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Glycosylation of sputum mucins is altered in cystic fibrosis patients

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Cystic fibrosis (CF) is characterized by chronic lung infection and inflammation, with periods of acute exacerbation causing severe and irreversible lung tissue damage. We used protein and glycosylation analysis of high-molecular mass proteins in saline-induced sputum from CF adults with and without an acute exacerbation, CF children with stable disease and preserved lung function, and healthy non-CF adult and child controls to identify potential biomarkers of lung condition. While the main high-molecular mass proteins in the sputum from all subjects were the mucins MUC5B and MUC5AC, these appeared degraded in CF adults with an exacerbation. The glycosylation of these mucins also showed reduced sulfation, increased sialylation, and reduced fucosylation in CF adults compared with controls. Despite improvements in pulmonary function after hospitalization, these differences remained. Two CF children showed glycoprotein profiles similar to those of CF adults with exacerbations and also presented with pulmonary flares shortly after sampling, while the remaining CF children had profiles indistinguishable from those of healthy non-CF controls. Sputum mucin glycosylation and degradation are therefore not inherently different in CF, and may also be useful predictive biomarkers of lung condition.

Key words: mucin/O-glycosylation/mass spectrometry/cystic fibrosis/pulmonary exacerbation

Introduction

Chronic pulmonary infection and inflammation is a hallmark of cystic fibrosis (CF), which results in progressive, irreversible, and often fatal deterioration of lung condition (Gibson et al. 2003). Lung disease in CF is typified by cycles of acute exacerbation, which although responding in most cases to aggressive antibiotic and antiinflammatory treatment, ultimately result in permanent lung damage. The correlation between the number and severity of pulmonary exacerbations and patient mortality (Liou et al. 2001) means there is a recognized need for improved measures of pulmonary condition in CF, for use in both monitoring recovery from, and predicting the onset of, acute exacerbations. The damage caused to lung tissue by recurring infection and inflammation is usually not adequately treated until individuals actually present with severe symptoms. While direct measures of pulmonary function, such as the forced expiratory volume in 1 s (FEV_1), and review of symptoms are routinely used to monitor lung condition, there is an increasing awareness that severe but localized lung disease, for example, as detected by high-resolution computer-assisted tomography (HRCT) scans, can precede changes in pulmonary function (Helbich et al. 1999; Tiddens 2002).

Airway mucus is the primary site of infection in patients with CF (Ramphal and Arora 2001), and mucins, which are high-molecular mass glycoproteins, are a major component of mucus at epithelial surfaces. Mucins are posttranslationally modified proteins with a huge diversity of oligosaccharide structures that are important in mediating interactions with other proteins, cells, and pathogens (Lamblin et al. 2001). A large body of work (summarized in Table I) has been collected over several decades describing the glycosylation of glycoconjugates from patients with CF in relation to disease status and compared with various non-CF controls, as well as from a variety of model systems. However, the exact differences in glycosylation which are associated with CF remain unclear. It is also unclear if these differences are a direct result of dysfunctional CF transmembrane conductance regulator (CFTR), or are rather secondary effects of chronic infection and inflammation. Two large, well-controlled studies (Chace et al. 1983; Davril et al. 1999) have reported that the sulfation and sialylation of respiratory mucins from patients with CF increases with the severity of lung disease, and also that respiratory mucins have higher sialylation in CF patients than in healthy non-CF controls. In contrast, several studies of glycoconjugates from a variety of in vitro cell or tissue culture systems have reported increased sulfate uptake and incorporation by cells lacking functional CFTR. However, other studies have reported large inter-sample variation, and/or not detected statistically significant differences. Analysis of in vivo airway mucin glycosylation in healthy non-CF individuals has been notoriously difficult, as healthy individuals cannot easily expectorate sputum without saline induction. Comparison of CF with non-CF mucin glycosylation in sputum has therefore largely used subjects with non-CF pulmonary disease as controls (Boat et al. 1976; Lamblin et al. 1977; Chace et al. 1985; Davril et al. 1999). Here, we report protein and glycosylation analysis of the high-molecular mass components of saline-induced sputum from adults with CF suffering an exacerbation

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Table I. Previous studies of glycosylation in CF

References	Subjects		Sample	Methods	Results
	CF	Non-CF			
Respiratory mucus					
Chace et al. 1983	33	10	BAL	Monosaccharide analysis	More sialic acid and less hexose in CF. Sialic acid, hexose, mucin concentration and sulfated mucin increase with severe CF disease
Davril et al. 1999	14	24	Sputum	Monosaccharide analysis	Sulfate and sialic acid increase with severity of infection in CF. More sulfate in CF than chronic bronchitis
Lamblin et al. 1977	2	3	Sputum, BAL	Monosaccharide analysis	More sulfation than sialylation in children with CF and chronic bronchitis. More sialylation than sulfation in adults with chronic bronchitis
Xia et al. 2005	2	2	Airway mucins	MALDI-TOF-MS, HPLC	More sialylation and sulfation in CF
Boat et al. 1976	1	2	Sputum, BAL	Chemical assays, liquid chromatography	Sulfated mucin more abundant in CF
Chace et al. 1985	1	1	Sputum	Chemical assays	More sulfate in CF than asthma
Other mucus					
Shori, Genter, et al. 2001	20	20	Saliva	Colorimetric and lectin staining	MUC5B has more NeuAcα2-3 and sialyl Lewis x in CF
Shori, Kariyawasam, et al. 2001	20	20	Saliva	Colorimetric and lectin staining	No differences in sulfation or sialylation in CF. Large inter-subject variation
Schulz et al. 2005	6	9	Submucosal gland secretion mucins	LC-ESI-MS/MS	No differences in glycosylation
Wesley et al. 1983	6	8	Intestinal mucus	Monosaccharide analysis	CF sugars are longer, with higher (Fuc, Gal, GlcNAc: NeuAc)
Carnoy et al. 1993	3	4	Saliva	Gel filtration, chemical assays.	More sialylation, sulfation, and fucosylation in CF
Cell/tissue culture					
Zhang et al. 1995	13	13	Bronchial epithelial xenograph secreted mucus glycoproteins	Radiolabeling	Sulfate incorporation higher in CF
Reid et al. 1999	10	10	Colon carcinoma cell culture epitope-tagged MUC1	Radiolabeling, antibody binding	No differences in glycosylation
Frates et al. 1983	9	8	Respiratory epitehelial tissue culture secreted mucus glycoproteins	Radiolabeling	More sulfation and higher mucus secretion in CF
Cheng et al. 1989	6	9	Nasal epithelial secreted and cell-bound glycoconjugates	Radiolabeling	Sulfate incorporation higher in CF
Leir et al. 2005	6	6	Airway epithelial cell culture MUC5AC repeat	MALDI-TOF-MS/MS, ESI-MS/MS	No differences in glycosylation
Mendicino and Sangadala 1999	4	4	Tracheal cell culture secreted mucins	Radiolabeling, ion exchange chromatography, monosaccharide analysis	Higher sulfate uptake and more sulfated high-molecular mass glycans in CF
Holmen et al. 2004	3	3	Bronchial epithelial cell culture secreted mucins	LC-ESI-MS/MS	No differences in glycosylation
Rhim et al. 2000	3	3	Airway epithelial cell culture membrane glycoconjugates	Chemical assays, glycosidase digestion	Increased Fucα1-3/4: NeuAc in CF

Continued

Table I. *Continued*

References	Subjects		Sample	Methods	Results
	CF	Non-CF			
Jiang et al. 1997	3	3	Bronchial epithelial cell culture cell surface glycans	Lectin binding	Differences between CF and derived non-CF cell lines are not CFTR dependent
Saiman and Prince 1993	2	3	Primary respiratory epithelial cells	Flow cytometry	More asialoGM1 in CF
Glick et al. 2001	2	2	Nasal and bronchial epithelial cell culture membrane glycoproteins	Radiolabeling, paper chromatography, glycosidase digestion	Increased Fucα1-3: (NeuAc and Fucα1-2) in CF
Kube et al. 2001	1	1	Tracheal epithelial cell culture. Cell surface glycoconjugates	Lectin binding	No differences in glycosylation
Animal models					
Thomsson et al. 2002	13	13	Mouse intestinal mucins	GC-MS, ESI-MS, hNMR	More blood group H Fuc in small intestinal mucins in CF
Hill et al. 1997	3	5	Mouse glycosaminoglycans	in vivo radiolabeling	Higher sulfate incorporation in CF

and requiring hospitalization, a subset of these CF subjects at discharge from hospital, children with CF with stable disease and preserved lung function, and healthy non-CF adults and children, with the aim of more clearly describing pulmonary mucin glycosylation associated with CF lung disease, and also to identify biomarkers associated with lung disease progression in CF.

