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THE BIOCHEMICAL CHARACTERISATION OF RESPIRATORY MUCUS & MUCINS IN NORMAL, ASTHMA & COPD

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WITH ALL MY LOVE

TO

MOMMY, DADDY,

PETER, CRAIG, NEIL

AND DALE

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Publications and Presentations

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This study was conducted from 2006 to 2008 under the supervision of Professor A.H.S Mall in the Division of Surgical Sciences, University of Cape Town.

As the candidate's supervisor, I have approved this dissertation for submission.

Name: Professor A. Mall

Signed: ______________________

Date: ________________________

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Declaration

I hereby declare that: (1) the above thesis is my own unaided work, both in conception and execution, and that apart from the normal guidance of my supervisor, I have received no assistance apart from that stated below; (2) except as stated below, neither the substance nor any part of the thesis has been submitted in the past, nor is it being, nor is it to be submitted for a degree from the University of Cape Town or any other University.

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Astrid Joan Trimmel

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Abstract

Introduction: Airway mucus hyper-secretion is the main cause of mortality and morbidity in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) and is the leading cause of death in South Africa. Mucus is a viscid, slimy visco-elastic gel-like material, which coats the epithelial tissue of gastrointestinal, reproductive and respiratory tracts. Mucus has defined rheological properties that enable it to be transported out of the lungs by mucociliary clearance. Mucins are high-molecular weight, heavily O-glycosylated glycoproteins that comprise approximately 2% of mucus and cause the mucus to be thick and tenacious. Mucins are divided into three broad categories namely, secreted gel-forming, secreted non-gel forming and membrane bound.

Methods: Sputum samples were collected in 6M GuHCl containing protease inhibitors from patients with asthma and COPD from hospitals around Cape Town and from a group of non-symptomatic volunteers who comprised a control group. Mucins were purified using caesium chloride density gradient ultracentrifugation and identified by western blot analysis using MUC antibodies to specific mucins in the respiratory tract. Ion-exchange chromatography was used to separate the mucins based on their charge and used to detect whether there are different glycoforms present between mucins.

Results: Mucins eluted at a density of 1.39 – 1.40g/ml in a CsCl density gradient ultracentrifugation. Mucins eluted as a broad peak from the Mono Q 5/50GL column. The PAS positive fractions were further examined for the presence of MUC5AC, MUC5B and MUC2. The results showed an inter-individual variation in all three groups. MUC5AC and MUC5B were found in both asthma and COPD. However, MUC2, a mucin that only presents in the disease state was found in asthma and in COPD and in three normal volunteers. The presence of MUC2 in asthma over and above MUC5AC and MUC5B (all three are secreted gel-forming mucins) could change the rheological properties of the mucus gel in the airways resulting in an even more tenacious secretion that aggravates the disease. The fact that MUC2 was found in asthma and COPD and in some normal volunteers shows that these diseases have progressed and are very severe.

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Conclusion: The presence of MUC2, a gel-forming mucin, in airway disease could be an indicator for disease severity for those already suffering from respiratory diseases and an indicator of disease for healthy volunteers that present with MUC2. The presence of MUC2 in only 3/10 normal healthy volunteers, 8/15 asthma and 7/15 COPD samples tested shows there is some inter-individual variation within each group.

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Abbreviations

AMPS	Ammonium persulfate
Bis	NN'-methylenebisacrylamide
BSA	Bovine serum albumin
CHAPS	3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate
COPD	Chronic Obstructive Pulmonary Disease
CsCl	Caesium Chloride
DTT	Dithiothreitol
dH_2O	Distilled water
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FEV1	Forced expiratory volume in one second
Gp	Glycoprotein
H	Hour
HPLC	High Pressure Liquid Chromatography
IAA	Iodoacetamide
Mbar	Milibars
Min	Minute (s)
Ml	Millilitre
MUC	Mucin
MWM	Molecular Weight Marker
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NEM	N-ethylmaleimide
O/N	Overnight
PAS	Periodic acid / Schiff's
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulphonyl fluoride
RT	Room temperature

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SDS	Sodium dodecyl sulphate
SSC	Sodium chloride/tri-sodium citrate buffer
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered Saline Tween
TEMED	N, N, N, N'- tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
V	Volt (s)
VNTR	Variable number of tandem repeats
W/V	Weight per volume
A280	Absorbance at wavelength of 280nm
ϱ	Density

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Glossary

Apomucin	Protein backbone of mucin structure
COPD	Chronic Obstructive Pulmonary Disease
GalNac	Acetylgalactosamine
Glycoform	Differently glycosylated forms of the same polypeptide
MUC	Family of large glycoproteins
Polydispersity	Molecule varies in degree of polymerization can vary in size and molecular weight
Sputum	Expectorated secretions from the respiratory tract
Viscosity	A measure of the resistance to flow, the ratio of shear stress to rate of shear strain.
VNTR	Variable Number of Tandem Repeats

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Chapter 1
Introduction

This study was concerned with the isolation, purification and biochemical characterisation of mucus and mucins found in the respiratory tracts of patients suffering from chronic airway diseases namely, asthma and COPD and of normal healthy volunteers.

The aim was to analyse these respiratory mucins by biochemical methods and to make a comparison between asthma and COPD and then to compare these respiratory mucins with those found in healthy individuals. This is the first study of its kind in South Africa focusing on patients currently attending respiratory clinics around Cape Town, and the first to compare asthma and COPD in a single study. No other respiratory complications were present in our sample group.

The First Chapter gives a brief overview of mucus and mucins found in the respiratory tract and elsewhere in the body. It explains where specific mucins are expressed and the condition in which this occurs.

Chapter 2 explains the materials and methodology used in this study. The methods used were caesium chloride density gradient ultracentrifugation to purify mucins, ion-exchange chromatography to separate mucins based on charge, agarose gel electrophoresis and western blot analysis to identify the mucins.

Chapter 3 to Chapter 7 show the results found in each group, from the isolation and purification steps to the identification of the mucins present in these groups.

Chapter 8 shows the amino acids found in the mucins from the 3 groups under investigation.

In Chapter 9, a conclusion is drawn regarding mucins present in the respiratory tract of healthy and diseased individuals.

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There is an up-regulation of respiratory mucins MUC5AC and MUC5B in diseased states. More interestingly MUC2, present in small amounts in chronic airways has been found in asthma, COPD and also in normal groups.

1.1 Literature review

Airway mucus hypersecretion is the main feature in respiratory disease that leads to morbidity and mortality (Kim 2003; Rogers 2004; Rose and Voynow 2006). The lung uses specialised secretions produced by the airways to provide a renewable and transportable protective layer to interact with, neutralise and remove inhaled toxic materials trapped in mucus (Samet and Cheng 1994).

1.2 The general role of mucus

Mucus is a viscid (viscous), lubricious viscoelastic gel-like material which coats the epithelial surface of the gastrointestinal, reproductive and respiratory tracts of the body and plays a significant role in the vertebrate (as a protective function) and invertebrate (as a lubricant) kingdoms (Reid and Clamp 1978; Sharma et al, 1998). Airway mucus is a tenacious solution with defined rheological and chemical properties that enable it to be transported out of the lungs by muco-ciliary clearance (Samet and Cheng 1994; Lamblin et al, 2001).

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Muco-ciliary clearance is a natural defence mechanism which the immune system uses against inhaled pathogens to keep the lower airways sterile (Lamblin et al, 2001). It involves the removal of excess mucus produced by the goblet cells that line both the upper and lower surface epithelial layer which is covered with cilia that beat rapidly in a co-ordinated fashion, propelling particles trapped in the mucus layer up to the pharynx and out by either swallowing or coughing, see Figure 1-1 (Rubin 2002). Figure 1-1 also shows the removal of mucus from the lungs by coughing, another natural defence mechanism the body uses.

Mucus is an essential component of the protective biofilm covering the mucosal surfaces of the body and has specialized functions depending on the organ (Rose et al, 2001; Rogers 2007). The major types of mucus producing organs are the airway/respiratory, digestive and reproductive systems (Gevers 1987). Mucus protects the airway epithelium from dehydration and inhaled infectious and toxic agents (Rose and Voynow 2006). Mucus consists of a mixture of mainly water (95%), glycoproteins (2%),

protein (1%), lipids (1%) and inorganic salts (1%) (Allen 1984; Rogers 2007; Ke et al, 2008).

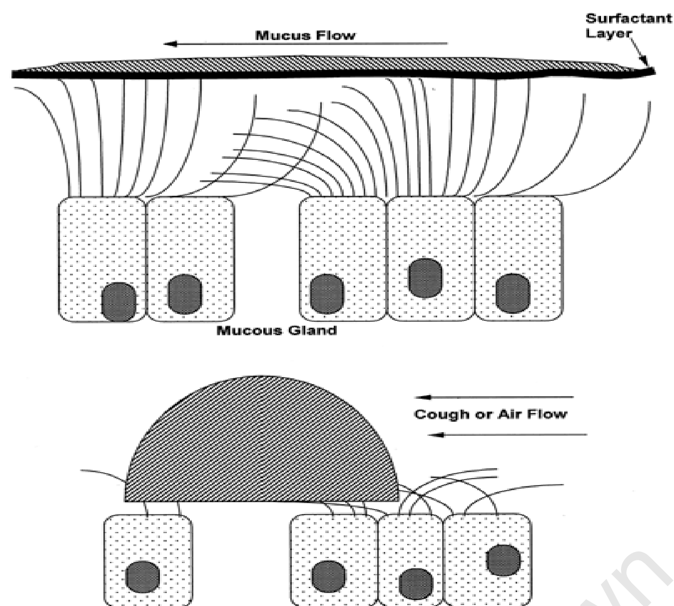

Figure 1-1: Shows the natural removal of mucus by ciliary clearance (top) and cough clearance (bottom) (Rubin 2002).

1.2.1 Mucus of the respiratory system

Airway mucus consists of large oligomeric gel forming glycoproteins (mucins) with varying molecular masses that range between 10 and 40 million Daltons. The polymer matrix of mucus is provided by large mucins, which in the airways, are produced by two different secretory cell populations: the surface epithelial goblet cells and the mucous cells in the submucosal glands (Hovenberg et al, 1996; Groneberg et al, 2002). Secretory cells include serous, mucosal and seromucosal cells of the submucosal glands and goblet cells in the airway surface epithelia (Rose 1992).

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In normal airways, the removal of the excess mucus, the presence of which is caused by the invasion of pathogens to the respiratory tract, is done by either coughing, ciliary motion or lymphatic drainage (Widdicombe 1978). As explained above, ciliary clearance is the removal of mucus by the action of cilia, which is aided by the secretory and membrane-tethered mucins as part of the innate immune defence system. Coughing depends on the viscosity, elasticity and thickness of the mucus. When the cilia are overloaded, the mucus will stimulate the cough receptors to induce coughing (Rose and Voynow 2006). The primary role of mucins in the respiratory tract is that of muco-ciliary clearance (Jany et al, 1991).

In healthy airways there is a low production of mucus that protects the airways efficiently, whereas in the diseased airways there is a high production of mucus (Thornton and Sheehan 2004; Thornton et al, 2007). Mucus protects the respiratory epithelium from dehydration in the normal airways (Kim S 2005). The major respiratory mucins are MUC5AC and MUC5B (Rose et al, 2001).

Mucus increases in viscosity in the diseased airways compared to normal and also differs in the cross linking, size, acidity and appearance (Rogers 2004). These differences lead to the formation of a mucus plug that is present only in the diseased airways. The increased mucus in diseased states leads to mucociliary clearance impairment and inflammation caused by inhaled insults (Kim 1997). Airway mucus hypersecretion is a key pathophysiological feature in many patients with asthma and chronic obstructive pulmonary disease (COPD) (Rogers 2000). Mucus and mucins are overproduced in airways of patients with chronic airway disease. Figure 1-2 shows the production of mucus and the excess mucus produced by goblet cells in the diseased conditions (Rose and Voynow 2006).

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Reduction in muco-ciliary clearance due to over-production of mucus leads to increased susceptibility to lung infections. These two chronic lower airway respiratory diseases, asthma and COPD, lead to lung function failure and ultimately death. In diseased states the glands are enlarged due to mucous cell hyperplasia which can be seen in mucus hypersecretory diseases such as asthma and COPD (Rogers 2004). Airway inflammation is considered to be the primary cause of airway disease such as asthma and COPD (Hargreave 1999). The inflammatory mediators produced by these respiratory diseases differ from each other. In asthma patients, there are more eosinophils produced than in COPD patients, where there are more neutrophils produced.

The common features of airway hypersecretion in these respiratory diseases are sputum production and goblet cell hyperplasia (Figure 1-2).

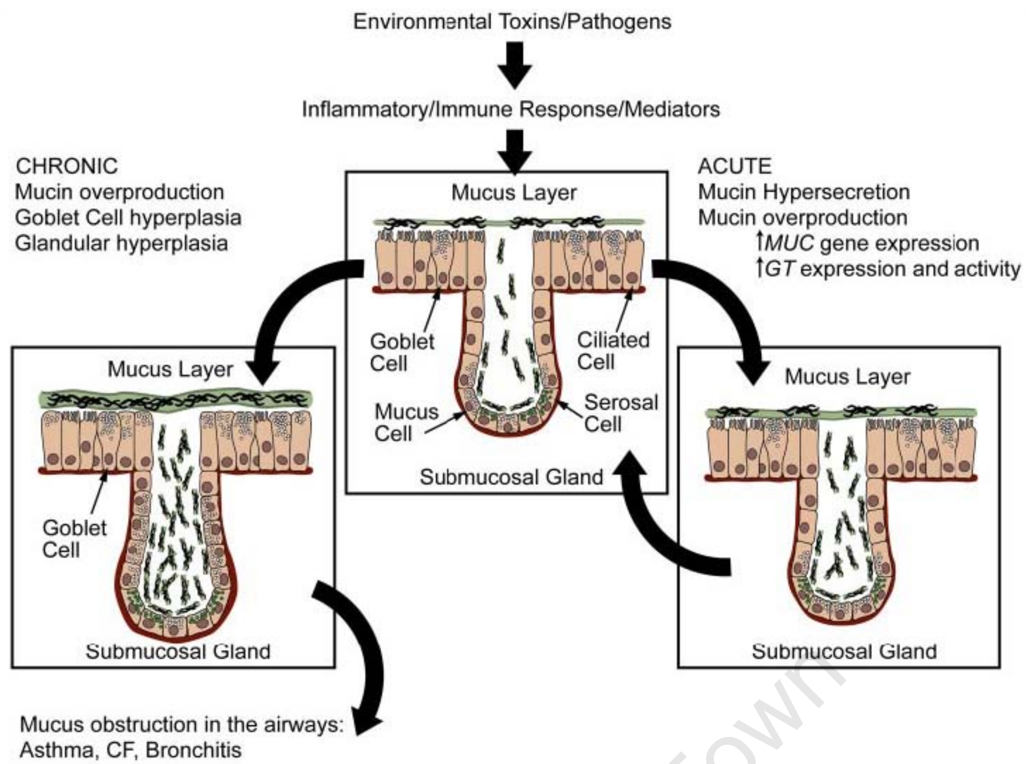

Figure 1-2: A schematic diagram of the airway secretory cells response to acute and chronic challenges (Rose and Voynow 2006).

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In healthy airways the mucus layer is thin and coats the cells to keep them hydrated and in an ideal environment, but this mucus is over-produced in chronic airways as a form of protection. This becomes harmful to the airways because of the excess mucus blocking the free flow of air in and out of the lungs.

1.2.2 Mucus of the gastrointestinal tract

Mucus forms a gel that provides a protective barrier and a stable unstirred layer at the enteric interface between the stomach and the duodenum. It shields the mucosa from strong acids, proteases and injurious action of food or foreign material (Gevers 1987; Taylor et al, 2005). Mucus in the gastrointestinal (GI) tract lubricates the passage of solids, undigested foods and faeces through the gut lumen and protects the tract from mechanical damage resulting from the removal of waste after digestion (Allen 1984). The mucus bicarbonate barrier in the stomach is responsible for the neutralisation of the lumen, allowing protection against acids and also acting as a barrier to pepsin digestion of the underlying gastric epithelia (Bhaskar et al, 1992; Allen and Flemstrom 2005). Mucins found in the GI tract are MUC5AC, MUC6 and MUC2.

1.2.3 Mucus of the reproductive system

Mucus produced from the cervix forms a protective barrier against infection, with a mucus plug at the mouth of the cervix during pregnancy for added protection (Sheehan and Carlstedt 1984; Lagow et al, 1999). The viscosity of this mucus depends on the timing of the menstrual cycle (Van Kooij et al, 1980). During mid-cycle, the mucus is more watery, thin and in strands, to facilitate the movement of sperm to the cervical tract (Argueso et al, 2002). During the rest of the cycle, it is viscous and tends to form a plug at the cervix. The primary functions of these mucins are to (i) aid in fertilization, (ii) prevent infection and (iii) act as a lubricant during sexual intercourse (Elstein 1978; Gevers 1987). There are eight types of mucin found in cervical mucus namely, MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6, MUC8 and MUC9 (Gipson et al, 1997; Lagow et al, 1999).

1.2.4 Mucus of the nasal cavity

Nasal mucus produced from the mucosa serves to protect the respiratory tract from environmental irritants. Together with foreign particles trapped by the mucus, it turns hard and is easily removed. Watery mucus leads to a runny nose and could be the first signs of a cold or infection. The major mucins found in the normal human nasal tissue are MUC5AC produced in the goblet cells in the epithelial surface and MUC5B from the submucosal glandular cells (Groneberg et al, 2003).

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1.3 Mucins (Mucous glycoproteins)

The isolation and characterisation of individual components of mucus are required to understand the relationship between mucin structure and physical properties of mucus (Chace et al, 1985). The substance that gives the mucus its viscous and gel-forming properties, mucins (mucous glycoproteins) are responsible for the rheological properties of mucus. Mucins are the major macromolecular glycoconjugates of mucus, the mucosal layer that protects and lubricates the epithelial lining of the body (Rose 1992; Thornton et al, 2007).

Mucins are comprised of approximately 80% carbohydrate and 20% protein. The carbohydrate is composed of five different monosaccharides namely; N-acetylgalactosamine (GalNac), N-acetylglucosamine (GluNac), galactose, fucose and sialic acid (Allen 1984; Van Klinken et al, 1998). The carbohydrates are important for mucin structure and contribute to the rheological and viscoelastic properties of the mucus secretions (Andrianifahanana et al, 2006; Thornton et al, 2007).

Mucins are high molecular weight glycoproteins, polydisperse in mass (2-4 x 106) and size (0.5-10μm). These linear molecules contain sites of extensive O-glycan attachments (mucin-like domains) (Rose et al, 1984; Thornton et al, 1997; Thornton and Sheehan 2004; Thornton et al, 2007), that are secreted by epithelial cells lining mammalian respiratory, gastrointestinal and reproductive tracts and provide the viscoelasticity required for efficient mucous-cilia interactions (Rogers 2004). Mucins have a protein backbone that contain numerous tandem repeats that are rich in serine and threonine residues which are sites for O-glycosidic linkage of N-acetylgalactosamine to the hydroxyl moieties of serine and threonine (Kim S 2005; Rose and Voynow 2006).

A generic diagram (Figure 1-3) depicts a mucin structure showing the interactions between the carbohydrates and the protein, with the O-glycosylation interaction between the hydroxyl amino acids and GalNac at the gene level. The carbohydrates are attached to the polypeptides by O-linkage and results in a stiffened large molecule that leads to mucins being space filling which aids in their gel making properties (Thornton and Sheehan 2004).

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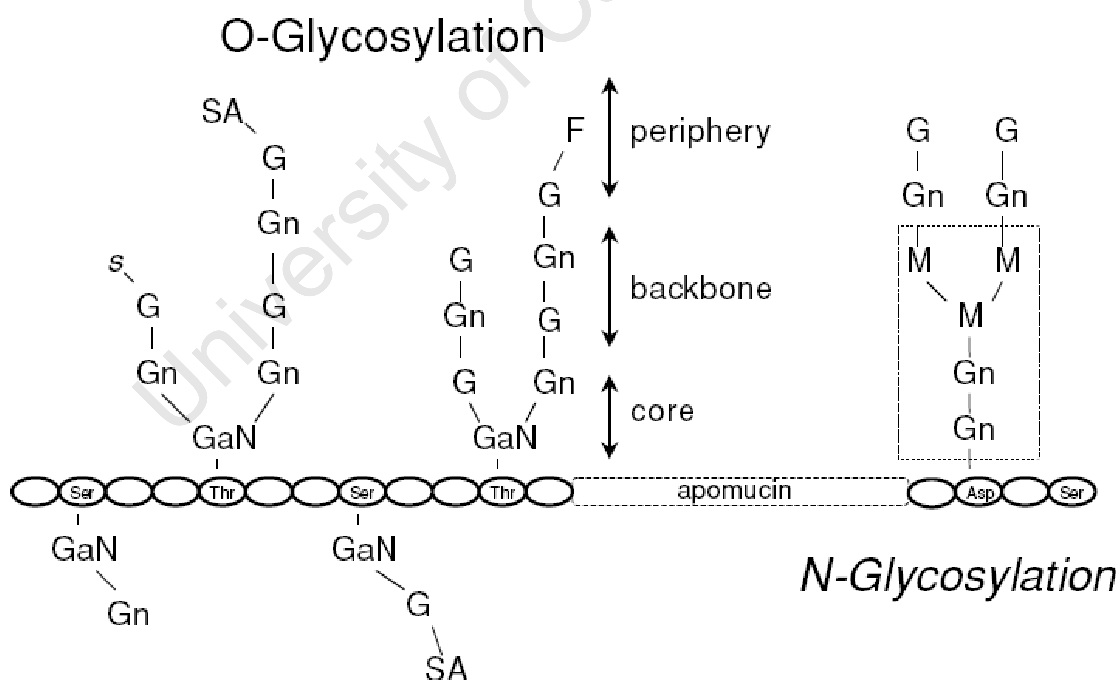

Figure 1-3: Shows a schematic representation of a mucin structure (Lamblin et al, 2001). The carbohydrate chain attachment linked to apomucin by O-glycosylation.

Mucin apoprotein (apomucin) is the primary gene product which is encoded by MUC genes which fall into three broad categories namely: gel-forming secreted mucins such as MUC2, MUC5AC, MUC5B, MUC6 and MUC19; non-gel forming and secreted MUC7, MUC8 and MUC9 and the rest that are membrane bound (Rose 1989; Audie et al, 1993; Thornton et al, 1996). Trans-membrane mucins give cells their polarity. Eight of the 21 mucin genes to date are well characterised. These mucins are organ-specific, for example, the stomach has MUC5AC, MUC6 and the colon has MUC2. Figure 1-4 shows examples of the different types of mucin found in the body.

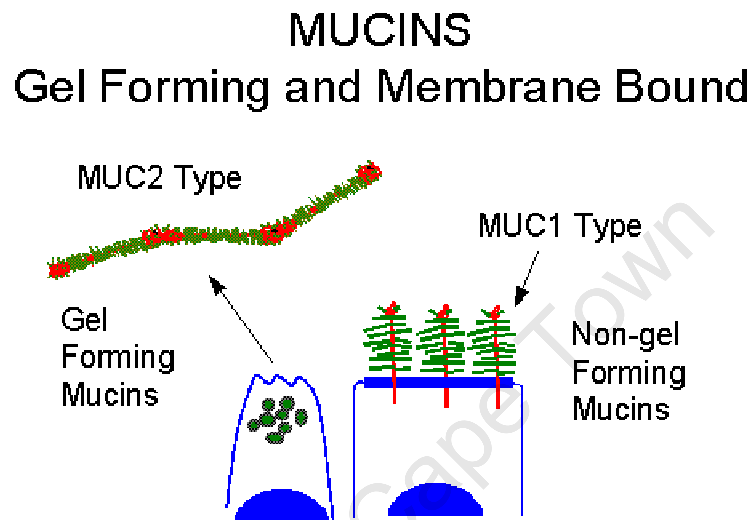

Figure 1-4: Shows secreted gel and membrane bound mucins (www.medkem.gu.se/mucinbiology/mucin.gif- June 2008).

