr   core 3   GlcNAcβ1-3GalNAcα - O - Ser/Thr

                    GlcNAc   GlcNAc
                      | |
                    β1-6   β1-6
                      | |

core 2   Galβ1-3GalNAcα - O - Ser/Thr   core 4   GlcNAcβ1-3GalNAcα - O - Ser/Thr

Short saccharide structures found in ocular mucins :-

Tn   GalNAcα - O - Ser/Thr

Sialyl Tn   Neu5Acα2-6GalNAcα - O - Ser/Thr

Fig. 3. (a) Hypothetical composite oligosaccharide structure found in mucins. Principal features include: O-glycosidic linkage of *N*-acetyl-*D*-galactosamine to serine and threonine residues on the polypeptide chain; CORE region, with core 4 as an example; BACKBONE of poly-*N*-acetyllactosamine type; PERIPHERAL groups such as sialic acid, sulphate and a blood group antigen. Many combinations based on these arrangements are possible: some examples are given in (b), including several that are directly relevant to the ocular mucins.

3. ORIGINS AND SUBCELLULAR LOCATION OF OCULAR MUCINS

3.1. Goblet Cells and Secreted Mucins

The precise cellular origin of each mucin cannot be determined from the mixture found in tears, conjunctival homogenates or whole eye washings. It is possible that shedding of membrane-bound mucins may contribute to the adherent gel layer of the tear film, but there is much more evidence for conjunctival goblet cells being the major producers of secreted ocular mucins.

Goblet cells can be detected in tissue sections, epithelial sheets and surface impressions, confirming their location in the conjunctiva and not the cornea. Their exact origin and life cycle have not been determined, but it is probable that they arise in the basal layers of the conjunctival epithelium, and mature as they approach the surface. Biomicroscopy of these cells *in vivo* and *in vitro* suggests that they are derived from other goblet cells within a small number of cell divisions (Chen *et al.*, 1994; Zieske, 1994; Dartt *et al.*, 1995; Kessler *et al.*, 1995; Wei *et al.*, 1996). It has been suggested that the maturation of mucin granules in goblet cells takes around 2 weeks (Aitken *et al.*, 1988). Identification of goblet cell precursors is hampered by them being devoid of secretory granules.

It is not yet possible to quantify total secreted mucin at the ocular surface and in tears, by either histochemical or biochemical methods. Goblet cell numbers, determined by impression cytology and histology, have been used to estimate the secretory status of the conjunctiva (Friend *et al.*, 1983; Kinoshita *et al.*, 1983). Histological methods reveal a considerable variation in goblet cell

numbers per unit area in normal control conjunctiva (Kessler *et al.*, 1995). Periodic acid Schiff (PAS) probably stains all goblet cells containing mucin. In human and canine conjunctiva, only a proportion of these goblet cells stains with

Alcian Blue [Fig. 4(a)], which detects sialylated or sulphated groups, depending on the pH (McGhee and Lee, 1985; Yamabayashi and Tsakahara, 1987; Adams and Dilly, 1989). Cross-reaction with anti-M1, antibodies to the peptide core of
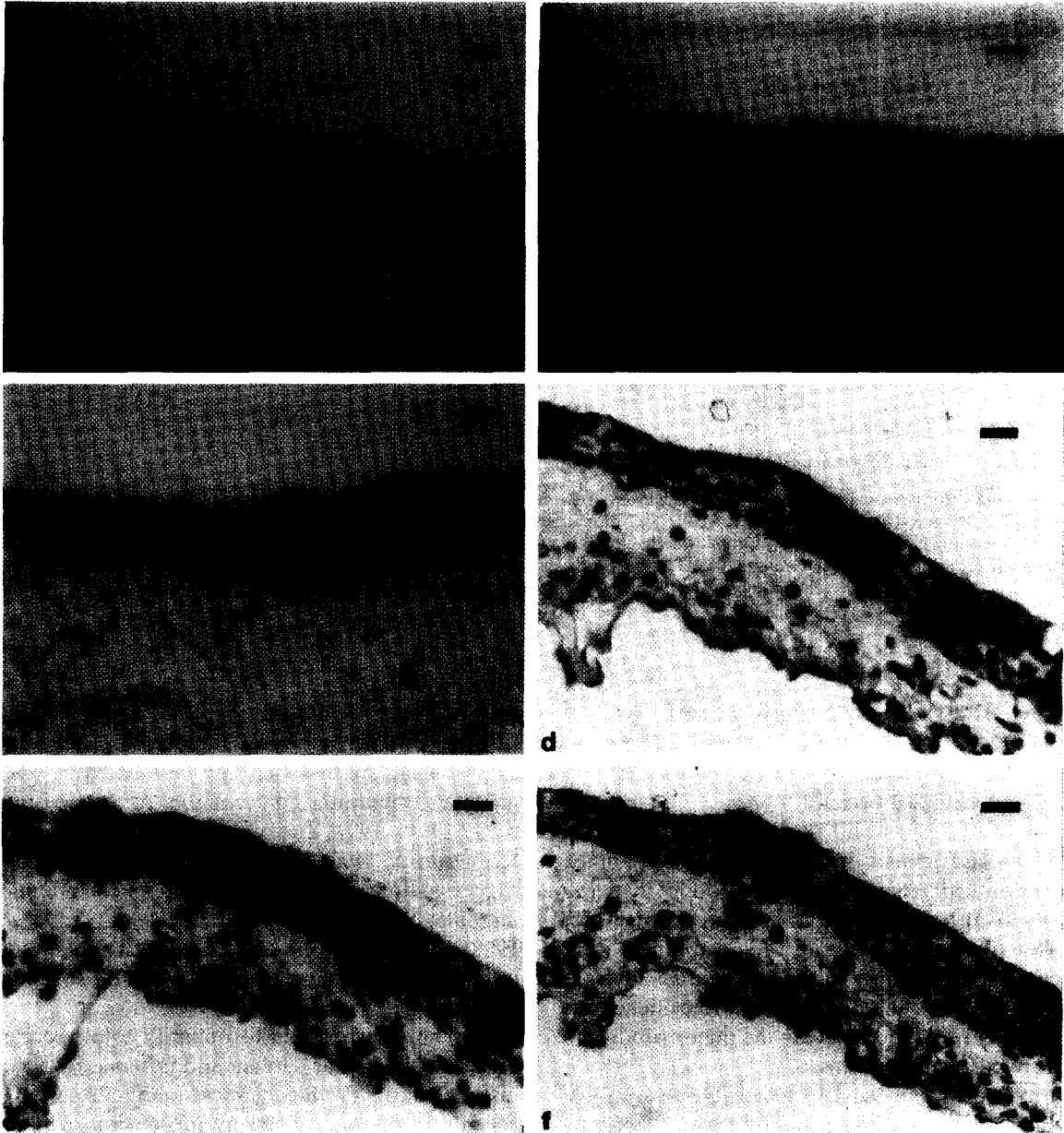
Fig. 4. Differential staining of goblet cells in canine and human conjunctiva. Canine conjunctiva stained with: (a) Periodic acid Schiff/Alcian Blue; (b) anti-M1 antibodies with Light Green counterstain; (c) polyclonal antibody PCcan1, prepared against purified canine ocular mucin. Serial sections of human limbal conjunctiva stained with: (d) Periodic acid Schiff; (e) anti-M1 antibodies; (f) antibody 1E3. Sections (d)–(f) were counterstained with haematoxylin (for all sections, bar = 2 μm).

gastric mucins (Bara *et al.*, 1986; Garcher *et al.*, 1994), gives a very similar picture to PAS in human [Fig. 4(d)], but not in canine conjunctiva [Fig. 4(b)]. An antibody to rabbit ocular mucin (Huang and Tseng, 1987) was also reported to bind to most goblet cells. We have found similar results with a polyclonal antibody (PCcan1) prepared against a fraction of canine ocular mucin [Fig. 4(c)]. Antibodies to human and rat membrane-bound mucin-like glycoproteins also showed reactivity with goblet cell membranes, but did not bind uniformly to all goblet cells (Gipson *et al.*, 1992; Watanabe *et al.*, 1993). Other antibodies, e.g. to the Tn antigen (GalNAcα-*O*-serine/threonine), stain a sub-population of goblet cells in histological sections and impression cytology specimens [Ellingham et al., in preparation, Fig. 4(f)]. Lectins (Gipson *et al.*, 1983; Kawano *et al.*, 1984; Versura *et al.*, 1986) also show differential binding to goblet cells. Shared epitopes on mucins and other glycoproteins, including blood group antigens, complicate the interpretation of stains for carbohydrate groups (Garcher *et al.*, 1994; Watanabe and Gipson, 1994).

Differential staining of mucin granules within and between goblet cells may be due to the segregation of different glycoproteins within cells or granules (Watanabe *et al.*, 1993) or to a maturation process (Tseng *et al.*, 1984; Watanabe *et al.*, 1993). Cell culture experiments may help to resolve these two processes. Goblet cells differentiate in conjunctival epithelium in both organotypic cultures (Kruse and Tseng, 1993; Chen *et al.*, 1994; Tsai *et al.*, 1994; Wei *et al.*, 1994, 1996) and in epithelial monolayers grown on plastic. We observed the disappearance of goblet cells from epithelial monolayers derived from human conjunctival explants after 7 days in culture. After 14 days in culture, cells appeared with large cytoplasmic granules that cross-reacted with anti-M1 (anti-mucin) antibodies, suggesting that they were goblet cells in culture (Ellingham *et al.*, in preparation) (Fig. 5).

In nude mice, sub-dermal implants of only a few rabbit conjunctival cells can produce cysts having either entirely stratified squamous or both goblet cell and squamous phenotypes (Wei *et al.*, 1993, 1994, 1996). Analysis of mucins within these cysts will help to differentiate which types of mucin are
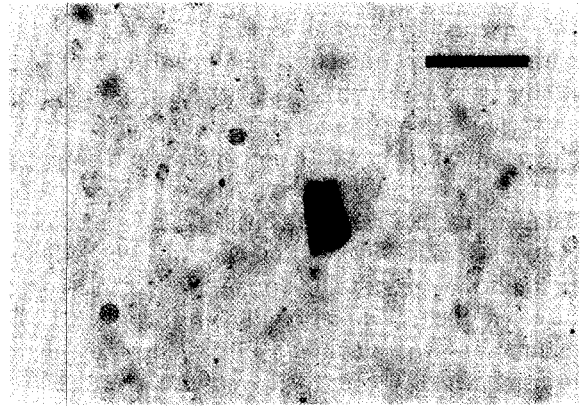
Fig. 5. Goblet-like cell in culture. Confluent conjunctival epithelial cells at 17 days, showing a cell stained positively with anti-M1 (bar = 10 μm).

derived from goblet cells, and which are derived from squamous cells. It will also provide a basis for examining whether staining differences between cells relates to cell maturation.

Corneal epithelial cells in culture also synthesize soluble mucins with electrophoretic properties similar to mucins from conjunctival epithelial tissue. As these corneal cultures are devoid of goblet cells, an alternative source of mucin and mechanism of secretion must be proposed (Ellingham *et al.*, in preparation).

3.2. Membrane-bound Mucins

Initial evidence for the existence of two distinct origins for ocular mucins came from electron microscopic observations in guinea-pig (Nichols *et al.*, 1983, 1985) and human conjunctivae (Dilly, 1985). A glycocalyx was visible, which was differentiated from an overlying layer of secreted mucus. Both components stained for carbohydrate, suggesting the presence of mucin in both membrane-bound and secreted form.

The detection in human conjunctival epithelium of small subsurface vesicles rich in carbohydrate (Greiner *et al.*, 1980; Dilly and Mackie, 1981; Dilly, 1985; Greiner *et al.*, 1985) suggested that not all surface mucin was derived from goblet cells. The material stored in these vesicles was reported to be externalized as part of the glycocalyx, which

was particularly prominent at the tips of the corneal and conjunctival epithelial microplicae (Dilly, 1985; Greiner *et al.*, 1985). Confirmation and characterization of rat and human glycocalyx mucin-like molecules, corresponding to the small vesicle pathway, have been provided using antibodies raised against corneal apical cells (Gipson *et al.*, 1992; Watanabe *et al.*, 1993, 1995). In addition, membrane-bound MUC1 has been detected in corneal and conjunctival epithelial cell membranes (Inatomi *et al.*, 1995). Currently, there is insufficient evidence to prove that the glycocalyx mucin-like molecules detected in the small vesicle pathway are identical to MUC1.

3.3. The Lacrimal Glands

Lacrimal gland secretions have been considered a source of mucins in the tear film. Evidence for this has not been forthcoming, in spite of histological investigations demonstrating an abundance of carbohydrate reactive material in the gland (Versura *et al.*, 1986; Ahmed and Grierson, 1989; Breipohl *et al.*, 1994). Earlier studies with a polyclonal antibody to semipurified human ocular mucin failed to show any cross-reactivity with the lacrimal gland (Moore and Tiffany, 1979). Furthermore, biochemical studies of lacrimal gland explants (Chao *et al.*, 1980) showed the synthesis of high molecular weight material representing IgA and IgG, but failed to demonstrate the presence of mucins. Confirmation of lacrimal gland mucins requires examination of MUC gene expression in the gland, and analysis of its secretions using techniques described in this chapter.

4. PURIFICATION AND IDENTIFICATION OF OCULAR MUCINS

Purification of intact mucins requires the protection of samples from degradation or aggregation during dispersal, storage and purification. The purity of the isolated products can be determined only if appropriate methods are available for the constituents of each mucosal system.

4.1. Collection and Extraction of Secreted Mucins

Collections of tears, surface aspirates and external ocular epithelia are suitable sources of mucin. Different sources may yield different mucin complements. Tear sampling methods may influence the quality and quantity of mucins collected, since mucin release from goblet cells can be triggered mechanically (Kessler *et al.*, 1995).

