Characterization of a carbohydrate epitope defined by the monoclonal antibody H185: Sialic acid O-acetylation on epithelial cell surface mucins

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Abstract

Sialic acids comprise a large family of derivatives of neuraminic acid containing methyl, acetyl, sulfate and phosphate among other groups, which confer specific physicochemical properties (e.g., hydrophobicity, resistance to hydrolases) to the molecules carrying them. Several years ago a monoclonal antibody, designated H185, was developed, which binds to cell membranes of human corneal, conjunctival, laryngeal, and vaginal epithelia, and whose distribution is altered on the ocular surface of patients with keratinizing disease. Recent findings using immunoprecipitation and immunodepletion techniques have demonstrated that, in human corneal epithelial cells, the H185 antigen is carried by the membrane-associated mucin MUC16. In this study, we show that the H185 epitope on human corneal cells and in tear fluid is an O-acetylated sialic acid epitope that can be selectively hydrolyzed in an enzyme concentration-dependent manner by sialidase from *A. ureafaciens*, and to a lesser extent by sialidases from Newcastle disease virus, *C. perfringens*, and *S. pneumoniae*. Binding of the H185 antibody was impaired by treatment of tear fluid with a recombinant 9-O-acetylesterase from influenza C virus. Two O-acetyl derivatives, $Neu5,7Ac_2$ and $Neu5,9Ac_2$, were identified in human tear fluid by fluorometric HPLC and electrospray mass spectrometry. Immunoprecipitation of the H185 epitope from human corneal epithelial cells revealed that $Neu5,9Ac_2$ was the major derivative on the mucin isolate. These results indicate that exposed wet-surfaced epithelia are decorated with O-acetyl sialic acid derivatives on membrane-associated mucins and suggest that O-acetylation on cell surfaces may protect against pathogen infection by preventing degradation of membrane-associated mucins.

Keywords

cell surface mucin; corneal epithelium; H185 antibody; MUC16; O-acetyl sialic acid

Introduction

Apical membranes on wet-surfaced epithelial cells contain a class of highly O-glycosylated, high molecular weight glycoproteins, termed mucins, which participate in the protection of the underlying epithelium by providing lubrication, maintaining hydration and preventing colonization by pathogenic microorganisms (Hollingsworth and Swanson, 2004). Essential to the functions of mucins are their O-glycans, which constitute 50-80% of their mucin mass. It has been proposed that O-glycans have specialized functions, as evidenced by the fact that different normal mucosal tissues within one individual attach different oligosaccharides to the same mucin protein core, reflecting the distinct requirements of the epithelia (Lan *et al.*,

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1990). The composition and sequence of the carbohydrates in the O-glycan chain of mucins are influenced by the specific profile of glycosyltransferases expressed by the cell, their level of activity and their subcellular distribution within the Golgi apparatus (Paulson and Colley, 1989).

Sialic acids occupy a strategic location at the outermost ends of O-glycans, N-glycans, and glycosphingolipids on cell membranes. They are composed of a 9-carbon acidic core, neuraminic acid (Neu)—generally containing N-acetyl (Neu5Ac) or N-glycolyl (Neu5Gc) groups at the 5-carbon position—which can additionally be modified at the 4, 7, 8, and 9-carbon positions (Schauer, 2000). A family of over 40 derivatives of neuraminic acid containing methyl, O-acetyl, sulphate and phosphate, among other groups, have been described in a tissue-specific manner in mammals, where they play important functions in biological recognition phenomena, mediating or preventing binding of cells to each other, and viruses and bacteria to their host cells (Varki, 1997;Schauer, 2000;Schauer *et al.*, 2001). These modifications may also prevent the fast degradation of sialylated glycoconjugates in the glycocalyx by exo-alpha-sialidases (EC 3.2.1.18) produced by certain bacteria, myxoviruses, and animal tissues (Rafelson, 1963). In this instance, the optimal susceptibility to several sialidases requires an intact exocyclic polyhydroxylated side chain (carbons in positions 7, 8 and 9) (Suttajit and Winzler, 1971). O-acetylation of one or more of the hydroxyl groups reduces the action of the sialidases (Corfield *et al.*, 1986).

Monoclonal antibodies that recognize the carbohydrate structure of cell surface glycoconjugates are useful tools for characterizing cell types in physiological and pathophysiological conditions (Kurosaka *et al.*, 1988). Several years ago, a mouse monoclonal antibody, designated H185, was developed, which recognizes a mucin-type glycoprotein in the plasma membrane of suprabasal cells in stratified squamous cells of human corneal, conjunctival, laryngeal, and vaginal epithelium (Watanabe *et al.*, 1995). Binding of the antibody was lost during drying epithelial diseases, such as dry eye and superior limbic keratoconjunctivitis (Watanabe *et al.*, 1997;Danjo *et al.*, 1998). Immunoprecipitation and immunodepletion techniques have shown that the H185 carbohydrate epitope is carried by the membrane-associated mucin MUC16 in human ocular surface epithelial cells (Argüeso *et al.*, 2003). This report presents the carbohydrate structure recognized by the H185 antibody as an O-acetylated derivative of sialic acid differentially cleaved by sialidases from different bacterial and viral sources and discusses its biological effects on membrane-associated mucins in stratified wet-surfaced epithelia.

Results

The H185 carbohydrate epitope in human tear fluid is differentially cleaved by sialidases of bacterial and viral origin

The characterization of the H185 carbohydrate was primarily carried out using human tear fluid, which contains high levels of the epitope. By western blot, protein extracts from immortalized human corneal (HCLE) or conjunctival (HCjE) epithelial cell lines contained lesser amounts of the carbohydrate epitope—average levels in the cell lines were 8.8% of those found in tear samples (data not shown). The H185 carbohydrate epitope in tear samples may originate from mucins either secreted or shed from the epithelial cell surface by specific proteases (Thathiah *et al.*, 2003). These proteases cleave the mucin ectodomain in a putative site outside the highly glycosylated, tandem repeat region (Thathiah *et al.*, 2003) and are unlikely to affect terminal carbohydrate antigenicity.