Results

The main objective of this study was to identify sputum mucin biomarkers that could be used to monitor the recovery of CF patients after pulmonary exacerbation, which would be complementary to the current widely used FEV_1 test. Since FEV_1 only measures secondary effects of infection, it may have difficulty detecting minor or localized residual infection in the lung. The discovery of biomarkers which provide additional information on patients' respiratory status would help in monitoring the success of treatment of exacerbated CF-patients before their release from hospital. This study was performed on CF adults with acute pulmonary exacerbation ($n = 19$) and on several of these patients also after discharge from hospital ($n = 13$) (Table II). Since sputum mucin expression and glycosylation may be directly related to mutations in CFTR, as well as to infection status, we included adult non-CF healthy subjects as controls ($n = 19$). CF children with preserved pulmonary function ($n = 4$), and non-CF healthy children ($n = 4$) were also included in this study in order to account for the effect of deterioration of lung function on mucin expression and glycosylation. Differences between the mucin characteristics of CF children and non-CF healthy children would suggest a direct change due to CFTR mutation. Differences in the mucin characteristics of CF children and CF adults would suggest that any such differences are an indirect effect of chronic infection.

Identification of high-molecular mass proteins in sputum from CF and control adults

High-molecular mass proteins in saline-induced sputum samples collected from control adults, CF adults with acute pulmonary exacerbation, and several of these CF subjects at discharge from hospital were separated by one-dimensional sodium dodecylsulfate–agarose polyacrylamide gel electrophoresis (1D SDS–AgPAGE) and stained for total carbohydrate content with periodic acid Schiffs reagent (PAS). 1D SDS–AgPAGE protein profiles of sputum from non-CF and CF adults with samples before and after hospital treatment are shown in Figure 1. Large and significant differences ($P = 2 \times 10^{-9}$) in the high-molecular mass protein profiles were observed between CF and control adults (Figure 1). Adult control subjects showed distinct major glycoprotein bands at approximately 4 MDa, with lower intensity bands from approximately 2 to approximately 3 MDa. In contrast, adult CF subjects with an exacerbation displayed several major mucin bands in the molecular mass range of approximately 1–4 MDa, with the bands at approximately 1.5 MDa being typically the most intense. There were no significant differences between the high-molecular mass protein profiles of CF subjects at acute pulmonary exacerbation and at discharge from hospital.

Duplicate gels for further protein and glycosylation analysis were electroblotted onto polyvinylidene fluoride (PVDF) and stained for acidic oligosaccharide content with the non-destructive Alcian Blue, which stained the same bands as PAS (Figure 2). These bands were excised, digested with trypsin, analyzed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), and identified by peptide mass fingerprinting (PMF). Protein identities were confirmed in several samples by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS) and SEQUEST. This analysis showed that the major protein bands were heavily glycosylated mucins

Table II. Patient characteristics

Subject	Gender	CFTR mutation	Age	Baseline FEV_1 (% predicted)		Δ FEV_1 (After hospitalization)
CF adults				Exacerbation	Discharge	
CF-4	Male	ΔF508/ΔF508	28	52.3		
CF-6	Female	Not determined[a]	Not determined	Not determined		
CF-10	Male	ΔF508/ND	26	17.8		
CF-11	Female	ΔF508/ΔF508	24	59.5	81.3	21.8
CF-12	Male	ΔF508/ΔF508	37	53.5[a]	62.3	8.8
CF-15	Male	ΔF508/ΔF508	21	50.4		
CF-16	Female	ΔF508/ΔF508	21	46.7	46.0	−0.7
CF-20	Female	ΔF508/G551D	31	28.3	35.4	7.1
CF-35	Female	ΔF508/ΔF508	32	59.1		
CF-37	Male	ΔF508/Not determined	31	47.8	40.6	−7.2
CF-38	Female	ΔF508/G542X	20	14.3	27.3	13.0
CF-39	Female	ΔF508/ΔF508	35	66.9	82.7	15.8
CF-40	Female	ΔF508/ΔF508	19	18.4	25.8	7.4
CF-41	Female	ΔF508/ΔF508	28	60.7	67.2	6.5
CF-43	Male	ΔF508/G542X	24	46.4	69.4	23.0
CF-44	Female	ΔF508/R560T	19	56.9	76.0	19.1
CF-45	Female	ΔF508/ΔF508	19	30.7	26.7	−4.0
CF-46	Female	ΔF508/ΔF508	21	55.6	60.5	4.9
CF-47	Female	ΔF508/G542X	38	39.4		
CF-48	Female	Not determined	Not determined	Not determined		
CF children						
CF-61	Female	ΔF508/ΔF508	12	102		
CF-63	Male	ΔF508/W1282X	12	97		
CF-64	Male	ΔF508/ΔF508	13	97		
CF-69	Female	ΔF508/ΔF508	11	86		
Control adults						
H-7	Male		26	90.2		
H-8	Female		21	96.3		
H-9	Female		25	98.7		
H-13	Male		23	94.1		
H-17	Male		32	114.5		
H-18	Male		20	122.4		
H-19	Female		39	103.5		
H-21	Female		23	98.3		
H-22	Female		22	116.2		
H-24	Female		19	102.2		
H-25	Female		33	92.2		
H-26	Female		21	105.1		
H-27	Female		23	108.8		
H-28	Female		20	102.7		
H-29	Male		21	110.2		
H-30	Male		30	107.5		
H-32	Female		31	86.6		
H-34	Female		31	94.6		

Continued

Table II. *Continued*

Subject	Gender	CFTR mutation	Age	Baseline FEV_1 (% predicted)	Δ FEV_1 (After hospitalization)
H-36	Male		32	98.2	
Control children					
H-70	Female		10	89	
H-72	Male		9	97	
H-73	Female		10	123	
H-75	Female		10	95	

[a]Sample not analyzed.

and mucin-like proteins. Although several distinct bands were present in most samples, these were found to contain the same major mucin species, MUC5B and MUC5AC, together with the mucin-like glycoprotein gp-340 in some samples (Figure 1). This indicates that there is no difference in the major mucin protein species present between CF and non-CF sputum.

Glycosylation analysis of sputum mucins from CF adults with an exacerbation and adult control subjects

O-Linked oligosaccharides are the major glycosylation modification on mucins, and are responsible for many of the biological and physical properties of these glycoproteins. *O*-Linked oligosaccharides were released by reductive alkaline β-elimination from the Alcian Blue-staining bands from sputum from each subject after separation by 1D SDS–AgPAGE and electroblotting to PVDF. These oligosaccharides were then analyzed by LC-ESI-MS/MS.