Guanidine hydrochloride (4–6 M) or urea (6–8 M) have been used to obtain dissociation of mucus samples (Carlstedt *et al.*, 1985). These reagents will dissolve most mucous gels, but some insoluble gels have been described (Carlstedt *et al.*, 1993, 1995). Where such material is present, addition of thiol reagents, such as dithiothreitol, mercaptoethanol or N-acetylcysteine, will induce dissolution. These reagents, however, convert native mucins into subunits, by reduction of disulphide bridges, precluding studies of native mucins.

Samples require immediate stabilization by inactivation of proteolytic enzymes. Guanidine inactivates many enzymes, but during subsequent purification, adherent enzymes may reactivate if it is removed. Inhibitor cocktails block proteolysis as a result of cytosolic, membrane-associated and microbial sources. The most effective inhibitors for mucin preparations are phenylmethylsulphonylfluoride (or diisopropylfluorophosphate) and soybean trypsin inhibitor. The general protease inhibitors EDTA, benzamidine and *N*-ethylmaleimide are also frequently used. For each mucosa, the optimal mixture can be identified by using the target mucins as substrate in proteolytic assays (Corfield and Paraskeva, 1993). When such experiments are not possible, a broad-spectrum cocktail of inhibitors can be substituted (Carlstedt *et al.*, 1985).

4.2. Density Gradient Centrifugation

Solubilized mucins can be separated from other proteins, glycoproteins, polysaccharides, proteoglycans and nucleic acids by isopycnic centrifugation. Mucins show a characteristic buoyant density (1.3–1.5 g/ml), depending, to some extent,

on the solubilizing mixture (Section 4.1). Centrifugation through a concentration gradient (rate zonal centrifugation) can effect separation on the basis of hydrodynamic volume (size in solution).

4.2.1. *Isopycnic centrifugation*

This technique represents the gold standard for purification of mucins (Carlstedt and Sheehan, 1984). Samples are centrifuged at high g values in the presence of a caesium salt (Creeth and Horton, 1977). This generates a density gradient in which each component segregates according to its buoyant density. The formation of a gradient, ranging from 1.25 g/ml to 1.6 g/ml, is appropriate for the separation of mucins. Depending on the g values employed, this will take a minimum of 24 hr. In practice, the complete separation of mucins from other contaminants, particularly nucleic acids and proteoglycans, is not achieved in one centrifugation. Removal of nucleic acids can be optimized by running the first gradient at 4–6 M guanidine hydrochloride, pooling the mucin rich densities and re-running the sample in a second gradient at 0.2 M guanidine hydrochloride. Reduced and alkylated subunits, or proteolytically cleaved mucin fragments, will have the same buoyant density as native material, with an identical resolution on gradients.

Membrane-bound mucins can be fractionated on density gradients (Baeckstrom *et al.*, 1991; Carraway and Hull, 1991) after isolation using suitable surface active agents. The inclusion of low concentrations (<0.1%) of detergents, such as Triton X100, octyl glucoside or CHAPS, shifts these mucins into the 1.3–1.5-g/ml buoyant density range. Without detergents, the presence of lipids (Scheiman *et al.*, 1992) may lead to artefactual mucin–lipid coacervation or the generation of micellar structures (Scheiman *et al.*, 1992; Fung *et al.*, 1995). These complexes fractionate at the lower densities associated with proteins and poorly glycosylated glycoproteins. Some mucin–lipid complexes may be physiological (Scheiman *et al.*, 1992; Fung *et al.*, 1995). They have been implicated in the removal of degraded mucins

from the ocular surface (Holly and Lemp, 1971, 1977; Tseng *et al.*, 1987). Figure 6 shows a gradient of canine ocular mucus where a buoyant mucin fraction is present, attributable to lipid complexation.

4.2.2. *Rate zonal centrifugation*

Mucin fractions derived from isopycnic density gradients are separated only on the basis of buoyant density. Separation by molecular size will resolve native mucins and subunits. This is of considerable relevance for studies of synthetic precursors, degradation products and their relative proportions. The technique is accomplished by centrifuging a mucin mixture through a salt gradient. Guanidine hydrochloride, or a similar chaotropic salt, forms the gradient and minimizes aggregation as a result of non-covalent interactions. The mucin sample is layered on to a preformed gradient and centrifuged for 4–6 hr at high g values. Larger mucins, containing a greater number of subunits, sediment more rapidly (Sheehan and Carlstedt, 1987; Thornton *et al.*, 1995). Thus, mucins of a different molecular size, or with different numbers of covalently bound subunits, are identified by their distribution across the gradient. Subsequent reduction and alkylation separates all subunits, and comparison with the previous profile confirms their size.

4.3. Gel Filtration

Size fractionation by gel filtration has been widely used to obtain partially purified mucins without the prior use of CsCl gradients (Carlstedt and Sheehan, 1984; Carlstedt *et al.*, 1985; Parker *et al.*, 1993). The limitations of this technique for mucin analysis should be considered. First, the fractionation range of most gel filtration media is not sufficient to resolve the very high molecular weights encountered in native mucins or their reduced and alkylated subunits. Second, the technique is unable to separate mucins from other high molecular weight contaminants, such as nucleic acids, proteoglycans and
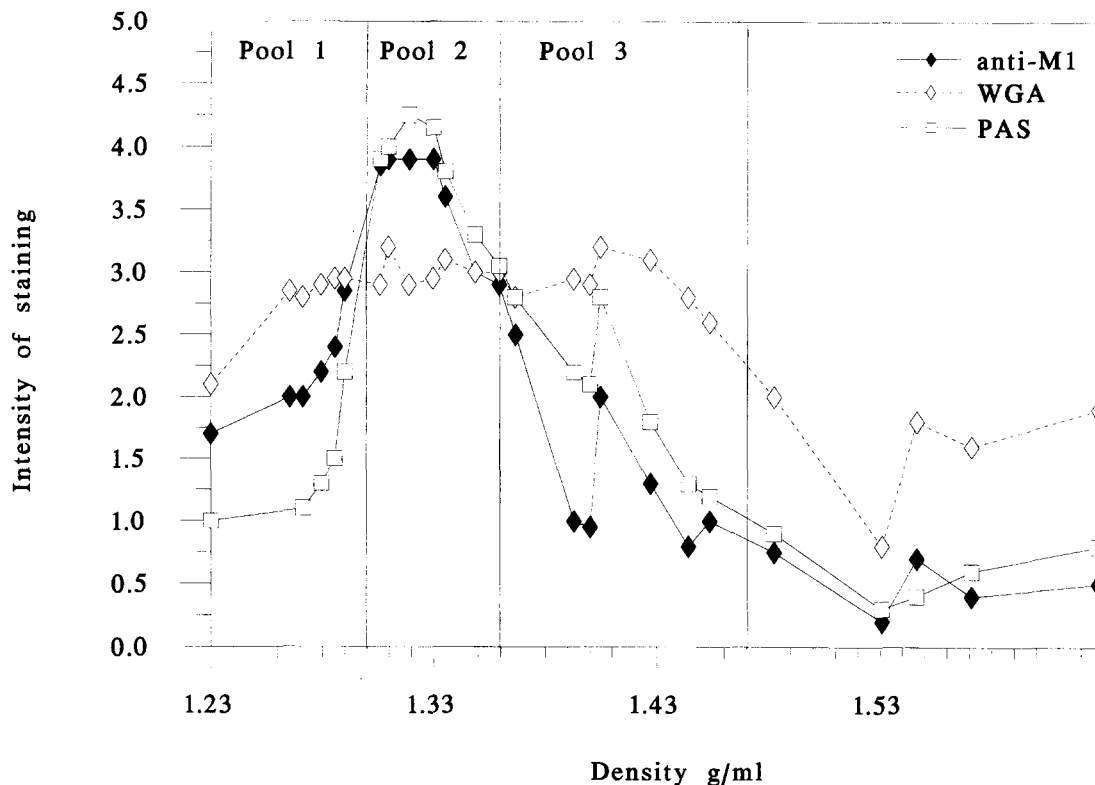
Fig. 6. Density gradient profile of canine ocular mucus (starting density: 1.41 g/ml). Slot blots of gradient fractions were stained with PAS (Periodic acid Schiff), anti mucin antibodies (anti-M1; Bara *et al.*, 1986) and wheatgerm agglutinin (WGA). The presence of low density material (<1.3 g/ml) suggests that there may be complexation with lipid.

polysaccharides. Third, lower molecular weight mucins will be discarded with contaminants. Some contaminants can be removed by pretreatment with nucleases and proteoglycan/polysaccharide degrading enzymes (Strous and Dekker, 1992; Jass and Roberton, 1994). However, this introduces a further variability into the purification process, and requires the removal of enzymes after each digestion.

Canine and human ocular mucus contains a range of mucins that can be partially separated by gel filtration (Fig. 7). Large mucins eluting in the excluded volume may constitute a mixture while the smaller mucins are fractionated. Separation and identification of the lower molecular weight mucins is achieved only if density gradient centrifugation (which is dependent on carbohydrate content and not molecular size) is performed first. Gel filtration has also been used to fractionate membrane-bound mucins.

Rapid analysis of mucin-rich fractions derived from metabolic labelling experiments in cell and organ culture can be undertaken by using gel filtration. In these experiments, only trace amounts of mucins are produced. As a result, losses resulting from adsorption on to labware surfaces may be significant. Good recoveries are achieved using all glass apparatus, whereas plastics should be checked for losses. Identification of mucins can be undertaken using specific enzyme digestion of suspected contaminants such as proteoglycans (Corfield and Paraskeva, 1993).

Cross-linked agarose (e.g. Sepharose, BioGel A) and agarose–polyacrylamide copolymers (Sephacryl) are suitable for gel filtration in high- or low-salt elution buffers. An assessment of recovery is necessary in all experiments, to ensure that no selective or non-specific losses are occurring. Despite the concentrating effects of isopycnic centrifugation and gel filtration,

(a)

(b)
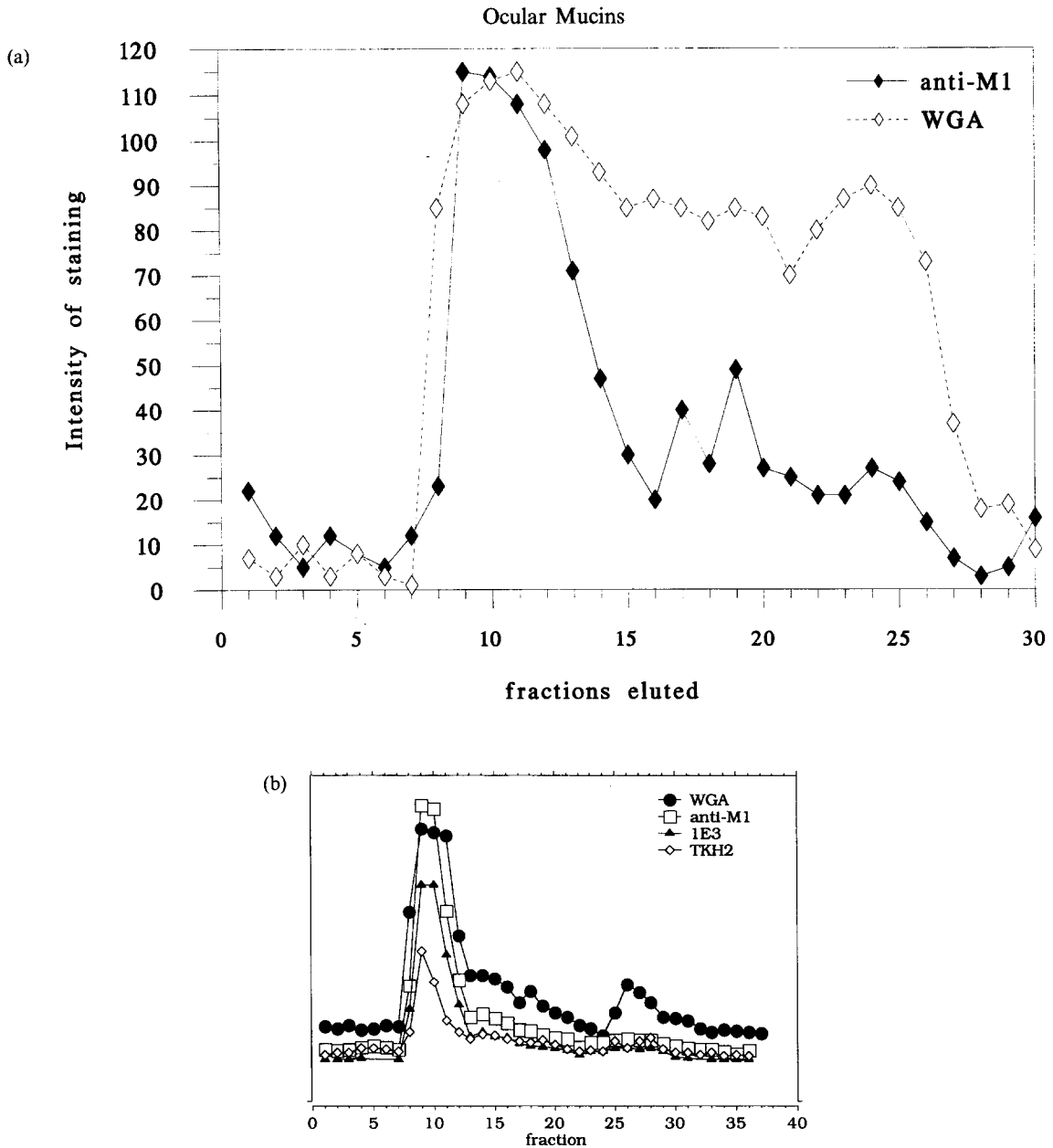
Fig. 7. Gel filtration profiles of secreted ocular mucin. (a) Canine ocular mucin (Pool 2; see Fig. 6) on Sepharose CL-4B. High MW excluded material (V_0 range = fractions 8–14) and lower MW included material are present. (b) Human conjunctival mucins from tissue homogenates (buoyant density 1.3–1.4 g/ml) on Sepharose CL-2B. Material positive for WGA, anti-M1, TKH2 and 1E3 is present in both the excluded and included volumes.

samples may be too dilute for further purifications. We find that using a buffer containing very dilute urea during gel filtration avoids the production of insoluble aggregates on lyophilation and is compatible with subsequent ion exchange or electrophoresis.

