THE BIOCHEMICAL AND MOLECULAR CHARACTERISATION OF RESPIRATORY MUCINS IN TB

URESHNIE GOVENDER

BSc (Hons)

Thesis submitted in fulfillment of the requirements for the degree of

Master of Science in Medical Biochemistry

In the division of Surgical Sciences

Department of Surgery

Faculty of Medicine

University of Cape Town

February 2006

WITH ALL MY LOVE

TO

MUMMY, DAD, LUCIAN, NEVS

AND LINUS

PREFACE

This study was carried out from 2004 to 2006 under the supervision of Professor A.H.S. Mall in the Division of Surgical Sciences, University of Cape Town.

I hereby certify that these studies are a true representation of my own work and have not been duplicated in any other form for a degree at another university. Where use has been made of the work of others, it has been duly acknowledged in the text.

Signed by candidate

Ureshnie Govender
February 2006

Signed by candidate

Prof. A.H.S. Mall
February 2006

ABSTRACT

The association of mucus and mucin hypersecretion with respiratory diseases such as COPD, cystic fibrosis and asthma has long been known. The role of the dominant respiratory mucins (MUC5AC and MUC5B) and MUC2 has been investigated in chronic airway diseases as it is the mucin glycoprotein that confers upon mucus its biological, rheological and physicochemical properties. Tuberculosis (TB) is a highly contagious respiratory disease that spreads via the pathogen *Mycobacterium tuberculosis*. TB is associated with diseases such as bronchiectasis and bronchorrhea whereby copious amounts of sputum (diseased mucus) are produced. According to the World Health Organization (WHO), stringent methods are required to curb the spread of TB as the HIV pandemic and the demographics of varying infected populations are strongly influencing the transmission and lack of resistance to TB. Within South Africa, specifically the Western Cape, TB has wreaked havoc especially amongst those of the lower socio-economic groups. However, despite the prevalence of the disease in South Africa and the known morbidity and mortality associated with mucus and mucin hypersecretion in respiratory diseases, little is known of the association between respiratory mucins and TB.

This is a novel study that investigated the association between respiratory mucins and TB at a biochemical and molecular level. The biochemical study involved the collection of sputum from healthy individuals, post mortem cadavers and suspected TB positive adults and children. Antibody detection was carried out on of crude mucin samples as it was proven unnecessary to purify the samples via CsCl ultracentrifugation. This resulted in a substantial reduction in expense and time as CsCl purification is a lengthy, expensive procedure.

MUC5B was identified in both the healthy controls and TB positive adults and children. In contrast MUC5AC was barely evident in the controls but presented in both the TB positive adults and children. MUC2 barely featured in the control group but was identified as glycoforms in the TB positive adults and children. The low charge doublet

glycoform appeared more distinctly in the 'possible' TB state and the high charge smear appeared more distinctly in the probable to definite TB stage. MUC5AC and MUC5B did not present as glycoforms.

An amino acid analysis presented with high values of glycine, threonine, serine, proline and alanine in the healthy controls. This was suggestive of a typical mucin amino acid profile. The combination of glycine, threonine, serine, proline and alanine were much lower in the PM group and TB adults and children. Differences in the amino acid composition were related to differences in the level of secretion of varying mucin types during a healthy and diseased state. The individual serine, threonine and proline values did not dominate the composition of the protein backbone. This was explained by incomplete deglycosylation prior to amino acid analysis. The combined serine, threonine and proline (STP) content and the serine and threonine combination predominated in the healthy volunteers (22.38 mol%). The TB positive adults and children presented with lower yet similar results (18.37 mol% and 17.17 mol% respectively). It is likely that the minor differences between the control group and TB positive group could be secondary to the TB mycobacteriums interaction with the sugar side chains.

In addition, molecular investigations were undertaken for the presence of known polymorphisms in the mucin genes, MUC2 and MUC5B, and the informativity of such polymorphisms in the South African population. Both the MUC2/PvuII polymorphism in exon 11 of the MUC2 gene and the VNTR of intron 36 of MUC5B were identified in the study sample. Generally the polymorphic C allele of MUC2/PvuII occurred less frequently than the G allele. Categorisation of the sample on the basis of TB status showed a higher C allele frequency in TB positive cases and revealed that allele C homozygotes were found only amongst TB positive cases (30%).

The MUC5B alleles represented variable numbers of the 59bp repeat sequence within intron 36. This differed from the European study as a greater number of repeats were identified in the Western Cape population. In comparison to the European study of Dessyn *et al* (1999) it appeared that the frequency of the 8 repeating unit was higher in

the Western Cape population whereas the 7 repeating unit dominated in the European population. Furthermore the 10 and 3 repeating unit found of this study did not present in the European study, and the 4 repeating unit of the European study was absent in this study group. The 10 repeating unit was of interest as it was seen only in a TB negative individual and not in the TB positive group. The 8/8 allele combination common to the study group presented predominantly in the TB positive individuals.

ACKNOWLEDGEMENTS

To my supervisor Professor A. Mall, thank you for your guidance and advice during the experimental and writing phase of this thesis. It has been a marvelous experience during which I have grown not only knowledge wise but as an individual.

To Prof Kahn, I will forever be grateful to you for your encouragement and belief in me. Your kindness and support has been unforgettable.

To Dr Robea Ballo for your endless patience, selflessness, and criticism during the writing of my thesis. Furthermore, for being a brilliant geneticist. I hope that a simple thank you suffices as words fail me.

I am grateful to my collaborators Dr Vanessa Burch of G.F. Jooste hospital and Dr Heather Zar of The Red Cross Childrens hospital for playing vital roles in the collection of samples and clinical data.

To Dr Alistair Hunter and Dr Andre Hendrikse of the Department of Radiobiology for their assistance during the ECL work. Working with you guys has been both insightful and absolute fun.

To Dr Jerry Rodrigues of the School of Molecular and Cell Biology for his help in the amino acid analysis.

To Dr Neil Ravenscroft and Dr Meredith Hearshaw for the attempts made at analyzing the sugar content and composition of the mucins.

Thank you to Prof D Swallow, Dr Sara Kirkham and Dr Annkatrin Herrmann for kindly supplying the polyclonal antibodies and for the technical advice.

I am forever grateful to the SAMRC, Cannon and Collins and UCT for the scholarships and bursaries afforded me during the last two years. The financial relief was a blessing that allowed me to focus on my work.

To Dominique Pepper of G.F. Jooste, Sr Nchukana of Red Cross Childrens Hospital, Mariette Ross of the UCT Lung Institute and Dr Denise Lourens and the Pathologists at the Salt River Morgue for their assistance in collecting mucus samples.