MUC5B/MUC5AC-containing bands dominated the one dimensional protein profile, and the oligosaccharide mass profiles for each of these bands from approximately 1 to approximately 4 MDa from within the same sample were very similar (Figure 2). Monosaccharide compositions and relative abundances of oligosaccharides from the dominant mucin band were therefore determined for all subjects. These data were used to determine the weighted average monosaccharide compositions of *O*-linked oligosaccharides from the dominant mucin band for each subject (Figure 3A and supplementary data).

Glycosylation analysis showed major differences between mucins from the sputum of CF adults with an exacerbation and of adult controls (Figure 3A). *O*-linked oligosaccharides typically consist of *N*-acetyllactosamine chains capped with various terminal epitopes. *N*-Acetyllactosamine chains consist of *N*-acetylglucosamine (GlcNAc) and galactose (Gal). There were statistically significant differences between the abundances of these monosaccharides, with sputum from CF adults with an exacerbation displaying 12% less *N*-acetylhexosamine ($P = 0.009$) and 18% less Gal ($P = 0.0006$) than adult controls. There were also large and statistically significant differences in the abundances of monosaccharides from terminal epitopes, with CF adults with an exacerbation having 1.7-fold less fucose (Fuc) ($P = 4 \times 10^{-7}$), 3.6-fold more sialic acid ($P = 3 \times 10^{-7}$), and 5.6-fold less sulfate ($P = 4 \times 10^{-9}$) compared with adult controls. These differences in sulfate and sialic acid abundance were also confirmed with Alcian Blue staining of whole dot-blotted samples at pH 0.1 (sulfate specific) and pH 1.0 (sulfate and sialic acid) (data not shown). As these adult CF subjects were suffering from severe acute pulmonary exacerbations, these changes may be a direct result of the CFTR mutation, or may be regulated responses to acute infection and inflammation. As terminal oligosaccharide epitopes mediate many biologically important interactions, such as bacterial and leukocyte adherence, these differences in the glycosylation of the dominant mucins in the sputum of CF subjects with an exacerbation are very likely to have substantial biological consequences.

Glycosylation analysis of adult CF sputum mucins after hospitalization

There were no significant differences ($P > 0.05$) between the monosaccharide compositions of MUC5B and MUC5AC in CF adults with an exacerbation and upon discharge from hospital (Figure 3A), even though most CF subjects showed improvement in FEV_1 (Table II). However, Student's *t*-test of sulfation showed a 2.33-fold decrease in the average sulfation value after hospital treatment ($P = 0.06$) relative to that of sputum from CF subjects with an exacerbation. This is suggestive of a real change after treatment of the pulmonary exacerbations, which may be useful as a longitudinal measure of lung condition. However, this reduction in sulfation in CF adults after hospital treatment further increased the sulfation difference compared with sputum glycosylation in adult control subjects (Figure 3A). This suggests that the glycans of the major tracheobronchial mucins are hyposulfated in all adults with severe CF lung disease, irrespective of pulmonary function.

Case studies of individual subjects

Although there were no statistically significant differences ($P > 0.05$) in either high-molecular mass protein profiles (Figure 1A) or mucin glycosylation profiles (Figure 3A), for CF subjects with acute pulmonary exacerbations and the same CF subjects at discharge from hospital, several individual CF adults did display large and intriguing changes in these protein and glycosylation profiles after hospitalization.

Subject CF-11 showed a dramatic change in mucin protein profile at discharge from hospital (Figure 1B). At exacerbation, this subject showed a mucin protein profile typical of all adult CF subjects with an exacerbation, with a low average molecular mass and large molecular mass spread of mucins. At discharge from hospital, however, this subject displayed an intense MUC5B- and MUC5AC-containing band at approximately 4 MDa, as typically seen in adult controls.

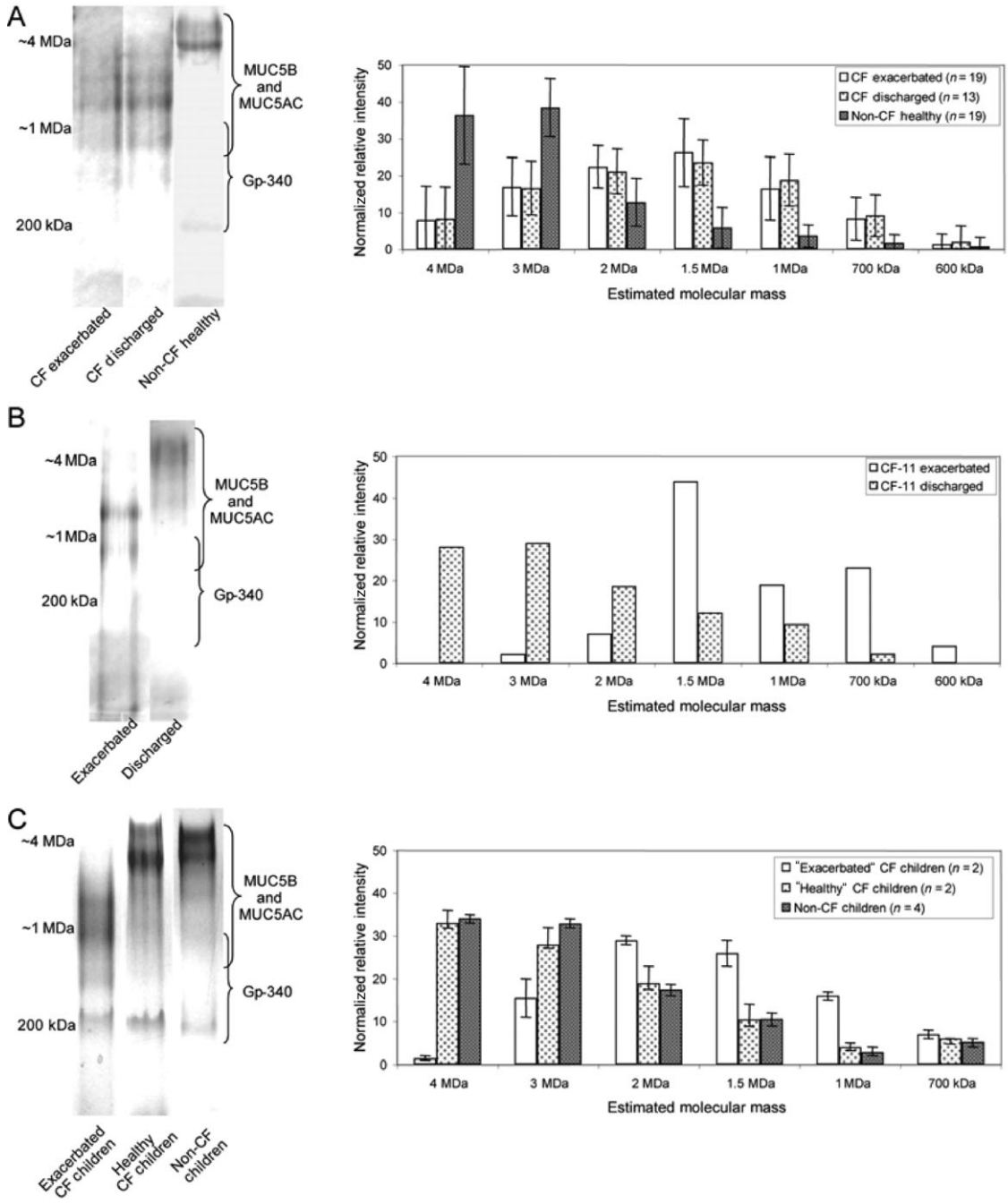

Fig. 1. High-molecular mass glycoproteins in sputum. Typical 1D SDS–AgPAGE profiles of sputum proteins; and the intensity of PAS-staining bands, determined by image analysis, across the molecular mass range that contained mucin bands. (**A**) CF adult subjects with an exacerbation (e.g., CF-16), the same CF subjects at discharge from hospital (e.g., CF-16D), and controls (e.g., H-9); (**B**) CF subject CF-11 at exacerbation and at discharge (i.e., after hospital treatment); and (**C**) CF children whose profiles resembled those of CF adults with pulmonary exacerbation (e.g., CF-64), CF children whose profiles resembled those of control adults (e.g., CF-63), and control children (e.g., H-72). Error bars show (A) standard error or (C) range.