ELISA was initially used to determine the susceptibility of the H185 epitope to hydrolysis by commercial sialidases from different microorganisms (Fig. 1A). Sialidase from *A. ureafaciens* (α2-3,6,8,9 specific) efficiently abolished H185 antibody binding to tear samples

in an enzyme concentration-dependent manner, indicating that a terminal sialic acid residue is involved in H185 antibody recognition. Digestion of tears with increasing concentrations of other bacterial sialidases, *S. pneumoniae* (α2-3 specific) and *C. perfringens* (α2-3,6 specific), minimally affected H185 antibody binding—binding was reduced by less than 25%—as compared to that of *A. ureafaciens.* Treatment with Newcastle disease virus sialidase (α2-3,8 specific) resulted in a 50-85% loss of reactivity. The effect of sialidases on H185 binding was further examined on agarose gels in western blot experiments. *A. ureafaciens* sialidase totally abolished H185 binding to a high molecular weight band (>250 kDa) on human tears, whereas *S. pneumoniae, C. perfringens* and Newcastle disease virus did not (Fig. 1B). The membrane-associated mucin MUC16, which has been shown to be a carrier of the H185 carbohydrate epitope in HCLE cells (Argueso *et al.*, 2003), was also detected as shed in tear fluid and migrated on agarose gels to the same position as the H185 epitope. Slight differences in the electrophoretic mobility (particularly after treatment with *A. ureafaciens* sialidase) were observed in the MUC16 bands, which may have resulted from changes in charge density due to loss of sialic acids, and may have depended on the hydrolysis rate of the enzymes. Additionally, an increase in OC125 antibody binding to MUC16 was observed after desialylation as compared to control (Fig. 1B), which could be explained by the susceptibility of certain mucin antibodies to sialylation (Argueso *et al.*, 2002). The hydrolytic effect of *A. ureafaciens* sialidase towards the H185 epitope was further confirmed by lack of H185 binding to apical cell membranes on islands of stratified cells in HCLE cultures after enzymatic treatment (Fig. 1C). These results indicate that epithelial mucins carrying the H185 epitope contain sialic acid moieties partially resistant to *S. pneumoniae, C. perfringens* and Newcastle disease virus sialidases, but labile to digestion with *A. ureafaciens* sialidase.

Identification of glycoconjugate-bound O-acetyl sialic acid in human tear fluid

The incomplete hydrolysis of the H185 sialic acid epitope by restricted linkage-specificity sialidases could be explained by the presence of one or several O-acetyl groups on the polyhydroxy side chain of the neuraminic acid molecule (Suttajit and Winzler, 1971;Corfield *et al.*, 1986). Because O-acetyl esters on sialic acids are susceptible to alkaline hydrolysis, we applied mild base treatment to human tears as described in *Materials and Methods,* and analyzed H185 antibody binding subsequently by ELISA and western blot. By ELISA, there was an average 62% decrease in H185 binding in three tear samples after de-O-acetylation for 30 min (Fig. 2A). H185 binding was not completely abolished after further treatment for up to 120 min. By western blot analysis, there was also a reduction of H185 antibody binding after alkaline hydrolysis (Fig. 2A, inset), suggesting the presence of O-acetyl groups as part of the sialic acid epitope recognized by the H185 antibody. Subsequent treatments of the de-O-acetylated samples with sialidases other than *A. ureafaciens* did not completely abolish H185 antibody binding, indicating that these sialidases are still unable to hydrolyze the de-O-acetylated H185 epitope under the conditions used in this assay. Treatment of human tears with recombinant 9-O-acetylesterase from influenza C virus resulted in a 90% reduction of H185 binding as determined by ELISA (Fig. 2B), indicating that the H185 carbohydrate epitope is dependent on 9-O-acetyl sialic acid.

The identification of O-acetyl sialic acid derivatives that could potentially constitute the carbohydrate epitope recognized by the H185 antibody was performed by fluorometric HPLC and tandem HPLC-electrospray mass spectrometry (MS) after digestion of tear fluid with *A. ureafaciens* sialidase. As shown in Fig. 3, crude tear fluid contains a mixture of sialic acids, of which 5-N-acetyl-neuraminic acid (Neu5Ac) is predominant. Two O-acetyl derivatives, Neu5,7Ac$_2$ and Neu5,9Ac$_2$, were also detected, constituting potential determinants of the H185 carbohydrate antigen. Electrospray MS on DMB-derivatized sialic acid peaks further confirmed the presence of Neu5,7Ac$_2$ and Neu5,9Ac$_2$ in three tear samples after digestion with sialidase from *A. ureafaciens*. The m/z ratio in the mass spectra for Neu5,7Ac$_2$ and

Neu5,9Ac$_2$ was 468, and was accompanied by two additional peaks corresponding to the sodium adduct (+22) and the dehydrated form (-18) (data not shown). A minor peak, with a retention time similar to that of the Neu5,7(8),9Ac$_3$ standard, was also detected by fluorometric HPLC but not by electrospray MS. The identity of the O-acetylated residues was confirmed through their susceptibility to alkaline treatment. Hydrolysis of the O-acetyl esters resulted in diminution of the peak area corresponding to the O-acetyl derivatives. This reduction was particularly evident in the case of Neu5,9Ac$_2$, approximately 70%, which correlates to the 62% decrease observed in H185 binding after de-O-acetylation of tears as determined by ELISA (Fig. 2A).

As compared to *A. ureafaciens* sialidase, digestion of tears with sialidases from *S. pneumoniae, C. perfringens* and Newcastle disease virus was less efficient (Fig. 4, step 1), correlating with the inability of these enzymes to completely hydrolyze the H185 carbohydrate epitope (Fig. 1A). Further addition of *A. ureafaciens* sialidase to the samples revealed the presence of remaining O-acetyl derivatives (Fig. 4, step 2), which may account for the H185 antibody binding observed after sialidase treatment in Fig. 1A. Results in Figure 4 also show that the sialidases from *S. pneumoniae, C. perfringens* and Newcastle disease virus were able to remove non-O-acetylated sialic acid (Neu5Ac) from tear samples. Interestingly, the sialidase from *S. pneumoniae* seemed to be less efficient than *C. perfringens* and Newcastle disease virus. Reduced efficiency of the *S. pneumoniae* sialidase, which only cleaves the α2-3 linkage, suggests that different sialic acid linkages within the mucin are present, α2-3 being a minor component. In this regard, O-acetylation does not seem to take place on α□3-linked sialic acids (Shi *et al.*, 1996;Lewis *et al.*, 2004).