To Nathalie Christians of G.F. Jooste for analysing the samples for the TB diagnosis.

To Gama Bandawe, Michelle Parker and Gemma Carvill for going the extra mile in assisting during the molecular aspect of my work. Thank you for your time, kindness and insight.

Thanks to Tarquin and Micheal Wyeth of Imago Visual (Medical graphics) for editing of figures within this thesis and for creating my posters for conferences. It has been an absolute pleasure working with you.

A very special thanks Zoe Lotz and Marilyn Tyler, my mothers in the lab. Thank you for your motivation, insight, strength, love and wealth of knowledge throughout my Masters and for always watching over and taking care of me.

Thank you to Habtom Habte for being a wonderful big brother. Your wise words, laughter and support will forever be treasured. Thank you for opening my eyes and mind to the world of politics.

To my family and colleagues within the Department of Surgery, Gert Engelbrecht, Lizette Fick, Jason Lewis, Erika Jansen van Rensburg, Ntato Chirwa and Astrid Trimmel. Thank you for your support and assistance during my thesis and for beautiful memories filled with fun and laughter.

Thank to the secretaries within the Department of Surgery for their assistance and efficiency: Ayeesha Isaacs, Michelle Ahsing, Brenda Fine, Yasheera Kannemeyer, Lwundlekazi Mavula, Mrs Sanger, Yvonne Monro.

Thank you to Adri Winckler and the postgraduate team. I wish that administrative teams would take a leaf out of your book.

To Prof Dennison, Dr Elliot and Dr Mervyn Beukes of the University of Kwazulu Natal. Thank you for kindness and insight during my academic years at The University of Natal and during my Masters at the University of Cape Town.

To My Medical residence family: Gaysheen Kistan, Tarang Peedikayil, Lloyd Joubert, Cornell Enssle, Louisa Basso, Doris Kleyenstuber, Nicholas Coetzer, Tanya Toussaint, Faatima Ahmed, Jason Ensor and Rhoan David, for their never ending support, encouragement, love and unforgettable memories.

To my girls Anja Reuter and Minerva Titus. Your strength, love, support and belief in me has been phenomenal. Thank you for sharing my burdens, for celebrating my achievements but most of all for never letting me give up.

To Gail Papli, Avishkar Jayraj, Sheldon Chetty, Ike Achilounu, Karabo Motsamai, Eulashnini Chuthunpersad, Kim Munisamy, Aletia Naidoo, Yegan Pillay, Tad Dorasamy, Lynette Mariah, Adrian Christian and Natasha Papli. Thank you for your faith and support in me and for teaching me that friends can also be family.

A special thank you to Jessica Subramani without whom I would never have had the courage to embark on this journey. Your love and belief in me gave me the strength to make it through to the end.

To uncle Caps and Aunty Raj and family, Aunty Selvi, Aunty Niri, Aunty Vani, Tivania and family, Kuben, Melissa, Ajay and family and to my dear friends Yohannes Ghebramariam and Gillian Christians. I thank you for your love, support and never ending faith in me.

To my family members: Rhumba, Uncle Mike, Reggie, Kuvesh, Rowan, Bash, Sooren and family and Shantham and family. I am beyond blessed to have love such as yours. Your unwavering faith, support and confidence in me gave me the strength to complete this thesis.

To the best grandparents in the world. Thank you for your prayers, love and support now and always.

To Linus, thank you for blessing me with your patience, moral support, love, strength, and insight and for sharing in the ups and downs of this thesis till the very end.

To my mother, father, Lucian and Nevelyn. This thesis is for you! I thank you for your undying love and support throughout everything I have achieved in life. I would not be who I am today if it were not for your guidance and backing. I love you dearly.

Lastly, I thank God for always watching over me and for giving me the faith, strength and support that allowed to complete this thesis.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AMPS	Ammonium persulfate
BCB	Brilliant Coomassie Blue
Bp	Base pairs
Bis	*NN'*-methylenebisacrylamide
BSA	Bovine serum albumin
cDNA	copy Deoxyribonucleic acids
CF	Cystic fibrosis
CsCl	Caesium Chloride
CsBr	Caesium bromide
COPD	Chronic obstructive respiratory disease
CXR	Chest x-rays
DNA	Deoxyribonucleic acids
dNTP's	deoxynucleotide triphosphates
DPB	Diffuse panbronchiolitis
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
HCl	Hydrochloric acid
HF	Hydrogen fluoride
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
Na_2-EDTA	Ethylenediaminetetra-acetic acid disodium salt
DNA	Deoxyribo nucleic acid
GuHCl	Guanidinium hydrochloride
MEC	Molecular Exclusion Chromatography
MWM	Molecular weight marker
NANA	N-acetylneuraminic acid
NEM	*N*-ethylmaleimide
PBS	Phosphate buffered saline
RFLP's	Restriction fragment length polymorphisms

PCR	Polymerase chain reaction
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS–PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
PTS	Proline Threonine Serine
TBE	Tris Borate buffer
TBST	Tris buffered Saline Tween
TFMS	Trifluoromethane sulfonic acid
UV	Ultraviolet
VNTR	Variable number of tandem repeats
(vWF)	von Willebrand factor
WHO	World health organisation

CHAPTER ONE

INTRODUCTION

1.1 Mucus secretions and distribution

Mucus has played significant roles in the vertebrate and invertebrate kingdom. This sticky, visco-elastic gel has served man and animals throughout the evolutionary process as the primary line of defence against both external and internal irritants (Moniaux *et al.*, 2001, Davies *et al.*, 2002, Rose and Voynow, 2006). It has acted as a lubricant preventing dessication in slugs, snails and earthworms (Negus, 1967) and has assisted amphibians and eels during respiration by allowing for gaseous exchange through the skin. It has further served invertebrates by acting as a distasteful external coating protecting them from predators and is known to have antibacterial properties in the secretions of oysters and acorn worms. Slugs have exploited the gel-sol properties of mucus thereby allowing for their motility (Denny, 1989) and a mucus layer in the frog palate allows, via the beating of cilia, for the movement of food to its gastrointestinal tract (Silberberg *et al.*, 1977). Mucus also provides nutrition to lower life forms. This situation is evident when organic material is trapped in the caudal mucus plug of a slug (Denny, 1989). With respect to more evolved life forms, such as man, mucus is found to lubricate the epithelial lining of the gastrointestinal, genitourinary and respiratory tracts (Taylor *et al.*, 2005). It has also been found in reduced quantities in the middle ear and lachrymal glands (Fitzgerald *et al.*, 1987) and in a soluble form in human gallbladder bile (Pearson *et al.*, 1982).