The average monosaccharide composition of the dominant mucin in the sputum of subjects CF-11, CF-41, and CF-44 also showed large changes after hospitalization (Figure 3B). For these three CF subjects, at discharge from hospital, the relative abundances of *N*-acetylhexosamine, Gal, Fuc, and sialic acid changed from resembling that typical of all CF subjects with an exacerbation, towards resembling controls, although only the increase in fucosylation was significant ($P = 0.016$). However, sulfation stayed at the low level typical of all CF subjects. Although these changes were observed only in a small subset of individuals, they suggest that monitoring mucin proteins and glycosylation of sputum, on an individual basis, may provide useful measures of lung condition.

Oligosaccharide structures on sputum mucins

To define sputum mucin glycosylation further, we selected three samples for detailed oligosaccharide structural characterization, CF subject 11 at exacerbation (CF-11) and discharge

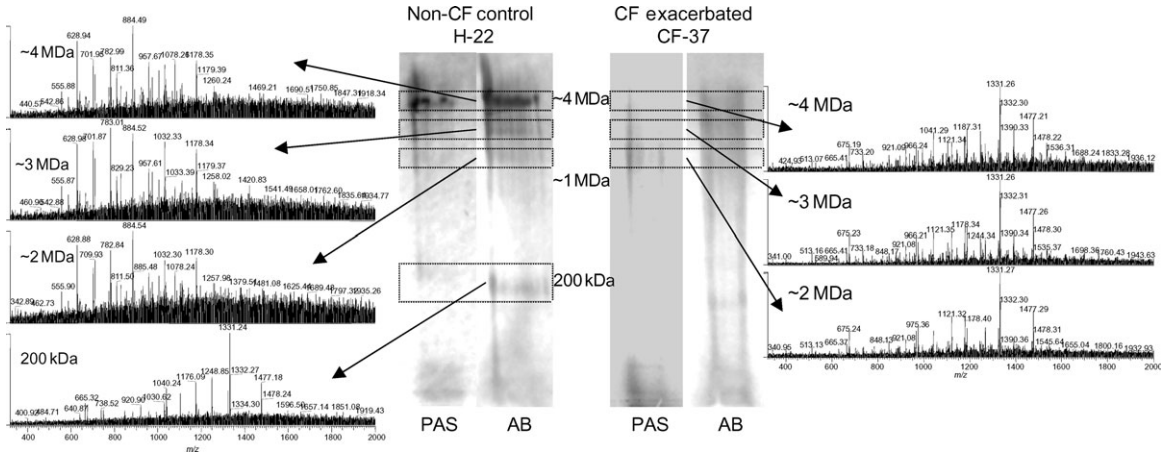

Fig. 2. High-molecular mass protein and glycosylation profiles. Proteins from saline-induced sputum were separated by 1D SDS–AgPAGE, and stained for carbohydrate (PAS) or acidic carbohydrate (AB). Oligosaccharides were released from Alcian Blue-stained bands by reductive β-elimination, and analyzed by LC-ESI-MS.

from hospital (CF-11D), and non-CF subject H-22 (Table III and Figure 4). This tandem mass spectrometry (MS/MS) analysis showed a very high diversity of *O*-linked oligosaccharides, which were distinctly different between the three samples. No sialylated structures were detected after α2-3-specific desialylation, in agreement with previous reports describing the presence of α2-3-linked sialic acid on secreted glycoconjugates and α2-6-linked sialic acid on cell surface glycoconjugates from human lung (Gagneux et al. 2003). The presence of abundant sulfated oligosaccharides in non-CF subject H-22 was confirmed, with mainly sulfated Gals (hexoses), and also sulfated GlcNAcs (*N*-acetylhexosamines) detected. In contrast, only one sulfated structure was detected from subject CF-11. Lewis a/x structures were abundant in all three samples, but were also detected as sulfo-Lewis a/x in non-CF subject H-22, and sialyl-Lewis a/x in CF-11 and CF-11D. The most striking difference between the glycosylation of sputum mucins from subjects CF-11 and CF-11D was the appearance of blood group epitopes (B and H) after hospitalization. As the blood group status of this individual cannot have changed, this difference may instead be due to changes in secretion or glycosyltransferase activity upon recovery. Hence, a measurement of blood group antigens in sputum may provide additional information about the infection status of CF patients.

Mucin analysis of sputum from children with CF and control children

Saline-induced sputum samples from children with CF, none of whom had a pulmonary exacerbation at the time of sample collection and for whom FEV_1 measurements indicated preserved respiratory function, were similarly analyzed. Sputum from control children was also analyzed. High-molecular mass protein profiles for these subjects are shown in Figure 1C, and mucin glycosylation monosaccharide composition data are shown in Figure 3C and supplementary data. Interestingly, two of these CF children (CF-64 and CF-69) were later diagnosed with pulmonary infections at 96 and 49 days, respectively, after sample collection (data not shown). At the time of sample collection, these same two children showed mucin protein and glycosylation profiles resembling those of CF adults with pulmonary exacerbations (Figures 1 and 3). This is in contrast to the other two CF children (CF-61 and CF-63), who showed mucin protein profiles resembling those of healthy controls, and glycosylation profiles intermediate between those of CF adults with an exacerbation and control subjects. Proteomic analysis of these same samples also indicated that these two "exacerbated" CF children also had an intense inflammatory response at the time of sample collection (Sloane et al. 2005). Although no causal association has been demonstrated and the sample size is small, these data suggest that changes in proteins and glycoproteins in saline-induced sputum from children with CF can provide early evidence of deterioration in lung condition, before manifestation of other clinical signs.

Statistical analysis of sputum mucin glycosylation differences

To correlate and compare all of the mucin glycosylation compositional data statistically, principal component anaylsis was performed with these data from all subjects in this study (CF adults at exacerbation and at discharge from hospital, and control adults and children) (Figure 5). Adults with CF were clearly differentiated from control adults without CF on the basis of sputum mucin glycosylation, based mainly on the degrees of fucosylation, sialylation, and sulfation. While control children were not separated from control adults, children with CF who were not suffering from pulmonary exacerbation were positioned intermediary to controls and CF adults with an exacerbation.

Discussion

The same major mucins, MUC5B and MUC5AC, were found to be present in the sputum of all subjects analyzed, including adults and children with and without CF, and with a range of severity of lung disease. However, there were differences found in the average apparent molecular mass and spread of molecular masses of the mucin species present (Figure 1), with pulmonary exacerbation correlating with MUC5B and MUC5AC being detected over a very large molecular mass range (approximately 1–4 MDa). It seems most likely that the lower molecular mass species, which are more abundant