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Lung mucus containing mucins in healthy airways contributes to the mucociliary defence system that protects the lungs against environmental influence (Rose and Voynow 2006). An airway mucin, such as MUC5AC as shown (Figure 1.5), is one of the major core proteins of secreted polymeric mucins found in the goblet cells of the airway surface epithelium (Kaneko et al, 2003; Cohn 2006; Thornton et al, 2007). MUC5B is the other major core protein which is a secreted polymeric mucin, found in the submucosal (tracheobronchial) glands specifically in the mucous glandular cells (Hovenberg et al, 1996; Sharma et al, 1998; Wickstrom et al, 1998; Thornton et al, 2007). A small amount of MUC2, a secreted mucin like MUC5AC but smaller in size, if present, will be detected in very low levels in ‘irritated’ airways (Rogers 2007).

The airways consist of two types of cells namely, goblet cells and the submucosal glands that synthesize mucins (Rose et al, 1984; Kim et al, 1997). Goblet cells found in the surface epithelium, upon interaction with environmental irritants, shows a marked increase of mucus produced for protection. The secreted mucins are a fundamental component of the mucosal barrier (Thornton et al, 2007). The second cell type is the submucosal glands, which consist of mucous and serous cells. Both these cell types produce secreted polymeric mucins.

1.4 Respiratory airway mucins

In the respiratory tract there are a number of cells that produce mucus which are situated in the surface epithelium namely, serous cells found in foetal and not adult airways, mucous cells which are present throughout the airways and Clara cells which are found on the periphery and only in the intrapulmonary regions (Reid and Clamp 1978).

There are six mucins found in airway secretions namely: (i) MUC1, a transmembrane mucin that is localised and tethered to the apical membrane of the surface epithelial cells and to serous glands by its C-terminal subunit. (ii) MUC2, a secreted intestinal mucin expressed in the surface epithelial cells. (iii) MUC4, a cell-associated mucin expressed in both the surface and glandular cells. (iv) MUC7, a secreted salivary mucin expressed solely in the glands. (v) MUC5AC, a large cysteine-rich, gel-forming, secreted mucin found in the surface epithelium layer and (vi) MUC5B, another gel-forming secreted mucin found in the submucosal glands of the respiratory tract (Groneberg et al, 2003). These major respiratory mucins were present in bronchial washes from normal patients and those that have chronic airway disease.

The oligomeric secreted mucin genes found in the respiratory tract of humans (MUC5AC, MUC5B, MUC2 and MUC6) are all clustered on chromosome 11p15.5. (Pigny et al, 1996).

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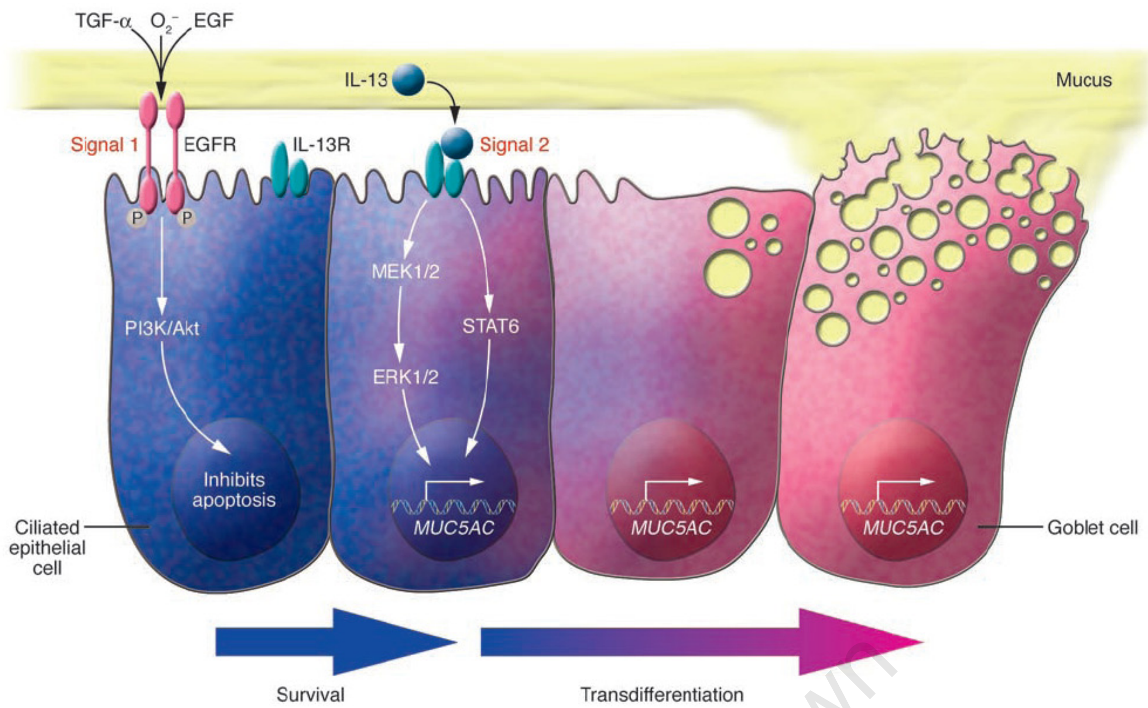

Figure 1-5: Mucus in chronic airway diseases (Cohn 2006).

1.5 Mucus and mucins in asthma

Asthma is a pulmonary disease in which there is an obstruction to the flow of air out of the lungs but unlike other respiratory diseases, this obstruction is reversible. Asthma is a chronic inflammatory airway disease that is characterized by inflammatory cells such as mast cells, eosinophils and T-lymphocytes, bronchial hyper-responsiveness and mucus hypersecretion (Groneberg et al, 2002).

Figure 1-6 represents the stages the lung undergoes when an asthma attack occurs. The airways become narrower during inflammation and there is an over-production of mucus. During a bronchospasm, air is prevented from passing through the trachea into the alveoli, thus blocking the passage of the oxygen to the lungs and preventing the individual from breathing.

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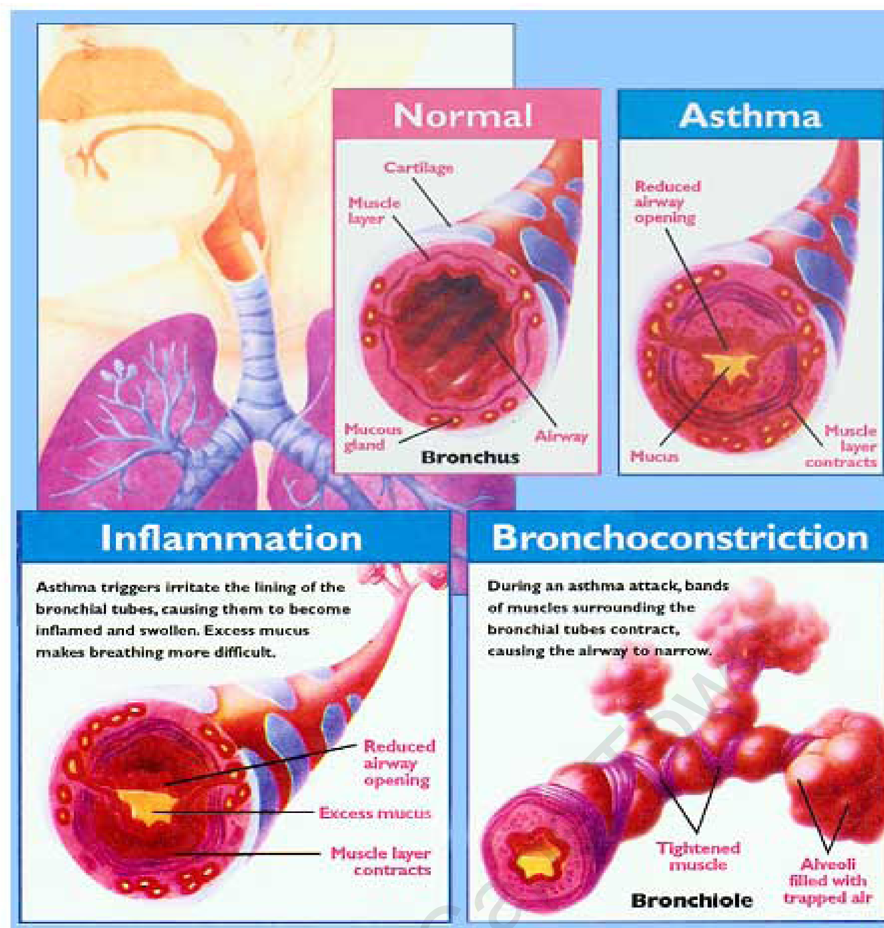

Figure 1-6: A representation of an asthma attack. The normal airway, airway with inflammation, bronchospasm and mucus production. (http://www.network-health.org/tpl/health_464.asp?contid=health_asthma_lungs – February 2009).

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Mucus hypersecretion is a main feature of asthma as seen by the increase in sputum production during an acute exacerbation which is associated with a decrease in FEV1 (Kim S 2005). The mucus in sputum of an asthma patient is very viscous and contains DNA, lactoferrin, eosinophils, cationic protein and plasma protein (Rogers 2004). Other pathological features of asthma are mucous plugging, goblet-cell hyperplasia, smooth muscle hypertrophy, submucosal gland hyperplasia, thick basement membrane and eosinophilic infiltration (inflammation) (Aikawa et al, 1992; Rogers 2004).

1.6 Airway remodelling

These pathological characteristics lead to mucus obstruction with increased luminal mucus which in turn leads to airway remodelling (Rose et al, 2001; Morcillo and Cortijo 2006). There is severe scarring of the epithelial lining that occurs during these attacks by the inhaled agents or inflammatory cells that are recruited to aid the airways in response to injury. The repair process involves epithelial cell migration, proliferation and integrity of the airway epithelium restoration. This damage control that the airways undergo is called airway remodelling.

Airway remodelling is the structural changes of the airways, which are characterised by the thickening of the airway wall, associated with loss of epithelial organisation, goblet-cell hyperplasia, sub-epithelial fibrosis, increased vascularisation, fibroblast and myofibroblast proliferation, and smooth-muscle hyperplasia and/or hypertrophy (Elias et al, 1999; Inman 2004; Bai and Knight 2005). Goblet-cell hyperplasia in the airways leads to different levels of severity of asthma which leads to specific inflammatory and immune response mediators that activate mucin gene regulation and airway remodelling (Rose and Voynow 2006). This abnormality in goblet cells contributes to the pathophysiological changes that occur in the normal function of the mucin stores and production (Rose et al, 2001).

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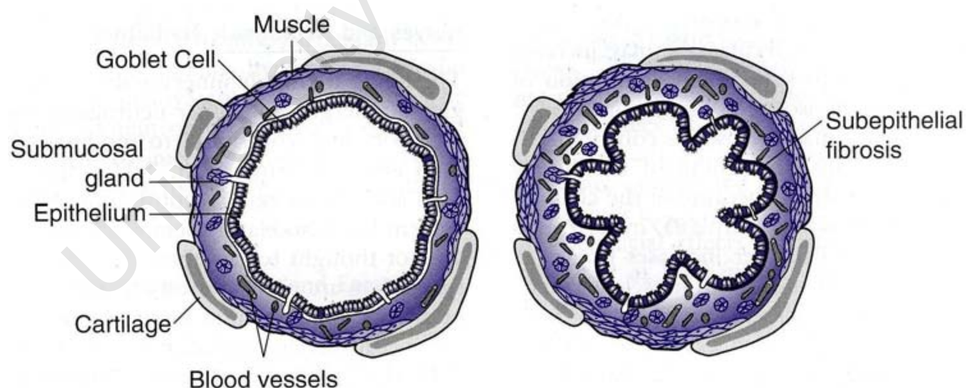

Figure 1-7: Airway remodelling (Kim S 2005).

The main cells that are associated with the changes in the epithelium and submucosa in the respiratory airways of people suffering from asthma are the eosinophils and the Th2-lymphocytes. Neutrophils have been found to be present in sputum of asthma sufferers. Airway remodelling is depicted in Figure 1-7 and Figure 1-8 (Bousquet et al, 2000; Kim S 2005).

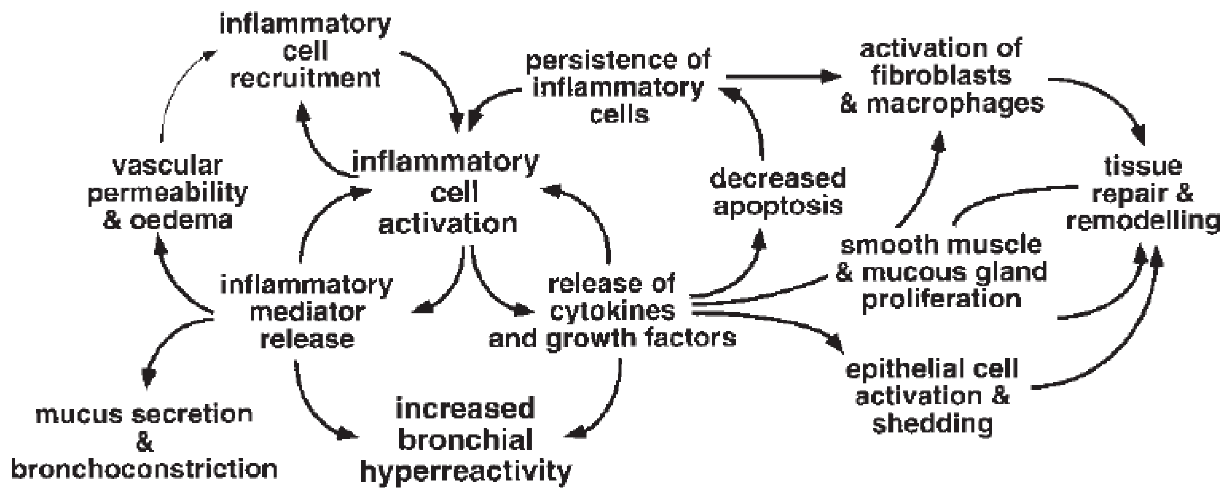

Figure 1-8: Mechanisms of acute and chronic inflammation in asthma and remodelling processes (Bousquet et al, 2000).

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1.7 Mucus and mucins in COPD

Chronic obstructive pulmonary disease (COPD) is one of the most common respiratory diseases in the world (Barnes 2003). COPD is a disorder that persistently obstructs bronchial airflow and consists of chronic bronchitis (hypersecretion of mucus, chronic cough and sputum production), chronic bronchiolitis (small airway disease/chronic inflammation of the peripheral airways) and emphysema (airspace enlargement due to alveolar destruction) (Rogers 2000; Barnes 2003; Kim S 2005; Minai et al, 2008).

It is a heterogeneous syndrome characterised by the relentlessly progressive limitation of airflow, associated with abnormal lung inflammation in response to noxious gases or particles and is poorly reversible (Barnes 2004; Hogg et al, 2004; Minai et al, 2008). The term "Obstructive" means that the person finds it difficult to exhale which leaves stale (deoxygenated) air in the lungs and reduces the amount of new (oxygenated) air the person can inhale with the next breath (Standifer et al, 2006). Figure 1-9 shows the effect of chronic bronchitis and emphysema on the airways in the lung.

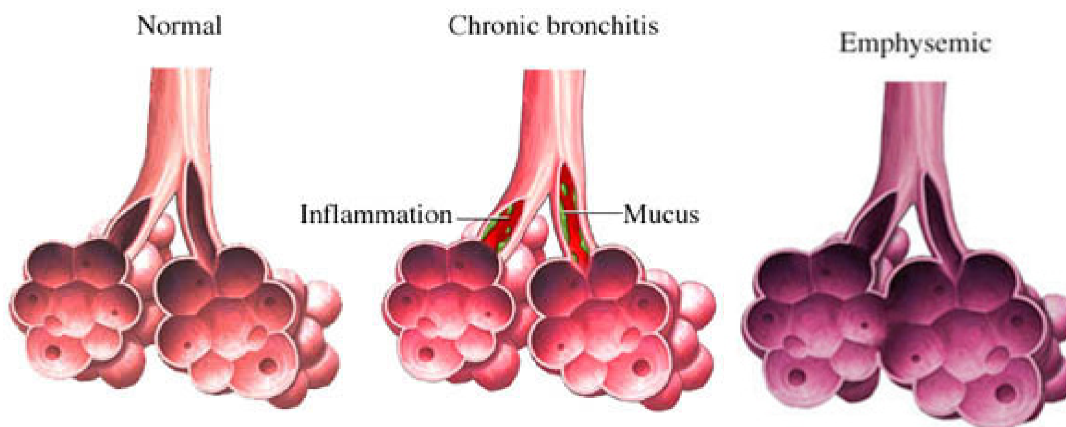

Figure 1-9: The effect of COPD on the airways. The picture depicts the effects of two of the diseases that comprise COPD, on the bronchus and alveoli of the lung. (http://healthguide.howstuffworks.com/COPD - June 2008).

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1.7.1 Pathogenesis of COPD

One of the main causes of COPD is smoke, especially cigarette smoke. The long-term exposure to cigarette smoke leads to an inflammatory response in the lower respiratory tract that is a progressively and poorly reversible airway obstruction and causes small airway remodelling (Hogg et al, 2004; Minai et al, 2008). COPD will reduce life expectancy and cause disabilities much faster if the patient continues to smoke, because the airflow limitation that results from this disease causes fixed narrowing of the small airways, emphysema and luminal obstruction with mucus production (Barnes et al, 2003; Barnes 2004).

There are inflammatory cells and inflammatory mediators that induce the expression and production of respiratory mucins, especially the production of MUC5AC and a small amount of MUC5B (Fabbri et al, 1998; Kim S 2005; Hogg et al, 2007). Other stimuli such as oxidative stress, bacterial exo-products due to infection and pro-inflammatory cytokines namely, TNF-α and IL-1β and the presence of cigarette smoke, all induce an inflammatory response in COPD, mucin hyper-production and mucus hypersecretion in the respiratory tract (Maestrelli et al, 2001; Takeyama et al, 2001; Perrais et al, 2002; Barnes et al, 2003; Dicpinigaitis 2003; Shao et al, 2004).

The symptoms of COPD are the presence of persistently abnormal lung function, cough, sputum production and breathlessness. Progression of COPD is associated with mucus over-production and

accumulation of inflammatory exudates in the lumen, which infiltrates the wall of the trachea by airway remodelling. The obstruction of small airways in COPD is associated with airway wall thickening by means of the remodelling process that aids in the repair of the lung tissue, damaged by the malfunction of mucociliary clearance, the natural and first line of defence (Hogg et al, 2004).

Chronic bronchitis is an inflammatory disease characterised by the presence of a persistent cough with sputum production on most days for at least three months in two consecutive years without any explanation (Samet and Cheng 1994; Jeffery 2000; Turato et al, 2001; Hogg 2008). The key features of chronic bronchitis are the mucus production and inflammation of the bronchial wall (Yoshida and Tuder 2007).

Chronic obstructive bronchiolitis (fibrosis) or small airway obstruction is another pathological hallmark of COPD, and is characterised by inflammation of the peripheral airways and airflow limitations, due to narrowing of and obliterating the lumen that leads to the deterioration and constriction of the tiny airways that provides oxygen to the body (Jeffery 2000; Turato et al, 2001).

Emphysema is a progressive disease which causes permanent lung destruction and airflow obstruction and reduces gaseous exchange with symptoms of dyspnoea (Turato et al, 2001; Barnes 2006; Minai et al, 2008). This disease reduces the quality of life attested by the worsening of pulmonary function, exercise capacity and continuous deterioration of functional status (Minai et al, 2008).

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1.7.2 Mucolytics

The mucolytics serve two functions, namely, (i) to increase the ability to expectorate sputum or (ii) to decrease mucus hypersecretion. They dissolve the mucus by hydrolysing glycosaminoglycans and reducing the viscosity of the mucus, making it thin and easier to cough out (en.wikipedia.org/wiki/Expectorant-July 2008). An example of a mucolytic drug, N-acetylcysteine, is one that is commonly used today. Other drugs that are used are carbocysteine and mecysteine hydrochloride which reduce the viscosity of bronchial secretions by cleaving disulphide bonds cross-linking mucus glycoprotein molecules (Dawson et al, 2002). Corticosteroids, which are commonly used to treat inflammatory diseases such as asthma, are not effective in treating COPD (Barnes et al, 2003; Barnes 2004).

1.8 Asthma and COPD

Asthma and COPD are two diseases that cause narrowing of the airways due to inflammation. The plugging of the airways is caused by the over-production of mucus in response to inflammation.

There are currently two hypotheses about the definition of asthma and COPD namely, the British and the Dutch Hypotheses. The British hypothesis claims that asthma and COPD are two different entities whereas the Dutch hypothesis claims they are different expressions of one disease.

According to the Dutch hypothesis, asthma and COPD are not completely distinct diseases and have common underlying mechanisms leading to the pathology of both these disorders (Van der Pouw Kraan et al, 2002; Bleecker 2004). Some similarities would be expected between these diseases as the lung responds in a limited manner to airway obstruction (Chhabra 2006). The similarities between these two chronic respiratory diseases are that they both cause airflow obstruction, chronic inflammation, mucus production, bronchoconstriction, are involved with the small airways and are consequences of gene-environmental interactions (Dodge et al, 1986; Barnes 2004; Bleecker 2004).

The differences between asthma and COPD for this study depended on the amount and type of mucus produced in response to these diseases. In COPD the mucus (Figure 1-10) is (i) less viscous and without marked plasma exudation, (ii) the ratio of mucin MUC5AC: MUC5B may be reduced, (iii) there is full release of mucin into the airway lumen, rather than tethering of mucus to epithelial cells as in asthma (Rogers 2000) and (iv) the mucus in COPD is more fluid because of the presence of neutrophils which cleave the mucus from the lumen, in contrast to asthma which produces eosinophils which cannot cleave it from the lumen (Rogers 2004). The neutrophils contain proteases that cleave the mucus thus allowing it to flow more easily. MUC5AC is more susceptible to proteolytic degradation than MUC5B as seen, for example, in patients that suffer with cystic fibrosis (Davies et al, 1999).

Another way to determine the differences between asthma and COPD is by looking at the systemic inflammatory markers present in the blood of patients suffering from these diseases. Higashimoto et al, 2008, 2008 showed that these diseases share common markers but certain systemic inflammatory markers may reflect differences in the serum especially when looking at TGF-β1 and α1-AT (Higashimoto et al, 2008; Higashimoto et al, 2008).

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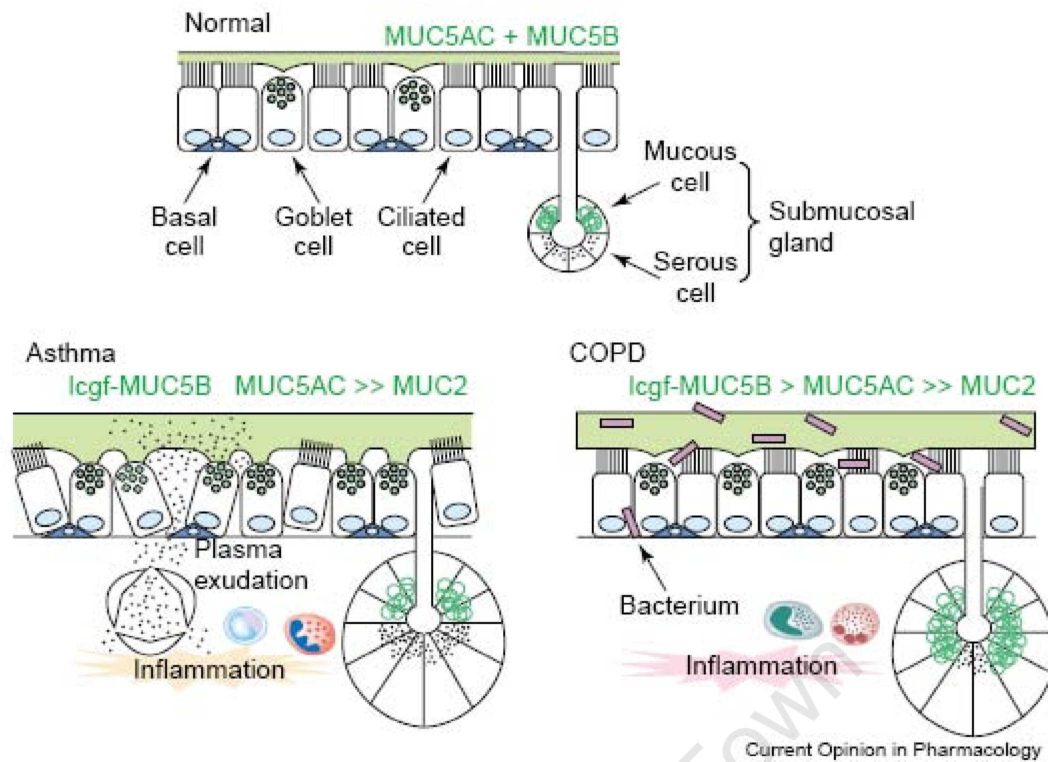

Figure 1-10: A schematic showing the differences between asthma and COPD response to inflammation compared to normal healthy subjects (Rogers 2004).