Neu5,7Ac$_2$ and Neu5,9Ac$_2$ were found in all tear samples (n=7) individually analyzed by fluorometric HPLC, and ranged 0.02-6.05% (average 2.4±2.1) of the total sialic acid content for Neu5,7Ac$_2$, and 0.49-4.21% (average 3.5±2.2) for Neu5,9Ac$_2$. The presence of the H185 epitope in each individual sample was confirmed by agarose gel immunoblot, resulting in one or two high molecular weight bands (Fig. 5). A subsequent densitometric analysis did not show a conclusive correlation between the intensity of H185 binding and the relative levels of NeuAc5,7Ac2 and NeuAc5,9Ac2 in these samples (e.g., the sample with the higher amount of NeuAc5,9Ac2 did not have the most intense band by western blot analysis), suggesting that despite the presence of O-acetyl derivatives in the sample, H185 antibody binding intensity may depend on a particular conformation of the epitope and its neighboring sugars.

All together, these data indicate that O-acetylated species are minor components of the total sialic acid in human tear fluid, but constitute potential antigenic determinants required for H185 antibody binding.

H185/MUC16 cell surface mucin in corneal epithelial cells contains O-acetyl sialic acid

Further analysis of O-acetyl sialic acids on a specific epithelial mucin was performed by immunoprecipitation of the H185 antigen from HCLE cells, previously identified on the membrane-associated mucin MUC16 (Argueso *et al.*, 2003). Analysis of the H185 immunoprecipitate by fluorometric HPLC revealed the presence of Neu5,9Ac$_2$ in the mucin isolate, but not Neu5,7Ac$_2$ (Fig. 6). The relative percentage of Neu5,9Ac$_2$ in the MUC16 molecule was low (6.2%) as compared to that of Neu5Ac (88.4%). Although this data indicates that 9-O-acetyl derivatives are present in MUC16, it should be noted that the 7-O-acetyl ester can migrate to carbon position 9 under physiological conditions or under the conditions of this analysis (Butor *et al.*, 1993;Lewis *et al.*, 2004). A low level of Neu5Gc (5.4%) was additionally found in the immunoprecipitate, most likely metabolically incorporated into the H185 mucin from exogenous glycoproteins present in the calf serum used to grow HCLE cells, as previously described (Tangvoranuntakul *et al.*, 2003;Bardor *et al.*, 2005). In these experiments, immunoprecipitation of the H185 mucin was confirmed by immunoblot (Fig. 6, inset). Taken

together, we have used immunoprecipitation followed by fluorometric HPLC to identify a mono-O-acetylated sialic acid derivative, Neu5,9Ac$_2$, on a cell surface-associated mucin in cultures of human corneal epithelial cells.

Discussion

The non-keratinized, human stratified squamous epithelia carry unique carbohydrate antigens on mucin-type glycoproteins, such as the one recognized by the H185 monoclonal antibody, whose distribution has been shown to be altered in the ocular surface of patients with keratinizing dry eye disease (Gipson and Argueso, 2003). This study shows that the H185 antibody recognizes an O-acetylated sialic acid epitope in mucosal secretions and on epithelial surfaces that is differentially cleaved by sialidases of bacterial and viral origin, and identifies membrane-associated mucins as carriers of O-acetyl sialic acid derivatives on exposed epithelial cell surfaces.

Previous analyses have shown that the H185 antibody has a restricted binding pattern, which in humans includes the stratified wet-surfaced epithelia of the cornea, conjunctiva, larynx, and vagina, in addition to the submucosal glands of the esophagus and large intestine, cells of eccrine sweat glands (Watanabe *et al.*, 1995), and the cervical epithelium (data not shown). No binding of the H185 antibody was detected in buccal mucosa, esophagus, ileum, colon, mammary epithelium, and liver, or in non-human species such as monkey, rabbit, rat, cow, and sheep (Watanabe *et al.*, 1995). By comparison, O-acetyl derivatives of Neu5Ac are widely expressed in almost all deuterostomal species in a tissue-specific manner, which in humans include mucosal tissues such as colon and nasal epithelia (Schauer *et al.*, 2001). Lack of H185 antibody binding to these tissues containing O-acetyl sialic acid derivatives suggests that, although the epitope defined by the H185 antibody involves an O-acetylated sialic acid, this structure is a co-determinant and not the entire epitope responsible for antibody recognition. Derivatives of sialic acid that act as co-determinants in antibody recognition have been previously described. Cheresh et al. found that the D1.1 antibody, which reacts with an O-acylated sialic acid epitope on a ganglioside on developing rat embryonic neuroectoderm, did not show any reaction with murine erythroleukemia cells, which are known to have O-acetylated sialic acids on their surface (Cheresh *et al.*, 1984). The unique conformation of the H185 binding domain may be modulated by neighboring sugars, or the presence of other modification(s) on the sialic acid residue not detected under the conditions of our analyses. The presence of carbohydrate moieties in close proximity to the sialic acid antigen site is relevant in defining antibody specificities (Khurana *et al.*, 1997). In mucin-type glycoproteins, the presence of clusters of terminal sialic acid epitopes enhances the reaction of carbohydrate-directed monoclonal antibodies, such as MLS 102, which recognizes a cancer-associated sialic acid antigen on human colonic mucins (Kurosaka *et al.*, 1988). The complete characterization of the molecular structure of the carbohydrate side chains on the H185 mucin may lead to a better understanding of the nature of the H185 antigenic determinant.

Membrane-associated mucins have been proposed as potential carriers of O-acetylated sialic acid derivatives in the glycocalyx of polarized epithelial cells (Maury *et al.*, 1995). In this report, we have identified O-acetyl sialic acid residues in H185 immunoprecipitated material, which in HCLE cells corresponds to the membrane-associated mucin MUC16 (Argueso *et al.*, 2003). Based on immunofluorescence data showing sporadic binding to conjunctival goblet cells (Watanabe *et al.*, 1995), it is possible to speculate that other mucins, such as the secreted gel-forming MUC5AC in goblet cells, may also contain O-acetyl sialic acid. Interestingly, we could not detect H185 antibody binding to the membrane-associated MUC1 immunoprecipitated from HCLE cells (Argueso *et al.*, 2003). Sialic acid O-acetylation on specific membrane-associated mucins may mediate a large number of adhesion events by generating unique patterns of cell surface carbohydrates. In this regard, the success of different

microorganisms to colonize an epithelium depend on their capacity to secrete exoproducts, such as sialidases, that modify the host cell surface microenvironment to expose binding sites and facilitate attachment to the epithelial cell surface. This is well illustrated with the *Pseudomonas aeruginosa* PA01 sialidase, whose activity on primary respiratory epithelial cells is two orders of magnitude greater than that of the *C. perfringens* enzyme, resulting in an increased adherence of *Pseudomonas* to respiratory cells treated with the PA01 strain (Cacalano *et al.*, 1992). O-acetylation on mucins can markedly slow or even block the release of sialic acid by sialidases (Varki and Diaz, 1983;Corfield *et al.*, 1986) and may facilitate the protection of the underlying epithelium. Our results show that bacterial and viral sialidases have a differential effect on the hydrolysis of the H185 epitope. The membrane-associated mucin MUC16, which carries the terminal H185 epitope in HCLE cells, contains a long glycosylated extracellular amino terminal domain (O'Brien *et al.*, 2002) that may extend above other membrane-associated mucins and glycoproteins in the glycocalyx, potentially dictating the microbial-host interactions in stratified wet-surfaced epithelia and preventing infection in intact exposed epithelia.