4.4. Ion-exchange Chromatography

Ion-exchange chromatography is a technique for identifying mucin glycoforms that are not resolved using other methods (Thornton et al., 1994, 1995). As many mucins show some variation in their

sulphate or sialic acid content, they can be fractionated on the basis of charge. Mucins are adsorbed on to a column matrix in salt-free buffer, then eluted with an increasing linear salt gradient (Podolsky and Isselbacher, 1984; Shimamoto *et al.*, 1989; Raouf *et al.*, 1991).

The best matrices are mild anion exchangers such as DE Sephadex, and Mono-Q (Podolsky and Isselbacher, 1984; Thornton *et al.*, 1994). The elution of adsorbed mucin species from ion-exchange resins is best accomplished using continuous salt gradients at suitable pH, since discontinuous gradients may lead to elution artefacts (Raouf *et al.*, 1991). Sample losses due to non-specific adherence of mucins to exchange matrices have been overcome by including urea and CHAPS in the equilibration and elution buffers (Thornton *et al.*, 1995).

Human ocular mucins, separated by density gradient centrifugation and gel filtration, have been reduced and radio-alkylated. The labelled subunits were successfully separated using Mono-Q and visualized on Western blots of agarose electrophoresis gels (Berry *et al.*, in press; Fig. 8).

4.5. Electrophoresis

Electrophoresis of mucins has been used to assess the complexity and contamination of mucin preparations. Most studies have employed sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE) under reducing and non-reducing conditions (e.g. Irimura *et al.*, 1988; Dekker *et al.*, 1991; Klomp *et al.*, 1994; McCool *et al.*, 1994). Demonstration of contamination in the molecular weight range below 200–300 kDa can be achieved reliably using this method, but polydisperse cationic mucins of a higher molecular weight are difficult to resolve. Even 3% polyacrylamide gels give a limited resolution, and much of the sample may not even enter the gel. The influence of acidic groups on the migration of high molecular weight mucin precursors in SDS–PAGE is unpredictable (Tytgat *et al.*, 1995), especially for mature mucins. This renders estimations of molecular size inaccurate.

Electrophoresis in 1% agarose has been introduced to improve the resolution of mucins

and their subunits. Migration differences are due to both size and charge, and comparisons must be made on this basis (Thornton *et al.*, 1995).

Western blotting has been used with both SDS–PAGE and agarose gel electrophoresis. The latter method has identified secreted and membrane-bound ocular mucins/mucin-like glycoproteins and their subunits (Fig. 8). Fluorometric systems have greatly increased the sensitivity of detection.

4.6. Mucin Detection Methods

Reagents for mucin detection include histochemical stains, lectins and antibodies. These can be used in direct liquid assays, in blotting assays and in ELISA.

Histochemical methods have been adapted to detect mucins during purification. PAS, Alcian Blue and high iron diamine detect glycol or cationic groups. Standard liquid assays based on these and other carbohydrate reactions are greatly hampered by salts, but the reagents are valuable for probing mucin fractions immobilized on polyvinylidine difluoride or other transfer membranes (Thornton *et al.*, 1989). Blotting concentrates the sample, ensures that salts are removed before probing and provides a stable archive.

Lectins are sensitive probes for carbohydrate moieties. Many lectins have specificities for glycosyl subgroups that are widely expressed in glycoconjugates. They do not detect mucins specifically but may be used as general reagents. We routinely use wheat-germ agglutinin in this way (Figs 8 and 9). Lectins with a specificity for sialic acids (*Sambucus nigra* and *Maakia amurensis*) may have more value in mucin detection. Other useful lectins may be identified by their differential or specific binding to goblet cell granules or cell membranes in tissue. Monoclonal antibodies to carbohydrate epitopes can be selected in the same way. Antibodies to sialyl–Tn and Tn (Kjeldsen *et al.*, 1988; Takahashi *et al.*, 1988) were found to be valuable in ocular mucin preparations [Fig. 7(b)].

Antibodies against mucin-specific epitopes are also useful during mucin purification. Many anti-mucin antibodies recognize peptide epitopes
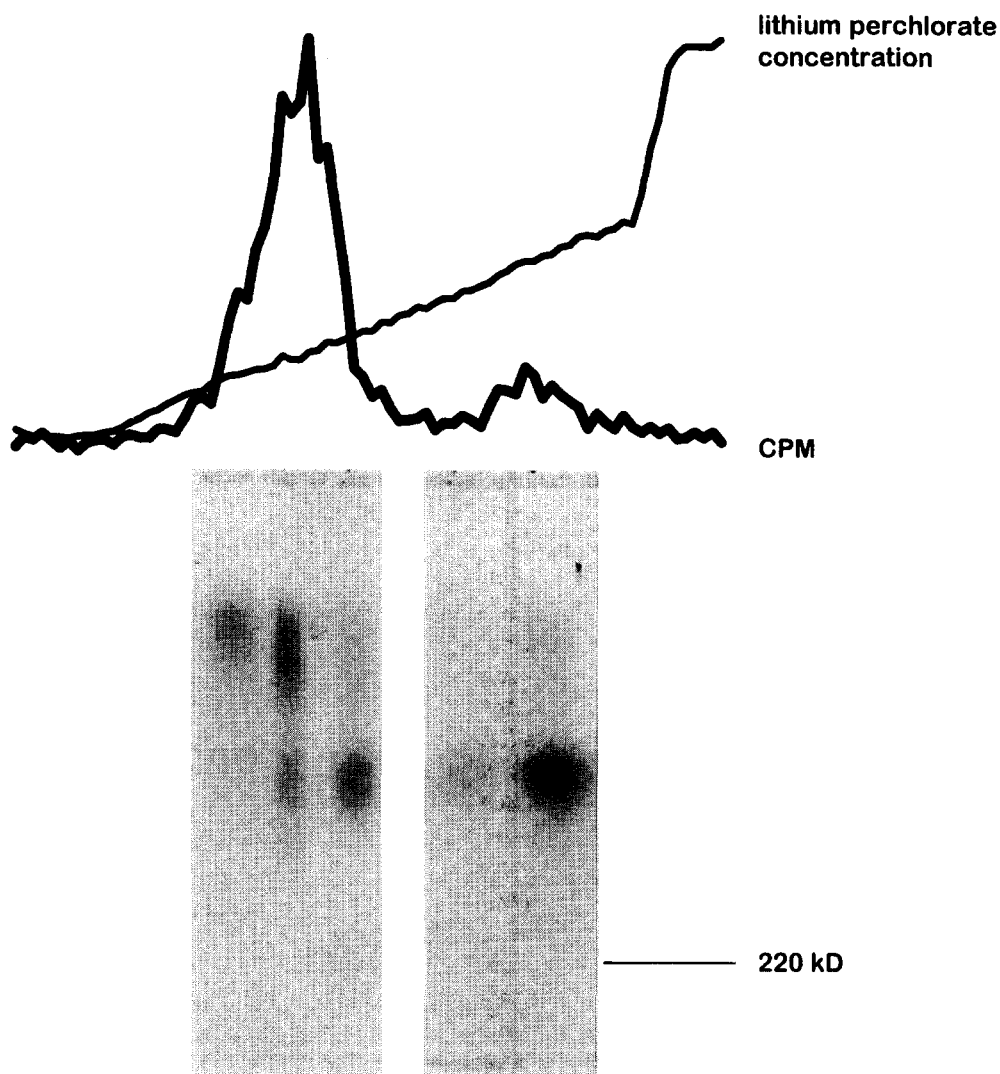
Fig. 8. Charge separation of human ocular mucin subunits by ion exchange chromatography. Upper panel: the elution profile on Mono-Q demonstrates the relatively low negative charge of most radiolabelled subunits, and shows the presence of a distinct population of higher charge (eluting at high salt concentrations). Lower panel: vacuum blots of 1% agarose gels, stained with WGA. Electrophoresis of the two peaks distinguishes bands of differing migration. Each blot is positioned under the corresponding area of the elution profile.

only after deglycosylation (Durrant *et al.*, 1994), a procedure that can destroy the peptide. The newly described anti-MUC2 and anti-MUC5AC antibodies (Carlstedt *et al.*, 1995) are valuable as they react with fully glycosylated native or subunit mucins. Antibodies to human gastric mucin peptide (anti-M1; Bara *et al.*, 1986) react with native and subunit canine ocular mucins, but only native human ocular mucins. Other useful reagents are listed in Table 1.

5. CHARACTERIZATION OF OCULAR MUCINS

5.1. Separation

We have isolated a number of mucin populations from human conjunctival tissue and total canine ocular surface mucus. The preparations have mucin characteristic buoyant density (Fig. 6), high MW on Sepharose gel filtration

200 kD
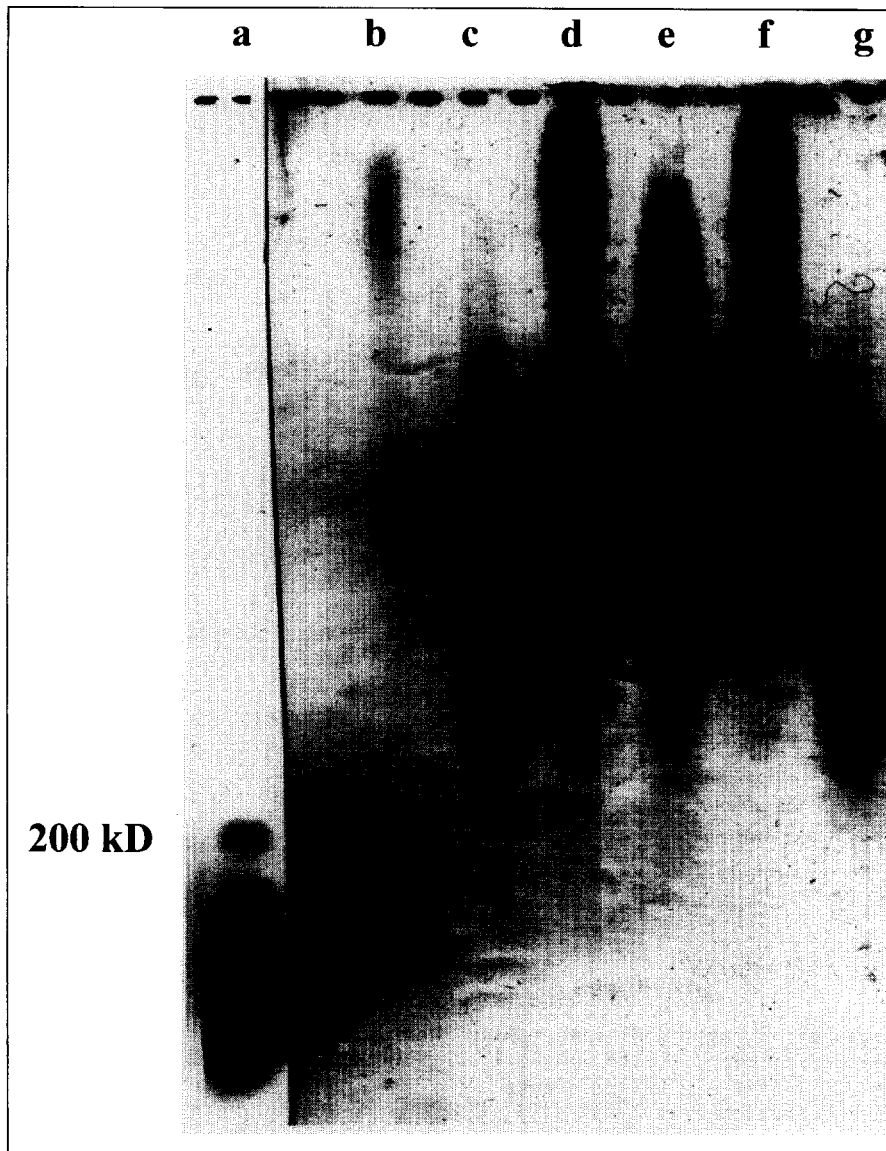
Fig. 9. (a) Electrophoretic mobility of normal canine ocular mucins (vacuum blot of 1% agarose gel, stained with WGA). Pools 1, 2 and 3 refer to mucins isolated by density gradient centrifugation (see Fig. 6). Lanes a, c and e: pools 1, 2 and 3 native material. Lanes b, d and f: pools 1, 2 and 3: reduced and alkylated. Lane g: high MW marker proteins. Material from pools 1, 2 and 3 show limited migration, indicating a very high MW for mucins in the normal canine eye. In each pool, reduction and alkylation lead to conversion into subunits: in pool 2, the subunit is of a very high MW; in pools 1 and 3, apparently similar subunits migrate substantially further. (b) Electrophoretic mobility of normal membrane-bound canine ocular mucins (vacuum blot of 1% agarose gel, stained with WGA). Lane a: standard (bovine submaxillary mucin—MW ~6 million Da). Lane b: standard (IgM—MW ~1 million Da). Lane c: normal canine ocular surface membranes, extracted with octyl glucoside. Lane d: material from lane c, reduced and alkylated. Two components of extremely high MW are resolved after octyl glucoside extraction of membranes (lane c). Upon reduction and alkylation of this material, the apparent MW of the larger component is decreased, causing it to comigrate with the smaller component (lane d). This change indicates subunit structure: a feature not previously recorded for membrane bound mucins or 'mucin-like' glycoproteins in any system.