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The aim of this study was to analyse by biochemical methods mucins found in the respiratory tract of patients suffering from respiratory diseases such as asthma and COPD and to make a comparison, firstly between these diseases and then with mucins found in normal healthy individuals. Attention was focused on the different charge forms of mucins and their association with normal healthy individuals and asthma and COPD patients.

In the case of asthma, patients who die of status asthmaticus have a condition where the mucus plugs the airways causing the patients to have difficulty in exhaling, resulting in asphyxiation. It is believed that MUC5B, a secreted gel-forming airway mucin, has two glycoforms of different charges. These different charge forms (glycoforms) are released from the airways at different times. It is hypothesised that the second glycoform causes the mucus to be so thick that it forms a plug occlusion resulting in death. This is not the case in COPD, where the mucus is very fluid and no mucus plug forms.

A further aim of this study was to look at these diseases simultaneously in a controlled setting, which has not been done before, and to confirm the differences as stated above. This study was conducted on

sputum samples collected from patients who attend the clinics around Cape Town, South Africa, for treatment of the above-mentioned respiratory diseases and not from post mortem samples as previously reported (Sheehan et al, 1995). Evidence from HPLC analysis, agarose gel electrophoresis and Western blot analysis showed that the major respiratory mucins found in people that suffer from asthma and COPD are MUC5AC and MUC5B. The presence of MUC2 found in a few of the diseased samples could be an indicator for disease progression. The up-regulation of MUC5AC was prominent in normal, asthma and COPD as it was seen in greater quantities than the other respiratory mucins. The fact that MUC5B was present in all 3 groups could be an indicator of disease and interestingly, the presence of MUC2 in these groups could be an indicator of disease severity.

Patients with asthma and COPD exhibit characteristics of airway hypersecretion namely, sputum production, luminal mucus, submucosal gland hypertrophy and goblet-cell hyperplasia (Rogers 2001). Our aim was to establish the differences between asthma and COPD by looking at the clinical data and biochemical processes that occur with the onset of inflammation and mucus hypersecretion, to look at these diseases and compare them to see if there was any novel mucin present and if there was any difference compared to normal states. A novel finding was the presence of MUC2 in some of the normal volunteers, which is quite interesting as MUC2 has not previously been found in normal healthy individuals.

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Chapter 2
Materials and Methods

2.1 Ethics

The University of Cape Town Research and Ethics Committee approved this study (Ethics approval number REC REF: 069/2006).

2.2 Materials

Guanidine hydrochloride (GuHCl), agarose (TYPE V), urea, bovine serum albumin, caesium chloride, lithium perchlorate, piperazine and Poly-L-Lysine were obtained from Sigma Company, St Louis, USA. LumiGLO Reserve Western blot kit was purchased from KPL, Whitehead Scientific. Sodium chloride, dialysis tubing, sterile water and nitrobond pure nitrocellulose (0.22μm) membrane were purchased from Argon Laboratory Services, RSA. Beckman International supplied ULTRA CLEAR™ centrifuge tubes (16 X 76mm). HPLC Mono Q™ 5/50 GL column (GE Healthcare, Tricorn) was supplied by Anatech, RSA. Ethylenediaminetetra-acetic acid disodium salt (Na2-EDTA), Tris (Hydroxymethyl) aminomethane, ammonium persulfate, iodoacetamide (IAA), Folins Ciocalteu's phenol reagent, acrylamide, NN'-methylenebisacrylamide (Bis), ammonium persulfate (AMPS), N-ethylmaleimide (NEM), sodium metabisulphite, sodium dodecyl sulphate (SDS), periodic acid and 2-mercaptoethanol were supplied by British Drug House (BDH), Dorset, UK. Sodium carbonate anhydrous, disodium hydrogen orthophosphate anhydrous and sodium dihydrogen orthophosphate were from SAARCHEM (Merck).

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2.3 Sample Collection

Asthma and COPD sputum samples were collected from patients attending the Victoria, GF Jooste and Groote Schuur Hospitals, all in the region of Cape Town. The asthma and COPD patients from the above-mentioned hospitals were either in the Respiratory Ward, Respiratory Outpatient Clinic or in the Casualty Unit. 'Normal' sputum samples were collected from healthy volunteers in the Department of Surgery Research Laboratory at the University of Cape Town, from colleagues, friends and family members. The sputum samples were collected on ice in 50ml tubes containing 5.0ml 6M GuHCl containing proteolytic inhibitors such as EDTA and PMSF.

2.4 Sputum induction for healthy volunteers

Sputum induction, a non-invasive technique was used to collect samples from healthy individuals. The volunteers were nebulised using 3% saline for a total time of 21 minutes with three breaks (7 minutes per induction), using an ultrasonic nebuliser (MED active Clearway 1000, Brescia, Italy). During the breaks, the volunteers had to blow their noses, rinse their mouths with water and try to cough to produce sputum. Most of the volunteers found this process difficult and produced more saliva than sputum (Pin et al, 1992; Bacci et al, 2002; Bathoorn et al, 2007).

2.5 Sputum from respiratory disease patients

A number of patients attending the above-mentioned hospitals were being nebulised for acute exacerbations and voluntarily gave their sputum for research, while the other patients spontaneously produced sputum by coughing only. In most cases, the COPD patients produced more sputum than the asthma patients. The asthma sufferers were exhausted after acute exacerbations and this resulted in low sputum production. Sputum collection was generally difficult with patients being too tired or in some cases too embarrassed to cough due to exacerbations, or patients being discharged before we could have access to them.

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2.6 Solubilisation of sputum

Weighed sputum samples in 6M GuHCl were mixed overnight at 4°C to allow sputum to dissolve in GuHCl. The more viscous sputa samples were homogenised using, a Junkel and Kunkel Ultra-Turrax (40 seconds, 9500min^{-1}, RT), to further disaggregate the sputum and increase its solubility in GuHCl. After sonication, the samples were centrifuged at 800g in a HITACHI, HIMAC centrifuge for 1h at 4°C to remove any cell debris and food particles (Allen 1977). The supernatant was stored at -20ºC until further purification and the pellet was discarded.

2.7 Purification of mucins

Respiratory mucins were purified according to the method described by (Creeth and Denborough 1970). Briefly, mucins were extracted with 4M GuHCl containing protease inhibitors such as 10mM EDTA and 1mM PMSF at pH 6.5, and adjusted to a density of 1.39 to 1.40g/ml with solid caesium chloride (CsCl). The samples were purified by a two-step isopycnic density gradient ultracentrifugation in CsCl, for 48h, twice at 105 000g at 4°C. This technique separates mucins from contaminants, such as lipids,

that fractionate at lower densities to that of mucin (Creeth and Denborough 1970; Starkey et al, 1974) and proteins that fractionate at higher densities to that of nucleic acid (Allen 1984). This technique has been established as the accepted method for the isolation and purification of mucins.

2.8 Analytical Procedures

After centrifugation, aliquots were taken from each of 8 fractions, starting from the top of the tube, for the determination of density by weighing (g/ml), protein content (Lowry et al, 1951) and mucin (glycoprotein) content by PAS method (Mantle and Allen 1978). The fractions that contained the pure mucin as indicated by the PAS positive peaks at a density of 1.39-1.40g/ml were pooled, dialysed against distilled water to remove CsCl and excess salts, lyophilized, weighed and stored at -20ºC.

2.8.1 PAS assay

Mucin detection was performed by the PAS assay and measured at a wavelength of 555nm (Mantle and Allen 1978).

2.8.2 Lowry assay

The amount of crude protein was quantitatively measured and the protein during purification was qualitatively measured using the LOWRY assay at a wavelength of 700nm according to Lowry et al (Lowry et al, 1951).

2.9 Reduction and alkylation

Freeze-dried purified mucin samples were reduced in 6M guanidinium chloride/0.1M Tris, pH 8.0 containing 10mM dithiothreitol (DTT) for 5h at 37°C. After reduction, the sulphydryl groups were carboxymethylated with iodoacetamide (IAA) to prevent re-formation of disulphide bonds. IAA was added to a final concentration of 25mM and the mixture was left overnight at room temperature in the dark (Thornton et al, 1994). After reduction and carboxymethylation, the purified reduced mucins in 6M guanidinium chloride from the asthma and COPD patients were dialyzed against 6M urea for 3 days to remove GuHCl salts and to ensure that the sample was in the correct buffer conditions for separation by anion-exchange HPLC. The samples from the normal group were reduced after purification, dialysed against water for 3 days, freeze-dried to concentrate the mucin sample as the amount of purified normal sputum was very low, and then made up to 10ml with 6M urea.

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2.10 Anion-exchange HPLC

Ion-exchange chromatography was used to separate, identify and quantify compounds based on their different charge properties. Mono Q is a strong anion exchanger with a charged group (O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-$N^+(CH_3)_3$) which aids in the separation of pure reduced mucin through ion-exchange chromatography (Morimoto et al, 1996; Handbooks and Biosciences 2004).

Ion-exchange chromatography is used to separate different species of purified mucins that contain a wide variety of negative charges due to the presence of anionic sialic acid (Allen 1984; Kirkham et al, 2002). MUC5AC is an acidic mucin and is therefore the first to elute using HPLC and a Mono Q 5/50GL column (Kirkham et al, 2002). Figure 2-1 shows the positively charged Mono Q column interacting with the negatively charged ions on the mucin.

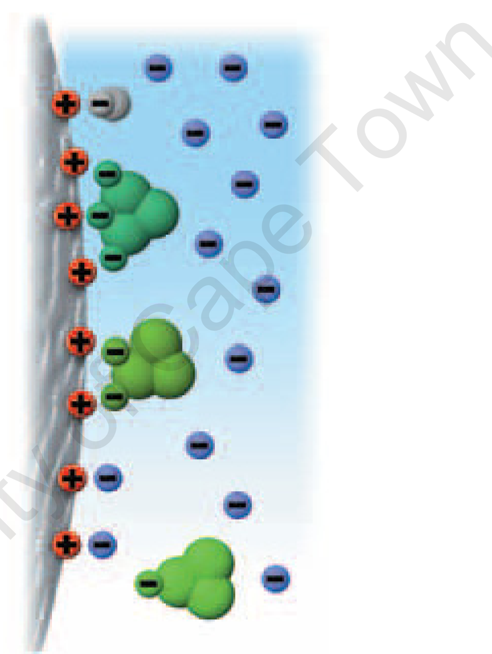

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Figure 2.1: Ion exchange (Handbooks and Biosciences 2004).

The separation of the mucin on the column is based on the solubility between the two liquid phases - the mobile or solvent phase and the stationary or bonded phase. In this case, the start buffer is the mobile phase and the elution buffer is the stationary phase. The 20ml loop and Mono Q 5/50GL column was primed for 45 minutes with the starting buffer of 10mM piperazine, pH 5.0 made up in 6M urea containing 0.05% CHAPS before the sample was injected into the column. Each sample of varying concentration of mucin (as the concentration of the sample depended on the amount of sputum produced by the patient and the amount of mucin recovered after purification) was made up to 10ml

with 6M urea and before each HPLC run, was centrifuged at 800g, at 4ºC for 10 minutes to ensure the mucin was in solution. This 10ml mucin sample was injected into the 20ml loop through the injection valve and the trigger was switched to activate the run. The trigger allowed the sample to move through the loop and onto the column. The mucin samples were then chromatographed on the Mono Q column eluted with 0 to 0.4M lithium perchlorate/10mM piperazine, pH 5.0 in 6M urea containing 0.05% CHAPS for a 60-minute run at a flow rate of 0.5ml/min and at a wavelength of 280nm (UV) to produce a linear gradient. The fractions collected were assayed with the PAS method to confirm the presence of mucin in the peak produced from the HPLC Mono Q column. The PAS assay, modified for the lesser volume (50μl) of the concentrated HPLC fractions was done using an ELISA plate and read on an ELISA plate reader at 540nm. This is the first time this HPLC Mono Q system has been used in our laboratory and is now well established.

2.10.1 Maintenance of Mono Q column

Cleaning of the column is essential as it reduces back-pressure, prevents colour change at the top of the column and increases sample recovery. Before and after each sample run, the column was washed with distilled water at the same flow rate. Regular cleaning entails changing the direction of flow so that contaminants do not flow the entire length of the column. The cleaning procedure of the column was as follows; 20 minutes with 2M NaCl, 40 minutes with 1M NaOH, another 20 minutes with 2M NaCl and finally a water rinse of at least 20 minutes until UV baseline is stable at a wavelength of 280nm and a flow rate of 0.5ml/min at RT.

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2.11 Agarose gel electrophoresis

Agarose gel electrophoresis is a method that aids in the characterisation of mucin, by separating them according to the different charge forms (Holden et al, 1971). Loading buffer containing 1xTAE/ 50% glycerol /bromophenol blue was added to the mucin samples which were applied to the gel. The concentration of each sample loaded onto the gel varied as the amount of sample collected could not be controlled. The amount of positive control however was consistent throughout the study with a concentration of 1mg/ml loaded onto each gel. The negative control used was sample application buffer (SAB) containing no mucin. The mucin samples (crude mucus, purified mucin and HPLC mucin fractions) and the positive and negative controls, in a total of 50μl, were loaded into each of the lanes of a 1.0% (w/v) agarose gel and electrophoresed at 65V and maximum amperes (400mA) for 3 hours in

1xTAE buffer containing 0.1% (w/v) SDS (40mM Tris acetate, 1mM EDTA, pH 8.0, containing 0.1% (w/v) SDS). The positive control used was PMP which is positive for MUC5AC, MUC5B and MUC2. On completion of electrophoresis, the gels were soaked in 0.6M sodium chloride/0.06M sodium citrate (4xSSC) or transfer buffer to remove excess tank buffer (1xTAE) and prepare the gel for vacuum blotting. By soaking the gel in 4xSSC, the gel is prevented from swelling during the vacuum blotting procedure. Variable amounts of sputa were collected and therefore this study depended on volume rather than quantity which was shown not to matter by Henke et al as volume equivalents could be used (Henke et al, 2004).

2.12 Western blot analysis

Western blot or immunoblot is a method used to identify and detect the presence of specific proteins in a sample. Mucins in the respiratory tract were identified and detected with the aid of antibodies specific to the lung (Rose and Voynow 2006). Nitrocellulose membranes with the pore size of 0.2μm were soaked in tank buffer until the membrane changed colour (white to colourless) or until the membrane was wet (approximately 5 minutes) (Thornton et al, 1989). The membrane was then placed on the vacuum blot apparatus and before applying the gel, 4xSSC containing 10% poly-L-lysine was vacuum blotted onto the membrane to ensure that the protein from the gel binds to the membrane via electrostatic interactions (Thornton et al, 1989). The gel was carefully placed on the membrane, ensuring that there were no air bubbles between the gel and the membrane as this would interfere with the transfer of protein and disrupt the western blot analysis. The gel was vacuum blotted for 2h at 40mbars and kept wet with 4xSSC to ensure complete transfer of proteins. After vacuum blotting the nitrocellulose membrane was washed with distilled water for 5 minutes and then incubated in blocking buffer (1% fat free milk powder in TBST) for 1h at RT, on the shaker. The blocking buffer was removed and the membrane was incubated in primary antibody for 2h at RT on the shaker or overnight at 4ºC. All primary and secondary antibodies were diluted in TBST. The membrane was washed 3 times for 5 minutes with 1xTBST (washing solution) to ensure removal of excess unbound primary antibody and then incubated with secondary antibody for 1h at RT on a shaker. The excess unbound secondary antibody was removed with three 10-minute washes of 1xTBST to ensure the membrane is free of any endogenous proteins. The membrane was stored in distilled water ready for visualisation and detection by chemiluminescence.

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In this study, antibodies to the major respiratory mucins found in the lung were used namely, MUC5AC (Hovenberg et al, 1996) and MUC5B (Thornton et al, 1994; Wickstrom et al, 1998). The Thornton group in Manchester kindly donated the MUC5AC and MUC5B antibodies. MUC5AC and MUC5B polyclonal rabbit antibodies were raised against a synthetic peptide that recognises specific sequences as seen in Table 2-1 under antigen recognition. The MUC2 polyclonal antibody that recognises the non-tandem region of the C-terminus was kindly donated by Professor D. Swallow, Department of Biology, University College London (Herrmann et al, 1999).

This mucin gene was found to have low levels of expression in the respiratory tract (Herrmann et al, 1999; Thornton et al, 2007). The secondary antibody a polyclonal goat anti-rabbit immunoglobulin/ HRP was purchased from DakoCytomation.

Table 2-1: Primary and secondary antibodies used in western blot analysis.

Primary antibodies		
Antibody type	Antigen	Dilutions
Polyclonal rabbit anti-MUC5AC/LUM5-1	A synthetic peptide RNQDQGPKFMC (Hovenberg et al, 1996)	1:10 000 (2h at RT or O/N at 4ºC)
Polyclonal rabbit anti-MUC5B/LUM5B-2	RNREQUGKFKMC, NH2 terminus of the MUC5B apomucin (Wickstrom et al, 1998)	1: 2000 (2h at RT or O/N at 4ºC)
Polyclonal rabbit anti-MUC2/ LUM2-3	NGLQPVRVEDPDGC, non-tandem repeat region of the C-terminus (Herrmann et al, 1999)	1: 2000 (2h at RT or O/N at 4ºC)
Secondary antibodies		
polyclonal goat anti-rabbit immunoglobulin/HRP		1: 20000 (1h at RT)

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2.12.1 Chemiluminescence

Enhanced chemiluminescence detection (ECL) was used to visualise the immunoreactivity between the antibodies and the mucin. ECL is a good detection method as it can detect femtomole quantities of mucin. After completion of the western blot analysis, the membrane was stored in distilled water until detection with chemiluminescence and visualisation by autoradiography using an X-ray machine or a chemi-imager. Chemi-imaging was done in the Department of Pathology, University of Cape Town, South Africa.

2.12.2 Membrane stripping

Once detection with the first primary antibody was complete, the membranes were incubated with stripping buffer (2% SDS, 100mM mercaptoethanol, 0.5M NaCl and 62.5mM Tris-HCl buffer at pH 6.7) for 1hour at 37ºC. The membranes were then washed twice for 10 minutes with distilled water and then incubated in 1xTBST for a further 10 minutes. The membranes were washed for another 10 minutes with distilled water to ensure that they were free of stripping buffer. Once the membranes were clean, they were incubated in blocking buffer for 1 hour on the shaker and then probed for the next primary antibody of interest (Henke et al, 2004; Femmeauburn 2005). Stripping was a cost-effective and time-saving method that was previously conducted in our laboratory by Govender, 2006 and worked effectively (Govender 2006).

2.13 Amino acid analysis

The amino acid content of purified respiratory mucins was analysed using a high pressure liquid chromatography (HPLC) system according to the methods of Klapper (1982). In this method samples were vacuum-dried and placed in a hydrolysis vessel containing constant boiling HCl and 1% (v/v) phenol before hydrolysing in the gas phase at 110ºC for 24h. The vessel was purged with nitrogen gas and sealed under vacuum. The vials were cooled and vacuum dried to remove the residual HCl. The dried samples were solubilised in citrate buffer pH 2.2 and injected into a HPLC column from Waters Associates, Medford, MA., filled with cation exchange resin (sulfonated polystyrene cross-linked with divinylbenzene) and eluted with a number of buffers ranging from a low (0.25M trisodium citrate, pH 3.05) to high (0.25M sodium nitrate, pH 9.5) pH. Detection of amino acids was performed using post column derivatization with O-phthalaldehyde (OPA), a fluorescent reagent that interacts with all the amino acids except proline. For proline detection, treatment of samples with sodium hypochlorite ahead of post column derivatization with OPA was required (Klapper 1982). The relative ratios of the individual amino acids for each sample were determined and compared to each other. Jeronimo Rodriquez at the Department of Cellular and Molecular Biology at UCT conducted the amino acid analysis.

University of Cape Town

Chapter 3
Patients' clinical details and sample collection for further biochemical analysis

3.1 Introduction

The biochemical characterisation of mucus and mucins in respiratory diseases namely, asthma and COPD, were analysed using anion-exchange chromatography and western blot detection methods. Mucins were isolated from the sputum samples collected in 6M GuHCl containing proteolytic inhibitors (EDTA and PMSF) using isopycnic density gradient centrifugation in CsCl, reduced with10mM DTT, and subsequently alkylated with 25mM IAA. Reduction and alkylation exposes the mucin epitopes for detection by Western blot analysis (Thornton et al, 1995). This work has been approved by the UCT Human Ethics Committee (see Chapter 2 for REC/REF number).

Table 3-1, 3-2 and 3-3 show quantitatively the amount of sputum collected in 6M GuHCl from the healthy volunteers and the patients suffering from respiratory diseases namely, asthma and COPD before purification and the amount of dry-weight mucin extracted from the sputum after purification. There were 10 healthy volunteers (N), 15 asthma and 15 COPD samples researched in this study.

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3.2 Data of sputum from healthy individuals

Sputum induction was conducted using 3% saline to aid in the expectoration of sputum from the lower airways of healthy volunteers (Bacci et al, 2002). This method was effective but required the participation of the volunteers. The amount of sputum collected varied from individual to individual as some had their airways irritated easier than others. Table 3-1 shows the varying amounts of sputum collected. This influenced the amount of purified mucin obtained for my investigations.

Table 3-1: Sputum from Healthy Individuals.

Volunteer	Gender & Age	Crude Sputum Wet Weight (g)	Total Protein (mg/ml)	Purified dry Mucin Mass (mg)	mg Mucin/mg Wet Weight
N1	F 55	20.0616	11.03	8.2	0.41
N3	F 33	7.9945	9.30	4.5	0.56
N4	F 30	12.8532	11.30	3.9	0.30
N5	F 24	3.7230	3.50	2.7	0.73
N6	F 26	3.0166	3.10	2.3	0.76
N7	M 61	2.1523	5.90	8.6	3.99
N8	M 27	8.5810	10.50	1.4	0.61
N9	M 21	6.2260	3.96	10.8	1.73
N12	M 28	1.4609	2.01	20.8	14.23
N14	M 38	1.8820	2.40	0.8	0.43

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To date there is only one volunteer who currently smokes (N9), two ex-smokers (N7 who last smoked 30 years ago and N12 who last smoked 10 years ago) and the remaining volunteers who have never smoked. There are an equal number of males and females and the ages range from 21 to 61years. Interestingly the mucin concentration of the current smoker (N9) and of the ex-smoker (N12) is higher than the mucin concentration found in the majority of non-smokers in this healthy volunteers (N) group.

3.3 Data of sputum samples from asthma patients

Sputum was collected from asthma patients being treated (nebulised) for an acute asthma attack.

Table 3-2: Sputum from respiratory disease patients suffering from asthma.