It is now well established, mainly in colorectal cancer, that alterations in the level of O-acetylated species on mucins accompany the development of disease (Schauer *et al.*, 2001). At the ocular surface, Corfield et al. have recently demonstrated a decrease in the level of O-acetylation in tear mucin secretions in a canine model of dry eye (Corfield *et al.*, 2005), which correlates with previous reports in humans showing lack of H185 antibody binding on the cell surface of apical conjunctival cells in patients with dry eye and superior limbic keratoconjunctivitis (Watanabe *et al.*, 1997;Danjo *et al.*, 1998). We hypothesize that lack of O-acetyl groups, known to confer a more hydrophobic character to sialic acid residues (Schauer, 2000), at the ocular surface in dry eye may alter the interaction of the cell surface glycocalyx with other components of the tear film, such as lipids and hydrophobic proteins, leading to tear film instability. Also, the presence of O-acetylated sialic acids on apical cell surface-associated mucins and their alteration may influence the adhesion of circulating leukocytes—which contain sialic acid-binding lectins (Varki, 1997)—present in tears during overnight eye closure (Wilson *et al.*, 1989;Sakata *et al.*, 1997). At the molecular level, the turnover of O-acetyl ester groups on sialic acids is thought to be regulated by the relative activities of two enzymes, sialate-O-acetyltransferases present within the lumen of the Golgi apparatus or Golgi-related organelles, and sialate-O-acetylesterases (Varki, 1999;Shen *et al.*, 2004). Exogenously, O-acetylesterase activity has been reported from bacterial and viral sources (Varki, 1999). Elucidating the relative activities of these enzymes at the ocular surface of patients with keratinizing disease may give insight into the pathophysiology of this disease.

In summary, this study indicates that O-glycans on cell surface mucins from human stratified squamous epithelia contain O-acetyl sialic acids at their non-reducing ends that are recognized by the H185 monoclonal antibody and which can be differentially cleaved by sialidases of bacterial and viral origin. We propose that chemical modifications of neuraminic acid on membrane-associated mucins prevent the fast degradation of the epithelial glycocalyx by microorganisms and mediate the interactions of apical wet surfaces with their extracellular milieu. Alterations in sialic acid O-acetylation, such as that observed at the ocular surface of dry eye patients, may result in cell surface instability and damage to the underlying epithelium.

Materials and Methods

Mucin Collection

Tear fluid and protein extracts from human corneal-limbal epithelial cell cultures were tested for H185 epitope content. The highest level of H185 antigen was found in tear fluid, which contains mucins either secreted or shed by the ocular surface epithelia (Gipson and Argueso, 2003). Therefore, analysis of the H185 carbohydrate epitope was primarily carried out using

tear samples. Tear fluid was collected, in compliance with institutional review board regulations, from the inferior fornix of normal subjects after instillation of 60 μl of sterile water onto the cul-de-sac, as described previously (Argueso *et al.*, 2002). Samples were centrifuged for 30 min at $16,000 \times g$ at 4°C. Protein concentration was determined using the MicroBCA protein assay reagent kit (Pierce; Rockford, IL), using a dilution series of bovine serum albumin (BSA) as standard. All samples were promptly frozen at −80°C until the time of analyses.

Cell culture

Telomerase-immortalized human corneal-limbal epithelial (HCLE) cells were plated at a seeding density of 5×10^4 cells/cm^2 on culture chamber slides (Lab-Tek, Naperville, IL) or T75 flasks (Corning Costar Corp., Cambridge, MA) and maintained at 37°C in 5% CO_2. Characterization of HCLE cell cultures has been previously reported (Gipson *et al.*, 2003). HCLE cultures were grown in a medium optimized for proliferation of keratinocytes (keratinocyte serum-free medium; Gibco-Invitrogen Corporation, Carlsbad, CA) to achieve confluence. After reaching confluence, cells were switched to DMEM/F12 supplemented with 10% calf serum and 10 ng/ml EGF for 7 d, which promotes stratification and differentiation of corneal cells.

Enzymatic treatments and de-O-acetylation

Sample digestions were performed with α2-3-sialidase from *Streptococcus pneumoniae* (Calbiochem, San Diego, CA), α2-3,6-sialidase from *Clostridium perfringens* (Calbiochem), α2-3,8-sialidase from Newcastle disease virus (Glyko, Novato, CA) and α2-3,6,8,9-sialidase from *Arthrobacter ureafaciens* (Glyko). Enzymatic treatments were carried out at 37°C in 50 mM sodium phosphate, pH 6.0 (*S. pneumoniae, C. perfringens, A. ureafaciens*) or 50 mM sodium acetate, pH 5.5 (Newcastle disease virus). Sialic acids were de-O-acetylated by alkaline hydrolysis as described by Toba et al. (Toba *et al.*, 2000). Briefly, 1-50 μg of total protein from human tears were treated with 0.2 M NaOH at 24°C for up to 120 min. The reaction was neutralized by addition of 3 M acetic acid. De-O-acetylation was also performed enzymatically using a recombinant 9-O-acetylesterase of influenza C virus kindly supplied by Dr. Reinhard Vlasak (Applied Biotechnology GmbH, Salzburg, Austria). Samples were digested with the esterase using conditions previously described (Hellebo *et al.*, 2004).