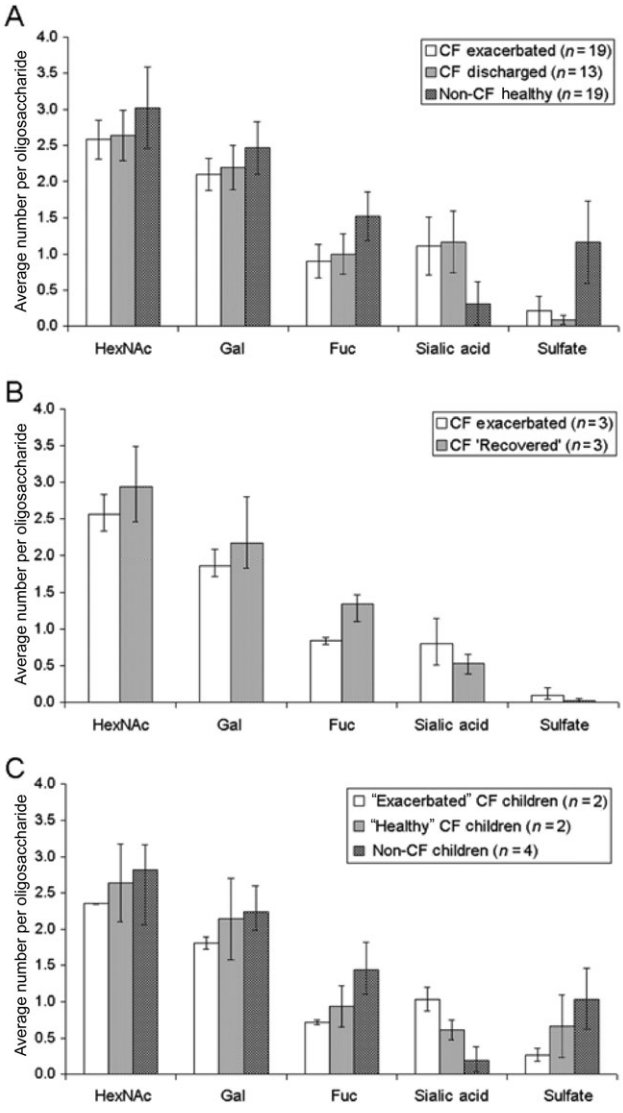

Fig. 3. Average monosaccharide composition of *O*-linked oligosaccharides from sputum mucins. *O*-Linked oligosaccharides were released from high-molecular mass sputum proteins by reductive β-elimination, detected and semi-quantified by LC-ESI-MS, and their monosaccharide compositions determined with GlycoComp. Sputum was analyzed from (**A**) CF subjects with acute pulmonary exacerbation, a subset of the same CF subjects at discharge from hospital, and non-CF healthy control subjects; (**B**) CF subjects CF-11, CF-41 and CF-44 at exacerbation and after hospital treatment; and (**C**) healthy non-CF children, and children with CF who were not suffering from an exacerbation. HexNAc, *N*-acetylhexosamine; error bars show (A) standard error or (B and C) range.

in CF subjects, are the result of protein degradation, perhaps by secreted leukocyte or *Pseudomonas aeruginosa* elastase, which are abundant in the lung mucus of CF subjects (Konstan and Davis 2002). Mucins form a cross-linked polymeric gel, which provides mucus with much of its viscosity and rheological properties. Degradation of this gel through proteolysis may affect these properties, and contribute to the reduction in mucus clearance reported in CF.

Our data show that the glycosylation of sputum mucins is significantly different in adults with CF compared with adult controls. CF adults suffering from acute pulmonary exacerbations displayed significantly less sulfation, more sialylation and less fucosylation than controls (Figure 3A). There were also indications that further reduced sulfation might be associated with improved pulmonary condition in CF subjects (Figure 3B), as has been previously reported (Davril et al. 1999). However, the glycosylation of CF patients on discharge from hospital does not resemble that of control subjects. In particular, the sulfation of mucin oligosaccharides in CF subjects was always comparatively very much lower than in controls.

These characteristics of glycosylation in CF and healthy controls, and their association with the severity of CF disease, are in agreement with some of the previous analyses of sputum mucin glycosylation but differ from other reports in the literature. The conflicting glycosylation phenotypes associated with CF found in other studies (Table I) could be due to differences in the types of samples, sputum collection protocols, analytical methods, or the clinical status of the subjects analyzed. Table I shows the range of reported changes in the glycosylation status of the respiratory cells in CF. When taken as a whole there is a confusing amount of contradictory data. In part, this could be due to the different experimental systems being studied. Since cell/tissue culture and different mammals are known to vary in their glycosylation phenotype, we chose to analyze the oligosaccharide profiles presented on mucins purified from sputum obtained from human subjects. If we focus on the other such studies (Table I) which used large sample numbers of clinically well-defined subjects (7, 8), similar increases in sialic acid content are observed. However, in these other studies, and apparently conflicting with our data, sulfation is shown to be high relative to controls and increases with severity of disease. These latter studies were based on compositional analysis of the high-molecular mass fraction of the sputum rather than the specific sugar structural analysis that we have carried out by mass spectrometry of the separated mucin oligosaccharides. It is conceivable that there are also sulfated proteoglycans present in the high mass fractions and that the non-CF disease bronchitis sputum used as a control also presents with altered sulfation relative to normal sputum.

Recently, a study of cells in the respiratory tract showed a difference in the sugar epitopes distributed along the human airways (Shinya et al. 2006) which implies that there are differences in the glycosylation machinery active in the diversity of epithelial cells and associated glands that contribute to the composition of the lung mucus. We have shown that the profile of the submucosal gland secretions, which comprise a portion of the total sputum mucins, does not differ significantly in CF subjects compared with non-CF controls (Schulz et al. 2005). Taken together with the hyposecretion of mucus from the epithelial glands caused by the lack of CFTR (Joo et al. 2006), it is not surprising that the oligosaccharide profile of the total sputum mucin complement differs in CF.

Intriguingly, the differences we find in the glycosylation of sputum mucins between CF and our healthy non-CF control adults (Figure 3A) shows that even when adults with CF show improvements in general health and pulmonary function, including increased FEV_1, their lung biochemistry is still fundamentally different from that of healthy non-CF individuals.

In the detailed oligosaccharide structural characterization of individual subjects, CF subject CF-11 showed changes in the

Table III. Sputum mucin oligosaccharide structures determined by mass spectrometry

Subject			Oligosaccharide structure	Oligosaccharide epitopes		Lactosamine chain type	Core
CF-11	CF-11D	H-22		Lewis type	Blood group		
Neutral							
	x[a]		Fuc(α1-2)Gal(β1-3)GalNAc		H		1
	x		Fuc(α1-2)Gal(β1-3)**[GlcNAc(β1-6)]**GalNAc[b]		H		2
	x		Fuc(α1-2)[Gal(α1-3)]Gal(β1-3)GlcNAc(β1-3)GalNAc		B	1	3
	x		Fuc(α1-2)Gal(β1-3)[Gal(β1-6)]GlcNAc(β1-3)GalNAc		H	1	3
	x		Fuc(α1-2)Gal(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]**GalNAc		H	2	2
	x		GlcNAc(β1-3)**[Fuc(α1-2)[Gal(α1-3)]Gal(β1-4)GlcNAc(β1-6)]**GalNAc		B	2	4
x			Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]**GalNAc		H	1, 2	4
	x		Fuc(α1-2)[Gal(α1-3)]Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3) GalNAc	Le a/x	B		1
	x		Fuc(α1-2)Gal(β1-3)**[Fuc(α1-2)[Gal(α1-3)]Gal(β1-4)GlcNAc(β1-6)]** GalNAc		B, H	2	3
	x		Fuc(α1-2)Gal(β1-3)**[Fuc(α1-2)[Gal(α1-3)]Gal(β1-3/4)[Fuc(α1-3/4)] GlcNAc(β1-6)]**GalNAc	Le a/x	B, H		2
	x		GlcNAc(β1-3)Gal(β1-3)**[Fuc(α1-2)[Gal(α1-3)]Gal(β1-3/4)[Fuc(α1-3/4)] GlcNAc(β1-6)]**GalNAc	Le a/x	B		2
x		x	Gal(β1-4)GlcNAc(β1-3)GalNAc			2	3
	x		Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)GalNAc	Le a/x			3
x		x	Gal(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]**GalNAc			2	2
x		x	GlcNAc(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]**GalNAc			2	4
x			Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)Gal(β1-3)GalNAc	Le a/x			1
x			Gal(β1-3/4)GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc				4
		x	Gal(β1-4)GlcNAc(β1-3/6)[Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc			2, Not determined[c]	4
x		x	Gal(β1-3/4)GlcNAc(β1-3)**[Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-6)]** GalNAc	Le a/x			4
		x	Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]** GalNAc	Le a/x		Not determined	4
x			Gal(β1-3/4)GlcNAc(β1-3)[Gal(β1-3/4)GlcNAc(β1-6)]Gal(β1-3)GalNAc				1
x			Fuc(α1-3)[Gal(β1-4)]GlcNAc(β1-3)Gal(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]** GalNAc	Le a/x			2
	x		Fuc(α1-3)[Gal(β1-4)]GlcNAc(β1-3)Gal(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]** GalNAc	Le a/x		2	2
x			Gal(β1-3/4)GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]Gal(β1-3/6) [Gal(β1-4)GlcNAc(β1-6)]** GalNAc			2	4
x			Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3/6)[Gal(β1-3/4)GlcNAc(β1-3/6)] Gal(β1-3)**Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc	Le a/x			4
Desialylated							
	x		Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]**GalNAc		H	1,2	4
	x		Fuc(α1-2)[Gal(α1-3)]Gal(β1-3)GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]** GalNAc		B	1, Not determined	4
	x		Fuc(α1-2)[Gal(α1-3)]Gal(β1-4)GlcNAc(β1-3)**[Fuc(α1-3/4)[Gal(β1-3/4)] GlcNAc(β1-6)]**GalNAc	Le a/x	B	2	4
	x		Fuc(α1-2)[Gal(α1-3)]Gal(β1-3/4) [Fuc(α1-3/4)]GlcNAc(β1-3)**[Gal(β1-3/4) GlcNAc(β1-6)]**GalNAc	Le a/x	B	Not determined	4
x	x		Gal(β1-3)**[Gal(β1-4)GlcNAc(β1-6)]**GalNAc			2	2
x			Gal(β1-4)GlcNAc(β1-3)Gal(β1-3)GalNAc			2	1
	x		Gal(β1-4)GlcNAc(β1-3)**[GlcNAc(β1-6)]**GalNAc			2	4
	x		Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)Gal(β1-3)GalNAc	Le a/x			1