1.2 The general role of mucus

Mucus a crude native secretion primarily protects the internal tracts of the body creating an interface or barrier between the epithelial surfaces within the host and its internal agents and the external environment. It is further specialised in function depending on the organ within which it is found.

1.2.1 Gastric mucus

A thick, stable layer of unstirred mucus lines the gastric epithelia and in doing so shields the mucosa (Venables, 1986) from strong acids, proteases and the injurious action of food or foreign material (Gevers, 1987, Allen and Flemstrom, 2005, Taylor *et al.*, 2005). The mucus bicarbonate barrier in particular is responsible for the neutralization of luminal acid and acts as a barrier to pepsin digestion of the underlying gastric epithelia (Allen and Flemstrom, 2005). Degraded gastric mucus in the gastric juices, serves to enhance the lubrication of food particles and gastric mucins buffer the stomach against a drop in pH and proteolytic enzymes (Taylor *et al.*, 1998).

1.2.2 Intestinal mucus

Intestinal mucus acts as a barrier to invasion by pathogenic micro-organisms by interacting with, aggregating or binding bacteria. It is known to increase in volume, in response to the presence of toxins and parasites, preventing them from attaching to sites on the epithelial lining (Neutra *et al.*, 1987). IgA within intestinal mucus is known to act as a barrier to *Candida* colonisation (Bai *et al.*, 2004). Mucin carbohydrate side chains of the insoluble mucus barrier are hypothesized to compete with pathogens for epithelial sites hence minimising or preventing colonisation (Allen *et al.*, 1990). The mucus barrier further serves to protect the colonic epithelium against mechanical injury (Taylor *et al.*, 2005) from hard faecal matter and provides a conducive environment and food source for natural colonic bacteria (Hoskins, 1978).

1.2.3 Gallbladder mucus

Mucus of the gallbladder protects the epithelium from the action of surface active chemicals (Gevers, 1987). Biliary mucins are suspected to be involved in the formation of gallstones. These mucins are located in the centre of gallstones and are believed to participate in the initial phase of gallstone development by a possible hydrophobic binding to cholesterol or interfering with the removal of seed crystals from the gallbladder (Baeckstrom, 1994).

1.2.4 Cervical mucus

The viscosity, water and protein composition of cervical mucus alters depending on the phase of the menstrual cycle (van Kooij *et al*, 1980). The levels of the hormones progesterone and oestrogen also vary during the menstrual cycle. These changes play vital roles during fertilization as the motility of spermatozoa is dependent on the viscoelasticity of the cervical mucus and the level of two hormones (Yurewicz *et al.*, 1987, Argueso *et al.*, 2002). During ovulation, oestrogen increases resulting in a more hydrated and less viscous mucus solution which is conducive to the transportation of spermatozoa. The reduction in viscosity could be attributed to the hydrophilic nature of mucin carbohydrates, which increase during the ovulatory phase and in doing so retain water in the endocervix (Wiggins *et al.*, 2001, Argueso *et al.*, 2002). The luteal phase results in the dominance of progesterone hence a less hydrated, more viscous mucus gel which greatly reduces the motility of the spermatozoa through the cervical mucus plug (Wolf *et al.*, 1977, Wiggins *et al.*, 2001).

1.2.5 Salivary mucus

Mucus assists in the mastication of food allowing for bolus formation (Gevers, 1987). Salivary mucins confer upon mucus its antimicrobial properties. They also behave as competitors for receptors on the buccal epithelium attempting to reduce colonisation by *Streptococci.* With the assistance of secretory IgA, salivary mucins serve to trap and aggregate infecting organisms (Kashket *et al.*, 1972, Bobek and Situ, 2003) hence allowing for phagocytosis by macrophages, the products of which are washed away by saliva (Neutra *et al.*, 1987). They are known to interact with and trap respiratory, cariogenic, and periodontal pathogens as well as *Candida albicans* and HSV and HIV-1 viruses. It has been proposed that human salivary mucin, MUC7, poses as a potent agent for antifungal therapy (Situ *et al.*, 2003, Wei and Bobek, 2005).

1.2.6 Respiratory Mucus

Tracheobronchial secretions exist in a gel and sol phase indicative of a double layer of mucus (Kim, 1997). The sol phase, in a normal state, forms a 2-5μ thick blanket that

extends from the alveoli to the trachea (Kaliner *et al.*, 1986). The gel phase (or superficial blanket) traps airway irritants allowing for their easy removal with the beating of cilia which exist within the sol phase (Gevers, 1987, Kim, 1997). This is termed mucociliary clearance and is one of the various roles of airway mucus (Leikauf *et al.*, 2002). It was hypothesized that the mucus blanket acts as a barrier preventing the transudation of the serum through the mucosa (Negus 1963, Negus 1967, Rogers, 2005). Respiratory mucus further defends the airways as a lubricant, humidifier, a barrier to destructive foreign organisms, and a neutralizing agent against toxic gases and provides a site for immunoglobulin action. This tenacious viscoelastic slime stems from the goblet cells of the respiratory tree (to the level of the alveolar ducts) and submucosal glands of the respiratory tract (which exists between the nose and the cartilaginous sector of the airways).

1.3 Composition of respiratory mucus

Mucus in general is a mixture of secreted proteins such as immunoglobulin A, lactoferrin, lysozyme and transudated proteins specifically albumin (Gevers, 1987). Water makes up 95% of mucus, salts and dialyzable components are approximately 1% and free proteins and mucin glycoproteins are in the 0.5-1% range. In airway diseases, mucus is comprised of the above mentioned components together with dead bacteria and leucocytes, DNA and lipids (Creeth, 1978, Kim, 1997). The water content of mucus is of paramount importance to the functioning of the mucus blanket. It has been illustrated that dehydration in birds resulted in a thicker more condensed mucus blanket which lead to a reduction in efficiency in particle transport of the nasal mucosa. A similar situation was discovered in dehydrated patients with chronic obstructive lung disease. Rehydration was found to clear the respiratory tract and reduce the viscosity of the sputum (Yeager, 1971). Immunoglobulins, specifically IgA, assist in immune function. IgG and IgM are also present in mucus secretions but IgA appears to play the pivotal role as it protects against various infections. Immunoglobulin IgE has been associated with allergic bronchial asthma and a lack thereof may increase ones susceptibility to infections. In general it appears that mucosal antibodies afford antimicrobial protection against various recurring infections. The antimicrobial activity of the mucus blanket is

further strengthened by the action of lactoferrin and lysozyme. Lactoferrin chelates iron thereby preventing bacterial growth and lysozyme hydrolyzes bacterial cell walls (Yeager, 1971).