Volunteer	Hospital	Gender & Age	Crude Sputum Wet Weight (g)	Total Protein (mg/ml)	Purified dry Mucin Mass (mg)	mg Mucin/mg Wet Weight
37	Victoria	F 34	1.7405	0.199	1.16	0.67
38	Victoria	M 57	1.2173	0.192	1.24	1.02
39	Victoria	M 44	2.5398	0.327	7.24	28.50
40	Victoria	M 51	2.3477	0.185	1.23	0.52
41	Victoria	F 47	7.1372	0.283	6.32	0.88
42	Victoria	F 74	2.4464	0.192	1.08	0.44
43	Victoria	F 50	3.6559	0.204	1.14	0.31
44	Victoria	M 40	1.4564	0.199	0.80	0.55
45	Victoria	M 72	13.429	0.386	19.72	1.47
46	Victoria	F 51	0.7483	0.219	1.25	1.67
47	Victoria	M 73	0	0.189	0.80	0
52	GSH	F 55	2.8504	0.259	4.90	1.42
54	GSH	M 50	1.0401	0.705	11.51	11.07
56	GSH	F 73	4.9952	0.200	5.80	1.16
57	GSH	M 62	3.3718	0.460	3.52	1.04

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There were adults only in this group. The ages ranged from 34 to 74 years. The majority of patients were from Victoria Hospital and there were more females than males in this group.

There was only one confirmed smoker in this group, patient (A42) and the rest are unknown.

GSH = Groote Schuur Hospital

3.4 Data of sputum samples from COPD patients

Sputum was collected from COPD patients being treated (nebulised) for an acute respiratory attack.

Table 3-3: Sputum from respiratory disease patients suffering from COPD.

Volunteer	Hospital	Gender & Age	Crude Sputum Wet Weight (g)	Total Protein (mg/ml)	Purified Dry Mucin Mass (mg)	mg Mucin/ mg Wet Weight
8	Victoria	M 54	5.3683	N/A	N/A	N/A
27	Victoria	M 56	14.277	0.448	0.05	0.003
28	Victoria	F 43	1.2363	0.216	2.31	1.72
29	Victoria	M 37	1.0433	0.271	1.52	1.46
31	Jooste	M 75	2.8474	0.443	1.04	0.36
36	Jooste	M 74	0.2795	0.207	1.94	6.95
48	GSH	F 44	9.7970	0.355	25.90	2.64
50	GSH	M 52	10.542	0.428	9.30	0.88
51	GSH	M 58	2.2878	0.173	6.50	2.84
53	GSH	F 59	4.8816	0.123	3.60	0.74
55	GSH	F 34	3.3825	0.109	6.60	1.95
58	GSH	M 82	1.1830	0.114	3.90	3.30
59	GSH	F 35	6.2107	0.060	1.00	0.16
61	GSH	M 87	1.0173	4.000	2.10	2.06
62	GSH	M 63	3.6006	7.400	7.80	2.17

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There were adults only in this group. The ages ranged from 34 to 87 years. The majority of patients were from GSH in Observatory and there were more males than females in this group. There were only 7 confirmed smokers in this group, patients' (C29, C36, C48, C51, C55, C61 and C62), and 1 confirmed non-smoker (C58) and the rest are unknown.

3.5 Clinical Data of patients with disease

Clinical data for patients with asthma and COPD was obtained from second tier hospitals Victoria and G.F. Jooste and GSH a tertiary hospital and teaching institution. These three hospitals serve patients from a huge community of people who come from poor socio-economic backgrounds and who live in sandy low-lying areas commonly known as the "Cape Flats". The predominant group living on the "Cape Flats" was previously classified as "Cape Coloured" because of their ethnic ancestry (African and Asian). The sample for this study was drawn from South African adult males and females.

3.5.1 Methods of collecting sputum from patients with disease

Visits and telephone calls were made to these hospitals to find patients, suffering from asthma and COPD, that had been admitted due to an acute respiratory attack or respiratory distress. The attending physician made the medical diagnosis of either asthma or COPD. The patients were asked to read and sign a consent form, shown in the appendix A and B. The procedure for producing sputum was explained to the patients who were asked to cough to try to produce sputum from the lungs.

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3.5.2 Complicating factors relating to sputum collection

Whilst the majority of patients expectorated unaided to provide us with sputum, a certain number of patients and most of the volunteers had to undergo sputum inductions in order to produce some sample. Many patients tried to co-operate but due to the fact that they were not feeling well and/or were being discharged refused to cough again. This resulted in small volumes of sample.

3.5.3 Reasons for patient admission

The patients suffering from respiratory diseases on admission to the hospital, presented with a productive cough, wheezing, chest pain or acute exacerbations. Their sputum colour changed from clear to either white, yellow or in some cases green, which are indicators of chest infection. The patients' folders and the attending physicians provided us with this information (personal communication with the attending physicians).

3.5.4 Information of patients with disease

The following information was retrieved from the patients' folders. Most patients were on the same medication namely, prednisone, asthavent and budeflam. The information retrieved from the patients'

folders was limited and we could only confirm that there were 7 smokers and 1 non-smoker in the COPD group and 1 smoker in the asthma group. The information regarding the other patients is unknown. Besides suffering from asthma and COPD, these patients had other ailments such as hypertension and dyspnoea. Three patients died during this study and one patient's disease progressed from asthma to COPD.

3.6 Discussion

The macroscopic observation of the crude sputa collected from the patients with asthma and COPD appears to be infected due to the difference in the colour (yellow and green) as compared to the sputa collected from the healthy volunteers (N) that remained clear. More purified mucin was extracted from the sputa collected from the asthma and COPD patients than from the normal volunteers which could be due to the infected crude sputa produced. According to the literature there is an up-regulation of mucins in diseased airways as compared to healthy airways (Thornton et al, 2007) as was observed in this study. The mucin concentration was greater in the COPD group compared to the asthma and normal groups. The normal group had the least mucin concentration as mucins are not up-regulated in healthy individuals (Rogers 2000).

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Chapter 4
Extraction, purification and isolation of mucins from human airways

4.1 Introduction

Sputum samples were collected from healthy volunteers (no known respiratory disease) and patients suffering from respiratory diseases namely, asthma and COPD. Sputum containing DNA, lipids, proteins and mucin, was suspended in 6M GuHCl containing proteolytic inhibitors (EDTA and PMSF). Isopycnic density gradient centrifugation was used to isolate mucins (Carlstedt et al, 1983). The mucins were extracted from the soluble sputa by a two-step caesium chloride isopycnic density gradient centrifugation at a buoyant density of between 1.39 - 1.40g/ml to remove all contaminants (DNA, lipids and proteins) and isolate the mucins. After two successive equilibrium density gradient centrifugations, each for 48h at 105 000g in a Beckman ultracentrifuge, mucins were collected by fractionation of the CsCl density gradient, from the top of the tube. The density of each fraction was measured, the PAS assay method was used to detect glycoproteins (Mantle and Allen 1978) and the Lowry method for the detection of proteins (Lowry et al, 1951). The PAS positive fractions containing the glycoproteins were pooled and dialysed overnight against distilled water to remove CsCl and GuHCl. After dialysis, the pure mucin was lyophilised and the dry weight of mucin (w/w) was calculated.

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Anion-exchange chromatography is an analytical technique that separates molecules based on charge. Purified mucins, reduced with DTT and carboxymethylated with IAA, were chromatographed on a Mono Q 5/50GL column eluted with a linear gradient of 0–0.4M lithium perchlorate, 10mM piperazine, pH 5.0, in 6M Urea containing 0.05% 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (elution buffer) (Kirkham et al, 2002).

The Mono Q column is positively charged and binds to the negatively charged mucins, which are eluted from the column with lithium perchlorate in the elution buffer. Purified reduced mucins from normal, asthma and COPD samples were separated by anion-exchange HPLC on a Mono Q column and fractionated into 15ml Kimble tubes at 0.5ml/min.

4.2 Results

4.2.1 CsCl centrifugation

Figure 4.1 illustrates the PAS, protein and density profile of the respiratory mucins in normal, asthma and COPD patients after the first and second CsCl centrifugation steps. Absorbance measurements at 700nm suggested the presence of trace amounts of protein under the mucin peak (Figure 4.1) and therefore the mucin containing peaks (Figure 4.1, fractions 3, 4 and 5, left panel) were pooled and subjected to a second CsCl centrifugation to separate the mucins (1.39-1.40g/ml) from the protein. Figure 4.1, right panel illustrates that the mucins are free of protein contaminants after the second centrifugation step. The PAS peak at a density of (1.39-1.40g/ml) showed the mucins to be pure (Figure 4.1, fractions 4-6, right panel).

4.2.2 HPLC analysis

Purified reduced respiratory mucin samples were made up in 10 ml of 6M urea and loaded onto the Mono Q column which separates mucins based on charge. Figure 4.2 (left panel) shows the mucins from normal, asthma and COPD patients eluting as a broad peak from the Mono Q column. Aliquots of these fractions were analysed for the presence of mucin using the PAS assay (Mantle and Allen 1978; Kirkham et al, 2002) and the PAS positive fractions were further analysed to identify the presence of the respiratory mucins. Figure 4.2, left panel is an HPLC profile and Figure 4.2, right panel is a PAS profile of the eluted fractions from the HPLC column of all 3 groups. The PAS profile (Figure 4.2, right panel) shows the presence of mucins in all 3 groups. The PAS positive fractions were further analysed by Western blot technique for the presence of MUC5AC, MUC5B and MUC2.

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4.3 Discussion

4.3.1 CsCl centrifugation purification

Mucus was solubilised in a mixture of 6M GuHCl containing proteolytic inhibitors (EDTA and PMSF) in order to minimize enzymatic degradation through endogenous proteolysis during the course of the experiments (Sheehan and Carlstedt 1984). GuHCl is a chaotropic agent that prevents mucin association by disrupting the structure of water and suppressing ionic and hydrogen-bonding interactions (Sheehan and Carlstedt 1984; Bromberg and Barr 2000). It also removes protein contamination, denatures proteins and suppresses the activity of the degradative enzymes (Carlstedt et al, 1983). The proteolytic inhibitors included EDTA, which inhibits metalloproteinases and PMSF, which inhibits serine proteases

(Carlstedt et al, 1983; Sheehan and Carlstedt 1984).

Isolation and purification by isopycnic density centrifugation in CsCl is a known method to successfully separate mucins from lower density lipids and proteins and higher density nucleic acids (Creeth and Denborough 1970; Creeth et al, 1977; Carlstedt et al, 1983). CsCl density gradients isolate the mucin at a density of 1.4g/ml and are compatible with the collection mixture of GuHCl and proteolytic inhibitors. CsCl dissociates non-covalently bound protein-glycoprotein interactions (Starkey et al, 1974). This method is important as it aids in the understanding of the structure-function relationship of mucins (Woodward et al, 1982) and is the accepted method to use when analysing mucins (Allen 1981). CsCl purification is a laborious practice that involves lengthy intermediate steps which require large quantities of reagents and involves two and sometimes even three ultracentrifugation steps for 48hr each. All samples were purified by the method of density gradient centrifugation in CsCl to extract and analyse the respiratory mucins. The removal of protein contaminants after CsCl density gradient ultracentrifugation was evident. SDS-PAGE analysis of purified mucin was conducted to test the purity of the mucin and to ensure the absence of protein contamination with Brilliant Coomassie Blue staining (not shown).

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Mucin samples after purification were dialysed, lyophilised and then reduced with 10mM DTT and subsequently alkylated with 25mM IAA. DTT is a reducing agent that breaks intermolecular and intramolecular disulphide bonds between cysteine residues of the glycoprotein, promotes unfolding of the peptide chain and aids mucins in forming subunits (Holden et al, 1971; Rose et al, 1979; Carlstedt et al, 1983). Iodoacetamide (IAA) is an alkylating agent that prevents mucin aggregation and keeps the molecule in a subunit form by binding covalently with cysteine, so the protein cannot aggregate to its natural polymeric form (Rose et al, 1979). Reduction and alkylation was carried out on the purified mucin samples to generate mucin subunits (Holden et al, 1971; Pearson et al, 1981; Thornton et al, 1995). Reduced mucin subunits, smaller in size and less viscous can be injected into the HPLC column for detection and analyses. Bronchial mucus needs to be reduced as it is very viscous and forms gels very easily (Houdret et al, 1981). Reduction is also important in antibody detection as it exposes the mucin epitopes for detection and interaction between mucin and antibody during western blot analysis (Thornton et al, 1995). These biochemical methods are important in the investigation of mucins found in the respiratory tract.

4.3.2 HPLC analysis

Mono Q is a strong anion exchanger with a positively charged group that binds easily to the negatively charged mucins that are eluted with lithium perchlorate ($LiClO_4$), which is a positively charged salt. The column and the salt compete to bind the mucin aiding in the elution of the mucin from the column. The purified reduced respiratory mucins from normal, asthma and COPD samples were solubilised in 6M urea and then subjected to ion-exchange chromatography (HPLC) using a Mono Q 5/50GL column. The mucins were eluted from the Mono Q column with a linear gradient of 0-0.4M lithium perchlorate (Thornton et al, 1996) for 60 minutes (Davies et al, 1996), (Dr Sara Kirkham from the University of Manchester, personal communication). We adapted the technique described by Thornton et al (1996) with some modifications. The modifications were the size of the loop (20ml), the extra tubing attached from the detector to the fraction collector and the time the fractions were collected (96 minutes).

We loaded 10ml of purified reduced mucin sample onto the Mono Q column through a 20 ml loop. The amount of sample loaded onto the column depended on the amount of sputum collected from the patients and volunteers. The concentrations ranged from approximately 0.01mg/ml to 20mg/ml (Chapter 3 Tables 3.1, 3.2 and 3.3). The mucin fractions (0.5ml/min) eluted as seen on the HPLC profile (Figure 4.2, left panel). The PAS profile (Figure 4.2, right panel) confirms the presence of mucin as indicated by the peak. This HPLC profile was different to that of Thornton et al (1996) very likely due to the modified method used in this study. The mucin was chromatographed for 96 minutes. A 96 well plate was used for the detection of the mucin in the fractions eluted from the Mono Q column using the PAS assay, and the absorbance was read at 540nm in an ELISA plate reader.

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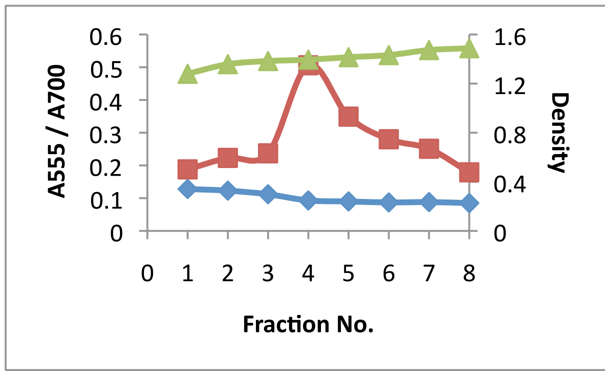
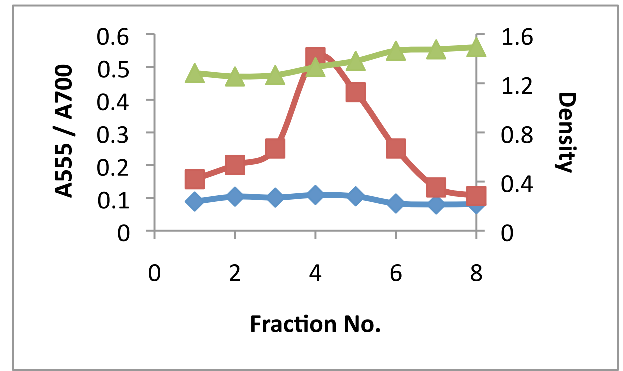
A limitation of this study was that the HPLC technique was not available in this laboratory and had to be established from the beginning with much troubleshooting, which was time-consuming and very costly. We were able to set up this modified HPLC system through personal communication with Dr Sara Kirkham from the University of Manchester.

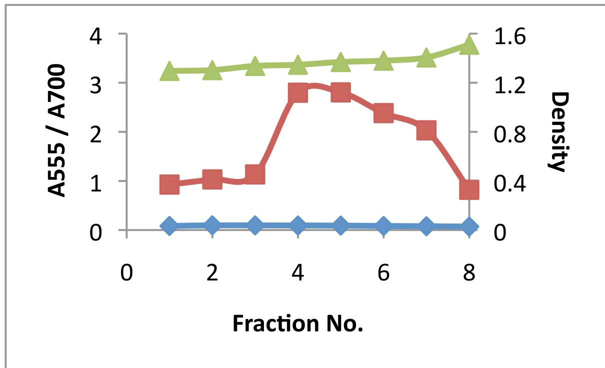
Once the mucins from the normal, asthma and COPD samples were extracted, purified and isolated by CsCl density gradient ultracentrifugation and HPLC Mono Q column analysis, the purified reduced mucins were ready for identification by agarose gel electrophoresis and Western blotting.

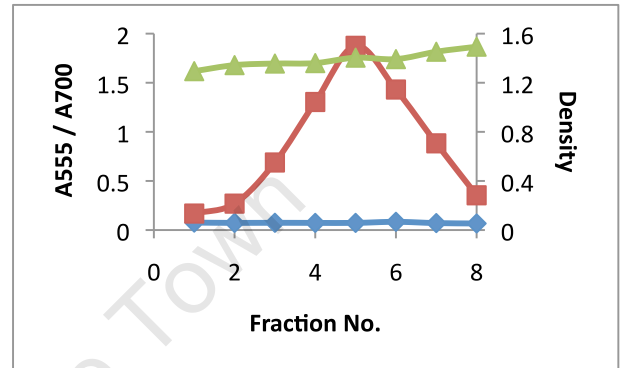




A

B





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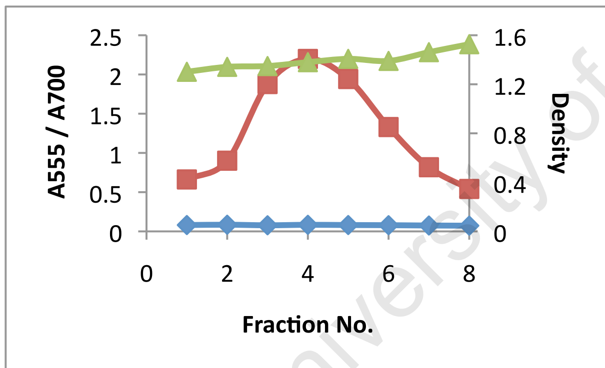

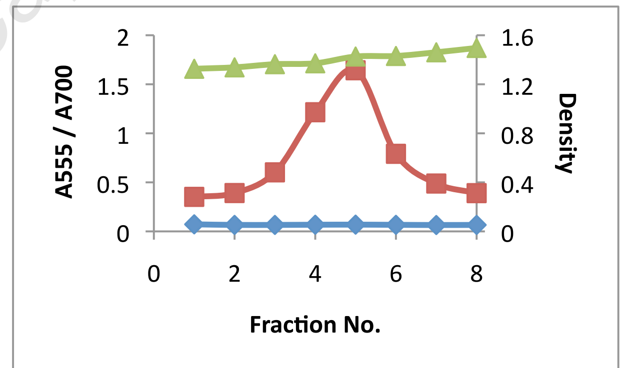

E

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Figure 4-1: First (panel on the left) and second (panel on the right) caesium chloride isopycnic density gradient purification of normal (A, B), asthma (C, D) and COPD (E, F) sample.

A PAS, protein and density profile of the first and second CsCl purification of respiratory mucus. The density of each fraction was measured (▲), following dialysis of aliquots of mucus samples against distilled water, each fraction was analysed for glycoproteins (■) PAS and protein (♦) Lowry. The results are expressed in absorbance values (PAS at 555nm and protein at 700nm) and density in g/ml.

(A) Mucin sample from a normal healthy volunteer after the first centrifugation step with CsCl.

(B) Mucin sample from a normal healthy volunteer after the second centrifugation step in CsCl.

(C) Mucin sample from an asthma patient after the first centrifugation step in CsCl.

(D) Mucin sample from an asthma patient after the second centrifugation step in CsCl.

(E) Mucin sample from a COPD patient after the first centrifugation step in CsCl.

(F) Mucin sample from a COPD patient after the second centrifugation step in CsCl.

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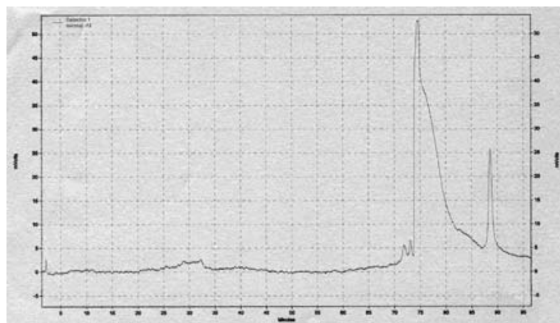

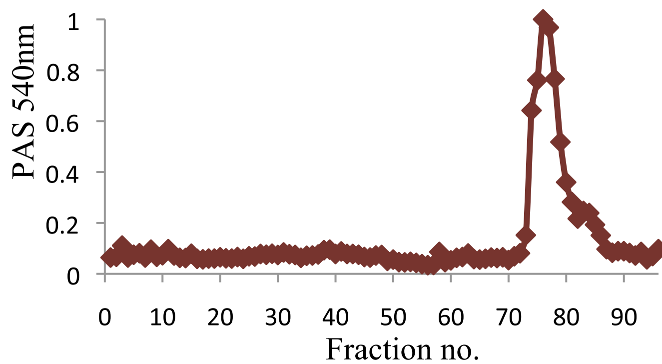

A

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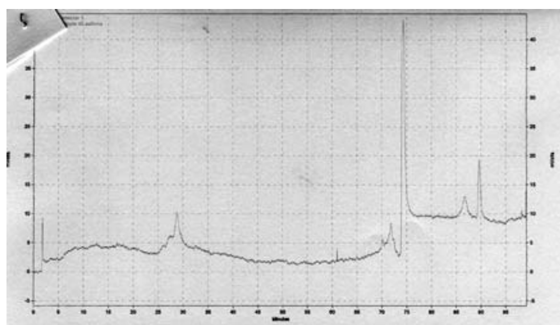

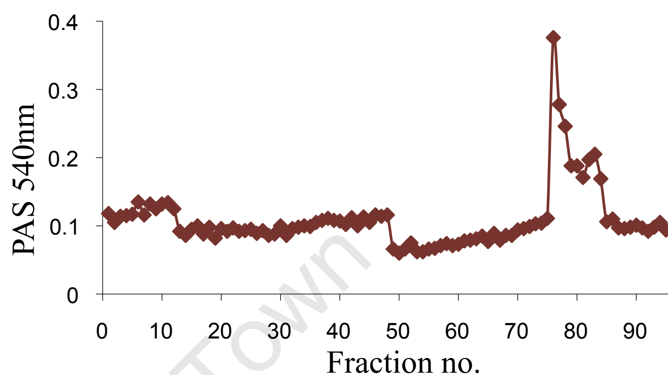

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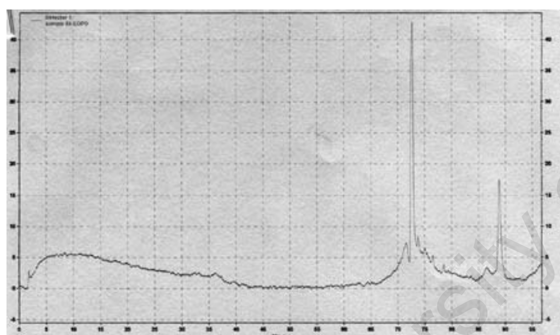

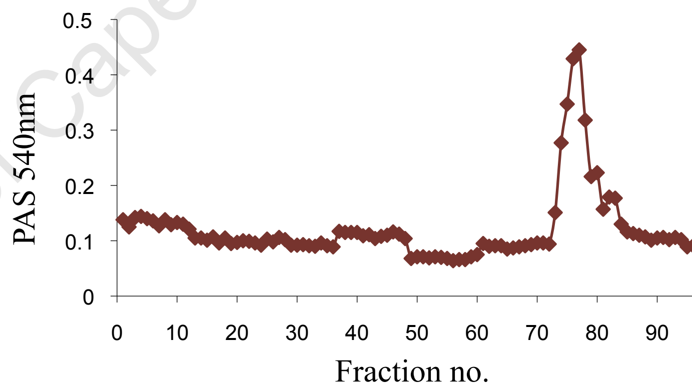

E

F

Figure 4-2: Mono Q elution (panel on the left) and PAS (panel on the right) profiles of reduced/ alkylated mucin subunits of mucus sample.