Continued

Table III. *Continued*

Subject			Oligosaccharide structure	Oligosaccharide epitopes		Lactosamine chain type	Core
CF-11	CF-11D	H-22		Lewis type	Blood group		
x			Gal(β1-3)**[Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-6)]**GalNAc	Le a/x			2
	x		Gal(β1-3/4)GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc			Not determined	4
x			Gal(β1-3)GlcNAc(β1-3)**[Gal(β1-3)GlcNAc(β1-6)]**GalNAc			1	4
x			Gal(β1-4)GlcNAc(β1-3/6)[Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc			2, Not determined	4
x			GlcNAc(β1-3)Gal(β1-3)**[Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-6)]**GalNAc	Le a/x			2
	x		Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)Gal(β1-3)**[GlcNAc(β1-6)]**GalNAc	Le a/x			2
x			Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]** GalNAc	Le a/x		Not determined	4
x	x		Gal(β1-3/4)GlcNAc(β1-3)**[Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-6)]** GalNAc	Le a/x		Not determined	4
	x		Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]** GalNAc	Le a/x		Not determined	4
x	x		Fuc(α1-3/4) [Gal(β1-3/4)]GlcNAc(β1-3)Gal(β1-3)**[Gal(β1-3/4) GlcNAc(β1-6)]**GalNAc	Le a/x		Not determined	2
x			Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-3) GalNAc	Le a/x		2	1
x			Fuc(α1-3/4)[Gal(β1-3/4]GlcNAc(β1-3)Gal(β1-3/4)[Fuc(α1-3/4)] GlcNAc(β1-3)Gal(β1-3)GalNAc	Le a/x			1
x			Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3/6)[Gal(β1-3/4)GlcNAc(β1-3/6)] Gal(β1-3) **[Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc	Le a/x		Not determined	4
Sulfated							
		x	Gal(β1-3/4)[HSO3(-3/6)]GlcNAc(β1-3/6)[Gal(β1-3/4)GlcNAc(β1-3/6)] GalNAc			Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)GlcNAc(β1-3)**[Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc			Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)GlcNAc(β1-3/6)[HSO3(-3/6)Gal(β1-3/4) GlcNAc(β1-3/6)]GalNAc			Not determined	4
		x	Gal(β1-3/4)GlcNAc(β1-3)**[HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)] GlcNAc(β1-6)]**GalNAc	Su Le a/x		Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3/6)[Gal(β1-3/4) GlcNAc(β1-3/6)]GalNAc	Su Le a/x		Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3/4) GlcNAc(β1-3)GalNAc	Su Le a/x		Not determined	3
		x	Fuc(α1-3/4)[Gal(β1-3/4)][HSO3(-6)]GlcNAc(β1-3/6)[HSO3(-3/6) Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc	Su′ Le a/x		Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3/6)[HSO3(-3/6) Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc	Su Le a/x		Not determined	4
x			Fuc(α1-3/4)[Gal(β1-3/4)[HSO3(-6)]GlcNAc(β1-3)Gal(β1-3)**[Gal(β1-3/4)] GlcNAc(β1-6)]**GalNAc	SuLe a/x			2
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)][HSO3(-6)]GlcNAc(β1-3/6) [Fuc(α1-3/4)[Gal(β1-3/4)]GlcNAc(β1-3/6)]GalNAc	Su Le a/x, Le a/x			4
		x	Fuc(α1-3/4)[Gal(β1-3/4)[HSO3(-6)]GlcNAc(β1-3)Gal(β1-3)**[HSO3(-3/6) Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc	Su′ Le a/x[d]		Not determined	2
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3)**[HSO3(-3/6) Gal(β1-3/4)GlcNAc(β1-6)]**GalNAc	Su Le a/x		Not determined	2
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3)**[HSO3(-3/6) Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-6)]**GalNAc	Su Le a/x		Not determined	2
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)][HSO3(-6)]GlcNAc(β1-3)Gal(β1-3/4) GlcNAc(β1-3/6)[Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc	Su Le a/x		Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3/4) GlcNAc(β1-3/6)[HSO3(-3/6)Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc	Su Le a/x		Not determined	4

Continued

Table III. *Continued*

Subject			Oligosaccharide structure	Oligosaccharide epitopes		Lactosamine chain type	Core
CF-11	CF-11D	H-22		Lewis type	Blood group		
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3/6)[HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3/4)GlcNAc(β1-3/6)]GalNAc	Su Le a/x		Not determined	4
		x	HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3/6)[HSO3(-3/6)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3)Gal(β1-3/4)[Fuc(α1-3/4)]GlcNAc(β1-3/6)]GalNAc	Su Le a/x, Le a/x			4

[a]x indicates structure detected.
[b]Bold letters indicate the C-6 branch of the core GalNAc.
[c]Lactosamine chain present, but type not determined. Assumptions: anomericities are predicted from previous structural studies of similar human oligosaccharides; all deoxyhexoses are Fuc; all hexoses are Gal; the core HexNAcol is a GalNAcol; all HexNAcs are GlcNAc except for those in sequences assigned as blood group A epitopes, which are GalNAc.
[d]Sulfated GlcNAc.

sugar structures detected at discharge from hospital, compared with at exacerbation (Table III), with the appearance of blood group epitopes with clinical recovery. This is in contrast to the lack of blood group structures detected from non-CF healthy adult H-22. Submucosal gland fluid secretions are more viscous in CF (Jayaraman et al. 2001) and have been reported to contain mucins bearing blood group epitopes (Schulz et al. 2005). It is therefore possible that the increase in abundance of blood group epitope-bearing mucins in the sputum of CF subject CF-11 is related to changes in the different gland secretions that comprise tracheobronchial mucus, including an overall accumulation of submucosal gland secretions.