Sputum, a pathological form of respiratory mucus, consists of bronchial mucus together with saliva, transudated serum proteins and inflammatory and desquamated epithelial cells (Kim, 1997). Investigations into nonpurulent chronic bronchitis sputa revealed the presence of various mucin glycoproteins in the gel phase (Davies *et al.*, 2002, Yeager, 1971). Mucins are considered the main components of mucus as they confer upon mucus its rheological and physicochemical properties.

1.4 Structure and composition of mucins

Mucins determine the viscoelasticity of mucus and in turn determine the mucociliary action of the mucus blanket (Rogers, 2004). They are high molecular weight glycoproteins which range in size from a hundred to several thousand kilodaltons. Structurally they are composed of a peptide backbone or apomucin which is heavily glycosylated with O-linked oligosaccharides (50-80% of the mucin weight) (Figure 1.1) (Voynow, 2002). The composition and length of the oligosaccharides determines the size and charge density and thus the heterogeneity of mucins (Kim *et al.*, 2003). The apomucin does have nonglycosylated regions which appear rich in cysteine. This is indicative of either inter or intra molecular disulphide bonding which results in the mucin polymers (Figure 1.1). The glycosylated regions have been described as taking on a bottle brush structure; the oligosaccharides represent the bristles and the apomucin the wire support (McLeod, 1992). Mucins, depending on the investigators, have been illustrated as two separate models. The windmill model, proposed by Allen (1978), claims that mucins are lengthened rod like structures that polymerize to form larger rods representative of windmills; which at high concentrations form a viscoelastic gel (Gevers, 1987). This structure was challenged by Carlstedt and Sheehan (1984) who proposed that mucins are linear peptides containing glycosylated and non-glycosylated regions which alternate to form subunits. These subunits are held together by disulphide bonds which result in flexible structures (McLeod, 1992). Electron microscopy of purified bronchial

mucins describes them as being "extended filamentous polypeptides which are somewhat stiffened by the presence of numerous carbohydrate side chains" (Slayter *et al.*, 1984).

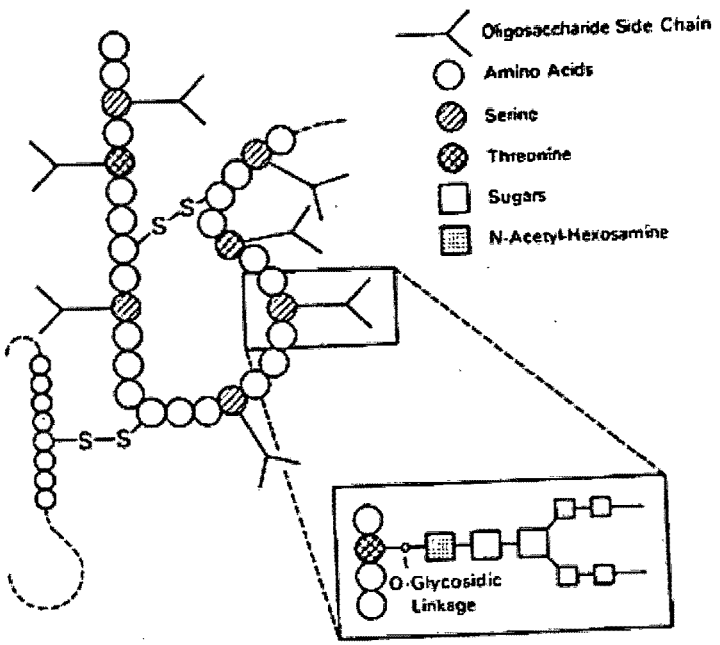


Figure 1.1 A schematic diagram of a human mucin glycoprotein illustrating the protein backbone and carbohydrate side-chains. The circles represent amino acids that make up the apomucin. Theonine and serine are the points at which O-glycosylation takes place within the tandem repeat. (-S-S-) represents inter and intra chain disulfide linkages. (Kaliner *et al.*, 1986).

1.4.1 Carbohydrate side chains

Human mucins in general comprise of the monosaccharides fucose, galactose, N-acetylneuraminic acid (sialic acid), N-acetylglucosamine and N-acetylgalactosamine. Each oligosaccharide varies in length between 1-20 monosaccharides. The terminal ends of oligosaccharides are identified by the presence of fucose, galactose, N-acetylneuraminic acid and N-acetylglucosamine (Lamblin *et al.*, 2001). The O-linked glycoproteins, specifically secreted or membrane proteins, are identified by an α-linkage of N-acetylgalactosamine to the hydroxylated amino acids serine or threonine (Piller and Piller, 1993, Carlson, 1977). N-acetylgalactosamine attaches specifically to the serine or threonine amino acids of the apomucin (Carlson, 1977). These types of O-glycans constitute approximately 90% of mucins (Piller and Piller, 1993).

The function of O-linked sugars remains unknown but it is speculated that they are involved in the secondary structure of heavily glycosylated glycoproteins (Piller and Piller, 1993). The presence of N-acetylgalactosamine alone results in the mucin polypeptide unfolding from a globular conformation to a linear structure. This extended, linear conformation in turn is vital for the gel formation of mucins, hence adding to their protective, hydrating nature (Hayase *et al.*, 1993, Piller and Piller, 1993). Sialic acids, specifically N-acetylneuraminic are biologically significant constituents of mucins (Spyridaki *et al.*, 1996). Sialic acid together with sulphate is responsible for the acidic nature of bronchial mucins as well as their negative charge (Kaliner *et al.*, 1986). Histochemical investigations have highlighted increases in the amounts of sulphated mucins during airway diseases (Buisine *et al.*, 1999). Bronchial mucins are highly glycosylated with marked heterogeneity which is resultant of the variation in oligosaccharide length, composition, structure, branching and acidity. This heterogeneity was illustrated when 88 varying carbohydrate chains were isolated from respiratory mucins of a single individual of blood type O. The terminal ends of human airway mucins also appear to have special features as they can act as receptors for various cells of the airway; the terminal sugar NeuAcα2-3Galβ1-3GalNAc behaves as a receptor for macrophage sialoadhesin. Airway mucin carbohydrates appear to present with epitopes that are possible sites of attachment for microbes and viruses. In providing such an

attachment they are believed to protect underlying mucosae (Lamblin *et al.*, 2001, Voynow, 2002).