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The graphs represent a mucin elution profile from the Mono Q 5/50GL column with 0.4M lithium perchlorate in the eluting buffer. Reduced mucin subunits were chromatographed on a Mono Q 5/50GL column. Lithium perchlorate concentration rises linearly from 0 to 0.4M in 60 minutes and then back to 0M in 5 minutes. Many Mono Q fractions contain more than one mucin species. Fractions eluted from the Mono Q column had similar charge densities for each group. Quantitative comparison between each Mono Q mucin elution profile is not possible.

(A) HPLC profile of a mucin sample from a normal healthy volunteer.

(B) PAS profile of a mucin sample from a normal healthy volunteer.

(C) HPLC profile of a mucin sample from an asthma patient.

(D) PAS profile of a mucin sample from an asthma patient.

(E) HPLC profile of a mucin sample from a COPD patient.

(F) PAS profile of a mucin sample from a COPD patient.

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Chapter 5
Identification of respiratory mucins in airways normal subjects (N) with no diagnosed disease by Western Blotting

5.1 Identification of mucins by Western Blotting

The objective of the study reported in this chapter was to characterise the mucins in the sputa of airways of normal individuals by Western blotting. Crude sputa from 10 'healthy' (symptom-free) volunteers (N) were collected after sputum induction and solubilized in cold 4M GuHCl and proteolytic inhibitors. The disulphide bonds of the mucins were then reduced with 10mM DTT and subsequently alkylated with 25mM IAA, to obtain mucin subunits with epitopes exposed for detection (Kirkham et al, 2002). Mucins were purified by density gradient centrifugation in CsCl, after which they were analyzed by HPLC analysis on a Mono Q 5/50GL column using a modified method of Thornton et al, (1996). Mucin identification was by agarose gel electrophoresis and Western blotting, again a slight modification of the method of Thornton et al, (1996) and developed in our laboratory (Trimmel et al 2008, in press).

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5.2 Results

Western blot analysis was performed to determine the identity of the mucins present in normal airways. Samples were loaded onto a 1% agarose gel and subjected to electrophoresis. Mucins were then transferred from the gel onto a nitrocellulose membrane by vacuum blotting and probed with polyclonal anti-sera that recognise protein epitopes on the VNTR region of MUC5AC, MUC5B and MUC2 (Chapter 2, Table 2-1), shown in the blots (Figures 5.1, 5.2 and 5.3), respectively. The same volume of material was loaded on all gels. Western blotting confirmed the presence of MUC5AC and MUC5B in normal airways although the amounts of MUC5B were far less on the gel (Figure 5.2) than MUC5AC (Figure 5.1), albeit for equal volumes of material. Surprisingly some of the normal sputa showed varying amounts of MUC2 (Figure 5.3), again for equal volumes.

The specificity of the MUC5AC antibody LUM 5-1, in the blots of samples from volunteers (N) (Figure 5.1) was confirmed against a purified sample of PMP (Figure 5.1 A-F, lanes 1). This appeared as a very large smear of material from the top of the gel to quite far down. Crude and untreated sputa samples from the normal subjects gave varying amounts of MUC5AC in the gels (Figure 5.1 A-F, lanes 2) appearing as a smear from the top of the gel. The purified mucin from the same sample of sputa, after

being reduced with DTT and loaded on the gel (Figure 5.1 A-F lanes 3), ran in approximately the same position as the purified and reduced samples eluted from the Mono Q column. The eluted fractions from the Mono Q column showed a slight charge gradient (Figure 5.1 A-F, lanes 4-11), more apparent in some cases (Figure 5.1 B and D, lanes 4-11) than in others (Figure 5.1, A, C, E and F). Sample application buffer was loaded in lanes 12 (Figure 5.1 A-F) and used as the negative control.

The presence of MUC5B (Figure 5.2) in the normal secretions was much less when compared to MUC5AC (Figure 5.1) and only found in 5 out of 10 secretions of the volunteers (N). The volunteers (N) that had MUC5B present (Figure 5.2 A-C), had lower levels of this mucin compared to MUC5AC for equal volumes of material loaded onto the gel (Figure 5.1 A-F). MUC5B was present in the positive (PMP) control in lanes 1 (Figure 5.2 A-C, arrows). A sample of crude sputa appeared as a band in the gel (Figure 5.2 A, lane 2), as a band just entering the gel (Figure 5.2 B, lane 2, arrow) and was absent from the gel (Figure 5.2 C, lane 2). A purified reduced sample of the same sputa, in lanes 3, was loaded onto the gel and electrophoresed as a single band (Figure 5.2 A), as a smear (Figure 5.2 B) and very interestingly, absent from the gel as shown in the blot (Figure 5.2 C), an indication of variation within a group. The purified reduced samples in lane 3 (Figure 5.2 A) electrophoresed in a similar position to the fractions eluted from the Mono Q column in lanes 9-11 (Figure 5.2 A) but differently to lanes 5-8 (Figure 5.2 A, arrows), which appeared earlier. The eluted fractions (Figure 5.2 B, arrows) from the Mono Q column in lanes 7-8 electrophoresed in a similar position to the purified reduced sample in lane 3 but differently to the eluted fractions in lanes 4-6. The eluted fractions from the Mono Q column showed a clear charge gradient for MUC5B (Figure 5.2 A and B, lanes 4-11). This gradient is similar to what was observed by Thornton et al (1996) when they looked at asthmatic reduced mucin (Thornton et al, 1996).

Sputa from normal controls were probed with the MUC2 antibody, LUM2-3 (Herrmann et al, 1999) on Western blots. MUC2 was present in only 3 out of the 10 volunteers (N). MUC2 was present in the positive control and appeared as a faint band (Figure 5.3 A, lane 1, as indicated by the arrow) but was absent in the sample. MUC2 was observed as a darker smear-band in lanes 1 (Figure 5.3 B-D, arrows) but this could be due to the over-exposure of these membranes which was required in order to visualise the bands in lanes 2-11. Interestingly, MUC2 in the crude sputa appeared as a band in the blot (Figure 5.3 B, lane 2) and was not seen in the other blots (Figure 5.3 C and D, lanes 2).The crude mucus could

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be present but cannot be seen because it is over-shadowed by the big smear of the positive control in lane 1. Interestingly, the purified reduced sample (Figure 5.3 B-D, lanes 3, arrows), electrophoresed similarly in all 3 gels as a distinct band indicating that the material could be highly charged because of its position towards the bottom of the gel. However, only sample N12 (Figure 5.3 C) in the group of normal volunteers had detectable MUC2 in the fractions that eluted from the HPLC.

5.3 Discussion

A defining feature of this entire study was the inter-individual variation of mucins from controls and patients within each group, rather than differences between groups. This is quite different from previous findings of Kirkham et al, (2002) who reported, for example, a difference in MUC5AC and MUC5B between healthy and diseased airways. An example of this would be the absence of MUC5B, a major respiratory mucin in 5 of the 10 normal volunteers used in this study (see Figure 5.2 C). In some instances crude sputum and even purified mucin from the crude sputum from one individual reacted with the antibody of MUC5B whilst another did not (see Figure 5.2 A-C). The occurrence of MUC2 in some of the controls is also an example of inter-individual variation within groups in this study (see Figure 5.3).

A major limitation of this study was the different amounts of sputa obtained from each patient. This in some cases was so little, that mucin purification was done with difficulty and because the final yield of purified mucin was so little in these instances, it made loading of equivalent amounts on the HPLC and on gels quite impossible. However, we tried to maintain some consistency by loading, as far as possible, equal volumes (50µl) of material on the gels. We thus had to resort to the loading of equal volumes of material on the HPLC Mono Q column and gels and characterise the mucins thereof. This qualitative approach to mucin characterization has been reported previously (Thornton et al, 1996; Govender et al, 2005; Govender 2006). Henke (2004) conducted a study on cystic fibrosis secretions where samples were loaded onto gels at either, the same concentration or the same volume and found that the intensity of the detected bands for MUC5AC and MUC5B were similar in appearance. This suggests that the intensity of the mucin smear-bands is not directly related to the concentration of the sample loaded onto a gel. The intensity of the bands in this study is related to the positive control (PMP) used throughout the study which was loaded at a standard concentration of 1mg/ml. It is hypothesised that a darker smear could be indicative of a greater number of epitopes on a single mucin (Henke et al, 2004).

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Appropriate positive and negative controls were used for the specific mucins on each gel. Material obtained from a patient with pseudomyxoma peritoneii (PMP) was used as a positive control (Figures 5.1, 5.2 and 5.3, lanes 1) for MUC5AC, MUC5B and MUC2 in each gel (O'Connell et al, 2002; O'Connell et al, 2002; Mall et al, 2007). PMP is an exudative disease that originates either in the appendix or ovary, resulting in an accumulation of viscous fluid in the abdomen. The mucins in this fluid were characterised in our laboratory (Mall et al, 2007). The presence of MUC5AC, MUC5B and MUC2 electrophoresed as smears on an agarose gel/western blot (Mall et al, 2007). Using the same extraction and purification methods as in this study, we found the mucins from a patient with PMP very difficult to purify because of the high protein content of the viscous fluid. However, we thought it would make a suitable positive control for all the mucins we examined in this study. Also, after detection of a specific antibody, membranes were stripped in stripping buffer (see Chapter 2) to detect the next antibody. Therefore, for our purpose it was convenient to use the same positive control. In this study sample application buffer was used as a negative control in all blots. The crude, unpurified non-reduced mucus with a higher molecular weight electrophoresed slower than the reduced samples and did not completely enter the gel due to its size. The purified reduced mucin shown in lanes 3 of all blots, was loaded onto the HPLC Mono Q column, which separates and elutes the fractions according to charge (Figure 5.1 A-F, lanes 4-11).

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MUC5AC is a known mucin component in gastric mucus secretions (Ho et al, 1993; Pinto-de-Sousa et al, 2004). MUC5AC was clearly present in the induced sputum of the 'healthy' volunteers in all the samples (10/10) (Figure 5.1 A-F, lanes 2-11). The elution of MUC5AC from the Mono Q column was similar to that reported by Kirkham et al, (2002) and quite in contrast to that of Govender (2006) who showed little or no MUC5AC in controls. Other researchers had a similar experience as Govender (John Sheehan, personal communication). The antibody LUM5-1 used to probe MUC5AC in this study was similar to previous studies (Govender 2006).

MUC5AC appeared as a dark smear-band with a clear charge separation between the fractions eluted from the Mono Q column for sputa collected from each of the 'healthy' volunteers (N) (Figure 5.1 A-F), with a strong signal for purified mucin from a patient with pseudomyxoma peritonei (PMP) used as a positive control (Figure 5.1 A-F). The position and appearance of MUC5AC on the western blot in normal secretions (Figure 5.1 A-F) is similar to that described by Thornton et al, (1996) where

MUC5AC was found to be the major respiratory mucin in the normal secretions and which was confirmed in this study (10/10) in the normal healthy volunteers.

MUC5B is present in smaller amounts in the normal airways (Figure 5.2 A-C) despite it being a major respiratory mucin (Thornton et al, 1997). The charge separation of MUC5B between fractions eluted (lanes 4-11) from the Mono Q column of N7 (Figure 5.2 A) and N12 (Figure 5.2 B) is more distinct than that for MUC5AC. The elution profile for MUC5B showed variation with some samples showing eluted fractions as a clear gradient when eluted from the Mono Q column (Figure 5.2 A and B), whilst others had no detectable eluted material (see Figure 5.2 C). The MUC5B blots that showed a charge gradient (Figure 5.2 A and B) could be suggestive of the presence of MUC5B glycoforms (Kirkham et al, 2002). MUC5B was present in 5 out of the 10 normal sputa collected.

Figure 5.3 A-D show the results for MUC2, an intestinal mucin that has been reported to be present only in small amounts in the respiratory tract of diseased patients (Davies et al, 1999). The presence of MUC2 in normals, though controversial and shown to appear in very small amounts (Davies et al, 1999), is largely accepted to be absent in normals by the major researchers in this field (Hovenberg et al, 1996). MUC2 was present in the positive control (PMP) (Figure 5.3 A–D, lanes 1 as indicated by the arrows). MUC2 was not detected in N7 (Figure 5.3 A) and was present in purified reduced mucin in lanes 3 of N9 (Figure 5.3 B), N12 (Figure 5.3 C) and N14 (Figure 5.3 D). However, the only sample that had detectable amounts of MUC2 in the fractions eluted from the HPLC Mono Q column was in N12 (Figure 5.3 C, lanes 4-11).

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Upon further investigation (after these experiments were completed) we discovered some interesting information about the individuals who kindly donated their sputa for this study in this group of controls. Control number N7 is a 61-year old male who is an ex-smoker who had MUC5AC (Figure 5.1 C), MUC5B (Figure 5.2 A) and no MUC2 (Figure 5.3 A). Control number N9 is a 21 year old male who is a current smoker and suffers from allergies. He had MUC5AC (Figure 5.1 D), MUC5B (not shown) and though he had MUC2 (which was purified) (Figure 5.3 B), there were no detectable amounts of MUC2 that eluted from the HPLC Mono Q column. This could be due to technical problems or it is possible that the mucins ran off the gel. Control number N12 was the most interesting individual in this group. He is a 28-year old male, an ex-smoker who is married to an ex-TB sufferer. He had high levels of

MUC5AC (Figure 5.1 E), MUC5B (Figure 5.2 B) and MUC2 (Figure 5.3 C), present in clear quantities. Even more interesting was the location of the bands at the bottom of the gel. This was comparable to the finding of Govender (2006), in whose study TB samples gave a similar electrophoretic profile (see Figure 5.4, lanes 7 and 9 as indicated by the arrows). It must be noted that Govender's study (2006) did not include elution of mucin from an HPLC column. The mucins were not even purified and only crude samples were used. However, the location and position of the bands of individual N12, who lives with an ex-TB sufferer and that of the sputa of the patients used by Govender in her study, were very similar. The confidential nature of this study does not allow us to reveal this finding directly to the patient but we are discussing with the University of Cape Town Ethics Committee the possibility of divulging this information to the doctor of individual N12.

Also of interest was control number N14. This is a 38-year old male who never smoked, has allergies and according to his physician, is susceptible to asthma and eczema. He had detectable amounts of MUC5AC (Figure 5.1 F), no MUC5B (Figure 5.2 C) but had some purified MUC2, but too little to have anything eluted from the HPLC column (Figure 5.3 D) and again present at the bottom of the gel.

In this study we used the Western blotting technique, a widely used analytical tool in detection and identification of mucins (Dunn 1986) and HPLC Mono Q analysis to isolate mucins according to charge (Thornton et al, 1996). This is the first study in this laboratory to show the presence of MUC5AC, MUC5B and MUC2 in normal airways after HPLC Mono Q analysis and Western blot detection. In the TB study conducted in this laboratory by Ureshnie Govender in 2004-2006 (Govender 2006), crude sputum was used to detect mucin type by Western blotting and autoradiography. We had available to us a supersensitive ECL detection kit, which required autoradiography or chemi-imaging for visualisation of the detected bands on the blot. Chemi-imaging was more sensitive and gave better visualisation than autoradiography. However, in the work of Govender (2006), MUC5AC and other mucins, up-regulated in disease were very easily detectable using autoradiography. MUC5AC sensitivity was low in the normal group of the previous study (Govender, 2006) and could not be detected in the crude sputa. It is suggested that for future TB studies, purified mucin should be used. In uninfected TB adults MUC5B and 2 glycoforms of MUC2 were detected (Govender 2006). However, in this study no glycoforms of MUC2 was detected.

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In these experiments on normal airways sputum electrophoresis was done on 1% agarose gels. Agarose gels appear to optimally separate large charged and uncharged glycoproteins (Tytgat et al, 1995). Exposure times on western blots for detection of respiratory mucins all varied as the method used depended on the concentration of the sample and the apparatus used to visualise the bands. A super-sensitive ECL detection kit was used as the purified reduced mucin was in low amounts and ECL lumiglo detects nanogram amounts of sample. The sensitivity to the ECL could be amplified by exposing the western blot to the reagents for longer than the allocated minute, or detection using either chemi-imaging or X-ray film for autoradiography and at different time periods. The nitrocellulose membranes were used repeatedly for different antibody detection by stripping the membrane with stripping buffer which saved time and money (a very important consideration in our laboratory). This buffer and/or the chemi-imager could have caused the distortion of the bands and bad background.

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In summary, we have shown that the major respiratory mucins MUC5AC (in all the controls) and MUC5B (in only 50% of the controls) are present in those subjects who have no known diagnosed respiratory or lung condition, which in this study we regarded as normals (N). Interestingly, we showed some MUC2 was present in N9, a current smoker, N14 a volunteer that never smoked and N12 an ex-smoker (living with an ex-TB patient). The electrophoretic profile of these MUC2 positive subjects was very similar to that of the crude mucus electrophoretic profile of TB patients (Govender 2006). Subject N12 also showed MUC2 material eluting from the HPLC Mono Q column. The fact that he lives with an ex-TB sufferer is the likely explanation of why he had more MUC2 material in his sputa, enough for us to obtain a positive result for the HPLC analysis. It should be noted that the incidence of TB in South Africa is probably one of the highest in the world (Zar et al, 2000). Almost everyone is exposed to TB although not everyone suffers from it or is diagnosed with it. Sadly, there are also multi-drug resistant cases of TB increasingly being recorded in South Africa (Zager and McNerney 2008). Due to the confidential nature of this study we are considering ways to get this information to the MUC2 positive cases in this study, to encourage them to be tested.

Finally, the steeper gradient of the HPLC eluted fractions of certain MUC5B eluted profiles is suggestive of the presence of a mixture of glycoforms of MUC5B in these sputa samples (Thornton et al, 1996).

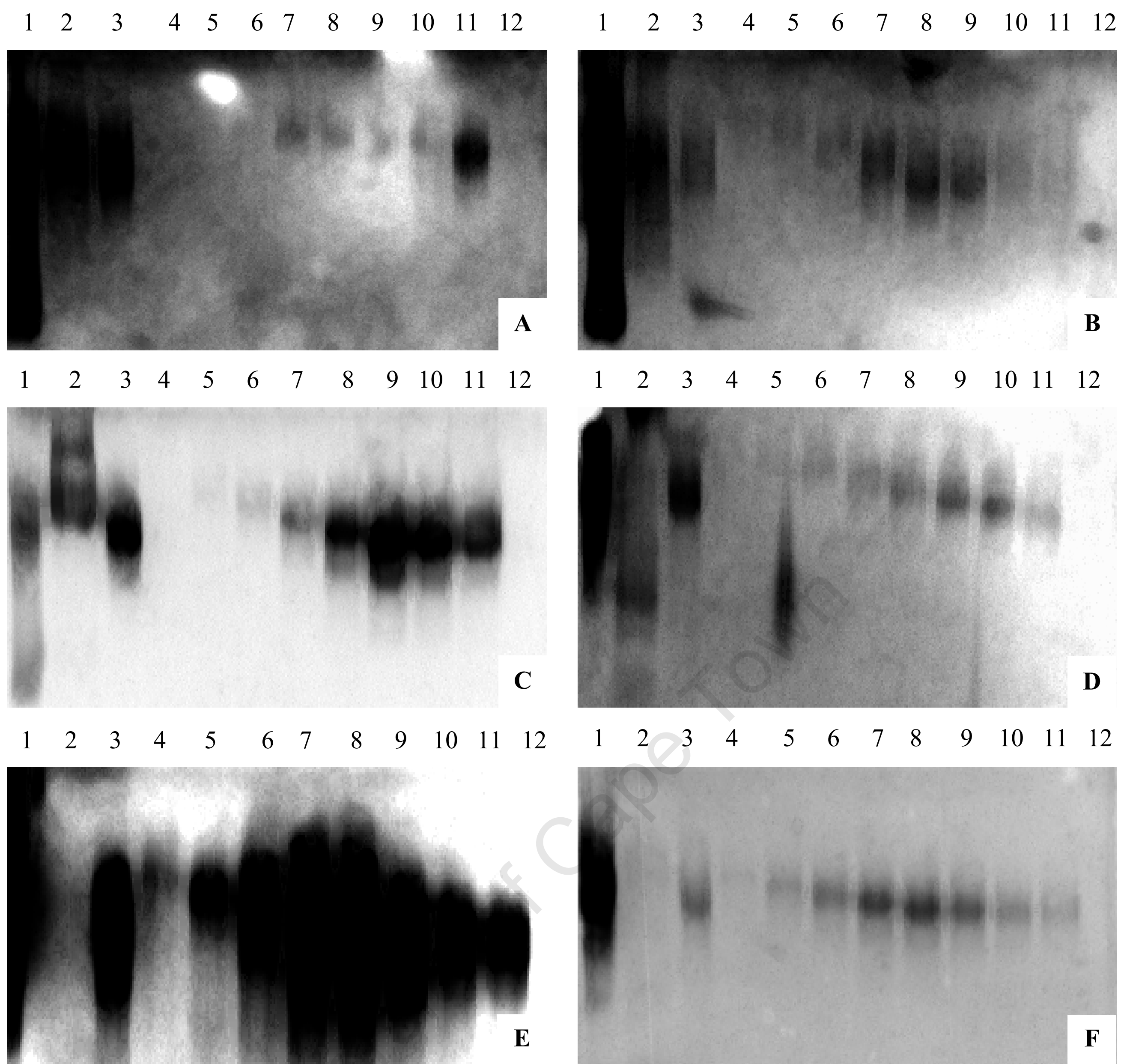

Figure 5-1: Agarose gel electrophoresis (a representative group of gels) of reduced and alkylated mucins probed for MUC5AC from the airway secretions of normal volunteers.

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Sputum induced with 3% NaCl from normal airways was solubilised in 6M GuHCl and then dialysed against 6M Urea. Mucins were reduced and alkylated and subjected to electrophoresis on a 1% agarose gel as descibed in the experimental section.

(A) Is a normal sample (N3) with a concentration of 0.45mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 1 minute. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 60-67 and lane 12, negative control, (sample application buffer SAB).

(B) Is a normal sample (N4) with a concentration of 0.39mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 3 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 66-73 and lane 12, the negative control, (SAB).

(C) Is a normal sample (N7) with a concentration of 0.86mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 30 seconds. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 74-82 and lane 12, negative control, (SAB).

(D) Is a normal sample (N9) with a concentration of 0.38mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 3 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 63-70 and lane 12, negative control, (SAB).

(E) Is a normal sample (N12) with a concentration of 0.18mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 1 minute. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, (SAB).

(F) Is a normal sample (N14) with a concentration of 0.1mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 1 minute. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 66-73 and lane 12, negative control, (SAB).