ELISA

Tear fluid (1 μg of total protein) diluted in PBS was coated on EIA plates (Corning Costar Corp.) overnight at 4°C. After washing with PBS, plates were incubated with 10% normal horse serum (NHS) in PBS for 30 min at room temperature and with the monoclonal mouse anti-human H185 antibody (undiluted hybridoma culture medium) for 1 h. The H185 antibody, which recognizes a human carbohydrate epitope on MUC16 (Argueso *et al.*, 2003), was obtained from culture supernatants of hybridoma cells as described previously (Watanabe *et al.*, 1995). Plates were then incubated with a 1:10,000 dilution of secondary sheep anti-mouse IgG peroxidase-conjugated antibody in PBS containing 10% NHS for 1 h. After washing, plates were incubated with the peroxidase substrate, tetramethylbenzidine (Sigma Co., St. Louis, MO), at room temperature. The reaction was stopped after 30 min by the addition of 0.5 M H_2SO_4 and the optical density (OD) of each well was read at 450 nm.

Immunoprecipitation

HCLE cells were scraped from T75 flasks after addition of 1 ml of 2% SDS in the presence of Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), homogenized, and centrifuged at $35,016 \times g$ (RCF) for 45 min. The resulting supernatant was recovered, and the protein concentration determined using the Pierce BCA Protein Assay (Pierce). For H185 mucin immunoprecipitation, the H185 antibody was incubated with anti-

mouse IgG agarose particles (Sigma) for 1 h at 4ºC as described previously (Argueso *et al.*, 2003). Five-hundred μg of HCLE protein was diluted in 500 μl of immunoprecipitation buffer (0.1 M NH$_4$HCO$_3$, 2.0 mM PMSF, 0.5 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM N-ethylmaleimide, and 0.02% NaN$_3$ and Complete Mini protease inhibitor cocktail), added to the conjugated beads, and incubated for 2 h on a rocker at room temperature. After three washes with a buffer containing 10 mM Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, 0.1% SDS, pH 7.4, the immunoprecipitate was resuspended in 120 μl of 2% SDS, boiled for 5 min, and the beads separated from the soluble H185 mucin and antibody by centrifugation. The supernatant was added to 10K molecular weight cut-off centrifuge tubes (Omega Nanosep 10K, Pall Corp., Ann Arbor, MI), filtered by centrifugation at 16,000 × g for 5 min, and the immunoprecipitated material on the membrane washed with sialidase buffer before enzymatic treatment with 20 mU of sialidase from *A. ureafaciens*. After sialidase treatment, the sialic acid derivatives were recovered by centrifugation and analyzed by fluorometric HPLC as described below. A control (no tear sample) was carried out to determine the sialic acid content of the antibodies used for immunoprecipitation. The efficiency of the H185 mucin immunoprecipitation was determined by immunoblot.

Agarose gel electrophoresis and immunoblotting

Tear samples (1-50 μg of total protein) or immunoprecipitated H185 mucin from HCLE cells were electrophoresed on 1% agarose gels (BMA, Rockland, ME) for 2 h 15 min at 50 V at 4ºC under reducing conditions (Thornton *et al.*, 2000). Proteins were transferred for 1.5 h to a nitrocellulose membrane by vacuum blotting in transfer buffer (0.6 M NaCl, 60 mM sodium citrate). Immunolabeling was performed using the H185 or OC125 (DAKO Corporation, Carpinteria, CA) antibodies as previously reported (Argueso *et al.*, 2003). Membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and protein bands detected after exposure to film (Hyperfilm, Amersham Biosciences, Buckinghamshire, UK).

Immunofluorescence microscopy

HCLE cultures grown for 7 d in cell culture chamber slides were fixed in 4% paraformaldehyde for 24 h. Cultures were then incubated with or without *A. ureafaciens* sialidase (0.5 units/ml in 50 mM sodium phosphate buffer, pH 6.0) for 2.5 h at 37°C. After blocking with 1% BSA, cultures were maintained overnight at 4°C with a 1:10 dilution of primary mouse monoclonal antibody H185. Cultures were then incubated with a 1:50 dilution of secondary donkey anti-mouse fuorescein-conjugated antibody (Jackson ImmunoResearch, WestGrove, PA) and coverslipped with antifade mounting medium plus propidium iodide (Vectashield; Vector Laboratories; Burlingame, CA) as previously described (Gipson *et al.*, 2003).

Fluorometric HPLC

Tear samples (1-50 μg of total protein) were added to 10K molecular weight cut-off centrifuge tubes and filtered by centrifugation at 16,000 × g for 5 min. Samples on the membrane were treated with 40 mU of sialidase from *A. ureafaciens*, or 50 mU of sialidases from *S. pneumoniae, C. perfringens* or Newcastle disease virus for 2 h at 37°C. Tear samples treated with sialidases that showed partial or low levels of H185 epitope digestion in the ELISA assay (*S. pneumoniae, C. perfringens,* Newcastle disease virus) were further digested on the membranes with *A. ureafaciens* sialidase for an additional 2 h. The released sialic acids were collected by filtration and lyophilized.

Sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and analyzed by reverse-phase fluorometric HPLC as described (Manzi and Varki, 1992). Fluorometric HPLC analyses were performed with a Dionex Bio-LC chromatography system (Sunnyvale, CA) using a TSK ODS-120T column (Tosoh Bioscience, Japan) at a flow rate of 0.9 mL/min. Samples were eluted with a linear gradient of acetonitrile (7-11%) in methanol

(7%) and H$_2$O over 30 min followed by 10 min at the final conditions. The excitation and emission wavelengths were 373 and 448 nm, respectively. The DMB-derivatized sialic acids were identified by comparing retention times to known standards (sialic acid reference panel, Glyko) that were similarly treated.

Electrospray MS

The identity of DMB-derivatized peaks observed in HPLC analysis was verified using both an on-line UV detector (373 nm) and a Thermo Finnigan LCQ mass spectrometer (San Jose, CA) operated in the positive ion mode using methods previously described (Lewis *et al.*, 2004). Sialic acid hydrolysates were analyzed by electrospray MS after reverse-phase HPLC separation as described above except that the column was eluted isocratically with acetonitrile (7%), methanol (8%), formic acid (0.1%), and H$_2$O.