1.4.2 Peptide backbone

The apomucin is a product of MUC genes (Rose *et al.*, 2001). It is approximately 20% of the glycoprotein and is comprised primarily of the amino acids proline, threonine and serine (PTS) (Kaliner *et al.*, 1986). O-glycosylated mucins share a common protein core which is characterised by a tandem repeat rich in PTS's (Rose *et al.*, 2001). The tandem repeat is the largest domain or region (Moniaux *et al.*, 2001) within a mucin molecule and is the site at which O-glycosylation takes place (Hovenberg *et al.*, 1997). The tandem repeat along with its O-glycans determines the size, mass and shape of mucins adding to their rheological, biological and physicochemical properties of mucins (Rose and Voynow, 2006). Due to heavy glycosylation these regions are protease resistant (Perini *et al.*, 1989). The non-glycosylated or naked regions are rich in cysteine (Wickstrom *et al.*, 1998) as they represent the disulphide bonds which hold the mucin subunits together. In addition to the PTS, human bronchial mucins are further dominated by glycine and alanine which altogether account for 50-60% of the protein backbone (Bhattacharyya *et al.*, 1990).

Apomucins vary according to the "amino acid sequence, the size and number of repeats between MUC genes and differs between human and rodent homologues of the same MUC gene". The varying and unique amino acid sequences determine the pattern of O-glycosylation, hence the heterogeneity between mucins (Voynow, 2002). Bronchial apomucins have been documented to share similarities with intestinal apomucins; their tandem repeats are rich in threonine and proline residues (Gerard *et al.*, 1990, Perini *et al.*, 1989). Similar apomucins are also common to glycoforms of mucins. MUC5B, a dominant gel forming mucin in the respiratory tract, has been documented to have a high and low charge glycoform (Wickstrom *et al.*, 1998).

1.5 Mucin genes

The mucin gene family consists of 20 members numbered according to their description (MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6-9, MUC11, MUC12, MUC13, MUC15-19, and MUC20 (Rose and Voynow, 2006). Of the 20 members, MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, MUC8, MUC11, MUC13, MUC15, MUC 19 and MUC20 have been detected at the mRNA level in the lower airways of healthy individuals (Rose and Voynow, 2006). MUC1, MUC2, MUC4, MUC5AC, MUC8 and MUC13 are expressed in the surface epithelium; the goblet cells produce mainly MUC5AC. MUC1, MUC2 and MUC8 are also expressed by the submucosal glands together with MUC7, MUC19 and the dominantly expressed MUC5B (Davies *et al.*, 2002, Rose and Voynow, 2006).

Recombinant DNA technology has categorised the 20 mucins into 3 groups depending on their structural similarities (Rose and Voynow, 2006). The secreted or gel forming mucins are MUC2, MUC5AC, MUC5B, MUC6 (Davies *et al.*, 2002) and the recently discovered MUC19 (Rose and Voynow, 2006). MUC7 is classified as a soluble mucin which is secreted but not gel forming mucin (Davies *et al.*, 2002) and MUC1, MUC3A, MUC3B, MUC4 MUC12, MUC13, MUC15-17, MUC18 and MUC20 are the membrane bound mucins (Moniaux *et al.*, 2001, Rose and Voynow, 2006). MUC 11 has also recently been associated with the membrane bound mucins (Davies *et al.*, 2002). According to Davies *et al* (2002), MUC8 is a membrane bound mucin. Rose and Voynow (2006) describe MUC8 as a secreted mucin that lacks the von Willebrand factor.

1.5.1 Soluble mucins

MUC7 is a secreted, non-cysteine rich mucin incapable of gel formation due to its simple structure (Voynow, 2002) (Figure 1.2). Its cDNA encodes for a 39kKDa protein comprising of 377 amino acids. It has about 6 or 7 tandem repeats each of which have 23 residues and appears to have just two cysteine residues both of which are found near the N-terminus of the molecule. MUC7 is a potent antimicrobial agent in the oral cavity. It has been reported to interact with a variety of oral micro organisms such as *Pseudomonas aeruginosa*, and is believed to be involved in the binding and inhibition of HIV-1 thereby

preventing oral transfer of the virus. These properties have been associated with the activity of the two cysteine residues. Furthermore, MUC7 has a histatin-like domain associated with anti-candidal properties (Moniaux *et al.*, 2001).

1.5.2 Membrane bound mucins

Membrane bound mucins are localized within the cell membrane (Rose and Voynow, 2006). The membrane bound mucins or cell associated mucins are composed of two domains: hydrophobic or transmembrane domains, which span the length of cell membranes, and mucin or cytoplasmic domains (Figure 1.2). MUC1 (Figure 1.2) was the first member of the group hence the most well defined membrane bound mucin (Davies *et al.*, 2002). MUC3, MUC4 (Figure 1.2) and MUC12 lie in a cluster on chromosome 7q22. The membrane bound mucins are further subdivided into four groups namely the membrane anchored form, the soluble form, the secreted form and those that lack the characteristic tandem repeat (Moniaux *et al.*, 2001). The soluble form is a result of proteolytic cleavage of the membrane bound form. The secreted form and the form lacking the tandem repeat are similar in that both have alternative splicing variants. MUC3 and MUC4 are known to exist as splice variants which lack the transmembrane domains and hence give rise to soluble forms of the two mucins (Davies *et al.*, 2002).

In general MUC1, MUC3, MUC4, MUC8, MUC11 and MUC12 are expressed at the epithelial surface by virtue of their transmembrane domains. MUC1 and MUC4 appear to extend further into the luminal surface in comparison to the other mucins; and MUC3 and MUC4 contain epithelial growth factor (EGF)-like domains in the extracellular portion of the transmembrane subunit (Davies *et al.*, 2002).

1.5.3 Secreted mucins

Secretory mucins are housed within granules or vesicles and are released at the apical surface of the cell when stimulated by secretagogues (Rose and Voynow, 2006). MUC2, MUC5AC, MUC5B (Figure 1.2) MUC6 and MUC19 are the secreted mucins or gel forming mucins. Their genes are located on chromosome 11p15.5 and appear to code for similar amino acid sequences which result in (Moniaux *et al.*, 2001) large oligomeric

configurations (Davies *et al.*, 2002). Their oligomeric structure is vital for gel formation (Voynow, 2002). Gel forming mucins are susceptible to solubilisation using chaotropic agents such as guanidinium chloride (GuHCl). Colonic MUC2 and salivary MUC5B are the exceptions as they strongly resist solubilisation in GuHCl. Thiol reduction of MUC2 produces mucin monomers and a range of oligomers suggesting that the MUC2 apomucin is held together by disulphide bonds and a yet unknown "reduction resistant-linkage" (Davies *et al.*, 2002). The secreted mucins share a sequence homology with the von Willebrand factor (vWF); they have cysteine rich domains at the amino and carboxyl termini which serve as sites for oligomerisation (Figure 1.2) (Voynow, 2002). They also have cysteine rich sequences interspersed within the apomucin (Figure 1.2) (Davies *et al.*, 2002). They are known to complex with other mucins and proteins via thiol, ionic and hydrophobic interactions (Rose *et al.*, 2001).