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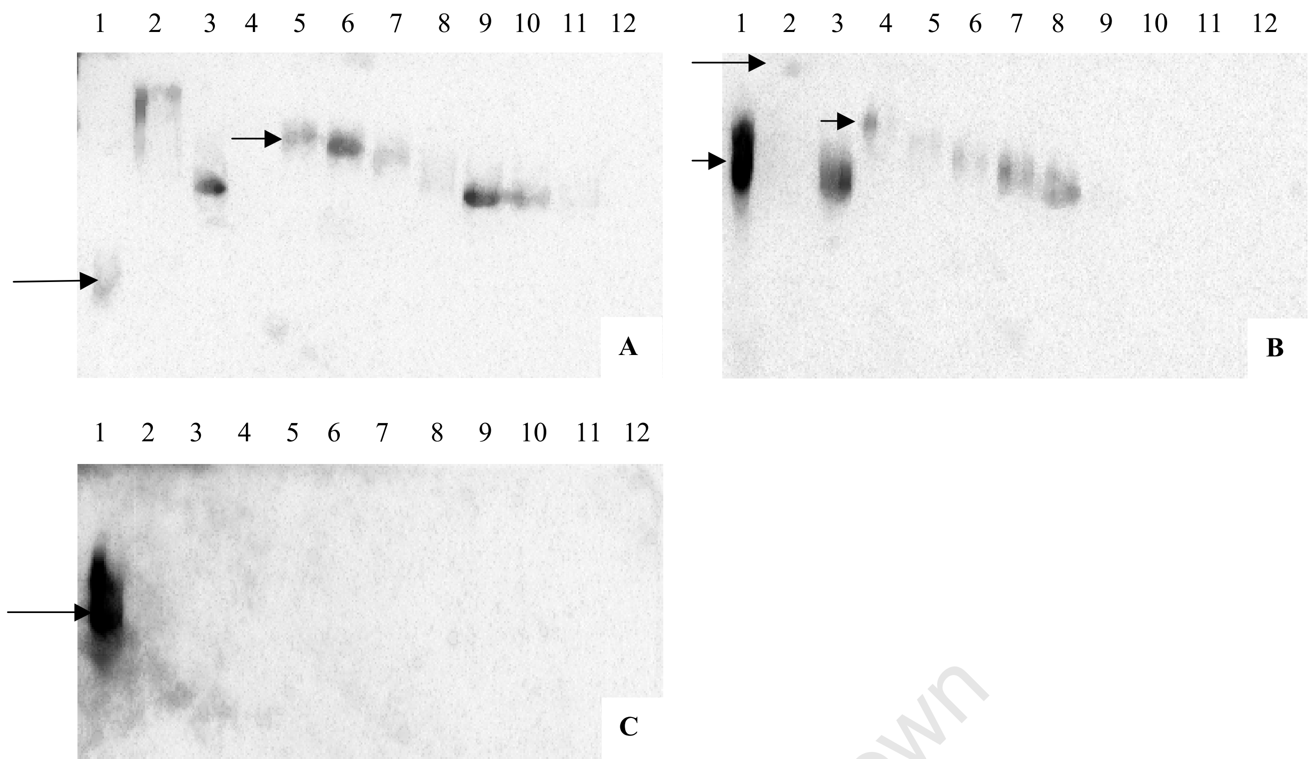

Figure 5-2: Agarose gel electrophoresis (a representative group of gels) of reduced and alkylated mucins probed for MUC5B from the airway secretions of normal volunteers.

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Sputum induced with 3% NaCl from normal airways was solubilised in 6M GuHCl and then dialysed against 6M Urea. Mucins were reduced and alkylated and subjected to electrophoresis on a 1% agarose gel as descibed in experimental section.

(A) Is a normal sample (N7) with a concentration of 0.86mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 3minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 74-82 and lane 12, negative control, (SAB).

(B) Is a normal sample (N12) with a concentration of 0.18mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 10 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, (SAB).

(C) Is a normal sample (N14) with a concentration of 0.1mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 5 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lane 4-11, HPLC fractions 66-73 and lane 12, negative control, (SAB).

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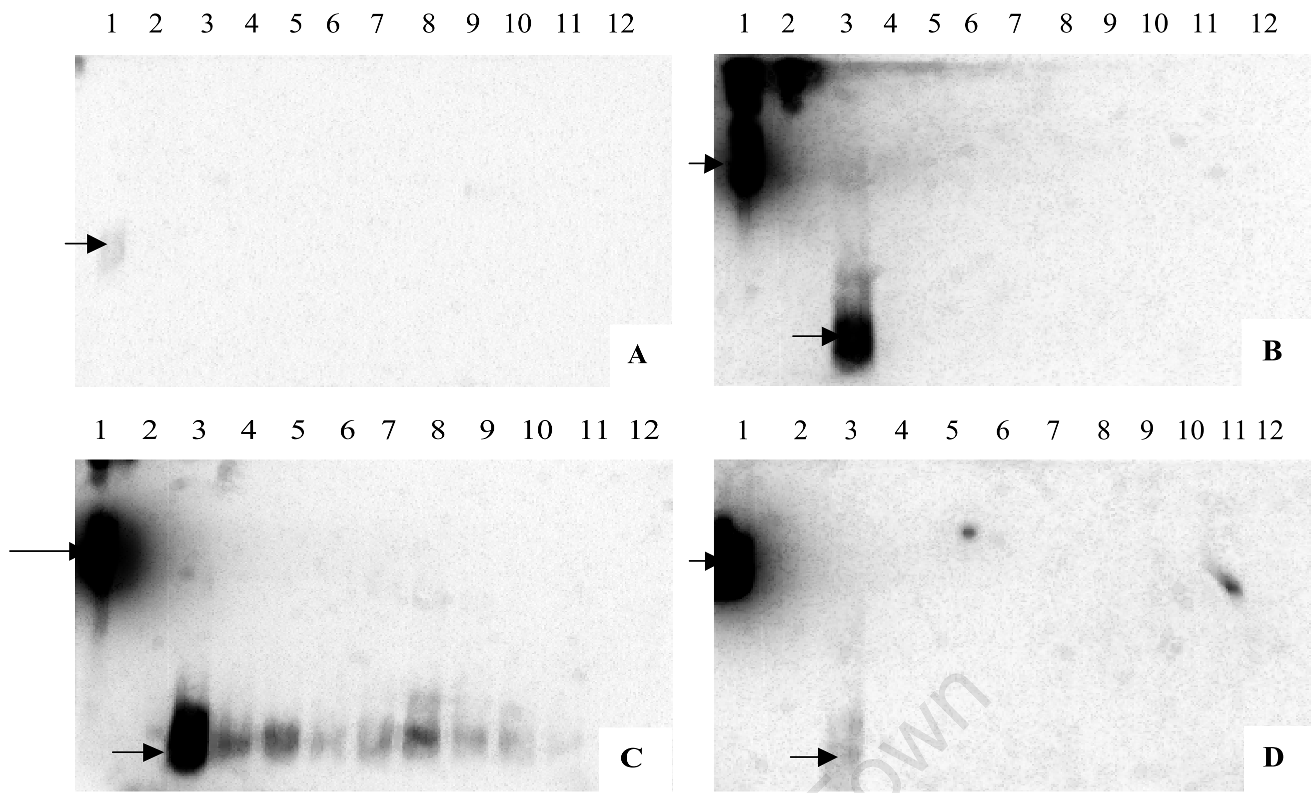

Figure 5-3: Agarose gel electrophoresis (a representative group of gels) of reduced and alkylated mucins probed for MUC2 from the airway secretions of normal volunteers.

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Sputum induced with 3% NaCl from normal airways was solubilised in 6M GuHCl and then dialysed against 6M Urea. Mucins were reduced and alkylated and subjected to electrophoresis on a 1% agarose gel as descibed in experimental section.

(A) Is a normal sample (N7) with a concentration of 0.86mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 3minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 74-82 and lane 12, negative control, (SAB).

(B) Is a normal sample (N9) with a concentration of 0.38mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 5 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 63-70 and lane 12, negative control, (SAB).

(C) Is a normal sample (N12) with a concentration of 0.18mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 5 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, (SAB).

(D) Is a normal sample (N14) with a concentration of 0.1mg/ml of purified mucin which has been exposed to ECL for 1 minute and detected by chemi-imaging after 10 minutes. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, reduced purified mucin; lanes 4-11, HPLC fractions 66-73 and lane 12, negative control, (SAB).

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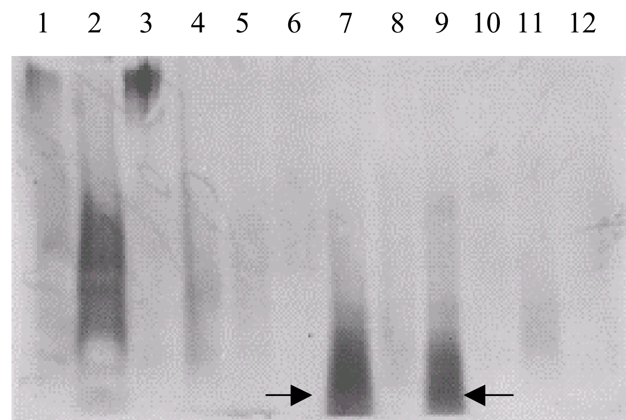

Figure 5.4: Agarose gel electrophoresis of TB sputa for detection of MUC2.

Lane 1: positive purified colon mucin; lane 2: saliva; lane 3, purified gastric mucin; lane 4-6, volunteer sputa control; lane 7 and 8, TB positive adults; lane 9 and 10, TB positive children; lane 11, 'possible' TB adult; lane 12, 'possible' TB child (Govender 2006).

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Chapter 6
Identification of respiratory mucins in airways of subjects with asthma (A) by Western Blotting

6.1 Identification of mucins by Western Blotting

This chapter describes the characterisation of mucins in sputa in airways of asthmatic patients by Western blotting. Crude sputa solubilised in 4M GuHCl and a cocktail of proteolytic inhibitors, 10mM EDTA and 1mM PMSF were collected from 15 asthma patients attending hospitals in the Cape Town region. The mucins were reduced with DTT and subsequently alkylated with IAA to obtain mucin subunits with epitopes exposed for Western blot detection (Kirkham et al, 2002). The reduced mucin subunits were loaded onto 1% agarose gels and electrophoresed to separate them on the basis of charge. Equal volumes (50μl) of mucin sample from individual patients were loaded onto the gel with the aim of detecting mucins of specific charge and possible glycoforms for each mucin type. Following electrophoresis the samples were vacuum-blotted onto a nitrocellulose membrane to confirm the presence of the major respiratory mucins, MUC5AC and MUC5B (Kirkham et al, 2002) and to investigate the possible presence of the minor MUC2 mucin component in asthmatic secretions. Western blots of purified, reduced asthma mucin samples were analysed with three polyclonal anti-sera that recognise protein epitopes on the VNTR regions of MUC5AC, MUC5B and MUC2.

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6.2 Results

MUC5AC, MUC5B and MUC2 are known mucin components of pseudomyxoma peritoneii (PMP) (O'Connell et al, 2002; Mall et al, 2007). PMP was used as a suitable positive control for the detection of the respiratory mucins being investigated. The specificity of MUC5AC antibody LUM 5-1, in the blots of samples from asthma patients (Figure 6.1) was confirmed against the purified PMP (positive control, Figure 6.1 A-H, lanes 1) and sample application buffer (negative control, Figure 6.1 A-H, lanes 12). Crude sputum was positive for MUC5AC and appeared as a broad smear-band (Figure 6.1 A-H, lanes 2), which penetrated the top of the gel due to the larger size of this unreduced material compared to the purified reduced mucin which easily entered the gels (Figure 6.1 A-H, lanes 3). The membranes of the Western blot were first probed with MUC5AC, then stripped and probed with MUC5B antibody and again stripped and probed with MUC2 antibody (Henke et al, 2004; Govender 2006). This was a useful method as the volume of the sputum samples collected was low; resulting in a low yield of

mucin and this method saved sample, time and was cost effective.

MUC5AC was present in all the asthma secretions (15/15) analysed. The charge variation is clear and greater between the HPLC fractions eluted from the Mono Q column (Figure 6.1 A-G, lanes 4-11, H lanes 4-9) of the asthma group than the HPLC fractions eluted from the Mono Q column of the normal and the COPD groups (Chapter 5, Figure 5.1 and Chapter 7, Figure 7.1). There is some variation in the gradients of the MUC5AC samples from the asthma group, with some mucin samples showing a slightly steeper gradient (Figure 6.1 A, C, F and G) than others (Figure 6.1 B, D, E and H). The intensity of the MUC5AC smear-bands in the asthma group is similar to the MUC5AC smear-bands of the normal group (all data not shown). The specificity of the MUC5AC antibody was supported, not only by the positive control (Figure 6.1 A-H, lanes 1), but also by the absence of this mucin in the sample application buffer (negative control, Figure 6.1 A-H, lanes 12). The position and appearance of MUC5AC on the western blot of the asthma secretions (Figure 6.1) is similar to that described by Thornton et al, (1996), where MUC5AC was found as the major respiratory mucin in asthma secretions.

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MUC5B was present in 14 of the 15 asthma samples (not all data shown) (Figure 6.2). Once again the specificity of the MUC5B antibody showed reactivity to the positive control (Figure 6.2 A-D, lane 1, arrows) and no reactivity to the negative control (Figure 6.2 A-D, lanes 12). The intensity of the bands for MUC5B (Figure 6.2, arrows) by comparison, was weaker than MUC5AC (Figure 6.1). MUC5B in the asthma group (Figure 6.2) showed similar electrophoretic mobility profiles to that of MUC5B in the normal group (Chapter 5, Figure 5.2).

MUC2 was present in 8 of the 15 asthma samples (not all data shown) (Figure 6.3). The location of the bands (Figure 6.3 A and B, lanes 2-11 and C, lanes 2-9) illustrated by the arrows, show that these mucins are low charge glycoforms of MUC2 unlike the high charge glycoforms seen in some of the normal group (Chapter 5, Figure 5.3) (Aksoy et al, 1999).

6.3 Discussion

Asthma is a respiratory disease that affects the lower respiratory tract (Dodge et al, 1986). There are two major respiratory mucins found in the airways of patients suffering from asthma, MUC5AC and MUC5B and some MUC2 (Davies et al, 1999). MUC5AC is produced from the goblet cells in the surface epithelial layer and MUC5B is produced from the submucosal glands. The production of mucus is essential in keeping the airways lubricated and clear of any pathogens. However, the over-production of the respiratory mucus is a key characteristic of asthma causing mucus plugging in the airways that can lead to morbidity and mortality of these patients (Sheehan et al, 1995; Rose and Voynow 2006). The major respiratory mucins MUC5AC and MUC5B were found to be present in the asthma group in this study. Also MUC2, that responds to inflammation has also been shown (Vinall et al, 2000) in 8/15. The sputa collected from patients with asthma were, upon observation, more tenacious causing difficulty for the patients to expectorate easily.

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MUC5AC was present in 15 out of 15 asthma samples collected. There is more of a charge variation in the asthma group (Figure 6.1) when compared to the normal group (Chapter 5, Figure 5.1), but MUC5AC expression appeared to be homogenous in both groups. The crude unreduced, non-purified mucus (Figure 6.1 A-H, lanes 2) appeared mostly on top of the gel because of its high molecular weight and presented with a broader smear-band than the reduced purified mucin (Figure 6.1 A-H, lanes 3).

The presence of MUC5B was observed in 14 of the 15 asthma samples (not all data shown). The reason for the absence of MUC5B in the one sample is unknown (not shown) as the clinical data for that patient is similar to those whose sputa had MUC5B. The fractions eluted from the Mono Q column as indicated by the arrows showed a charge gradient between these eluted fractions (Figure 6.2, A, B, D, lanes 4-11 and C, lanes 4-9) indicating that the mucin population separated by HPLC Mono Q column is based on charge. The presence of MUC5B in secretions of asthmatic patients (14/15) was greater than the MUC5B in 'healthy' normal volunteers (5/10). This is possibly an indicator of inter-individual variation within the asthma and the normal groups.

MUC2 was present in 9 of the 15 asthma samples collected. MUC2 was not as highly charged as the MUC2 found in the normal (Chapter 5, Figure 5.3) and TB samples (Chapter 5, Figure 5.4). MUC2 mucin was detected in a higher percentage of secretions from asthmatic patients (8/15) than from induced

sputum of healthy normal volunteers (3/10). This suggests that MUC2 should be further analysed in a larger cohort of patients with asthma. The presence of MUC2 is an indicator of inflammation in the airways (Davies et al, 1999). The intensity of the bands for MUC2 (Figure 6.3, arrows) was not as great as the intensity of the bands for MUC5AC (Figure 6.1). The explanation for this could be due to the technique used, for example, stripping of the membranes for re-probing for each respiratory mucin detected and the varying concentrations of the samples. The positioning of the bands is also of interest as they differ from the normal group (Chapter 5, Figure 5.3), indicating that the MUC2 in the asthma group (Figure 6.3) is less charged because it is higher up in the gel when compared to the highly charged MUC2, which is lower in the gel of sputa samples from the normal group that have MUC2. However, we have given a possible explanation of why some of the normal volunteers have MUC2.

The electrophoresis on asthma samples was done on 1% agarose gels, which optimally separates the large charged and uncharged mucins that have been analysed by HPLC (Tytgat et al, 1995). Visualisation of antibody reaction was done by chemi-imager and autoradiography. Both methods were used as they were easily accessible. The ECL super-sensitive kit was used for detection of the bands of all the asthma samples as the concentrations of these samples varied and small amounts could therefore be detected. The electrophoretic differences observed in all lanes of the blots are largely indicative of the intrinsic negative charges of the mucins, which were also observed by Kirkham et al (2002, 2008). The mucins differed in terms of electrophoretic mobility, which was attributed to differences in their charge densities (Thornton et al, 1995). In this study no glycoforms of MUC5AC and MUC5B were present in the asthma group, which is similar to what was observed in the normal group.

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The literature shows that MUC5AC is the dominant mucin in asthma airways (Hovenberg et al, 1996; Ordonez et al, 2001; Kirkham et al, 2002). Methodological differences could probably explain the differences in results obtained from this study when compared to that in the literature (Kirkham et al, 2008). The results presented here show that the electrophoretic profile of the MUC5AC is similar to the MUC5B in the asthmatic airways, which differs from what was previously reported in the literature (Kirkham et al, 2002; Govender 2006). We do, however, show that MUC5AC and MUC5B are the predominant species in mucus from the asthma airways. Comparative analyses of mucin levels are more challenging than simply identifying specific mucins because of the inherent difficulties in solubilising mucus or sputum samples (Rose 1989) and this was reflected in this study.

In summary, mucins produced from the asthma group presented with the respiratory mucins under investigation namely, MUC5AC, MUC5B and MUC2. The airways response to mucus hypersecretion was observed by the presence of both MUC5AC and MUC5B in nearly all the asthma samples. MUC5B presented in more asthma samples (14/15) compared to the normal group (5/10) and the COPD group (11/15), which could be significant. However, a larger study would be required to determine this. This could possibly be one of the reasons why the mucus of asthmatic patients is so viscous. MUC2 which was present in 8 out the 15 asthma samples, is an indicator of inflammation and disease in the airways and could also be an indicator of disease severity (Davies et al, 1999).

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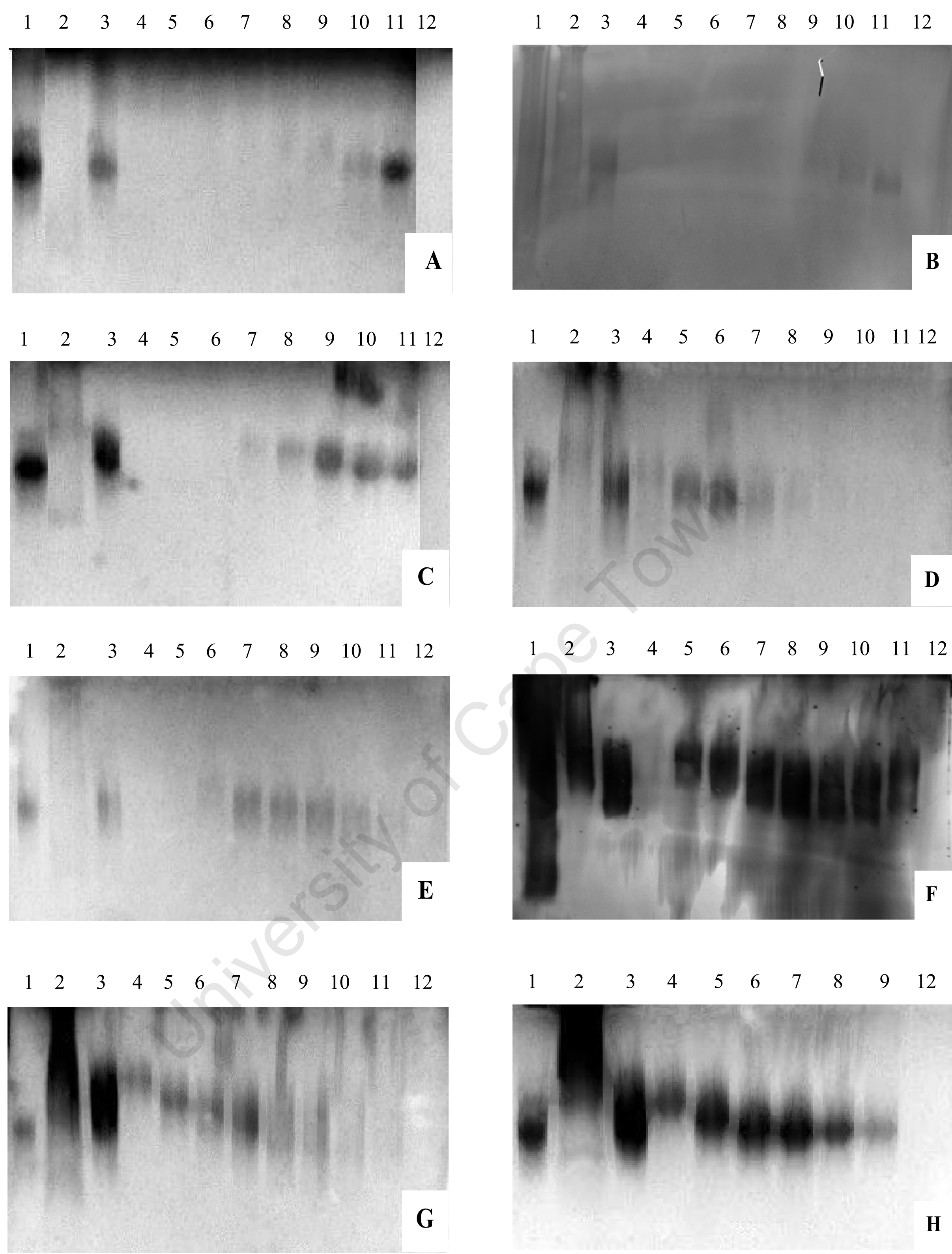

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Figure 6-1: Agarose-gel electrophoresis (a representative group) of reduced and alkylated mucins probed for MUC5AC from airway secretions of an asthma patient.

A 1% (w/v) agarose gel with aliquots of selected fractions was electrophoresed for 3h at 65V and 400mA in 1xTAE to separate mucins based on charge after anion-exchange analysis and subsequently blotted onto nitrocellulose as described in experimental section. Bands were visualised using ECL western detection kit after different exposure times using a chemi-imager and autoradiography.

(A) An asthma sample (A38), with a concentration of 0.93mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lane 4-11, HPLC fractions 78-84 and lane 12, negative control. Bands were visualised after 2 minutes with a chemi-imager.

(B) An asthma sample (A41), with a concentration of 3.95mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 59-66 and lane 12, negative control. Bands were visualised by chemi-imaging after 2 minutes.

(C) An asthma sample (A42), with a concentration of 0.72mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 75-82 and lane 12, negative control. Bands were visualised by chemi-imaging after 2 minutes.

(D) An asthma sample (A43), with a concentration of 0.88mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-84 and lane 12, negative control. Bands were visualised by chemi-imaging after 45 seconds.

(E) An asthma sample (A44), with a concentration of 0.53mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 110-117 and lane 12, negative control. Bands were visualised by chemi-imaging after 2 minutes.

(F) An asthma sample (A45), with a concentration of 12.33mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 85-94 and lane 12, negative control. Bands were visualised by X-ray film using autoradiography after 2 minutes.