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Abbreviations

BSA

bovine serum albumin

DMB

1,2-diamino-4,5-methylenedioxybenzene

DMEM/F12

Dulbecco's modified eagle's medium/ Ham's nutrient mixture F12

ELISA

enzyme-linked immunosorbent assay

HCLE

human corneal-limbal epithelium

HPLC

high-performance liquid chromatography

MS

mass spectrometry

Neu5Ac

5-N-acetyl-neuraminic acid

Neu5,7(9)Ac$_2$

5-N-acetyl-7(9)-O-acetylneuraminic acid

Neu5,7(8),9Ac$_3$

5-N-acetyl-7(8),9-di-O-acetylneuraminic acid

Neu5Gc

5-N-glycolyl-neuraminic acid

NHS

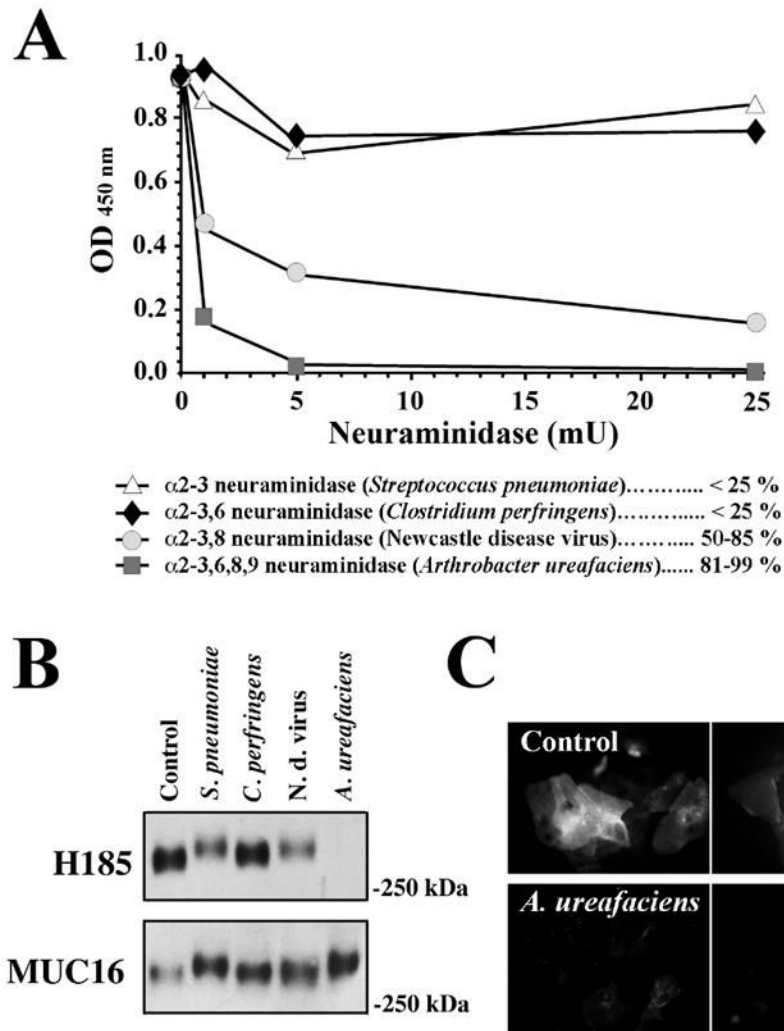normal horse serum

PBS

phosphate buffered saline

Fig 1. Differential effect of bacterial and viral sialidases on H185 antibody binding
A, In ELISA experiments, 1 µg total protein collected from human tear fluid was enzymatically digested for 1 h at 37°C with 1, 5 and 25 mU of sialidase from *S. pneumoniae*, *C. perfringens*, Newcastle disease virus and *A. ureafaciens*. Reduction in H185 binding (%) was compared to control (0 mU sialidase). *B,* Effect of sialidases on H185 and MUC16 antibody binding to tear fluid (25 µg of total protein) as demonstrated by western blot. *C.* Binding of the H185 antibody to apical cell membranes of stratified HCLE cultures (*top images*) is reduced after digestion with sialidase from *A. ureafaciens* (*bottom images*). Bar = 25 µm.