MUC5AC and MUC5B exist in a range of size (2-30x10^6 kDa) in the respiratory and cervical tracts. Disulphide cleavage of the two MUCs yields mucin subunits (2-3 x10^6 kDa) indicative of mucin monomers, and proteolytic digestion of reduced subunits yields protease resistant mucin domains (3-5 x105 kDa). According to electron microscopy intact MUC5AC mucins are long, linear threads composed of 18 monomers. MUC5B appears as a mass of entangled filament like structures (Davies *et al.*, 2002).

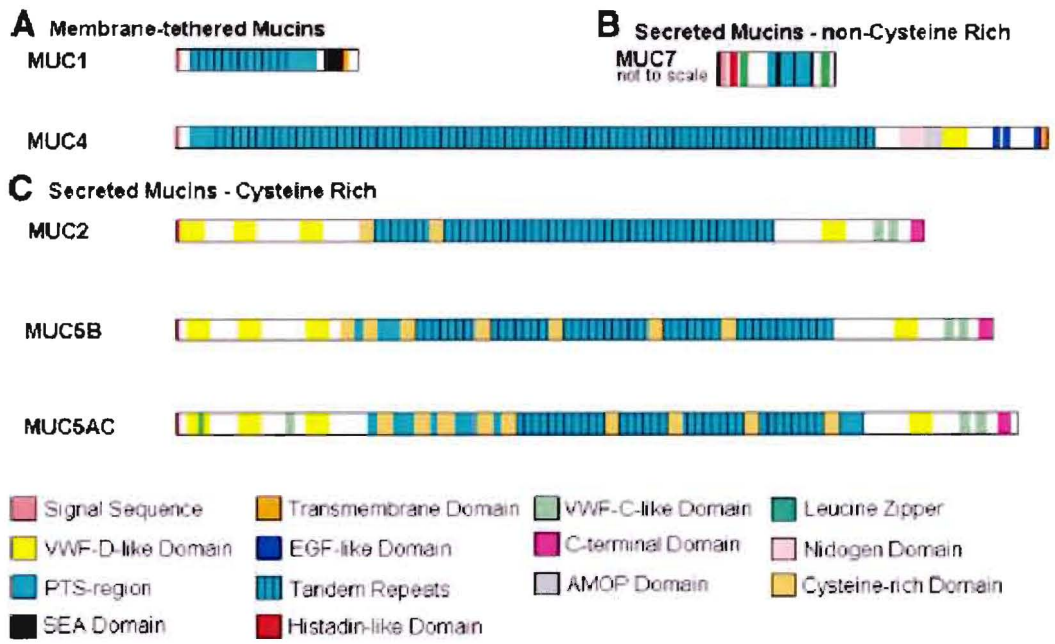


Figure 1.2 Schematically illustrates the majors domains within the soluble, membrane bound and secreted mucins. A. Membrane tethered mucins represented by MUC1 and MUC4, B. Secreted, noncysteine rich mucin represented by MUC7 and C. Secreted cysteine rich mucins represented by MUC2, MUC5AC and MUC5B. PTS, Proline Threonine Serine; SEA, Extracellular domain associated with O-glycosylation, EGF, Epidermal growth factor like domain, VWF, Von Willebrand factor; AMOP, adhesion associated extracellular domain. (Rose and Voynow, 2006)

1.6 Characterisation of mucins

Mucin characterisation allows one to differentiate between varying mucin types and to determine the presence of specific and dominant mucins in varying secretions. It further provides insight into the presence of novel mucins and mucin glycoforms. Physical methods were employed in characterisation studies but had proven to be challenging due to the large molecular weight of glycoproteins, their polydispersity and increased viscosity in solution. The tenacious nature of mucins added to this challenge via intermolecular forces which enabled mucins to readily form gels at moderate concentrations and to strongly adhere to supporting media (Holden *et al.*, 1971).

Methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) appeared promising during initial mucin studies but were soon found to have some limitations. SDS-PAGE gels are based on the principle that proteins are separated depending on their size. This principle falls short when attempting to characterise large hydrodynamic mucins. The polyacrylamide gel is incapable of separating high molecular weight mucins such as MUC5AC, MUC5B and MUC2, the movement of which through a gel becomes retarded resulting in the mucins remaining in the stacking gel or at the beginning of the running gel. SDS-PAGE gels were first modified into SDS-agarose gels (Tytgat *et al.*, 1995). Holden *et al* (1971) had studied the effect of physical degradation and disulphide bond cleavage on canine tracheal mucins by incorporating 1-2% agarose into a 7.5% acrylamide gel system. The high porosity of the agarose facilitated entry of the large molecular weight mucins. Mucin studies such as those by Carlstedt, Thornton and Sheehan, throughout literature, illustrates the advantage in using agarose gels without acrylamide during mucin characterisation (Sheehan *et al.*, 1996, Thornton *et al.*, 1996). SDS-PAGE gels are used to determine the purity of CsCl purified mucins; the gel should appear protein free with purified mucins remaining at the origin of the running gel.

1.7 Respiratory mucins

Characterisation of a normal human adult lung revealed the presence of 12 MUC genes identified in terms of their mRNA. These MUCs are MUC1, MUC2, MUC4, MUC5AC,

MUC5B, MUC7, MUC8, MUC11, MUC13, MUC15, MUC 19 and MUC20. Their expression is hypothesized to vary during development (Lamblin *et al.*, 2001). In the normal adult lung they are expressed between the upper and lower respiratory tract and are hence specific to certain cell types. MUC1, MUC2 and MUC4 are found in all superficial airway epithelial cells (Voynow, 2002). Mucin gene regulation studies were initially carried on the MUC2 cDNA and promoter as they were present before the MUC5AC and the MUC5B genes were cloned and characterized (Rose and Voynow, 2006). MUC2 is generally expressed at low levels, specifically during bacterial invasion (Kim *et al.*, 2003). *Pseudomonas aeruginosa*, a known pathogen of cystic fibrosis, activates NFκB in the MUC2 promoter hence inducing MUC2 expression (Lamblin *et al.*, 2001, Leikauf *et al.*, 2002) which is followed by the up regulation of MUC5AC (Lamblin *et al.*, 2001). As previously mentioned MUC7 is expressed in serous cells of the submucosal gland, MUC5AC is expressed in the goblet cells and MUC5B in the submucosal glands. Antibody detection has identified MUC5AC and MUC5B as the dominant glycoproteins in a healthy respiratory tract (Voynow, 2002, Lamblin *et al.*, 2001). Irritated or diseased airways have also illustrated the presence of MUC5AC, MUC5B and at times MUC2 in their secretions, with either MUC5AC or MUC5B appearing more prominent (Davies *et al.*, 2002).