Fig. 9(b). *Caption on opposite page.*

5.2. Glycosylation

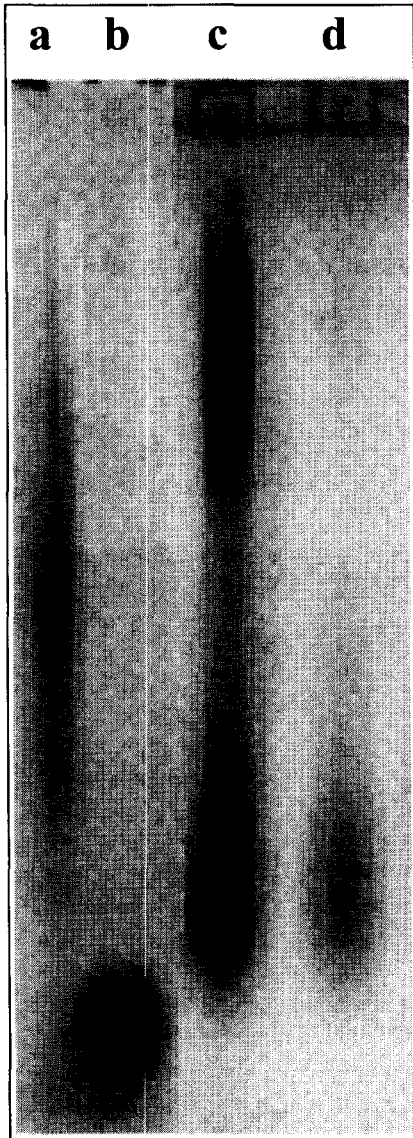Shared carbohydrate epitopes obligate the separation of mucins from other glycoconjugates before oligosaccharide analysis. The low proportion of carbohydrate and significant mannose content of partially purified ocular mucins (Moore and Tiffany, 1981; Chao *et al.*, 1983, 1988) suggest co-purification with non-mucin components. Rabbit ocular mucins purified using density gradient centrifugation have 84% carbohydrate, and no mannose, suggesting a higher purity (Tseng *et al.*, 1987).

Lectin and antibody staining of tissue sections has indicated the presence of hexosamines, galactose, and sialic acids in goblet cell vesicles. Ocular mucins are characterized by high proportions of *N*-acetyl galactosamine, *N*-acetyl glucosamine and galactose, variable amounts of sialic acids and low amounts of fucose (Moore and Tiffany, 1981; Tseng *et al.*, 1987; Chao *et al.*, 1988). The release of total oligosaccharides by β-elimination yields short chains only (Moore and Tiffany, 1981; Chao *et al.*, 1988).

Radiolabelling of the oligosaccharide side chains from human and canine mucins by β-elimination in the presence of borotritide suggests very short oligosaccharides. The prevalence of GalNAc-ol and Neu5Acα2-6GalNAc-ol was confirmed by gel filtration on Biogel P4 (Fig. 10) and oligosaccharide thin-layer chromatography. Cross-reaction with anti-Tn (1E3) and anti-Neu5Acα2-6GalNAc-peptide (anti-sialyl-Tn, TKH2) was observed throughout purification [Hicks *et al.*, unpublished; Berry *et al.*, in press; Fig. 7(b)]. Purified bovine and ovine salivary gland mucins show a similar pattern of short oligosaccharides (Tettamanti and Pigman, 1968; Corfield *et al.*, 1991a). Such short oligosaccharide chains require few glycosyltransferases for synthesis: these pathways are therefore amenable to study.

As an alternative to β-elimination, oligosaccharides may be released by hydrazinolysis and examined electrophoretically (Bigge *et al.*, 1995; Starr *et al.*, 1996). Glycosidase digestion, NMR and mass spectrometry can be used for the absolute determination of oligosaccharide structure (Dell *et al.*, 1993; Hounsell, 1995).

(Fig. 7) and are polydisperse on agarose gel electrophoresis (Fig. 9). They are susceptible to reduction yielding high MW subunits (Fig. 9) and bind anti-mucin antibodies. Ion exchange chromatography further separates several sub-populations within the largest human mucins (Fig. 8).

Table 1. *Specificity of probes used to detect mucins*

Name of probe	Specificity	Type
Wheat germ agglutin (WGA)	GlcNAc, sialic acid	Le
Ricinus communis toxin (RCA$_{60}$)	D-GalNAc, β-D-Gal	Le
Peanut agglutinin (PNA)	β-Gal(1–3)GalNAc	Le
Ulex europaeus (UEA I)	α-L-Fuc	Le
Maakia amurenis (Mal II)	α-2,3 linked sialic acid	Le
Sambucus nigra (SNA)	α-2,6 linked sialic acid	Le
Anti-M1	Human gastric mucin core protein	Mab
1E3	GalNAc-peptide (Tn)	Mab
TKH2	Sialyl-Tn	Mab
Periodic acid Schiff (PAS)	Vicinal glycol groups	C
Alcian Blue (AB)	Negative charge (SO$_3^{-}$, COO^{-})	C

Le, lectin; Mab, monoclonal antibody; C, chemical reagent.

Tn and sialyl–Tn, constitutively expressed in normal ocular mucins, are considered cancer markers in other mucosae (Singhal *et al.*, 1991; Fonseca *et al.*, 1994; Jass and Roberton, 1994; Itzkowitz *et al.*, 1995), and used in cancer therapy (O'Boyle *et al.*, 1992; Longenecker *et al.*, 1993). Therapeutic use of antibodies against epitopes that

are normal constituents of ocular tissue may affect its physiology.

5.3. Molecular Biology

The major secreted mucins in human ocular tissues are MUC5AC (Gipson and Inatomi, 1997)
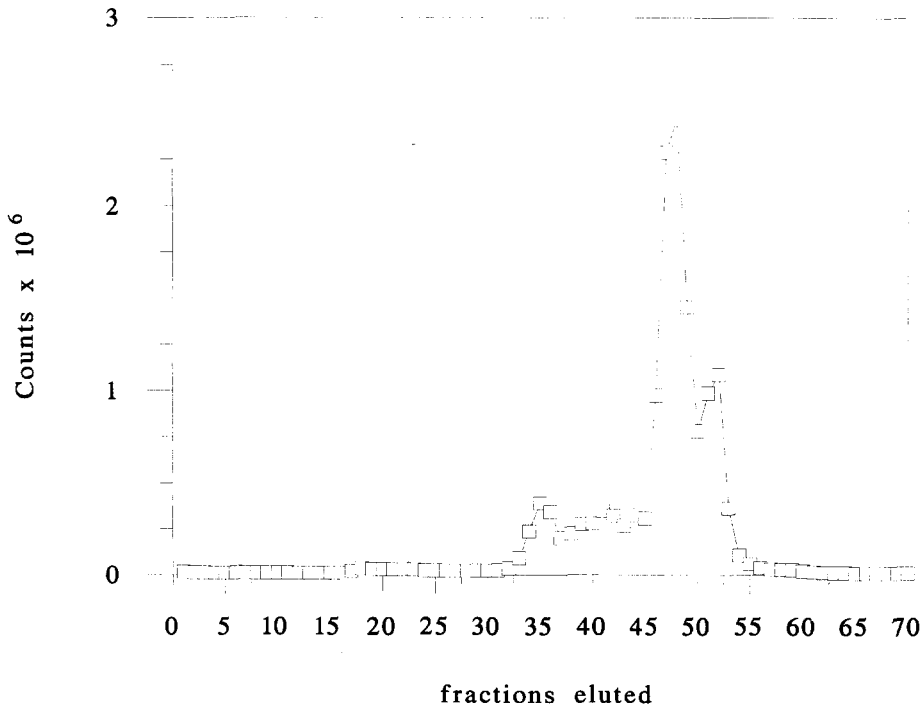

Fig. 10. Example of size-fractionation on Biogel P4 of oligosaccharides released from normal canine secreted ocular mucin (pool 2; see Fig. 6) by β-elimination. The majority of oligosaccharides are very short, the major peaks coeluting with the standards GalNAc-ol and Neu5Acα2–6GalNAc-ol (not shown), which elute in the range 46–48 ml and 51–53 ml, respectively.

and perhaps MUC4 (Jumblatt *et al.*, 1995; Gipson and Inatomi, 1997). MUC2 expression has been reported in human tears and conjunctiva (Bolis *et al.*, 1995; Jumblatt *et al.*, 1995), but *in-situ* hybridization experiments failed to confirm this (Gipson and Inatomi, 1997; Ellingham *et al.*, unpublished). The description of the full complement of mucin genes expressed at the ocular surface remains an immediate objective.

6. FUNCTION OF MUCINS IN THE TEAR FILM

Mucins lubricate the ocular surface and form a physical protective barrier that may also act as a matrix for smaller molecules. They optimize the refractive surface of the eye, and provide structures to which micro-organisms and immune cells can bind both specifically and non-specifically. Secreted and membrane-bound mucins are likely to interact to achieve these functions. The nature of interactions within the gel layer of the tear film is not well defined, but is likely to involve many other molecules, such as IgA, IgM, lysozyme, hyaluronan, amylase and lactoferrin (Fullard and Tucker, 1994; Kijlstra and Kuizenga, 1994).

The relationship between mucins in aqueous tears and in the gel layer awaits clarification. It is not yet clear whether the gel and aqueous phases exist in tears as separate entities. Mucins initially present in the gel phase might be solubilized by blinking. However, biochemical modification or degradation may be required for solubilization to occur. Soluble or aqueous tear mucins may be secreted as a distinct subgroup. Evidence suggesting this has been reported in the human gastric mucus layer (Ota and Katasuyama, 1992).

6.1. Tear Film Rheology

The eyelids and ocular surface are constantly in relative motion. The delicate extraocular muscles that move the eyes demand minimal frictional forces opposing them, especially during saccadic movements. During a blink, the eyelids must be able to move rapidly across the eye and spread secreted mucus. At the same time, it is necessary to avoid mechanical damage to the ocular surface due to the shear forces generated.

The rheological properties of tears are believed to be largely due to mucins. Solutions of mucins or hyaluronan (a normal component of the tear film) show non-Newtonian behaviour (Hamano and Mitsunaga, 1973; Tiffany, 1991, 1994b; Holly and Holly, 1994). This means that when a shear force is applied, e.g. during blinking, the viscosity of the solution falls. Newtonian behaviour is shown by solutions in which viscosity is independent of shear rate. Mucins form networks of entangled linear polymers. They separate during shearing, and re-entangle once the shear rate is reduced.

It is important that interpretation of tear film rheology measurements should be made in relation to the method used for sample collection. The aqueous and gel phases of the tear film may contain different concentrations and types of mucin, and the relative proportions of the two phases may vary with the sampling techniques employed.

6.2. Tear Film Stability

Between blinks, when the eye is open, the aqueous surface layer tends to evaporate. Without blinking and continued secretion, the tear film breaks up and dry spots can be seen on the ocular surface (Fig. 11). The mechanism of dry spot formation in normal eyes is unknown. It is possible that local shear forces generated at the margins of dry spots displace a proportion of the gel layer.

Tear film break-up time is thought to be influenced by mucins (Holly and Lemp, 1971, 1977): instability of the tear film being observed in dry eye syndromes in which there are mucin abnormalities (Bron, 1985, 1994). The presence of mucin in tears has been shown to lower the surface tension (Holly and Lemp, 1971, 1977) by interaction with tear lipids. The stability of the tear film is also influenced by: blink reflex; surface irregularities causing incongruity and loss of mucus layer; composition of the lipid
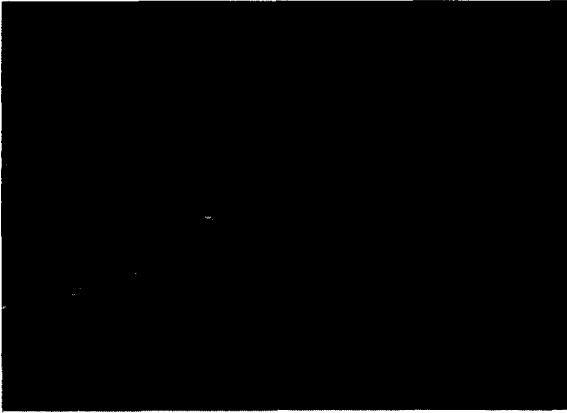
Fig. 11. Breakup of the precorneal tear film in a dog with KCS. The area of the corneal epithelium underlying the resulting 'dry spot' has a granular appearance (magnification: ×50).

layer influencing evaporation; and changes in the concentration of other tear film components (Bron, 1985, 1994).

The rate of formation of the ocular glycocalyx may influence the time taken for the tear film to break up. The glycocalyx develops as squamous cells reach the epithelial surface (Gipson *et al.*, 1992). It has been proposed that such sites presenting an immature glycocalyx, or other mucin depleted regions, may contribute to the initiation of dry spots (Sharma and Coles, 1990).

The anchoring of the gel layer to the ocular surface may be explained in part by molecular entanglement. Very high molecular weight (MW) mucin-like glycoproteins can be detected in octyl glucoside extracts of cell membranes derived from the canine ocular surface [Hicks, Carrington and Corfield, unpublished observation; Fig. 9(b)]. These membrane-bound molecules probably extend well beyond the conventional thickness of the glycocalyx, rendering entanglement with the secreted mucins possible.

6.3. Interactions with Micro-organisms

Mucins allow non-specific interactions with many particulates and chemicals at the ocular surface, enabling their neutralization or removal. The bulk carbohydrate properties of mucins are complemented by the expression of specific carbohydrate structures, which act as ligands in

cell or pathogen adhesion (Rogers *et al.*, 1986; Shimizu and Shaw, 1993; Schultze and Herrler, 1994; Yolken *et al.*, 1994; Lasky, 1995; Van Klinken *et al.*, 1995).

Whereas the ocular surface supports a commensal flora, it is generally accepted that secreted mucins protect epithelia by binding and entrapping pathogens. Part of the defensive function of the preocular surface involves anti-bacterial and anti-viral mechanisms. Ocular mucins, rich in sialic acid, may present themselves as binding partners in such mechanisms: influenza virus, coronavirus and rotavirus commonly bind to receptor sialic acids in intestinal mucins (Rogers *et al.*, 1986; Chen *et al.*, 1993; Schultze and Herrler, 1994; Yolken *et al.*, 1994). However, sialic acids also afford mucins protection from microbial proteolytic degradation (Nieuw-Amerongen *et al.*, 1995).