(G) An asthma sample (A46), with a concentration of 0.78mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-83 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

(H) An asthma sample (A47), with a concentration of 0.53mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-9, HPLC fractions 76-81 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

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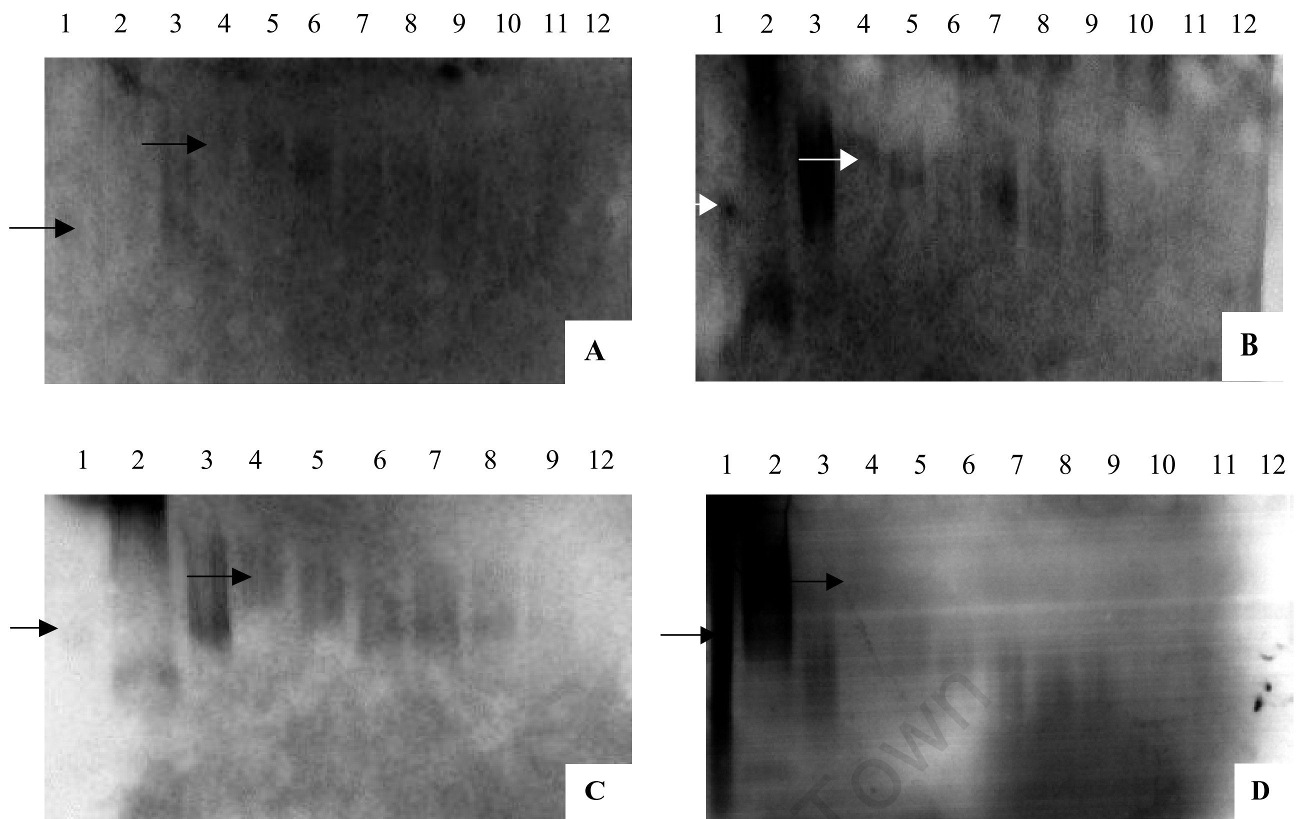

Figure 6-2: Agarose-gel electrophoresis (a representative group) of reduced and alkylated mucins probed for MUC5B from airway secretions of an asthma patient.

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A 1% (w/v) agarose gel with aliquots of selected fractions was electrophoresed for 3h at 65V and 400mA in 1xTAE to separate mucins based on charge after anion-exchange analysis and subsequently blotted onto nitrocellulose as described in experimental section. Bands were visualised using ECL Western detection kit after different exposure times using chemi-imager.

(A) An asthma sample (A44), with a concentration of 0.53mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 110-117 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

(B) An asthma sample (A46), with a concentration of 0.78mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-83 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

(C) An asthma sample (A47), with a concentration of 0.53mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-9, HPLC fractions 76-81 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

(D) An asthma sample (A52), with a concentration of 0.49mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-81 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

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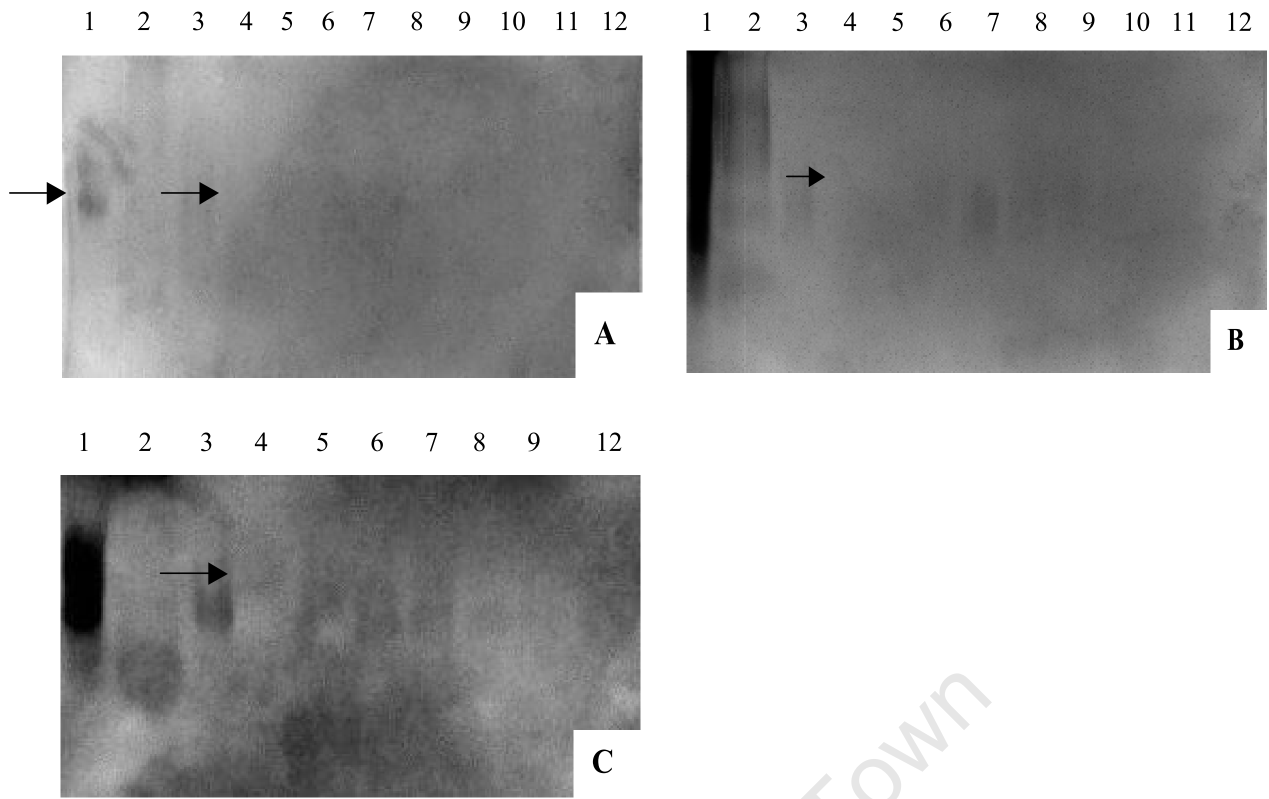

Figure 6-3: Agarose-gel electrophoresis (a representative group) of reduced and alkylated mucins probed for MUC2 from airway secretions of an asthma patient.

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A 1% (w/v) agarose gel with aliquots of selected fractions was electrophoresed for 3h at 65V and 400mA in 1xTAE to separate mucins based on charge after anion-exchange analysis and subsequently blotted onto nitrocellulose as described in experimental section. Bands were visualised using ECL Western detection kit after different exposure times using chemi-imager.

(A) An asthma sample (A43), with a concentration of 0.88mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-84 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

(B) An asthma sample (A52), with a concentration of 0.49mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-81 and lane 12, negative control. Bands were visualised by chemi-imaging after 1 minute.

(C) An asthma sample (A47), with a concentration of 0.53mg/ml of purified mucin. Lane 1, positive control; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-9, HPLC fractions 76-81 and lane 12, negative control. Bands were visualised by chemi-imaging after 3 minutes.

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Chapter 7
Identification of respiratory mucins in airways of subjects with COPD (C) by Western Blotting

7.1 Identification of mucins by Western Blotting

The objective of this chapter was to characterise mucins in sputa in airways from COPD patients by Western blotting. Crude sputa from 15 patients suffering from COPD and attending hospitals in the Cape Town region were solubilised in 4M GuHCl and proteolytic inhibitors. Equal volumes of sputa samples from a group of COPD patients were loaded onto a 1% agarose gel with the aim of detecting mucins of specific charge and the possible glycoforms present for each mucin type. Following electrophoresis the samples were vacuum blotted onto a nitrocellulose membrane to confirm the presence of the dominant respiratory mucins MUC5AC and MUC5B and the minor MUC2 component of respiratory mucus (Kirkham et al, 2002).

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7.2 Results

Using mucin specific anti-sera, MUC5AC was observed in all 15 COPD sputa samples (Figure 7.1). MUC5B was present in 11/15 (Figure 7.2) and MUC2 was present in 7/15 (Figure 7.3) of the COPD sputa samples (not all data shown). In the airways of patients suffering from COPD, proteases from neutrophils, the predominant inflammatory cells, cleave off mucins produced in goblet cells of the surface epithelium (Groneberg and Chung 2004; Rogers 2004). Patients with COPD produced more sputum and thus expectoration of the sample was easier.

Western blotting was performed to identify the presence of mucins in sputa samples of patients with COPD. Mucin samples were loaded on a 1% agarose gel and subjected to electrophoresis. Mucins were then transferred from the gel to a nitrocellulose membrane and probed with rabbit anti-MUC5AC, rabbit anti-MUC5B and rabbit anti-MUC2 polyclonal antibodies (Chapter 2). Western blotting confirmed the presence of MUC5AC (Figure 7.1), MUC5B (Figure 7.2) and MUC2 (Figure 7.3) in COPD sputa samples. MUC5AC was present in all the sputa samples (15/15) analysed in this study, MUC5B in 11/15 while MUC2 was present in fewer (7/15).

The PMP sample used as a positive control was present in all the blots (Figure 7.1, A-F, lanes 1, arrows).

PMP was a suitable control for mucins found in COPD patients as it is positive for MUC5AC, MUC5B and MUC2 (O'Connell et al, 2002; O'Connell et al, 2002; Mall et al, 2007). Sample application buffer was used as a negative control in all the blots for this study (Figure 7.1, 7.2 and 7.3 lanes 12).

MUC5AC was positive in the crude sputa in the blots (Figure 7.1 A-F, lanes 2) and appeared as a large smear-band of material from the top of the gel penetrating quite far into the gel. There was no distinct band for the crude sputa, only a large smear. The purified reduced mucin of the same sputa sample (Figure 7.1 A-F, lanes 3) gave a more distinct band for MUC5AC and electrophoresed in a position similar to those fractions eluted from the Mono Q column (Figure 7.1 A-E, lanes 4-11 and F, lanes 4-7, arrows). The fractions eluted from the Mono Q column electrophoresed with a slightly steeper gradient (Figure 7.1 B, C and D, lanes 4-11) than the others (Figure 7.1 A and E, lanes 4-11 and F, lanes 4-7, arrows).

The presence of MUC5B (Figure 7.2) in the COPD secretions was less when compared to MUC5AC (Figure 7.1) (not all data shown). MUC5B was present in the positive control in all the blots (Figure 7.2, A-D, lanes 1). The crude unreduced sputa presented as a smear (Figure 7.2 A, B and D lanes 2) and as a single band slightly entering the gel (Figure 7.2 C, lane 2, arrow). The presence of MUC5B in the purified reduced mucin was observed (Figure 7.2 A-C, lanes 3) as a single band in the gel. Interestingly, MUC5B was observed as two bands indicating the presence of two glycoforms of MUC5B in COPD (Figure 7.2 D, lane 3). This however was only observed in 1 out of the 15 COPD samples. The purified reduced mucins (lanes 3) electrophoresed slightly different to the reduced samples eluted from the Mono Q column. The fractions eluted from the Mono Q column showed a slight charge gradient (Figure 7.2 A and B, lanes 4-11 and D, lanes 4-7, arrows) and a more distinct gradient was observed (Figure 7.2 C, lanes 4-11, arrows). Sample application buffer was used as a negative control (Figure 7.2 A-D, lanes 12).

Trace amounts of MUC2 were present in 7 of the 15 COPD sputa samples (Figure 7.3) compared to both MUC5AC (15/15) and MUC5B (11/15) (not all data shown). The positive control (Figure 7.3 A-C, lanes 1, arrows) gave two bands for MUC2. This is interesting as PMP always presented as a single band (Mall et al, 2007). This could be due to some protein contamination of the laboratory sample. Crude sputa was observed as a smear from the top of the gel and entering far inside the gel

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(Figure 7.3 A-C lanes 2, arrows). The purified reduced samples are illustrated as single defined bands on the blot (Figure 7.3 A-C, lanes 3, arrows). There are slight bands showing the presence of MUC2 in the fractions eluted from the Mono Q column during HPLC analysis (Figure 7.3 A, lanes 4-11, arrows). The MUC2 bands were very weak but visible in the blot (Figure 7.3 B, lanes 4-11 and C, lanes 4-7, arrows). The negative control used in this study was sample application buffer (Figure 7.3 A-C, lanes 12).

7.3 Discussion

Chronic obstructive pulmonary disease (COPD) is a respiratory disease that affects the lower airways and is comprised of pulmonary emphysema, chronic bronchitis and chronic bronchiolitis (Rogers 2000; Yoshida and Tuder 2007). COPD patients invariably produce more sputum than patients suffering from any other respiratory disease (Aikawa et al, 1989; Rogers 2000). This fact is reflected in this study (see Chapter 3), where the most sputa was produced from the COPD patients. Western blotting was conducted to determine the identity of the respiratory mucins found in the COPD sputa. This analytical tool is widely used in the detection of mucins (Dunn 1986). Agarose gel electrophoresis with a slightly modified method of Thornton et al, (1996) was the method of choice in the separation of mucins after HPLC analysis using the Mono Q column in this study. Agarose gels appear to optimally separate large charge and uncharged glycoproteins.

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Suitable positive and negative controls were used in this study. Material from a patient suffering from pseudomyxoma peritoneii (PMP) was used throughout this study (see Chapter 5 and Chapter 6) as a positive control as it has high reactivity with the respiratory mucins of interest namely, MUC5AC, MUC5B and MUC2 (Mall et al, 2007). Interestingly, this positive control showed two bands when probed with MUC2 which previously presented as a smear regardless of the anti-sera that was being analysed (Mall et al, 2007). It is possible that some protein material has been detected here (Mall et al, 2007). Sample application buffer was consistently used throughout this study, as the negative control. There was no distinct crude sputa band present as the unreduced non-purified mucus in its intact form could not enter the gel easily due to its large size in all the blots. The purified reduced mucins were separated by agarose gel electrophoresis and subsequently detected by Western blotting with anti-sera to the respiratory tract mucins. Electrophoresis is expected to separate lower molecular mass proteins from mucins (Kirkham et al, 2002).

The MUC5AC fractions eluted from the Mono Q column (Figure 7.1) are consistent with the literature in the positioning of the bands and with a slight charge gradient (Figure 7.1 A, B, E and F) and in some cases there was a more distinct gradient (Figure 7.1 C, D). There was also homogeneity of the bands showing that these fractions all belong to the same species of MUC5AC mucin (Kirkham et al, 2002). The fractions eluted from the Mono Q column for MUC5B (Figure 7.2) had a slightly greater charge gradient than MUC5AC (Figure 7.1).

MUC5B was present in 11 out of the 15 COPD samples collected and was observed as faint (Figure 7.2 A, B and D) but detectable bands (Figure 7.2 C). The charge separation of MUC5B between the fractions eluted in lanes 4-11 from the Mono Q column (Figure 7.2 C, arrows) was more distinct than that of the other MUC5B samples represented (Figure 7.2 A, B and D). Interestingly, one sample showed the presence of 2 bands indicating the presence of MUC5B glycoforms in the purified reduced sample, before the mucin was eluted from the Mono Q column as seen in lane 3 (Figure 7.2 D). Glycoforms of MUC5B present in the mucus of the respiratory tract of a patient who died of status asthmaticus has been reported previously (Thornton et al, 1997).

There were low levels of expression in all the mucin samples that were positive for MUC2 (Figure 7.3 A-C) (not all data shown). MUC2 was present in the crude sputa, purified reduced mucin and the fractions eluted from the Mono Q column (Figure 7.3 A-C). There was a slight charge gradient visible.

MUC5AC mucin in this study is consistent with the literature which describes it as a homogenous species compared to the heterogenous MUC5B mucin (Kirkham et al, 2002). Mucus hypersecretion is a contributory factor in the progression of COPD (Rogers 2000; Kirkham et al, 2008). This study shows that airway mucus is not a single substance but is comprised of variable amounts of MUC5AC, MUC5B and MUC2 glycoproteins (Thornton and Sheehan 2004). The results obtained from this study showed no overall significant change in the type of mucin found in the respiratory tract of COPD patients, except that MUC2 was present in fewer numbers (7/15) when compared to MUC5AC and MUC5B.

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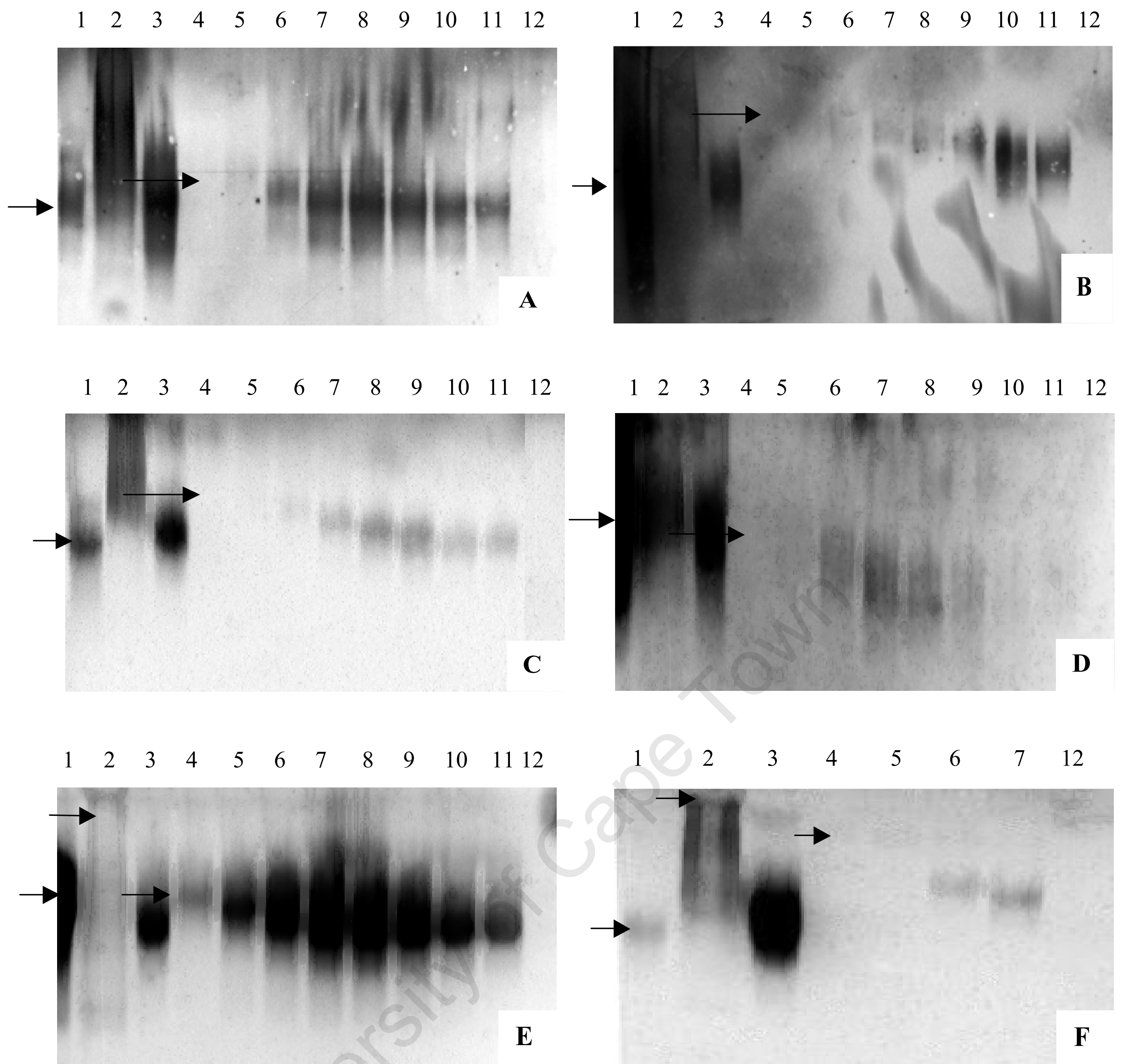

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Figure 7-1: Agarose gel electrophoresis (a representative group of gels) of reduced and alkylated mucins probed for MUC5AC from the airway secretions from COPD patients.

A 1% (w/v) agarose gel with aliquots of selected fractions was electrophoresed for 3h at 65V and 400mA in 1xTAE to separate mucins based on charge after anion-exchange analysis and subsequently blotted onto nitrocellulose as described in Experimental section. Bands were visualised using ECL Western detection kit after different exposure times using an autoradiograph machine and by chemi-imaging.

(A) A COPD sample (C31), with a concentration of 0.69mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-83 and lane 12, negative control, SAB; after 1 minute exposure to X-ray film and detected using autoradiography.

(B) A COPD sample (C51), with a concentration of 6.5mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 61-68 and lane 12, negative control, SAB; after 1 minute exposure to X-ray film and detected using autoradiography.

(C) A COPD sample (C58), with a concentration of 3.9mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, SAB; 30 seconds exposure to a chemi-imager.

(D) A COPD sample (C61), with a concentration of 0.21mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 75-82 and lane 12, negative control, SAB; after 1 minute exposure to a chemi-imager.

(E) A COPD sample (C62), with a concentration of 0.78mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, SAB; after 10 seconds exposure to a chemi-imager.

(F) A COPD sample (C55), with a concentration of 6.6mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-7, HPLC fractions 73-76 and lane 12, negative control, SAB; after 1 minute exposure to a chemi-imager.

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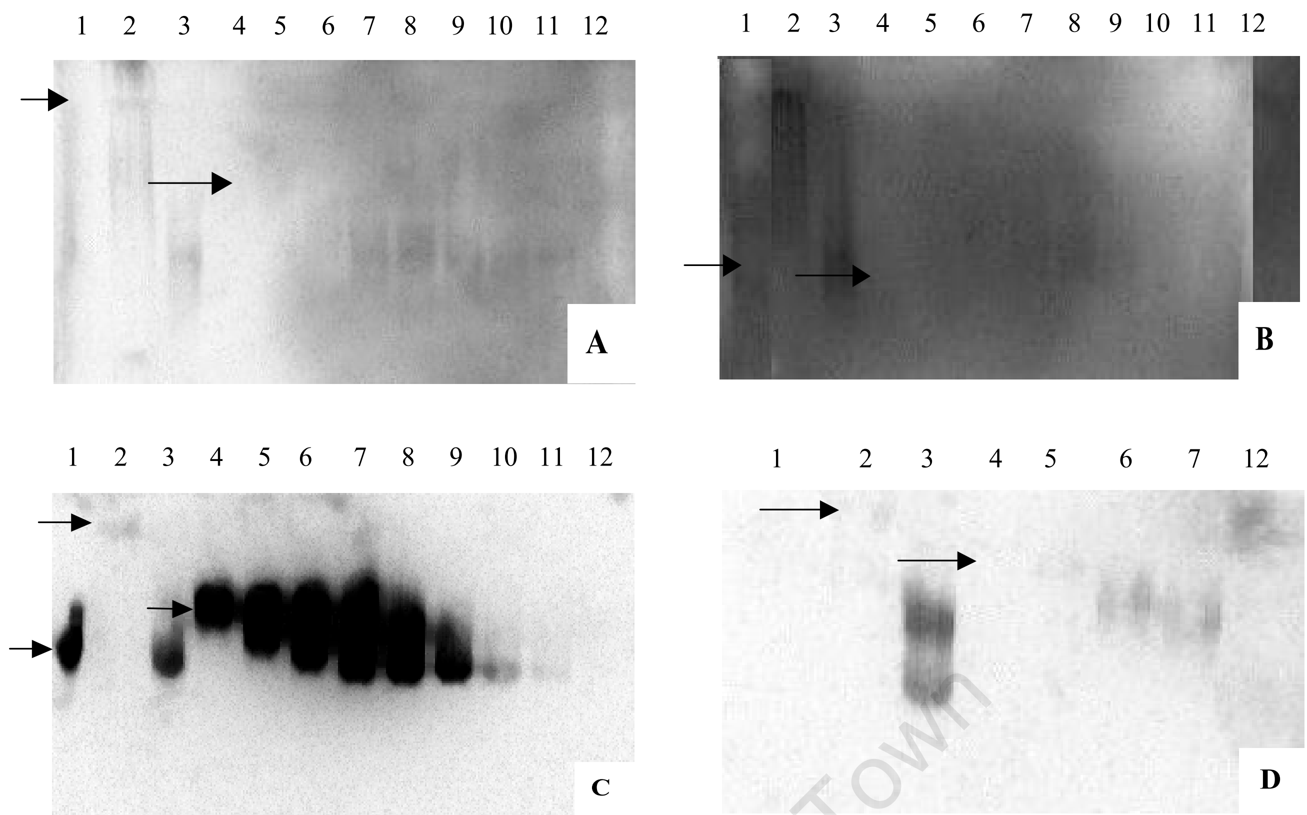

Figure 7-2: Agarose gel electrophoresis (a representative group of gels) of reduced and alkylated mucins probed for MUC5B from the airway secretions from COPD patients.