A. Alkaline hydrolysis
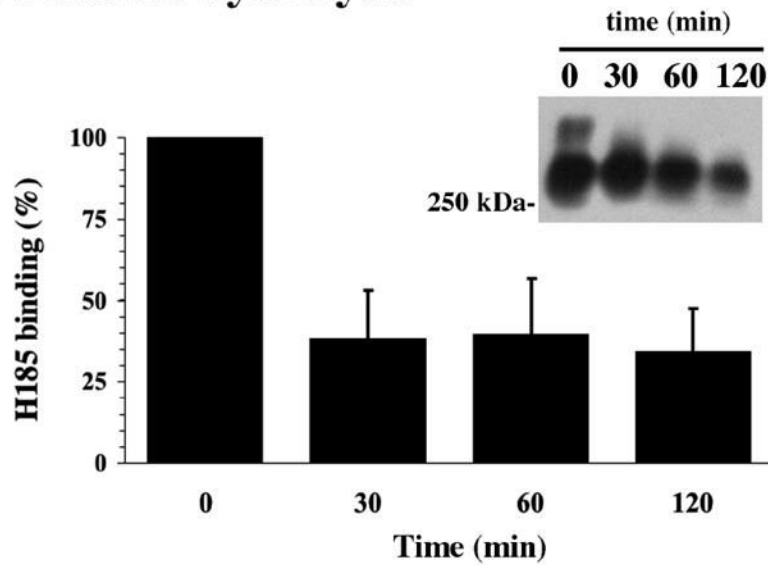
## B. Recombinant 9-O-acetylesterase
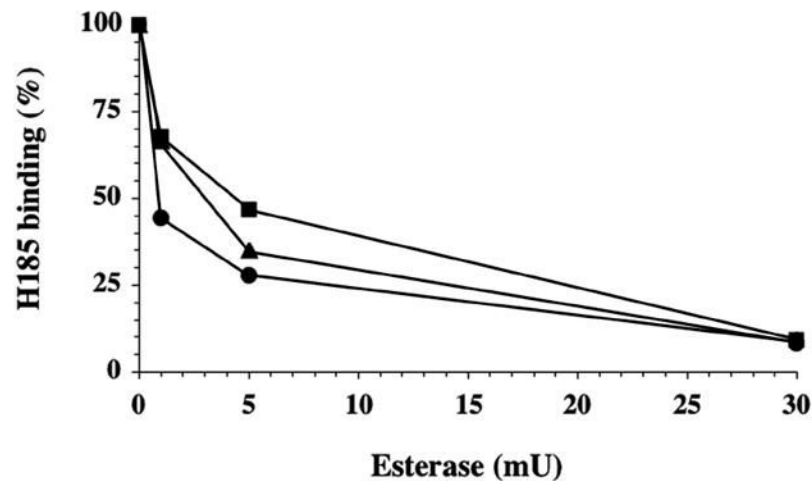
Fig 2. Effect of de-O-acetylation on H185 antibody binding
A, O-acetyl esters on sialic acids were removed from tear fluid by alkaline hydrolysis. A reduction of H185 antibody binding was determined by ELISA and western blot (*inset*). *B,* Three tear samples containing 1 μg of total protein each were incubated with 9-O-acetylesterase from influenza C virus. A 90 % reduction in H185 binding, as determined by ELISA, was observed after incubation with 30 mU esterase.
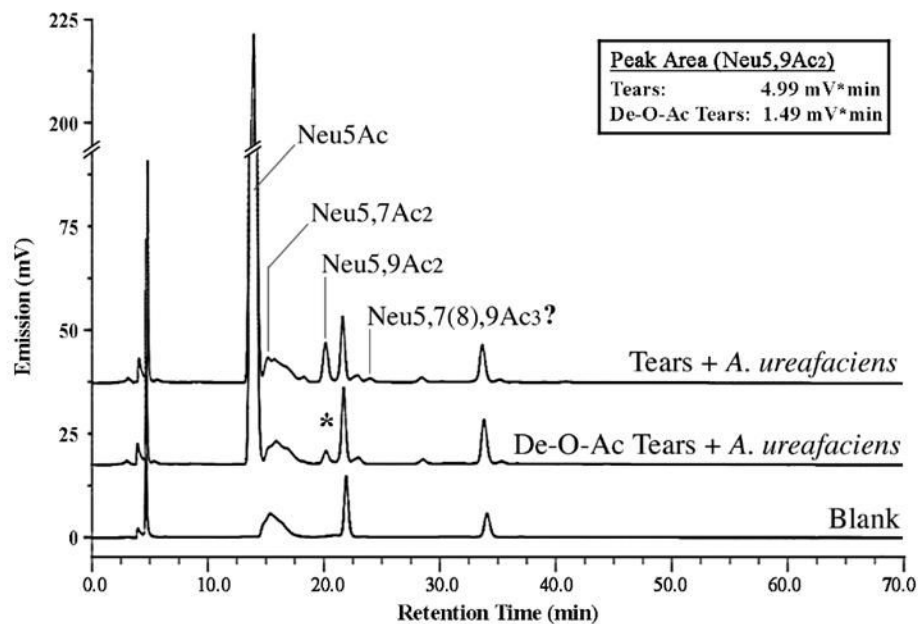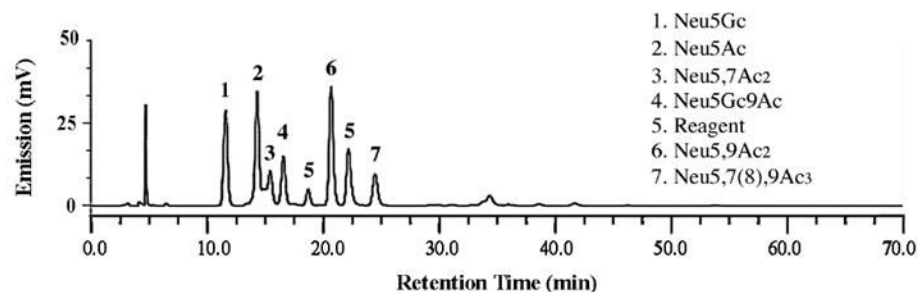
Fig 3. Identification of glycoconjugate-bound O-acetyl sialic acid derivatives in human tear fluid by fluorometric HPLC

Sialic acids were hydrolyzed using sialidase from *A. ureafaciens*. In addition to a major peak corresponding to Neu5Ac, two peaks corresponding to $Neu5,7Ac_2$ and $Neu5,9Ac_2$ were identified by fluorometric HPLC and confirmed by tandem HPLC-electrospray MS (see *Results*). An additional peak, with a retention time similar to that of the $Neu5,7(8),9Ac_3$ standard, was detected by fluorometric HPLC but not by electrospray MS. De-O-acetylation of the sample by alkaline hydrolysis reduced the peak area of $Neu5,9Ac_2$, which correlates with a diminution in H185 binding after alkaline hydrolysis (Fig. 2). The profile of sialic acids is shown relative to standard sialic acids (*bottom*).
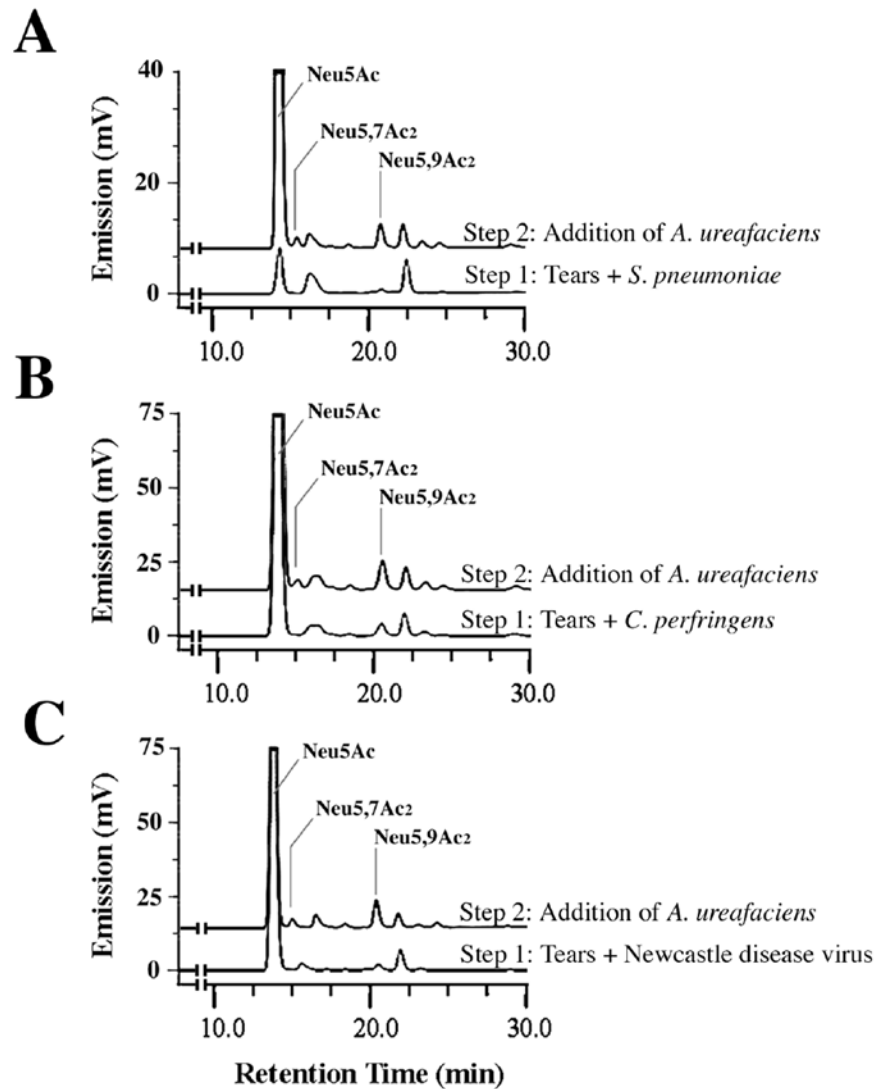
Fig 4. Differential effect of bacterial and viral sialidases in sialic acid hydrolysis, as determined by fluorometric HPLC