Pseudomonas aeruginosa, an aetiological agent in corneal ulcers, is known to bind scarified and immature intact corneal epithelium (Laibson, 1972; Hazlett *et al.*, 1995). Several carbohydrate epitopes capable of binding *P. aeruginosa* have been identified in the cornea, and implicated in the pathogenesis of corneal ulcers. These include 2–6-sialylated glycoprotein (Hazlett *et al.*, 1995), N-acetylmannosamine (Hazlett *et al.*, 1987), asialo-GM1 (Hazlett *et al.*, 1993; Gupta *et al.*, 1994) and sialic acids (Hazlett *et al.*, 1986). We have found predominant expression of NeuSAcα2–6GalNAc-*O*-Thr/Ser   and   GalNAc-*O*-Thr/Ser in secreted ocular mucins. This may suggest a 2–6-sialylated receptor similar to that found by Hazlett *et al.* (1995).

Demonstration that mucins too are binding partners supports the hypothesis that they also play a role in protection of the ocular surface (Fleiszig *et al.*, 1994) by blocking microbial binding sites before pathogens have penetrated the mucin barrier. Exposure of the corneal cell surface in wounding experiments has demonstrated that *P. aeruginosa* adherence at these sites is increased. A protective 'anti-adherence' mechanism has also been shown (Klotz *et al.*, 1989). In general, the diversity of oligosaccharide structures present within mucins offers a considerable scope for specific interactions (Ramphal and Pyle, 1983; Ritchings *et al.*, 1995) with potential microbial

pathogens. The interactions between ocular flora and pathogens remain to be fully explored.

7. MUCIN METABOLISM

Mucin synthesis requires considerable subcellular organization and integration. Incomplete knowledge of the structure, assembly, packaging and secretion of these molecules precludes a reliable overview of the biosynthetic processes for any mucosal system. Synthesis of mucins by the classical and the non-goblet cell pathways (Section 1) may share many processes, but probably differs in the modes of storage, targeting and secretion.

7.1. Biosynthesis of Secreted Goblet Cell Mucins

Studies in other mucosae show early dimerization or polymerization of mucin precursor polypeptides following translation. This is considered important for packaging of mucins into vesicles, and may involve disulphide linkages in von Willebrand domains as found in the salivary mucin MG1, in MUC2 and MUC 5AC. The addition of N-linked oligosaccharide chains targets the mucin precursors to the correct subcellular compartments for subsequent O-glycosylation (Dekker and Strous, 1990; Hilkens et al., 1992). Separation of different mucins into vesicles destined for distinct constitutive and stimulated pathways has been demonstrated in cultured colonic cells (McCool et al., 1995).

After subcellular sorting of a mucin polypeptide to the correct vesicles, transfer of the first monosaccharide N-acetyl-D-galactosamine occurs, followed by further O-glycosylation to yield the tissue specific oligosaccharide complement (Carlstedt et al., 1985; Schachter and Brockhausen, 1992; Brockhausen, 1993). This O-glycosylation of apomucin acceptor substrates is an integrated process involving a series of de-novo and salvage pathways generating nucleotide-sugars. These activated monosaccharides are the substrates for glycosyltransferases. O-glycosylation is thought to involve multi-enzyme complexes integrated with nucleotide sugar antiporters. These

antiporters ensure a sufficient supply of nucleotide sugars for glycosylation (Carlstedt et al., 1985, Schachter and Brockhausen, 1992; Brockhausen, 1993). Compartmentalization at each stage allows the optimal interaction of a selected group of glycosyltransferases with the mucin apoproteins, giving rise to fully glycosylated mucins. Other post-translational processes may result in the final product for secretion. These include sulphation, acylation and phosphorylation.

The majority of these processes remain to be elucidated at the enzymic level in the eye. However, the simplicity of glycosylation in ocular secreted mucins (Section 5.2.) suggests a less complex system than that found at many other mucosal surfaces.

7.2. Mucin Secretion

Mucins are stored in goblet vesicles under conditions that regulate their water binding. The precise nature of this packaging is not fully understood, but is thought to include ion-binding (Verdugo, 1991) and disulphide bridge formation related to von Willebrand domains (Gum et al., 1992; Gum, 1995).

Preocular secreted mucin is a mixture of distinct subtypes. These may arise from separate populations of vesicles within individual goblet cells, or from subpopulations of goblet cells expressing different mucins. Regulation of the proportion of different mucins may occur at the level of biosynthesis, release from individual secretory granules or release from individual goblet cells.

The mechanism of mucin secretion is not fully understood, but consists of extrusion of vesicle contents, hydration and creation of an extracellular gel. Progress has been made in the identification of stimuli that are involved in triggering this secretion. Neural control of ocular mucin secretion has been elegantly shown in experiments with corneal wounding, which leads to the release of mucins from conjunctival cells (Kessler et al., 1995). Direct innervation of conjunctival goblet cells has been shown through neurons containing synaptophysin and vasoactive intestinal polypeptide (VIP) (Dartt et al., 1995). Stimulation of goblet cell secretion may also be effected by

diffusion of neurotransmitters from the variety of nerves in conjunctival epithelium and stroma. Parasympathetic, serotonergic, dopaminergic and sympathetic nerves may trigger secretion (Dartt, 1994; Kessler and Dartt, 1994; Kessler *et al.*, 1995). This pattern is distinct from that seen in other mucosae (Forstner, 1995). Osmolarity has also been identified as a factor controlling goblet cell vesicles and the overall integrity of goblet cells in conjunctival tissue (Huang *et al.*, 1989). Hypertonic solutions applied to the rabbit conjunctiva disrupt the surface epithelium, reduce goblet cell numbers and induce abnormal discharge of mucin granules—intact vesicles being shed under these conditions (Huang *et al.*, 1989). A paracrine 'mucin stimulating factor', as yet uncharacterized, has also been demonstrated in tears (Franklin and Bang, 1980).

Shedding of membrane-bound mucins from the glycocalyx may add to the secreted mucins of the tear film (Ligtenberg *et al.*, 1992; McGuckin *et al.*, 1995; Zhang *et al.*, 1996). Identification of glycocalyx mucins is incomplete (Watanabe *et al.*, 1993, 1995), and the mode of release has not yet been identified. In human conjunctiva, we have found high-molecular-weight (>300 kDa) mucin-like molecules with a glycosylphosphatidyl inositol (GPI) membrane anchor (Corfield *et al.*, 1991b). In other tissues, GPI-anchored molecules are attached to apical membranes (Lisanti *et al.*, 1988, 1989). The anchor comprises phosphatidyl inositol, linked to a characteristic glycan and terminating in ethanolamine. The ethanolamine is, in turn, attached to the C terminus of the target molecule (Ferguson, 1992).

Release of GPI-anchored molecules from surface membranes may be controlled by the action of cellular phospholipases, cleaving at the phosphodiester bonds between inositol and diacylglycerol. Cellular and subcellular location of these molecules in the eye has not been determined, but will be of great interest with regard to glycocalyx targeting, the small vesicle pathway and membrane shedding.

The GPI-membrane anchor is different to the transmembrane peptide anchor identified for MUC1. At present, it is not possible to ascribe these GPI-anchored molecules to any MUC products or to other glycoproteins in their size range.

7.3. Mucin Degradation

The mechanism of mucus turnover at the ocular surface is unclear, and several fundamental questions remain unanswered. The presence of a mucus thread in the lower conjunctival fornix, containing insoluble mucus, proteins, lipids and desquamated cells (Tseng *et al.*, 1987), has been suggested as a mechanism for mucin elimination from the ocular surface. Although previous studies have used such mucus threads as a principal source of mucin (Moore and Tiffany, 1979; Chao *et al.*, 1983), the relation between mucin within the thread and the gel layer of the tear film is not yet clear. It is not known whether mucin is removed from the ocular surface as an insoluble complex, or as soluble mucin after proteolytic degradation.

Examination of human tear fluid revealed mucins with a different electrophoretic mobility than those derived from tissue homogenates. The difference in mobility suggests that larger mucins do not appear as soluble products in the aqueous component of the tear film (Fig. 12). The faster-migrating components may be smaller mucins or degraded mucin fragments. Studies of purified ocular mucins degraded by commensal or pathological bacteria may shed light on the manner of mucin removal from the ocular surface.

There is very little evidence for the presence of specific mucin-degrading enzymes in the tear film, although proteolytic activity has been detected (Sack *et al.*, 1992; Hamdi *et al.*, 1995) as has hyaluronidase. Hyaluronan has been demonstrated in the ocular surface epithelia (Madsen *et al.*, 1989; Rittig *et al.*, 1992) and in tears (Frescura *et al.*, 1994). Interactions of mucins with this glycopolymer may be significant in tear film rheology: proteoglycan-degrading enzymes, therefore, may also be involved in turnover of the tear film gel layer.

8. OCULAR MUCINS IN DISEASE

There are relatively little data on the biochemical changes in ocular mucins in disease. The number of goblet cells in the conjunctiva has been assessed using standard histology, flat preparations of conjunctival biopsies and impression cytology
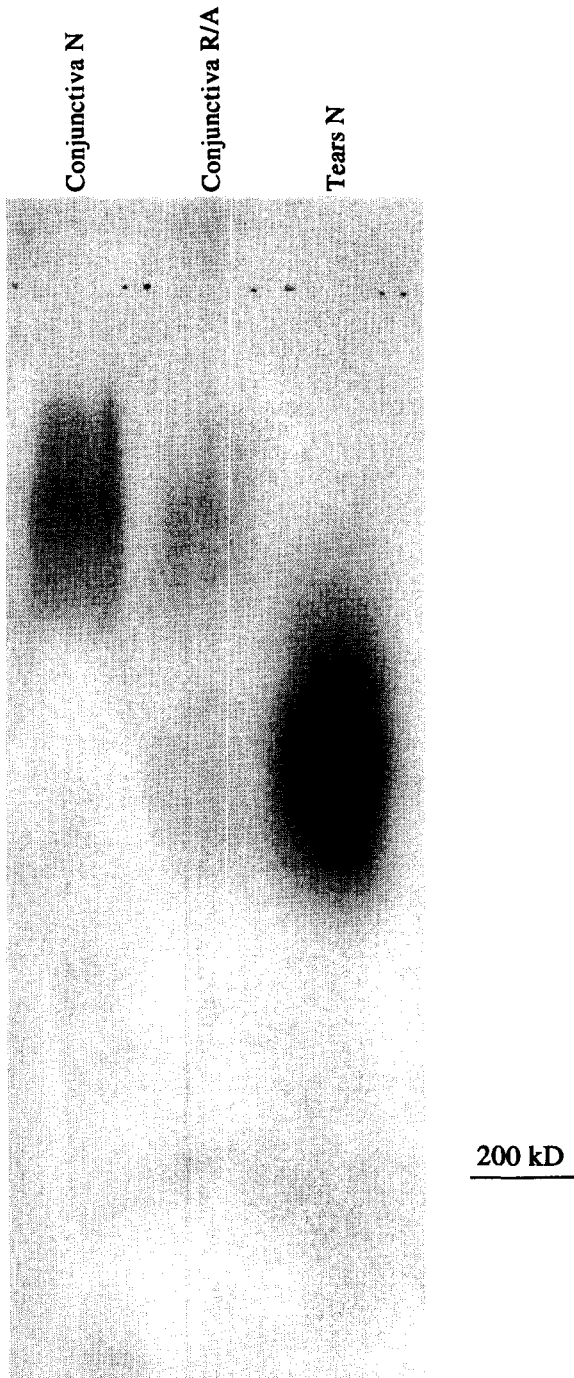
Conjunctiva N

Conjunctiva R/A

Tears N

200 kD

Fig. 12. Electrophoretic mobility of mucins in human tears (vacuum blot of 1% agarose gel, stained with WGA). Excluded fractions of conjunctival homogenate and tears (buoyant density 1.3–1.35 g/ml) were compared. Reduction and alkylation of conjunctival mucin give rise to a component that has a similar mobility to mucin found in native tears (N = native mucin; R/A = reduced and alkylated mucin).

(Nelson *et al.*, 1983; Tseng, 1985; Adams and Dilly, 1989). In each case, stains chosen to identify mucin are used to assess goblet cell numbers. Differential staining of goblet cells may complicate quantitative assessment (Section 3.1).

Flat preparations have the advantage of providing a sheet of conjunctiva in which the number of goblet cells present an accurate representation of the number *in vivo*. However, adjustment for shrinkage and stretching of the tissue must be made when calculating density. This method shows a considerable normal variation in the number of goblet cells in the conjunctivae of rats of very similar age and weight and which have been housed in identical conditions (Kessler *et al.*, 1995). Biopsy techniques have the obvious disadvantage of excisional tissue loss, and in human studies, ethical considerations must be made.

Impression cytology has the advantage of being simple and non-invasive. It can be performed repeatedly and extensively on the same subject, and a particular area of conjunctiva may be sampled more than once. Although it is straightforward to define the area sampled, it is uncertain as to what proportion of the goblet cells leave an impression on the filter. The wide variation in goblet cell impressions from normal controls (Rolando *et al.*, 1994) makes comparisons difficult.

Vernal allergic conjunctivitis, which may be associated with increased mucous discharge, has been studied with impression cytology. Goblet cells are found to be present in increased numbers and produce impressions that are characteristically smaller than in normal controls (Aragona *et al.*, 1996).