1.8 Mucus and mucins in Respiratory diseases

Mucus hypersecretion is strongly associated with respiratory diseases such as chronic bronchitis, bronchiectasis, asthma and cystic fibrosis (Buisine *et al.*, 1999). It has also proven to be an irritation to the sinuses. It impedes the action of the mucociliary blanket and leads to a reduction in airflow. This in turn results in stagnant mucus which behaves as an ideal medium for bacterial colonisation and viral infection (Ho *et al.*, 1993, Adler and Li, 2001). Mucus hypersecretion during chronic airway diseases is promoted by metaplasia and hyperplasia of mucus secreting cells (Figure 1.3) (Vilar *et al.*, 2003). Literature has underlined the central role of mucus in the pathogenesis of severe airway obstruction and asphyxiation in fatal asthma (Hovenberg *et al.*, 1996). Acute degranulation of hyperplastic goblet cells occurs during mild and moderate asthma exacerbations (Rose, 1992) and the numbers of mucus secreting cells increase in chronic

bronchitis after the inhalation of tobacco smoke (Figure 1.3) (Jeena *et al.*, 2002).
The large gel forming mucins MUC5AC and MUC5B are responsible for the rheological (flow) properties of respiratory mucus (Fontenot *et al.*, 1995, Bradshaw *et al.*, 2002, Fontenot *et al.*, 1996) and knowledge of the types of mucin, their cellular origin, synthesis, secretion and eventual fate in the respiratory tract in disease may therefore provide insights into aspects of the pathogenesis of these conditions. Airway irritation, carcinogenesis, chronic bronchitis and cystic fibrosis, do not appear to be associated with production of novel glycoprotein moieties (Jeena *et al.*, 2002). However, in asthma (Fontenot *et al.*, 1996) and chronic bronchitis (Jeffrey and Li., 1997), the predominant respiratory mucin MUC5B can exist as two glycoforms (Fontenot *et al.*, 1996, Jeffrey and Li, 1997), with different densities of charge and different lengths of glycosylated domains (Fontenot *et al.*, 1996). The existence of two glycoforms of the MUC5B gene product raises questions as to whether they are both present in the normal situation, whether their level changes between normal and diseased conditions, and if they are the product of the same or different cells. MUC2 has been detected in the respiratory tract during inflammation associated with asthma (Fontenot *et al.*, 1996). It is hypothesized that the presence of MUC2 causes changes in the physicochemical properties of the various gels on the respiratory epithelium, which could impact on treatment in the future. Longer MUC2 alleles are also present in atopic non-asthmatics, in comparison to asthmatics (Fontenot *et al.*, 1996), suggesting that specific glycoforms could have protective properties against certain diseases.

Mucus overproduction is a major source of morbidity and mortality to airway diseases and leads to a severe loss of pulmonary function, yet the role of mucus and mucus secreting cells in the respiratory tract is poorly understood. In fact determining the levels and the quantity of mucus *in vivo* is problematic. Various obstacles are encountered during sample collection from volunteers and diseased patients. This leads to the use of post mortem samples which in turn present wih problems dues to proteolytic degradation. It is likely that mucins are the key to understanding airway pathologies yet the knowledge surrounding these glycoproteins is also limited.

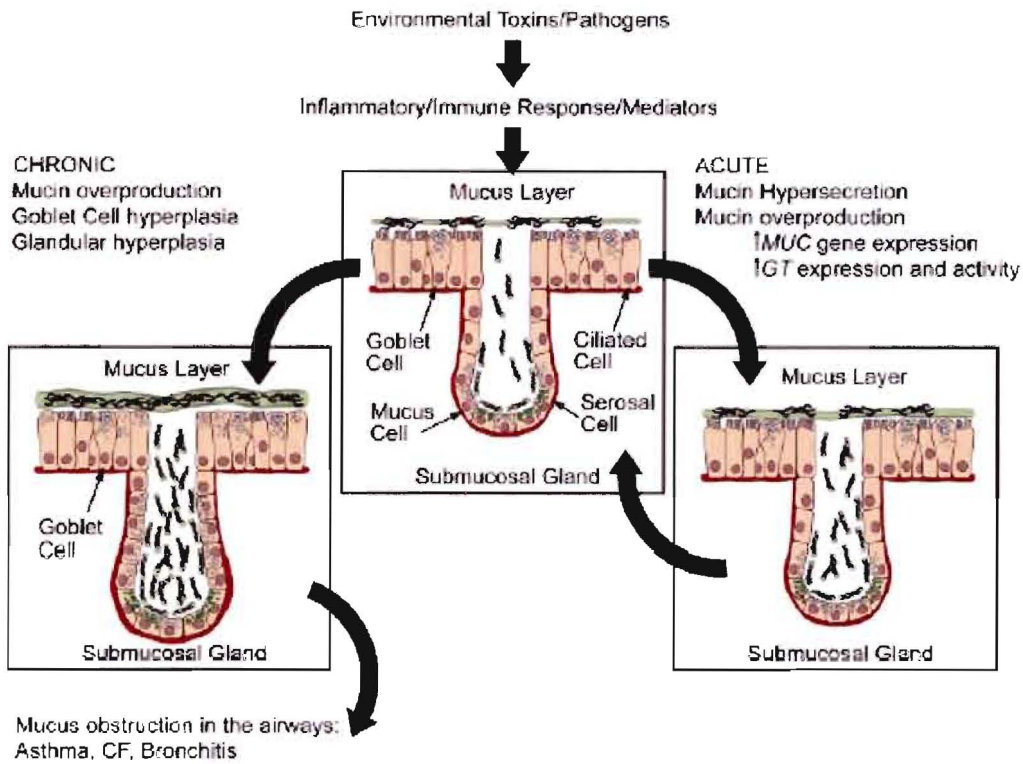


Figure 1.3 Illustrates the impact of external and internal irritants to the airways. When healthy human airway epithelia (middle) are exposed to pathogens or immune mediators the result is either chronic or acute mucin hypersecretion. Chronic mucin overproduction leads to airway diseases such as asthma were hyperplasia and metaplasia reflect a division in goblet cells, differentiation of progenitor cells and/or transdifferentiation of airway epithelial cells (bottom left). During an acute attack (bottom right) external pathogens or mediators activate mucin secretion from goblet cells and/or submucosal glands. Certain mediators also upregulate MUC gene transcription hence maintaining the hypersecretion and overproduction of mucin. An acute attack can be controlled by anti-inflammatory mediators and mechanisms resulting in a the airways reverting to a normal healthy state. (Rose and Voynow, 2006)