The monosaccharide composition (Figure 3) and structural epitopes (Table III) of oligosaccharides detected on CF sputum mucins are consistent with the presence of abundant high-affinity ligands for bacteria including *P. aeruginosa*, such as Lewis a, Lewis x, Lewis y, sialyl-Lewis x, and other fucosylated and sialylated epitopes (Ramphal and Arora 2001). This increased binding may then contribute to increased pulmonary infection and biofilm development in CF patients.

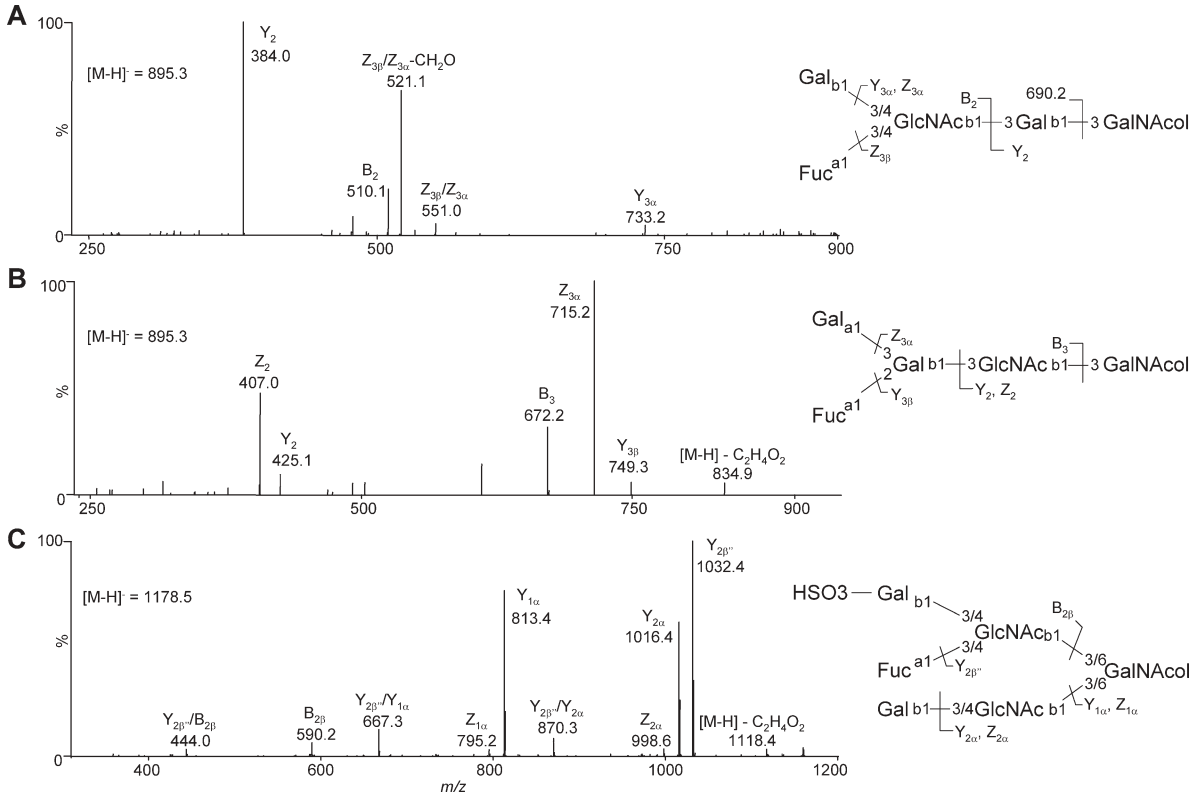

Fig. 4. LC-ESI-MS/MS spectra and assigned structures of typical *O*-linked oligosaccharides released from sputum mucins of CF subject CF-11 (**A**) with acute pulmonary exacerbation or (**B**) at discharge from hospital, and (**C**) non-CF healthy subject H-22.

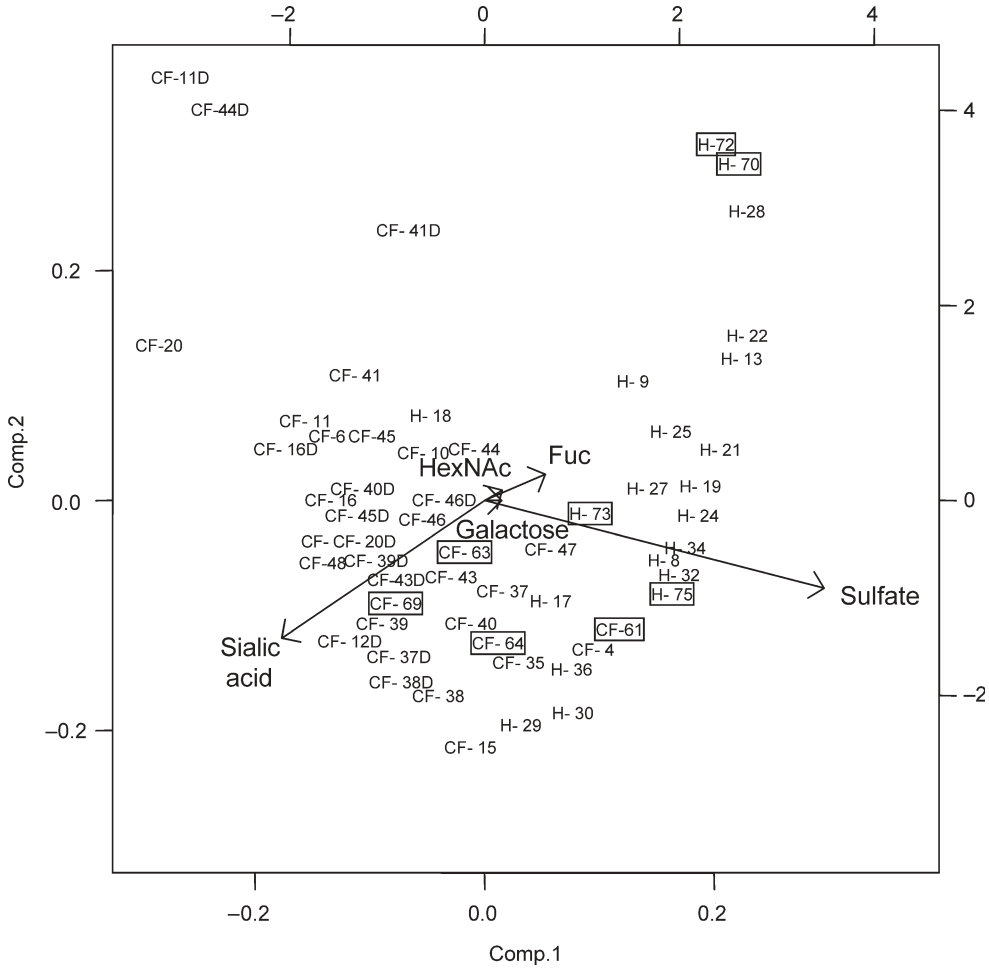

Fig. 5. Principal component analysis of the average monosaccharide composition of *O*-linked oligosaccharides from sputum mucins. CF adults with acute pulmonary exacerbation (CF-#), the same subjects at discharge from hospital (CF-#D), control adults (H-#), CF children (boxed text, CF-#), and control children (boxed text, H-#).

These oligosaccharide epitopes are also high affinity ligands for leukocytes (Lowe 2002). The continued high abundance of this "inflammatory" glycosylation in CF adults compared with controls suggests that this may contribute to the continued predisposition to both chronic infection and inflammation in adults with CF, even in overtly stable subjects with improved pulmonary function.