Dry eye in humans results in various patterns of epithelial change reflected in squamous metaplasia and loss of goblet cells. The term 'dry eye' covers many tear film abnormalities (Bron, 1985, 1994; Bron *et al.*, 1985), and the symptoms of dry eye in man are common to several surface pathologies. It is not always clear whether ocular surface changes cause, or are a result of, dry eye. Loss of goblet cells is associated with secondary dry eye symptoms and decreased tear film break up time (Toda *et al.*, 1995). However, increased tear osmolarity, as in meibomian and lacrimal gland diseases, causes a loss of goblet cells (Gilbard *et al.*,

1987, 1988). Return to normal tear osmolarity, by applying a hypotonic artificial tear solution, increases the number of goblet cells (Gilbard and Rossi, 1992). In addition to the changes in osmolarity, the pathological mechanisms in dry eye may include the loss of hyaluronan (Inoue and Katakami, 1993), retinoids (Driot and Bonne, 1992; Gilbard and Rossi, 1992; Ubels *et al.*, 1995) or cytokines such as epidermal growth factor (Inoue and Katakami, 1993).

Not all areas of the human conjunctiva are equally affected in dry eye, and different pathologies cause different regional effects. In keratoconjunctivitis sicca (KCS), reduced numbers of goblet cells were observed in the interpalpebral bulbar conjunctiva, whereas both this area and the inferior tarsal region had reduced goblet cell numbers in ocular cicatricial pemphigoid (Nelson and Wright, 1984). The superior bulbar conjunctiva revealed no change in KCS patients (Petroutsos *et al.*, 1992). Both the inferior tarsal and the superior bulbar regions are protected from exposure to the atmosphere and are less likely to show surface pathology through drying than the inferior bulbar conjunctiva. In contrast, the whole conjunctiva will be affected by a primary ocular surface disease, such as ocular cicatricial pemphigoid. Some KCS patients with Sjogren's syndrome

may have a primary conjunctival immune dysfunction resulting in a non-selective loss of goblet cells in all regions of the conjunctiva (Pflugfelder *et al.*, 1990) differing with the pattern described by Nelson for ocular cicatricial pemphigoid (Nelson and Wright, 1984).

The pathological changes in dry eye may result in structural alterations to the mucins secreted by the conjunctival epithelial cells. Lectin staining of mucus granules in dry eye patients has suggested a reduction in sialic acids, N-acetyl-glucosamine, N-acetyl-galactosamine and galactose-N-acetylgalactosamine, and an increase in glucose and mannose (Versura *et al.*, 1986). Whether or not the results indicate mucin-related changes cannot be concluded from these lectin binding studies.

The dog is a particularly suitable model for the study of changes in ocular mucin secretion in disease because affected dogs produce a copious secretion (Fig. 13). Differences in electrophoretic mobility exist between normal and KCS mucin samples (Hicks, Carrington and Corfield, unpublished). Some alteration in the relative proportions of oligosaccharides may also occur (Hicks, Carrington and Corfield, unpublished). The impact of such chemical changes on the rheology of the tear film has not been assessed, but is likely to be of pathological significance.

Fig. 13. Beagle dog with keratoconjunctivitis sicca: a copious accumulation of abnormal ocular mucus is visible (photograph courtesey of S. M. Crispin).

9. SUMMARY STATEMENT AND FUTURE DIRECTIONS

The ocular surface is covered by a mixture of membrane-anchored and secreted mucins that interact with the tear film and ocular epithelia. The study of ocular mucins is still in its infancy, and more questions are raised here than are answered.

We know that secreted ocular mucins, both canine and human, show multiple subunits and have short oligosaccharide chains. However, the total number of mucins that are normally present is unknown. We do not know any details of mucin biosynthesis in the normal or diseased eye. Membrane-bound mucins and mucin-like molecules are present in the ocular glycocalyx, but it is not yet clear how or if they are shed into the tear film. The ocular surface sustains a bacterial flora, but we do not yet know which micro-environmental factors select the commensals, and whether they contribute to mucin degradation. The detailed manner in which spent mucin is eliminated from the ocular surface is also unclear, since this may involve more than the mucus thread alone.

This chapter presents sensitive biochemical methods that ensure the isolation and purification of the whole complement of ocular mucins. An understanding of the detailed biochemistry of preocular mucins may hold the key to understanding and manipulating their physiology.

REFERENCES

Adams, G. G. and Dilly, P. N. (1989) Differential staining of ocular goblet cells. *Eye* **3:** 840–844.

Ahmed, A. and Grierson, I. (1989) Cellular carbohydrate components in human, rabbit and rat lacrimal gland. *Graefe's Arch. clin. exp. Ophthalmol.* **227:** 78–87.

Aitken, D., Friend, J., Thoft, R. A. and Lee, W. R. (1988) An ultrastructural study of rabbit ocular surface transdifferentiation. *Invest. Ophthalmol. Vis. Sci.* **29:** 224–231.

Allen, A. and Pearson, J. P. (1993) Mucus glycoproteins of the normal gastrointestinal tract. *Eur. J. Gastroenterol. Hepatol.* **5:** 193–199.

Aragona, P., Romeo, G. F., Puzzelo, D., Micali, A. and Ferreri, G. (1996) Impression cytology of the conjunctival epithelium in patients with vernal conjunctivitis. *Eye* **10:** 82–85.

Baeckstrom, D., Hansson, G. C., Nilsson, O., Johansson, C., Gendler, S. J. and Lindholm, L. (1991) Purification and characterisation of a membrane bound and a secreted mucin-type glycoprotein carrying the carcinoma associated sialyl-Le[a] epitope on distinct core proteins. *J. biol. Chem.* **266:** 21537–21547.

Bara, J., Gautier, R., Daher, N., Zaghouani, H. and Decaens, C. (1986) Monoclonal antibodies against oncofetal mucin M1 antigens associated with precancerous colonic mucosae. *Cancer Res.* **46:** 3983–3989.

Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M. and Parekh, R. B. (1995) Nonselective and efficient fluorescent labelling of glycans using 2-amino benzamide and anthranilic acid. *Analyt. Biochem.* **230:** 229–238.

Bolis, S., Devine, P. and Morris, C. A. (1995) Detection and characterisation of human ocular mucins. *Invest. Ophthalmol. Vis. Sci.* **36:** S421 (abstract 1933).

Breipohl, W., Spitznas, M., Sinowatz, F., Leip, O., Naib-Majani, W. and Cusumano, A. (1994) Galactose-binding sites in the acinar cells of the human accessory lacrimal gland. *Adv. exp. Med. Biol.* **350:** 45–48.

Brockhausen, I. (1993) Clinical aspects of glycoprotein synthesis. *Crit. Rev. clin. Lab. Sci.* **30:** 65–151.

Bron, A. J. (1985) Prospects for the dry eye. *Trans. Opthalmol. Soc. UK* **104:** 801–826.

Bron, A. J. (1994) Non-Sjögren dry eye: Pathogenesis, diagnosis and animal models. *Adv. exp. Med. Biol.* **350:** 471–488.

Bron, A. J., Tiffany, J. M., Kaura, R. and Mengher, L. (1985) Disorders of the tear film lipids and mucous glycoproteins. In: *External Eye Disease* (D. L. Easty and G. Smolin, eds), pp. 63–105. Butterworth, London.

Carlstedt, I., Herrmann, A., Hovenberg, H., Lindell, G., Nordman, H., Wickström, C. and Davies, J. R. (1995) 'Soluble' and 'insoluble' mucins—identification of distinct populations. *Biochem. Soc. Trans.* **23:** 845–851.

Carlstedt, I., Herrmann, A., Karlsson, H., Sheehan, J., Fransson, L.-A. and Hansson, G. C. (1993) Characterisation of two different glycosylated domains from the insoluble mucin complex of rat small intestine. *J. biol. Chem.* **268:** 18771–18781.

Carlstedt, I. and Sheehan, J. K. (1984) Macromolecular properties and polymeric structure of mucus glycoproteins. In: *Mucus and mucosa.* CIBA Foundation Symposium Vol. 109, pp. 157–172. Pitman, London.

Carlstedt, I., Sheehan, J. K., Corfield, A. P. and Gallagher, J. T. (1985) Mucous glycoproteins: a gel of a problem. *Essays Biochem.* **20:** 40–76.

Carraway, K. L. and Hull, S. R. (1991) Cell surface mucin-type glycoproteins and mucin-like domains. *Glycobiology* **1:** 131–138.

Chao, C. C. W., Butala, S. M. and Herp, A. (1988) Studies on the isolation and composition of human ocular mucin. *Exp. Eye Res.* **47:** 185–196.

Chao, C. C. W., Vergnes, J.-P. and Brown, S. L. (1983) Fractionation and partial characterisation of macromolecular components from human ocular mucins. *Exp. Eye Res.* **36:** 139–150.

Chao, W. C. C., Vergnes, J.-P., Freeman, I. L. and Brown, S. I. (1980) Biosynthesis and partial characterisation of tear film glycoproteins. Incorporation of radioactive precursors by human lacrimal gland explants *in vitro*. *Exp. Eye Res.* **30:** 411–425.

Chen, C. C., Baylor, M. and Bass, D. M. (1993) Murine intestinal mucins inhibit rotavirus infection. *Gastroenterology* **105**: 84–92.

Chen, W. Y. W., Mui, M. M., Kao, W. W. Y., Liu, C. Y. and Tseng, S. C. G. (1994) Conjunctival epithelial cells do not transdifferentiate in organotypic cultures: Expression of k12 keratin is restricted to corneal epithelium. *Curr. Eye Res.* **13**: 765–778.

Corfield, A. P., do Amaral Corfield, C., Veh, R. W., Wagner, S. A., Clamp, J. R. and Schauer, R. (1991a) Characterisation of the major and minor mucus glycoproteins from bovine submandibular gland. *Glycoconj. J.* **8**: 330–339.

Corfield, A. P., Myerscough, N., Berry, M., Clamp, J. R. and Easty, D. L. (1991b) Mucins synthesised in organ culture of human conjunctival tissue. *Biochem. Soc. Trans.* **19**: 352S.

Corfield, A. P., Myerscough, N., Gough, M., Brockhausen, I., Schauer, R. and Paraskeva, C. (1995) Glycosylation patterns of mucins in colonic disease. *Biochem. Soc. Trans.* **23**: 840–845.

Corfield, A. P. and Paraskeva, C. (1993) Secreted mucus glycoproteins in cell and organ culture. In: *Glycoprotein Analysis in Biomedicine* (E. F. Hounsell, ed.), Vol. 14, pp. 211–232. Humana Press, Totowa.

Creeth, J. M. and Horton, J. R. (1977) Macromolecular distribution near the limits of density-gradient columns: Some applications to the separation of glycoproteins. *Biochem. J.* **161**: 449–458.

Dartt, D. A. (1994) Regulation of tear secretion. *Adv. exp. Med. Biol.* **350**: 1–9.

Dartt, D. A., McCarthy, D. M., Mercer, H. J., Kessler, T. L., Chung, E. H. and Zieske, J. D. (1995) Localisation of nerves adjacent to goblet cells in rat conjunctiva. *Curr. Eye Res.* **14**: 993–1000.

Dekker, J., Aelmans, P. H. and Strous, G. J. (1991) The oligomeric structure of rat and human gastric mucins. *Biochem. J.* **277**: 423–427.

Dekker, J. and Strous, G. J. (1990) Covalent oligomerisation of rat gastric mucin occurs in the rough endoplasmic reticulum, is N-glycosylation dependent, and precedes initial O-glycosylation. *J. biol. Chem.* **265**: 18116–18122.

Dell, A., Khoo, K. H., Panico, M., McDowell, R. A., Etienne, A. T., Reason, A. J. and Morris, H. R. (1993) FAB-MS and ES-MS of glycoproteins. In: *Glycobiology* (M. Fukoda and A. Kobata, eds), pp. 187–222. IRL Press, Oxford.

Dilly, P. N. (1985) On the nature and the role of the subsurface vesicles in the outer epithelial cells of the conjunctiva. *Br. J. Ophthalmol.* **69**: 477–481.

Dilly, P. N. (1994) Structure and function of the tear film. *Adv. exp. Med. Biol.* **350**: 239–247.

Dilly, P. N. and Mackie, I. A. (1981) Surface changes in the anaesthetic conjunctiva in man, with special reference to the production of mucus from non-goblet-cell sources. *Br. J. Ophthalmol.* **65**: 833–837.

Driot, J. Y. and Bonne, C. (1992) Beneficial effects of a retinoic acid analog, cbs-211 a, on an experimental model of keratoconjunctivitis sicca. *Invest. Ophthalmol. Vis. Sci* **33**: 190–195.

Durrant, L. G., Jacobs, E. and Price, M. R. (1994) Production of monoclonal antibodies recognising the peptide core of MUC2 intestinal mucin. *Eur. J. Cancer.* **30A**: 355–363.

Ferguson, M. A. J. (1992) Glycosyl–phosphatidylinositol membrane anchors: The tale of a tail. *Biochem. Soc. Trans.* **20**: 243–256.

Fleiszig, S. M. J., Zaidi, T. S., Ramphal, R. and Pier, G. B. (1994) Modulation of *Pseudomonas aeruginosa* adherence to the corneal surface by mucus. *Infect. Immunol.* **62**: 1799–1804.

Fonseca, I., Rosa, J. C., Felix, A., Therkildsen, M. H., Mandel, U. and Soares, J. (1994) Simple mucin-type carbohydrate antigens (T, Tn and sialosyl-Tn) in mucoepidermoid carcinoma of the salivary glands. *Histopathology* **25**: 537.

Forstner, G. (1995) Signal transduction, packaging and secretion of mucins. *A. Rev. Physiol.* **57**: 585–605.

Forstner, J. F. and Forstner, G. G. (1994) Gastrointestinal mucus. In: *Physiology of the Gastrointestinal Tract* (L. R. Johnson, ed.), pp. 1245–1283. Raven Press, New York.

Franklin, R. M. and Bang, B. G. (1980) Mucus stimulating factor in tears. *Invest. Ophthalmol. Vis. Sci.* **19**: 430–432.