Sialidases from *S. pneumoniae* (*A*)*, C. perfringens* (*B*) and Newcastle disease virus (C), which showed partial or low levels of H185 epitope hydrolysis by ELISA (Fig1A), were less efficient in hydrolyzing Neu5,7Ac$_2$ and Neu5,9Ac$_2$ from human tears than *A. ureafaciens* sialidase (*step 1*); further treatment of the digested tears with *A. ureafaciens* sialidase revealed the presence of remaining Neu5,7Ac$_2$ and Neu5,9Ac$_2$ derivatives in the samples (*step 2*).
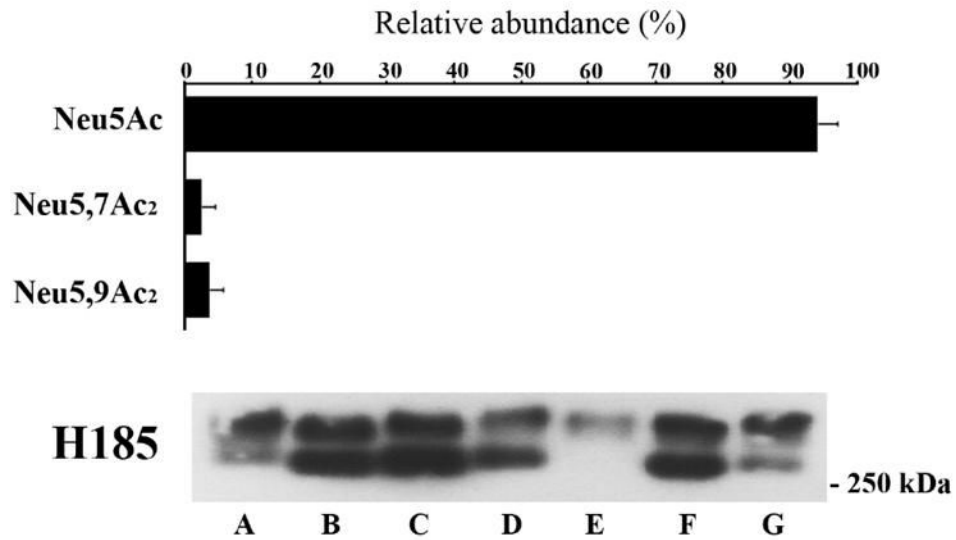
Fig 5. Relative abundance of sialic acid derivatives isolated from human tear fluid
Seven human tear samples were hydrolyzed using sialidase from *A. ureafaciens* and individually analyzed by fluorometric HPLC. Shown is the relative abundance of individual sialic acids as a percentage of total sialic acid (values are mean ± SD, n=7). The presence of the H185 carbohydrate epitope in each sample (A-G) analyzed by fluorometric HPLC is confirmed by western blot (5-50 μg of total protein). The presence of two molecular weight bands migrating over 250 kDa may result from mucin polymorphism (individual mucin alleles are expressed co-dominantly), or the presence of different mucin gene products or glycoforms containing the H185 carbohydrate epitope in the sample.
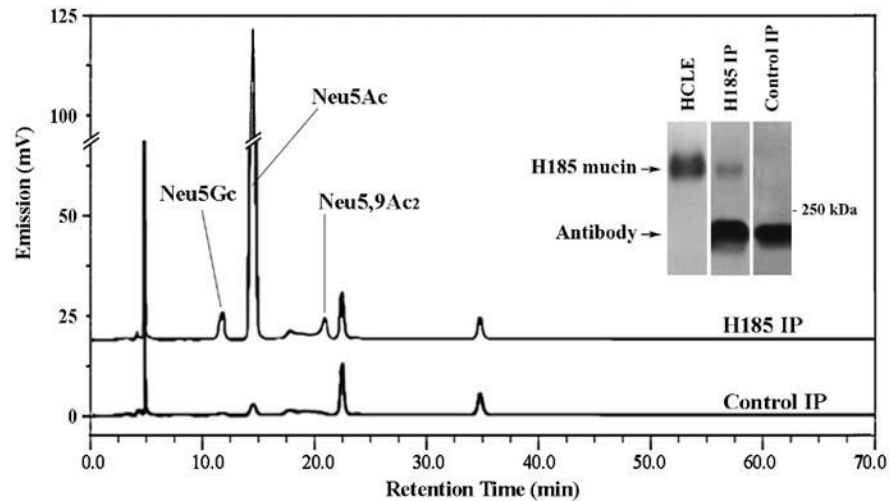
Fig 6. Identification of sialic acid derivatives after H185 antigen immunoprecipitation
The H185 antigen, which in HCLE cells is carried by the membrane-associated mucin MUC16 (Argueso *et al.*, 2003), was immunoprecipitated from HCLE cultures as described in *Materials and Methods*. Sialic acids in the H185 mucin immunoprecipitate (H185 IP) were hydrolyzed using *A. ureafaciens* sialidase, labeled with DMB and analyzed by fluorometric HPLC. A control (no tear sample) was carried out to determine the sialic acid content of the antibodies used for immunoprecipitation. The efficiency of the H185 mucin immunoprecipitation was assessed by immunoblot (*inset*). The low molecular weight band corresponds to antibodies used in the mucin immunoprecipitation assay.