It is unclear if the observed differences in the glycosylation of sputum mucins between CF and non-CF adults are inherent, or are a result of chronic pulmonary infection and inflammation. Children with CF have not yet been overly exposed to the cycles of pulmonary exacerbation characteristic of adult CF lung disease, and therefore allow this question to be investigated, although the small sample size precludes statistical comparison. Control children displayed high-molecular mass protein and glycosylation profiles that were essentially the same as those of control adults (Figures 1 and 3). In contrast, the protein and glycosylation profiles of children with CF showed more population heterogeneity. It is striking that such variation should exist in children with stable disease and preserved lung function. The two CF children with profiles resembling those of CF adults with pulmonary exacerbation, CF-64 and CF-69 (Figures 1 and 3), were diagnosed with pulmonary flares of infection 96 and 49 days after sampling, respectively. Their mucin protein and glycosylation profiles may reflect the severe but localized lung disease often detected in patients with CF using HRCT analysis (Helbich et al. 1999; Tiddens 2002), which are not detected by current standard measures of pulmonary health but which may predict the onset of pulmonary exacerbation. In contrast, the profiles of other CF children resembled those of healthy non-CF subjects, indicating that the glycosylation of sputum mucins is not inherently different in CF.

We have found clear differences in the glycosylation of the major mucins in sputum from CF patients, compared with non-CF healthy controls. This is in contrast to previous investigations showing that the glycosylation of pure secretions from submucosal glands (Schulz et al. 2005) and epithelial tissue culture (Holmen et al. 2004; Leir et al. 2005) cannot distinguish between CF and non-CF. This therefore suggests that there is infection/inflammation-dependent glycosylation of epithelial secretions in CF, which can even affect patients in their early childhood. These specific changes in protein and/or glycosylation components of sputum could be used to generate specific antibodies for immunoassay-based measurements. Such rapid diagnostic format measurements

may provide early indicators of the likely onset of pulmonary exacerbation in both children and adults with CF, before diagnosis would be possible with current methods.

Materials and methods

Patient characteristics

Saline-induced sputum was collected from control adults 18–45 years ($n = 19$), with FEV_1 more than 80% predicted, adults with CF and an acute clinical exacerbation (Dakin et al. 2001; Rosenfeld et al. 2001) with FEV_1 less than 60% predicted, and requiring hospitalization ($n = 19$), and again from 13 of these CF adults at the time of discharge from hospital (Table II). Subjects with CF were excluded from this study if they had any other coexisting acute or chronic illnesses, while control subjects were excluded if they had any existing acute or chronic illness. Saline-induced sputum was also collected from control children ($n = 4$) and children with CF ($n = 4$) with stable disease and preserved pulmonary function. Sputum was qualified using a criterion of a squamous cell count less than 80%. All CF subjects had at least one copy of the ΔF508 mutation in the CFTR gene. Subjects were specifically recruited for this study, and subject number allocation was based on order of recruitment, irrespective of CF or control status. Missing subject numbers correspond to subjects who were excluded from this study as a result of not satisfying inclusion criteria. Sputum from these excluded subjects was not analyzed in this study. The Institutional Human Research Ethics Committees approved human subject recruitment and research involving human samples for these studies. Written consent was obtained from all subjects (or their legal guardians) participating in this study. These studies were conducted in accordance with the World Medical Association Declaration of Helsinki regarding ethical principles for medical research involving human subjects.

Sample processing, protein gel electrophoresis and image analysis

Chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless specified otherwise. Saline (3% w/v) induced sputum was collected from subjects using previously described methods (Gershman et al. 1996). Sputum samples were liquefied using methods similar to those previously described (Fahy et al. 1995), in the presence of a cocktail of protease inhibitors (Roche Biomedical Laboratories, Inc., Burlington, NC). Liquefied samples were reduced and alkylated with 10 mM dithiothreitol and 25 mM iodoacetamide in sample loading buffer, then concentrated using 100 kDa MW cutoff spin columns (Millipore, Bedford, MA) and subsequently separated by 1D SDS–AgPAGE as previously described (Schulz et al. 2002). Equal volumes of processed sputum (equivalent to 10 μL of whole sputum) were separated by 1D SDS–AgPAGE for each sample. Duplicate gels were then either stained for the presence of total oligosaccharides (PAS), or electroblotted to Immobilon™ PVDF P^{SQ} membrane (Millipore) and stained for the presence of acidic oligosaccharides (Alcian Blue) as previously described (Schulz et al. 2002). The molecular mass spread of the intensity of glycoprotein staining, normalized to the total amount of staining within each sample, was determined from spot densitometry data of adjoining bands down the region of staining, and was obtained with an AlphaImager 3300 (Alpha Innotech, San Leandro, CA).

Protein identification

Proteins present in Alcian Blue-staining bands were identified as previously described (Wilson et al. 2002; Grinyer et al. 2004). Briefly, protein bands were excised from the PVDF membrane, digested with trypsin, and the resulting peptide mixture analyzed using an Axima CFR (Kratos, Manchester, UK) for MALDI-TOF-MS and an LCQ DECA (ThermoElectron, San Jose, CA) for MS/MS (LC-ESI-MS/MS). Proteins were then identified by PMF using BioinformatIQ™ (Proteome Systems, Sydney, Australia), or with fragmented peptide ion pattern matching using SEQUEST (ThermoElectron).

Oligosaccharide analysis

O-linked oligosaccharides were released from Alcian Blue-stained protein bands on the PVDF membrane by reductive β-elimination and analyzed by graphitized carbon liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) with an LCQ DECA XP (ThermoElectron) as described previously (Schulz et al. 2002; Karlsson et al. 2004). For each oligosaccharide mass profile, oligosaccharide ions with intensities greater than 10% of the most abundant ion were included in the analysis. This limit included approximately 10–100 different oligosaccharides, dependent on the characteristics of the particular subject and glycoprotein. To provide a measure of oligosaccharide component abundance, the base–peak chromatogram intensity was summed for the entire isotopic distribution of each selected ion. Ions with the same m/z but differing retention times represent different oligosaccharide species and so were summed independently. The monosaccharide composition of each oligosaccharide was determined from MS data using GlycoComp (Proteome Systems), in combination with MS/MS data if required. The intensities obtained for each oligosaccharide ion isomer were normalized within each oligosaccharide mass spectrum, and the weighted average monosaccharide composition was determined. Principal component analysis of monosaccharide composition data was performed using SPLUS® 6.1 (Insightful Corporation, Seattle, WA). For estimation of sulfation and sialylation, reduced and alkylated whole sputum samples were dot-blotted to PVDF membrane and stained with Alcian Blue in either 10% H_2SO_4 (pH 0.1) or 10% acetic acid (pH 1.0). For detailed oligosaccharide structural characterization, released oligosaccharide alditols were separated into acidic and neutral fractions by small-scale anion exchange chromatography and the acidic fractions were desialylated with *Streptococcus pneumoniae* α-(2-3) sialidase (Glyko, Noveto, CA). All fractions were desalted with graphitized carbon prior to analysis by LC-ESI-MS/MS as previously described (Karlsson et al. 2004). Oligosaccharide structural characterization from MS/MS fragmentation data was performed using GlycosidIQ (Proteome Systems) (Joshi et al. 2004), and all assigned structures were manually confirmed.

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Conflict of interest statement

B Schulz holds stock in Proteome Systems, Ltd. Coauthors A Sloane, J Jarry, and R Lindner are employees and stock holders of Proteome Systems, Ltd.

Abbreviations

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; 1D SDS–AgPAGE, one-dimensional sodium dodecyl sulfate-agarose–polyacrylamide gel electrophoresis; FEV_1, forced expiratory volume in 1 s; HRCT, high-resolution computer assisted tomography; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; MALDI-TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; MS/MS, tandem mass spectrometry; PAS, periodic acid Schiffs reagent; PMF, peptide mass fingerprinting.; PVDF, polyvinylidene fluoride.

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