Frescura, M., Carrington, S., Berry, M., Easty, D. and Corfield, A. P. (1994) Evidence of hyaluronan in human tears and secretions of conjunctival cultures. *Biochem. Soc. Trans.* **21**: 228S.

Friend, J., Kiorpes, T. C. and Thoft, R. A. (1983) Conjunctival goblet cell frequency after alkali injury is not accurately reflected by aqueous tear mucin content. *Invest. Ophthalmol. Vis. Sci.* **24**: 612–621.

Fullard, R. J. and Tucker, D. (1994) Tear protein composition and the effects of stimulus. *Adv. exp. Med. Biol.* **350**: 309–314.

Fung, D. C. K., Somerville, M., Richardson, P. S. and Sheehan, J. S. (1995) Mucus glycoconjugate complexes released from feline trachea by a bacterial toxin. *Am. J. Resp. Cell Molec. Biol.* **12**: 296.

Garcher, C., Bara, J., Bron, A. and Oriol, R. (1994) Expression of mucin peptide and blood group ABH and Lewis-related carbohydrate antigens in normal human conjunctiva. *Invest. Ophthalmol. Vis. Sci.* **35**: 1184–1191.

Gendler, S. J. and Spicer, A. P. (1995) Epithelial mucin genes. *A. Rev. Physiol.* **57**: 607–634.

Gilbard, J. P. and Rossi, S. R. (1992) An electrolyte-based solution that increases corneal glycogen and conjunctival goblet-cell density in a rabbit model for keratoconjunctivitis sicca. *Ophthalmology* **99**: 600–604.

Gilbard, J. P., Rossi, S. R. and Gray, K. L. (1987) A new rabbit model for keratoconjunctivitis sicca. *Invest. Ophthalmol. Vis. Sci.* **28**: 225–228.

Gilbard, J. P., Rossi, S. R., Gray, L. A., Hanninen, K. R. and Kenyon, K. R. (1988) Tear film osmolarity and ocular surface disease in two rabbit models for keratoconjunctivitis sicca. *Invest. Ophthalmol. Vis. Sci.* **29**: 374–378.

Gipson, I. K. and Inatomi, T. (1997) Mucin genes expressed by the ocular surface epithelium. *Progress in Retinal and Eye Research* **16**: 81–98.

Gipson, I. K., Riddle, C. V., Kiorpes, T. C. and Spurr, S. J. (1983) Lectin binding to cell surfaces: comparisons between normal and migrating corneal epithelium. *Devl. Biol.* **96**: 337–345.

Gipson, I. K., Spurr-Michaud, S. J., Tisdale, A. S., Kublin, C., Cintron, C. and Keutmann, H. (1995) Stratified squamous epithelia produce mucin-like glycoproteins. *Tissue and Cell* **27**: 397–404.

Gipson, I. K., Yankauckas, M., Spurr-Michaud, S. J., Tisdale, A. S. and Rinehart, W. (1992) Characteristics of a

glycoprotein in the ocular surface glycocalyx. *Invest. Ophthalmol. Vis. Sci.* **33:** 218–227.

Greiner, J. V., Kenyon, K. R., Henriquez, A. J., Korb, D. R., Weidman, T. A. and Allansmith, M. R. (1980) Mucus secretory vesicles in conjunctival epithelial cells of wearers of contact lenses. *Arch. Ophthalmol.* **98:** 1843–1846.

Greiner, J. V., Weidman, T. A., Korb, M. R. and Allansmith, M. R. (1985) Histochemical analysis of secretory vesicles in non-goblet conjunctival epithelial cells. *Acta Ophthalmol.* **63:** 89–92.

Gum, J. R. (1992) Mucin genes and the proteins they encode: structure diversity and regulation. *Am. J. Respir. Cell Molec. Biol.* **7:** 557–564.

Gum, J. R. (1995) Human mucin glycoproteins: varied structures predict diverse properties and specific functions. *Biochem. Soc. Trans.* **23:** 795–799.

Gum, J. R., Hicks, J. W., Toribara, N. W., Rothe, E.-M., Legace, R. E. and Kim, Y. S. (1992) The human MUC2 intestinal mucin has cysteine-rich sub-domains located both upstream and downstream of its central repetitive region. *J. biol. Chem.* **267:** 21375–21383.

Gupta, S. K., Berk, R. S., Masinick, S. and Hazlett, L. D. (1994) Pili and LPS of *Pseudomonas aeruginosa* bind to the glycolipid asialo-GM1. *Infect. Immunol.* **62:** 4572–4579.

Hamano, H. and Mitsunaga, S. (1973) Viscosity of rabbit tears. *Japan. J. Ophthalomol.* **17:** 290–299.

Hamdi, H. K., Berjis, F., Brown, D. J. and Kenney, M. C. (1995) Proteinase activity in normal human tears: male–female dimorphism. *Curr. Eye Res.* **14:** 1081–1086.

Hazlett, L. D., Masinick, S., Barrett, R. and Rosol, K. (1993) Evidence for asialo-GM1 as a corneal glycolipid receptor for *Pseudomonas aeruginosa* adhesion. *Infect. Immunol.* **61:** 5164–5173.

Hazlett, L. D., Moon, M. M. and Berk, R. S. (1986) *In vivo* identification of sialic acid as the ocular receptor for *Pseudomonas aeruginosa. Infect. Immunol.* **51:** 687–689.

Hazlett, L. D., Moon, M. M., Strejk, M. and Berk, R. S. (1987) Evidence for *N*-acetylmannosamine as an ocular receptor for *P. aeruginosa* adherence to scarified cornea. *Invest. Ophthalmol. Vis. Sci.* **28:** 1978–1985.

Hazlett, L., Rudner, X. W., Masininck, S., Ireland, M. and Gupta, S. (1995) In the immature mouse, *Pseudomonas aeruginosa* pili bind a 57-kD (alpha 2–6) sialylated corneal epithelial cell surface protein: a first step in infection. *Invest. Ophthalmol. Vis. Sci.* **36:** 634–643.

Hilkens, J., Ligtenberg, M. J. L., Vos, H. L. and Litvinov, S. V. (1992) Cell-membrane associated mucins and their adhesion-modulating property. *Trends biol. Sci.* **17:** 359–363.

Holly, F. J. (1973) Formation and rupture of the tear film. *Exp. Eye Res.* **15:** 515–525.

Holly, F. J. and Holly, T. F. (1994) Advances in ocular tribology. *Adv. exp. Med. Biol.* **350:** 275–284.

Holly, F. J. and Lemp, M. A. (1971) Wettability and wetting of corneal epithelium. *Exp. Eye Res.* **11:** 239–250.

Holly, F. J. and Lemp, M. A. (1977) Tear physiology and dry eye. *Surv. Ophthalmol.* **22:** 69–87.

Hounsell, E. F. (1995) H-1 NMR in the structural and conformational analysis of oligosaccharides and glycoconjugates. In: *Progress in Nuclear Magnetic Resonance Spectroscopy* (J. W. Emsley, J. Feeny and L. H. Sutcliffe, eds), Vol. 27, pp. 445–474. Elsevier, Amsterdam.

Huang, A. J., Belldegrun, R., Hanninen, L., Kenyon, K. R., Tseng, S. C. and Refojo, M. F. (1989) Effects of hypertonic solutions on conjunctival epithelium and mucin-like glycoproteins. *Cornea* **8:** 15–20.

Huang, A. J. W. and Tseng, S. C. G. (1987) Development of monoclonal antibodies to rabbit ocular mucin. *Invest. Ophthalmol. Vis. Sci.* **28:** 1483–1491.

Inatomi, T., Spurr-Michaud, S., Tisdale, A. and Gipson, I. K. (1995) Human corneal and conjunctival epithelia express MUC1 mucin. *Invest. Ophthalmol. Vis. Sci.* **36:** 1818–1827.

Inoue, M. and Katakami, C. (1993) The effect of hyaluronic acid on corneal epithelial cell proliferation. *Invest. Ophthalmol. Vis. Sci.* **34:** 1313–1315.

Irimura, T., Carlson, D. A. and Ota, D. M. (1988) Electrophoretic analysis of four high molecular weight sialoglycoproteins produced by metastatic human colon carcinoma cells. *J. Cell. Biochem.* **37:** 1–9.

Itzkowitz, S. H., Marshall, A., Kornbluth, A., Harpaz, N., McHugh, J. B. D., Ahnen, D. and Sachar, D. B. (1995) Sialosyl-Tn antigen: initial report of a new marker of malignant progression in long standing ulcerative colitis. *Gastroenterology* **109:** 490–497.

Jass, J. R. and Roberton, A. M. (1994) Colorectal mucin histochemistry in health and disease: A critical review. *Pathol. Int.* **44:** 487–504.

Jumblatt, M. M., Geoghegan, T. E. and Jumblatt, J. E. (1995) Mucin gene expression in human conjunctiva. *Invest. Ophthalmol. Vis. Sci.* **36:** S997.

Kawano, K., Uehara, F., Sameshima, M. and Ohba, N. (1984) Application of lectins for detection of goblet cell carbohydrates of the human conjunctiva. *Exp. Eye Res.* **38:** 439–447.

Kessler, T. L. and Dartt, D. A. (1994) Neural stimulation of conjunctival goblet cell mucous secretion in rats. *Adv. exp. Med. Biol.* **350:** 393–398.

Kessler, T. L., Mercer, H. J., Zieske, J. D., McCarthy, D. M. and Dartt, D. A. (1995) Stimulation of goblet cell mucous secretion by activation of nerves in rat conjunctiva. *Curr. Eye Res.* **14:** 985–992.

Kijlstra, A. and Kuizenga, A. (1994) Analysis and function of the human tear proteins. *Adv. exp. Med. Biol.* **350:** 299–308.

Kim, Y. S., Chang, S. K. and Gum, J. R. (1995) In: *Inflammatory Bowel Diseases* Falk Symposium 85 (G. N. J. Tytgat, J. F. W. M. Bartelsman and S. J. H. van Deventer, eds), pp. 388–393. Kluwer Academic, Dordrecht.

Kinoshita, S., Kiorpes, T. C., Friend, J. and Thoft, R. A. (1983) Goblet cell density in ocular surface disease: A better indicator than tear mucin. *Arch. Ophthalmol.* **101:** 1284–1287.

Kjeldsen, T., Clausen, H., Hirohashi, S., Ogawa, T., Iijima, H. and Hakomori, S. (1988) Preparation and characterisation of monoclonal antibodies directed to the tumor associated O-linked sialosyl-2–6 α-*N*-acetylgalactosaminyl (Sialosyl-Tn) epitope. *Cancer. Res.* **48:** 2214–2220.

Klomp, L., Van Rens, L. and Strous, G. J. (1994) Identification of a human gastric mucin precursor: N-linked glycosylation and oligomerisation. *Biochem. J.* **304:** 693–698.

Klotz, S. A., Au, Y. and Misra, R. P. (1989) A partial-thickness epithelial defect increases the adherence of *Pseudomonas*

aeruginosa to the cornea. *Invest. Ophthalmol. Vis. Sci.* **30**: 1069–1074.

Kruse, F. E. and Tseng, S. G. (1993) Growth factors modulate clonal growth and differentiation of cultured rabbit limbal and corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* **34**: 1963–1976.

Laibson, P. R. (1972) Annual review: cornea and sclera. *Arch. Ophthalmol.* **88**: 553–574.

Lasky, L. A. (1995) Sialomucins in inflammation and hematopoiesis. *Adv. exp. Med. Biol.* **376**: 259–260.

Ligtenburg, M. J. L., Kruijshaar, L., Buijs, F., van Meijer, M., Litvinov, S. V. and Hilkens, J. (1992) Cell-associated episialin is a complex containing two proteins derived from a common precursor. *J. biol. Chem.* **267**: 6171–6177.

Lisanti, M. P., Caras, I. W., Davitz, M. A. and Rodriguez-Boulan, E. (1989) A glycophospholipid membrane anchor acts as an apical targeting signal in polarised epithelial cells. *J. Cell Biol.* **109**: 2145–2156.

Lisanti, M. P., Sargiacomo, M., Graeve, L., Saltiel, A. R. and Rodriguez-Boulan, E. (1988) Polarised apical distribution of glycosylphosphatidylinositol-anchored proteins in a renal epithelial cell line. *Proc. natn. Acad. Sci. U.S.A.* **85**: 9557–9561.

Longenecker, B. M., Reddish, M., Koganty, R. and MacLean, G. D. (1993) Immune responses of mice and human breast cancer patients following immunisation with synthetic sialyl–Tn conjugated to KLH plus detox adjuvant. *Ann. N.Y. Acad. Sci.* **690**: 276–291.

Madsen, K., Schenholm, M., Jahnke, G. and Tengblad, A. (1989) Hyaluronate binding to intact corneas and cultured endothelial cells. *Invest. Ophthalmol. Vis. Sci.* **30**: 2132–2137.

McCool, D. J., Forstner, J. F. and Forstner, G. G. (1994) Synthesis and secretion of mucin by the human colonic tumour cell line LS180. *Biochem. J.* **302**: 111–118.

McCool, D. J., Forstner, J. F. and Forstner, G. G. (1995) Regulated and unregulated pathways for MUC2 mucin secretion in human colonic LS180 adenocarcinoma cells are distinct. *Biochem. J.* **312**: 125–132.

McGhee, C. N. J. and Lee, W. R. (1985) Quantification and enzymatic identification of conjunctival surface mucus. *Trans. Ophthalmol. Soc UK* **104**: 446–449.

McGuckin, M. A., Hurst, T. G. and Ward, B. G. (1995) Heterogeneity in production, secretion and glycosylation of MUC1 epithelial mucin by primary cultures of ovarian carcinoma. *Int. J. Cancer.* **63**: 412–418.

Montreuil, J., Vliegenthart, J. F. G. and Schachter, H. (1996) *Glycoproteins and Disease*. Elsevier, Amsterdam.

Moore, J. C. and Tiffany, J. M. (1979) Human ocular mucus. Origins and preliminary characterisation. *Exp. Eye Res.* **29**: 291–301.