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A 1% (w/v) agarose gel with aliquots of selected fractions was electrophoresed for 3h at 65V and 400mA in 1xTAE to separate mucins based on charge after anion-exchange analysis and subsequently blotted onto nitrocellulose as described in Experimental section. Bands were visualised using ECL Western detection kit after different exposure times using an autoradiograph machine and by chemi-imaging.

(A) A COPD sample (C31), with a concentration of 0.69mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-83 and lane 12, negative control, SAB; after 1 minute exposure to X-ray film and detected using autoradiography.

(B) A COPD sample (C58), with a concentration of 3.9mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, SAB; after 1 minute exposure to a chemi-imager.

(C) A COPD sample (C62), with a concentration of 0.78mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, SAB; after 10 seconds exposure to a chemi-imager.

(D) A COPD sample (C55), with a concentration of 6.6mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-7, HPLC fractions 73-76 and lane 12, negative control, SAB; after 1 minute exposure to a chemi-imager.

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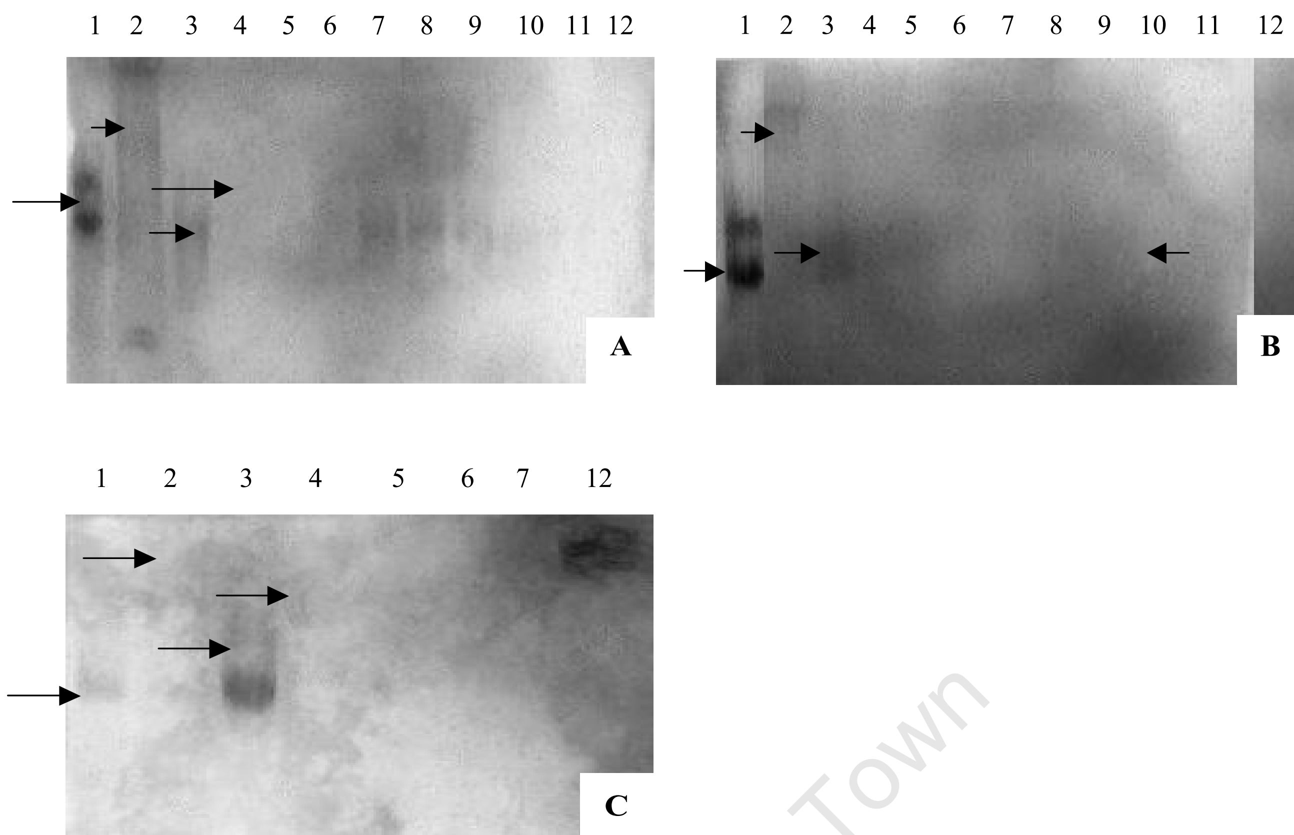

Figure 7-3: Agarose gel electrophoresis (a representative group of gels) of reduced and alkylated mucins probed for MUC2 from the airway secretions from COPD patients.

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A 1% (w/v) agarose gel with aliquots of selected fractions was electrophoresed for 3h at 65V and 400mA in 1xTAE to separate mucins based on charge after anion-exchange analysis and subsequently blotted onto nitrocellulose as described in Experimental section. Bands were visualised using ECL Western detection kit after different exposure times using an autoradiograph machine and chemi-imager.

(A) A COPD sample (C31), with a concentration of 0.69mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 76-83 and lane 12, negative control, SAB; after 1 minute exposure to X-ray film and detected using autoradiography.

(B) A COPD sample (C58), with a concentration of 3.9mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-11, HPLC fractions 74-81 and lane 12, negative control, SAB; after 1 minute exposure to a chemi-imager.

(C) A COPD sample (C55), with a concentration of 6.6mg/ml of purified mucin. Lane 1, positive control, PMP; lane 2, crude sputum; lane 3, purified reduced mucin; lanes 4-7, HPLC fractions 73-76 and lane 12, negative control, SAB; after 1 minute exposure to a chemi-imager.

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Chapter 8
Amino acid analysis

8.1 Introduction

The amino acid composition of the apomucins of the normal, asthma and COPD groups was determined by HPLC analysis of the purified reduced mucins of each group. One sample of each group was analysed for its amino acid composition by hydrolysing these samples to remove the carbohydrate chains from the mucin peptide and then placed on a HPLC column to elute the primary amino acids. Amino acid analysis was done to determine the composition of the respiratory apomucins from the normal, asthma and COPD groups under investigation during this study.

8.2 Results

Serine and threonine combined appeared at 23.32 mol % in the normal group, at 21.16 mol % in the asthma group and at 23.55 mol % in the COPD group. The serine and threonine levels in the normal and COPD groups (23.38 mol % and 23.55 mol % respectively) compared closely to each other and combined showed to be slightly higher than the asthma group (21.16 mol %).

Serine, threonine and proline (PTS) combined appeared at 35.61 mol % in the normal group, at 36.33 mol % in the asthma group and at 34.42 mol % in the COPD group. The PTS mol % was approximately similar for each group and there was only a 1 mol % difference between each group.

Glycine, threonine, serine, proline and alanine constituted 52.87 mol % of the normal group, 54.68 mol % of the asthma group and 51.92 mol % of the COPD group.

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Table 8.1: Amino acid composition (mol %) of the normal, asthma and COPD mucins.

Amino acid	Normal (mol %)	Total protein (N)	Asthma (mol %)	Total protein (asthma)	COPD (mol %)	Total protein (COPD)
Asp	7.08	6.80	7.46	7.30	7.01	7.2
Thr	13.04	12.8	11.88	11.8	14.11	14.2
Ser	10.28	10.00	9.28	9.20	9.44	9.60
Glu	10.64	10.30	13.10	13.00	11.85	12.00
Pro	12.29	12.29	15.17	15.17	10.87	11.20
Gly	9.81	9.50	11.60	11.50	10.55	10.70
Ala	7.45	7.20	6.75	6.60	6.95	7.10
Val	4.94	4.60	4.83	4.70	4.36	4.50
Met	1.19	0.90	1.07	0.90	0.59	0.80
Ile	2.84	2.50	2.28	2.10	2.35	2.50
Leu	6.21	5.90	5.66	5.50	5.26	5.40
Tyr	2.34	2.10	1.85	1.70	1.43	1.60
Phe	2.67	2.30	2.56	2.40	1.92	2.10
His	20.76	20.50	14.89	14.8	18.21	18.30
Lys	3.70	3.40	3.69	3.60	2.37	2.50
Arg	5.89	5.60	6.52	6.40	3.81	4.00

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8.3 Discussion

As shown in the Table 8.1 the mucins found in all 3 groups contained a higher amount of threonine, serine, proline, glutamic acid, glycine and histidine in the peptide portion of the respiratory mucin. These results are similar to the findings of Woodward et al, (1982) which states that (threonine, serine, proline, glycine and alanine) account for 51 mol % of the amino acid residues in the respiratory mucus and are characteristic of the typical mucin composition. Amino acid analysis reveals high levels of serine, threonine and proline levels which is consistent with a mucin-like macromolecule. This amino acid analysis compares well with Habte et al who found similar results (Habte et al, 2007).

CHAPTER 9
CONCLUSION

Airways mucus hypersecretion is the main feature in respiratory diseases that leads to mortality and morbidity (Kim 2003; Rogers 2004; Rose and Voynow 2006). The lung uses specialised secretions produced by the airways to provide a renewable and transportable protective layer to interact with, neutralise, and remove inhaled toxic materials trapped in mucus (Samet and Cheng 1994). The major limitation of this study was the variation in the concentration of each sample collected from each group. To overcome this limitation, qualitative analyses of these mucins, after purification, were done by dissolving the freeze-dried sample in 10 ml with 6M urea. Aliquots of 50μl of each fraction eluted from the HPLC Mono Q column were further analysed for identification of the mucins present in the samples in this study. This approach has previously been successful as reported by the group of Thornton et al (1997) in Manchester, United Kingdom.

The two respiratory diseases of interest investigated in this study were asthma and COPD, two of the leading causes of death due to respiratory arrest, in South Africa. This study was concerned with the isolation, purification and biochemical characterisation of mucus and mucins found in the respiratory tract of patients suffering from chronic airway diseases namely, asthma and COPD and then the comparison of these mucins to the ones found in the airways of normal healthy volunteers. This study is largely a qualitative one. The idea arose from an earlier finding that the mucus found in people suffering from asthma was more tenacious than in other respiratory diseases.

The methods used in this study were caesium chloride density gradient ultracentrifugation in order to isolate and purify the mucins obtained from the three groups (normal, asthma and COPD). Once the mucins were purified, they were reduced with DTT and alkylated with IAA in order to keep the mucin its subunit form and therefore making it easier to analyse. The mucin subunit is smaller and therefore moves more easily through the gels and columns. The mucins were then separated according to charge using a HPLC Mono Q 5/50GL column to determine if there was any charge differences found in these respiratory mucins. The mucins, once separated by ion-exchange chromatography, were further analysed for mucin identification using agarose gels and western blot analysis. HPLC Mono Q column chromatography for mucins was used n our laboratory as a method for mucin analysis for the first time.

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We now plan to widen our studies and include samples from other lung diseases such as TB and lung cancer.

The mucins investigated were MUC5AC and MUC5B, the two major respiratory mucins. MUC2 was also investigated, as it has also been found in the respiratory tract of diseased patients (Davies et al, 1996). Mucin from a patient with pseudomyxoma peritoneii (PMP) was used as a positive control as it was found, in our laboratory, to have all the mucins of interest, namely, MUC5AC, MUC5B and MUC2 (Mall et al, 2007).

Purified PMP mucin was used as an effective positive control and used consistently throughout this study at a concentration of 1mg/ml and a loading of 50μl, which is approximately 50μg, as it was positive for all the respiratory mucins under investigation, namely, MUC5AC, MUC5B and MUC2. The same positive control was used throughout this study to keep consistency and to serve as a standard and also because the membranes were stripped with stripping buffer after each antibody detection to save sample, time and costs (Govender 2006). We could not load a standard concentration of sample, like the Thornton laboratory did, as the concentration of each sample we loaded onto each gel depended on the amount of mucin purified from each sputum sample collected. The reason the concentrations were not consistent was that there were difficulties faced when collecting sputa samples, for example, some of the patients were too tired to cough and when they coughed they produced more saliva than sputa. We did however, have some consistency by loading 50μl of 1mg/ml positive (PMP) control and 50μl of each sample. This allowed us to have equivalent volumes of material loaded on the gel for all groups. Henke (2004) conducted a study on cystic fibrosis secretions where samples were loaded onto gels at either, the same concentration, or volume and the intensity of the detected bands for MUC5AC and MUC5B were similar in appearance. This suggests that the intensity of the mucin smear-bands is not directly related to the concentration of the sample loaded onto the gel. The intensity of the bands in this study is related to the positive control (PMP) used throughout the study, which was loaded at a standard concentration of 1mg/ml. It is hypothesized that a darker smear-band could be indicative of a greater number of epitopes on a single mucin (Henke et al, 2004).

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The amino acid analysis revealed high levels of serine, threonine and proline namely, 35.61 mol % in normal, 36.33 mol % in asthma and 34.42 mol % in COPD group, which is consistent with a

mucin-like macromolecule. The amino acid analysis presented with a high combined value of glycine, threonine, serine, proline and alanine in all 3 groups namely, 52.87 mol % in normal, 54.68 mol % in asthma and 51.92 in COPD group, which is indicative of a typical mucin amino acid profile. The amino acid profile of the mucin in all 3 groups is similar to results shown by Habte et al, 2007 and Govender, (2006) where approximately 51% of the amino acids comprise a typical mucin (Woodward et al, 1982). The individual serine, threonine and proline values did not dominate the amino acid composition and interestingly there were high levels of histidine. These samples were collected during spring (high allergy season), which could be a explanation for the high histidine levels.

MUC5AC was shown in all sputa samples collected, it was present in 5 females and 5 males in the normal group, 7 females and 8 males in those suffering from asthma and 4 females and 11 males in those suffering from COPD. MUC5AC has been reported as one of the major respiratory mucins in respiratory diseases such as asthma and COPD and has also been shown to be present in the airways of normal healthy volunteers (Rose and Voynow 2006; Thornton et al, 2007). MUC5AC presented as a single species in this and previous studies, with a more homogeneous charge distribution than the other respiratory mucins. The intensity of the MUC5AC bands in this study was similar to that of the positive (PMP) control. The intensity and the presence of the MUC5AC bands in the 3 groups were greater than the intensity and presence of the bands for MUC5B and MUC2. This could be that MUC5AC is produced from the lungs first as a defence mechanism or that the patient/volunteer did not cough up enough mucus to produce all 3 mucins at equal intensity (Thornton et al, 2007).

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MUC5B was shown in 2 females and 3 males in the normal group, 6 females and 8 males in the asthma group and 3 females and 8 males in the COPD group. The low charge glycoform of MUC5B that was present in one sample (C55) in this study was similar to that of Thornton (Thornton et al, 1997; Thornton et al, 2000). The positioning of the bands on the gel was the same in these studies. We found the presence of glycoforms without trypsin digestion. It is suggested for future studies that trypsin digestion be used on these reduced purified mucins as it aids in better separation (Thornton et al, 1997; Davies et al, 1999).

MUC2 was only shown in 3 males in the normal group, 3 females and 5 males in the asthma group and 2 females and 5 males in the COPD group. The presence of MUC2 in normal healthy volunteers is

very controversial as the normal healthy respiratory tract has small amounts of MUC5AC and MUC5B and no trace of MUC2 (Thornton et al, 1994). The presence of MUC2 in the airways of normal healthy volunteers, could be due to other influences and irritations to the airways, for example environmental factors. The climate in South Africa and especially in Cape Town could play a serious role in causing distress or irritation to airways of healthy individuals because of the infamous wind "Black South Easter".

The presence of MUC5AC mucin was investigated using an antiserum raised against a synthetic peptide based on the sequence of the MUC5AC apoprotein (Hovenberg et al, 1996). MUC5AC is the predominant mucin in all three groups as it is observed in 100% of all the samples investigated during this study. MUC5B was present in most cases, which confirms that it is another major mucin. More MUC5B was seen in asthma and this could be the reason the mucus is so tenacious. MUC2, when compared to MUC5B and MUC5AC, is a minor mucin as it was present in only a few samples.

This study shows that mucus does not consist of a single substance but is comprised of variable amounts of MUC5AC, MUC5B and to a certain extent MUC2 glycoproteins. There were no significant differences found between the two diseases regarding the presence or absence of the major respiratory mucin MUC5AC, but there was a difference in the presence or absence of the other respiratory mucin MUC5B and to a small extent MUC2. Another approach to determine if there are differences between these groups would be to load known concentrations of sample and to compare the intensity of the bands produced on the gels. The intensity is measured by reflective densitometry, which is not available in this laboratory. It has been suggested by Thornton and Sheehan that MUC5AC is involved in mucociliary clearance of mucus (Thornton and Sheehan 2004).

This thesis will contribute to the field of respiratory medicine as the presence of MUC2 could be used as a marker for the presence of disease whether it is an allergy or a respiratory disease. People who have MUC2 present in their airways should be tested by their physician for any complicating or associating factors. We have also shown that there are no major differences between the 3 groups, but that there are inter-individual variations within these 3 groups, for example, N14, which has the presence of MUC2 could mean that this volunteer is susceptible to allergies and asthma (according to his physician's diagnosis).

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Future work on this study could be to analyse a bigger cohort of sputum samples from each group. Follow up examinations of the normal volunteers that have MUC2 glycoforms associated with TB, if tested positive could confirm that the MUC2 in normal airways is a marker for respiratory disease. The high number of paitents' with MUC5B in asthma could explain why the mucus is so tenacous in these patients.

We have now established a technique to use for the future, the first Mono Q HPLC in this region for investigating the possible mucin glycoforms that may be secreted in a variety of respiratory and other diseases.

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Appendix

A. Consent Information Form

UNIVERSITY OF CAPE TOWN

DEPARTMENT OF SURGERY

2008-03-28

Dear Patient,

We are doing a study on the mucus produced in your lungs. We wish to find out what exactly the mucus is made of and whether it is different in asthma and COPD and normals.

You will be expected to undergo sputum induction which is the removal of mucus by breathing in aerosol saline; this procedure is harmless. We will place the mucus in a container, keep it on ice and use it for our study.

Please note:

1. We will need to take your folder number (NOT YOUR NAME) from which we will get details of your age, gender and origin. All this information will be kept **absolutely confidential** and we promise not to use your sample for any other purpose than that which we have described.
2. You have a right to say that you do not wish to take part in this study and be assured that it will not affect the treatment you receive in hospital.
3. If you agree to participate in this research project now and then decide at a later time that you do not wish to continue, that is also fine. That decision will once again not affect your right to treatment and care. We will **NOT** test for HIV/AIDS.
4. It is possible that we will publish the findings of this study but your name will not be mentioned.

If there are any queries, please refer them to your clinician. If you think of any queries at a later date, please don't hesitate to contact me at the telephone number below.

Professor A Mall

Tel: 021-4066232/6227

Patient information and consent:

I, _______________ give permission that my sputum obtained by sputum induction can be used for research purposes in the investigation of mucus (phlegm) in normals.

Signature: ______________________

Folder Number	DOB	Gender	Disease	Medication
	/ /	M or F	COPD or Asthma	Yes or No

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B. Consent Laboratory Form

REQUEST FOR BIOCHEMICAL ANALYSIS

Research Laboratory
Division of General Surgery
OMB Groote Schuur Hospital
UCT Medical School, Observatory 7925

Tel: (021) 406 6232/6227 Fax: (021) 448 6461

Please fill in all the information requested:

Surname: First Name(s):

Sex: M ☐ F ☐

Date of Birth Year: ______ Month: _______ Day: _______

Ethnic Origin: ______________________________________

Contact Address: Hospital/Clinic where samples are taken

Town: _______________________ Fax: _________________

Tel: _________________

Reason for referral (clinical diagnosis): ………………………..

Additional disorders (apparent or previously treated):

……………………………………………………….

Smoker / Non-smoker ………………..

For Laboratory use only:

Bronchial Lavage number ……………………

Date Received: YY ______ DD ______ Folder No.: _______

CONSENT FOR BIOCHEMCIAL ANALYSIS AND STORAGE

1. I, ____
   give permission that a sample of the bronchial lavage be taken for research purposes in the investigation of mucus (phlegm) in asthma and COPD.

2. I give permission that a portion of the sample be stored indefinitely for:
   a) Research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

3. I authorize / do not authorize my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the researchers.

ALL OF THE ABOVE HAS BEEN FULLY EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED

____

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C. Solutions

6M GuHCl

Guanidinium hydrochloride (287g)

10mM EDTA (1.86g)

1mM PMSF (0.087g) (dissolve in few drops of ethanol)

1M Tris-HCl pH 8.0

Put on heating block to dissolve at 37°C

Make up to 500ml, put on heating block to dissolve at 37°C

Periodic Acid Solution

For 50 tubes

7% acetic acid (10ml)

Periodic acid (20μl)

Decolorize Schiff's Reagent

For 50 tubes

Sodium Metabisulphite (100mg)

In Schiff's reagent (6ml)

Incubate at 37ºC for 30 minutes or until solution is colourless

Lowry Reagents

For 50 tubes

Solution A

2 % anhydrous Na_2CO_3 (2g)

Made up in 0.1M NaOH (100ml)

Solution B

1% CuSO4 (0.25g $CuSO_4$ in 25ml dH_2O)

2% Sodium tartrate (0.5g sodium tartrate in 25ml dH_2O)

Make up to 1:2 for working solution B

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Solution C

1ml solution B to 50 ml solution A

Folin Reagent

Dilute 1 in 2 with distilled water

TBST (Washing Solution)

10mM Tris-HCl (1.21g)

150mM NaCl (8.76g)

0.05% Tween 20 (0.5g)

Adjust to pH 8.0 and make up to 1L with distilled water.

SSC (Transfer Buffer)

To make 20xSSC

Sodium Chloride (176g)

Tri-Sodium Citrate (88.2g)

Adjust to pH 7.0 and make up to 1L with distilled water

Working solution 4XSSC

Dilute 20XSSC 1 in 5 with distilled water

1% Blocking Buffer

In 100ml

Fat free milk powder (1g)

1x TBST (100ml)

Non-Reducing Sample Application/Loading Buffer:

Glycerol (40-50%)

Bromophenol blue

In 1x TAE

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50xTAE Buffer

Tris base (242 g)

Glacial acetic acid (57.1 ml)

0.5 M Na_2 EDTA (100 ml)

Adjust pH to 8.0 and make up to 1L with dH_2O

1xTAE Buffer

50x TAE (20ml)

10% SDS (10ml)

Make up to 1L with distilled water.

10% SDS

10g SDS

In 100ml dH_20

Stripping Buffer

100mM mercaptoethanol (149μl)

2% SDS (20ml of 10% SDS)

0.5M NaCl (62.5g)

Make up to 100ml in 1M Tris-HCl pH 6.7

Start Buffer for HPLC

In 500ml

6M Urea (180.10g)

0.05% CHAPS (0.00025g)

10mM Piperazine (0.4307g)

Elution Buffer for HPLC

In 500ml

6M Urea (180.10g)

0.05% CHAPS (0.00025g)

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10mM Piperazine (0.4307g)

0.4M Lithium Perchlorate (21.278g)

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