Moore, J. C. and Tiffany, J. M. (1981) Human ocular mucus. Chemical studies. *Exp. Eye Res.* **33**: 203–212.

Nelson, J. D., Havener, V. R. and Camerson, J. D. (1983) Cellulose acetate impressions of the ocular surface. *Arch. Ophthalmol.* **101**: 1869–1872.

Nelson, J. D. and Wright, J. C. (1984) Conjunctival cell densities in ocular surface disease. *Arch. Ophthalmol.* **102**: 1049–1051.

Nichols, B., Dawson, C. R. and Togni, B. (1983) Surface features of the conjunctiva and cornea. *Invest. Ophthalmol. Vis. Sci.* **24**: 570–576.

Nichols, B. A., Chiappino, M. L. and Dawson, C. R. (1985) Demonstration of the mucous layer of the tear film by electron microscopy. *Invest. Ophthalmol. Vis. Sci.* **26**: 464–473.

Nieuw-Amerongen, A. V., Bolsher, J. G. M. and Veerman, E. C. I. (1995) Salivary mucins: protective functions in relation to their diversity. *Glycobiology* **5**: 733–740.

O'Boyle, K. P., Zamore, R., Adluri, S., Cohen, A., Kemeny, N., Welt, S., Lloyd, K. O., Oettgen, H. F., Old, L. J. and Livingston, P. O. (1992) Immunisation of colorectal cancer patients with modified ovine submaxillary gland mucin and adjuvants induces IgM and IgG antibodies to sialylated Tn. *Cancer Res.* **52**: 5663–5667.

Ota, H. and Katasuyama, T. (1992) Alternating laminated array of two types of mucin in the human gastric surface mucous layer. *Histochem. J.* **24**: 86–92.

Parker, N., Finnie, I. A., Raouf, A. H., Ryder, S. D., Campbell, B. J., Tsai, H. H., Iddon, D., Milton, J. D. and Rhodes, J. M. (1993) High performance gel filtration using monodisperse highly cross-linked agarose as a one-step system for mucin purification. *Biomed. Chromatogr.* **7**: 68–74.

Petroutsos, G., Paschides, C. A., Karakostas, K. X. and Psilas, K. (1992) Diagnostic tests for dry eye disease in normals and dry eye patients with and without Sjogren's syndrome. *Ophthalmic Res.* **24**: 326–331.

Pflugfelder, S. C., Huang, A. J. W., Feuer, W., Chuchovski, P. T., Pereira, I. C. and Tseng, S. C. G. (1990) Conjunctival cytologic features of primary Sjögren's syndrome. *Ophthalmology* **97**: 985–991.

Podolsky, D. K. and Isselbacher, K. J. (1984) Glycoprotein composition of colonic mucosa: specific alterations in ulcerative colitis. *Gastroenterology* **87**: 991–998.

Prydal, J. I., Artal, P., Woon, H. and Campbell, F. W. (1992) Study of the human precorneal tear film thickness and structure using laser interferometry. *Invest. Ophthalmol. Vis. Sci.* **33**: 2006–2011.

Prydal, J. I., Kerr Muir, M. G. and Dilly, P. N. (1993) Comparison of tear thickness in three species determined by the glass fibre method and confocal microscopy. *Eye* **7**: 472–475.

Ramphal, R. and Pyle, M. (1983) Evidence for mucins and sialic acid as receptors for *Pseudomonas aeruginosa* in the lower respiratory tract. *Infect. Immunol.* **41**: 339–344.

Raouf, A., Parker, N., Iddon, D., Ryder, S., Langdon-Brown, B., Milton, J. D., Walker, R. and Rhodes, J. M. (1991) Ion-exchange chromatography of purified colonic mucus glycoproteins in inflammatory bowel disease: absence of a selective subclass defect. *Gut* **32**: 1139–1145.

Ritchings, B. W., Almira, E. C., Lory, S. and Ramphal, R. (1995) Cloning and phenotypic characterisation of fleS and fleR, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. *Infec. Immunol.* **63**: 4868–4876.

Rittig, M., Lutjen-Drecoll, E. and Prehm, P. (1992) Immunohistochemical localisation of hyaluronan synthase in cornea and conjunctiva of cynomolgus monkey. *Exp. Eye Res.* **54**: 455–460.

Rogers, G. N., Herrler, G., Paulson, J. C. and Klenk, H. D. (1986) Influenza C virus uses 9-*O*-acetyl *N*-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. *J. biol. Chem.* **261**: 5947–5951.

Rolando, M., Brezzo, V. and Calabria, G. (1994) Ocular surface changes induced by repeated impression cytology. *Adv. exp. Med. Biol.* **350**: 249–254.

Roussel, P., Lamblin, G., Lhermitte, M., Houdret, N., Lafitte, J.-J., Perini, J.-M., Klein, A. and Scharfman, A. (1988) The complexity of mucins. *Biochimie* **70:** 1471–1482.

Sack, R. A., Tan, K. O. and Tan, A. (1992) Diurnal tear cycle: evidence for a nocturnal inflammatory constitutive tear fluid. *Invest. Ophthalmol. Vis. Sci.* **33:** 626–631.

Schachter, H. and Brockhausen, I. (1992) The biosynthesis of serine (threonine)-*N*-acetylgalactosamine-linked carbohydrate moieties. In: *Glycoconjugates* (H. J. Allen and E. C. Kisailus, eds), pp. 263–332. Marcel Dekker, New York.

Scheiman, J. M., Kraus, E. R. and Boland, C. R. (1992) Regulation of canine gastric mucin synthesis and phospholipid secretion by acid secretagogues. *Gastroenterology* **103:** 1842–1850.

Schultze, B. and Herrler, G. (1994) Recognition of cellular receptors by bovine coronavirus. *Arch. Virol. Supplementum* **9:** 451–459.

Sharma, A. and Coles, W. H. (1990) Physico-chemical factors in tear film breakup. *Invest. Ophthalmol. Vis. Sci.* **31:** 552.

Sheehan, J. K. and Carlstedt, I. (1987) Rate zonal centrifugation of cervical mucins. *Biochem. J.* **245:** 757–776.

Shimamoto, C., Deshmukh, G. D., Rigot, W. L. and Boland, C. R. (1989) Analysis of cancer-associated colonic mucin by ion-exchange chromatography: evidence for a mucin species of lower molecular charge and weight in cancer. *Biochim. biophys. Acta* **991:** 284–295.

Shimizu, Y. and Shaw, S. (1993) Mucins in the mainstream. *Nature* **366:** 630–631.

Singhal, A., Fohn, M. and Hakomori, S.-I. (1991) Induction of α-*N*-acetylgalactosamine-*O*-serine/threonine (Tn) antigen-mediated cellular immune response for active immunotherapy in mice. *Cancer Res.* **51:** 1406–1411.

Starr, C., Masada, R. I., Hague, C., Skop, E. and Klock, J. (1996) Fluorophore-assisted-carbohydrate-electrophoresis, FACE, in the separation, analysis and sequencing of carbohydrates. *J. Chromatogr.* **720:** 295–321.

Strous, G. J. and Dekker, J. (1992) Mucin-type glycoproteins. *Crit. Rev. Biochem. Molec. Biol.* **27:** 57–92.

Takahashi, H. K., Metoki, R. and Hakomori, S. (1988) Immunoglobulin G3 monoclonal antibody directed to Tn antigen (Tumor-associated α-*N*-acetylgalactosaminyl epitope) that does not cross-react with blood group A antigen. *Cancer Res.* **48:** 4361–4367.

Tettamanti, G. and Pigman, W. (1968) Purification and characterisation of bovine and ovine submaxillary mucins. *Arch. Biochem. Biophys.* **124:** 41–50.

Thornton, D. J., Devine, P. L., Hanski, C., Howard, M. and Sheehan, J. K. (1994) Identification of two major populations of mucins in respiratory secretions. *Am. J. Respir. Crit. Care Med.* **150:** 823–832.

Thornton, D. J., Holmes, D. F., Sheehan, J. K. and Carlstedt, I. (1989) Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. *Analyt. Biochem.* **182:** 160–164.

Thornton, D. J., Howard, M., Devine, P. L. and Sheehan, J. K. (1995) Methods for separation and deglycosylation of mucin subunits. *Analyt. Biochem.* **227:** 162–167.

Tiffany, J. M. (1990) Measurement of the wettability of the human corneal epithelium: II. Contact-angle method. *Acta Ophthalmol.* **68:** 182–187.

Tiffany, J. M. (1991) The viscosity of human tears. *Int. Ophthalmol.* **16:** 371–376.

Tiffany, J. M. (1994a) Composition and biophysical properties of the tear film: knowledge and uncertainty. *Adv. exp. Med. Biol.* **350:** 231–238.

Tiffany, J. M. (1994b) Viscoelastic properties of human tears and polymer solutions. *Adv. exp. Med. Biol.* **350:** 267–270.

Toda, I., Shimazaki, J. and Tsubota, K. (1995) Dry eye with only decreased tear break-up time is sometimes associated with allergic conjunctivitis. *Ophthalmology* **102:** 302–309.

Tsai, R.-F., Ho, Y.-S. and Chen, J.-K. (1994) The effects of fibroblasts on the growth and differentiation of human bulbar conjunctival epithelium in an *in vitro* conjunctival equivalent. *Invest. Ophthalmol. Vis. Sci.* **35:** 2865–2875.

Tseng, S. C. G. (1985) Staging of conjunctival squamous metaplasia by impression cytology. *Ophthalmology* **92:** 728–735.

Tseng, S. C. G., Hirst, L. W., Maumenee, A. E., Kenyon, K. R., Sun, T.-T. and Green, W. R. (1984) Possible mechanisms for the loss of goblet cells in mucin-deficient disorders. *Ophthalmology* **91:** 545–551.

Tseng, S. C. G., Huang, A. J. W. and Sutter, D. (1987) Purification and characterisation of rabbit ocular mucin. *Invest. Ophthalmol. Vis. Sci.* **28:** 1473–1482.

Tytgat, K. M. A. J., Swallow, D. M., Van Klinken, B. J. W., Buller, H. A., Einerhand, A. W. C. and Dekker, J. (1995) Unpredictable behaviour of mucins in SDS/polyacrylamide-gel electrophoresis. *Biochem. J.* **310:** 1053–1054.

Ubels, J. L., McCartney, M. D. and Lantz, W. K. (1995) Effects of preservative-free artificial tear solutions on corneal epithelial structure and function. *Arch. Ophthalmol.* **113:** 371–378.

Van Klinken, B. J. W., Dekker, J., Buller, H. A. and Einerhand, A. W. C. (1995) Mucin gene structure and expression: Protection vs. adhesion. *Am. J. Physiol.* **269:** G613–G627.

Verdugo, P. (1991) Mucin exocytosis. *Am. Rev. Respir. Dis.* **144:** S33–S37.

Versura, P., Maltarello, M. C., Cellini, M., Carmazza, R. and Laschi, R. (1986) Detection of mucus glycoconjugates in human conjunctiva by using the lectin-colloidal gold technique in TEM. II. A quantitative study in dry eye patients. *Acta Ophthalmol.* **64:** 451–455.

Watanabe, H., Fabricant, M., Tisdale, A. S., Spurr-Michaud, S. J., Lindberg, K. and Gipson, I. K. (1995) Human corneal and conjunctival epithelia produce a mucin-like glycoprotein for the apical surface. *Invest. Ophthalmol. Vis. Sci.* **36:** 337–344.

Watanabe, H. and Gipson, I. K. (1994) Detection of blood group differences in human corneal epithelium using a monoclonal antibody and lectins. *Arch. Ophthalmol.* **112:** 667–673.

Watanabe, H., Tisdale, A. S. and Gipson, I. K. (1993) Eyelid opening induces expression of a glycocalyx glycoprotein of rat ocular surface epithelium. *Invest. Ophthalmol. Vis. Sci.* **34:** 327–338.

Wei, Z.-G., Sun, T.-T. and Lavker, R. M. (1994) Cells form corneal, limbal, and conjunctival epithelia grown *in vitro* can faithfully mimic tissues of origin when implanted subcutaneously into athymic mice. *Invest. Ophthalmol. Vis. Sci.* **35:** 1559.

Wei, Z.-G., Sun, T.-T. and Lavker, R. M. (1996) Rabbit conjunctival and corneal epithelial cells belong to

two separate lineages. *Invest. Ophthalmol. Vis. Sci.* **37**: 523–533.

Wei, Z.-G., Wu, R. L., Lavker, R. M. and Sun, T. T. (1993) *In vitro* growth and differentiation of rabbit bulbar fornix and palpebral conjunctival epithelia: implications on conjunctival epithelium transdifferentiation and stem cells. *Invest. Ophthalmol. Vis. Sci.* **34**: 1814–1828.

Wolff, E. (1954) *Anatomy of Eye and Orbit*. Blakiston, New York.

Yamabayashi, S. and Tsakahara, S. (1987) Histochemical studies on the conjunctival goblet cells; I (Alcian blue) AB, (periodic acid-Schiff) PAS staining and PAS-AB staining. *Ophthalmic Res.* **19**: 137–140.

Yolken, R. H., Ojeh, C., Khatri, I. A., Sajjan, U. and Forstner, J. F. (1994) Intestinal mucins inhibit rotavirus replication in an oligosaccharide-dependent manner. *J. Infect. Dis.* **169**: 1002–1006.

Zhang, K., Baeckstrom, D., Brevinge, H. and Hansson, G. (1996) Secreted MUC1 mucins lacking their cytoplasmic part and carrying sialy-Lewis a and x epitopes from a tumor cell line and sera of carcinoma patients can inhibit HL-60 leukocyte adhesion to E-selectin-expressing endothelial cells. *J. Cell. Biochem.* **60**: 538–549.

Zieske, J. D. (1994) Perpetuation of stem cells in the eye. *Eye* **8**: 163–169.