1.9 Tuberculosis (TB)

Mycobacterium tuberculosis is a highly contagious, airbourne pathogen responsible for TB. The bacilli are contained and hence spread in droplets between individuals in close contact with each other or within closed quarters (Munch *et al.*, 2003). Once inhaled droplets, at 5μm in diameter (Middleton *et al.*, 2002), they travel through the respiratory tract to its primary site of infection, the lung tissue. The TB bacilli are capable of invading other parts of the body by disseminating from the lung via the lymphatic or blood system to the pleura, lymphatics, bone, genito-urinary system, minenges, peritoneum or skin (Raja, 2004).

1.9.1 The impact of TB

During the mid-seventeenth century consumption, what is now known as pulmonary TB was identified as the source behind 1 in 5 deaths by the London Bills of Mortality. It was renamed the White Plague of Europe, a name that stayed up until the 19th century. Beyond this period TB became pandemic resulting in 7 million deaths per year; a mortality rate that increased with time (Fenhalls, 2004). TB has been the worlds leading infectious disease since its discovery and appears to continue on that unrivalled path. The mortality rate stands at 3 million deaths annually, estimating 5 deaths per minute. It is approximated that 8-10 million people are infected per year with the highest incidence rate (290 cases /100 000 individuals) lying in 9 of 10 African countries (Walzl, 2004) and more than half the cases lying in five South East Asian countries (Uplekar *et al.*, 2001). The World Health Organisation (WHO) claimed in 2003 that if TB is not effectively controlled one billion people will be newly infected by 2020 due to the influence of the HIV pandemic, the movement of populations, demographics and drug resistance (Kanduma *et al.*, 2003). Africa boasts the highest annual growth rate of TB (6%) (Walzl, 2004).

TB appears to victimize women and children. In 2000, children represented 1.3 million of the estimated 8.4 million new TB cases globally. Children also accounted for 450 000 of the 3 million deaths attributed to TB in that year (Jeena *et al.*, 2002, Madhi *et al.*,

2002). Studies related to children hospitalized with community acquired pneumonia in an urban area of the Western Cape in South Africa reported that TB occurred in approximately 8% of children, irrespective of their HIV status (Zar *et al.*, 2000). In 1998, mortality related to TB was lead by women. Approximately three-quarters of a million women died of TB and about 3 million women contracted the disease (Uplekar *et al.*, 2001).

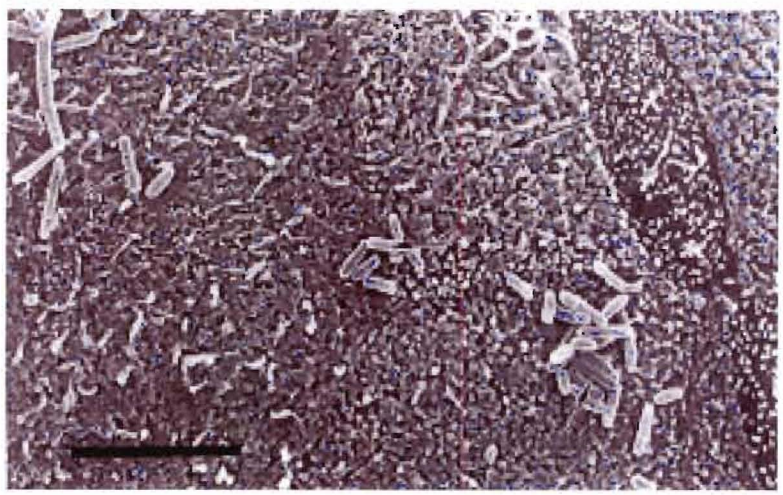
Despite efforts to control TB, it appears to be on a rampage, especially HIV related TB. Between 1993 and 1998 the TB cases in South Africa increased from 249 to 326 cases per 100 000 individuals. This illustrated a 31% increase (Munch *et al.*, 2003). The Western Cape Province in particular hosts the greatest number of TB cases in South Africa. A study carried out in the Cape Town suburbs of Ravensmead and Uitsig associated TB with socioeconomic classes prone to unemployment, overcrowding and various poverty related factors (Munch *et al.*, 2003). The lack of medical resources and the prevalence of HIV-AIDS, amongst other factors, have resulted in the poor management of TB especially amongst those of lower socio-economic status (Bradshaw *et al.*, 2002), who constitute the majority of the population.

1.9.2 TB and respiratory mucosal surfaces

The understanding of the pathogenicity of *Mycobacterium tuberculosis* is limited but studies conducted by Middleton *et al* (2002) report that mycobacteria have been illustrated to penetrate the respiratory mucosa. They are known to adhere to M-cells, which are epithelial cells involved in transporting particles across the mucosal barrier. M-cells are found above lymphoid tissue; this is of significance as it is suspected that bronchial-associated lymphoid tissue could be the primary entry site for *Mycobacterium tuberculosis* into the airways. The mycobacteria are also known to adhere to fibronectin and in doing so are further suspected to colonise mucosal surfaces. The role of fibronectin in bacterial colonisation was first illustrated when *M. Bovis* BCG was found to adhere only to damaged areas (Figure 1.4b) of the bladder epithelium. *M. Bovis* and *M. avium* have been shown to complex with a fibronectin by means of the fibronectin attachment protein (FAP). Similarly, *Mycobacterium tuberculosis* is suspected of

binding to fibronectin via the potential adhesion the 85B component of the antigen 85 (Middleton *et al.*, 2002).

Using an organ culture model Middleton *et al* (2002, 2003) have demonstrated that *Mycobacterium tuberculosis* adheres to "a particular form of mucus and occasionally to healthy unciliated cells, degenerate extruded cells and between cells with separated tight junctions" (Figure 1.4a). They also suggest that the attachment of the mycobacteria to airway cells could result in endobronchial infection observed in diseased states and in an animal model.

1.4a 

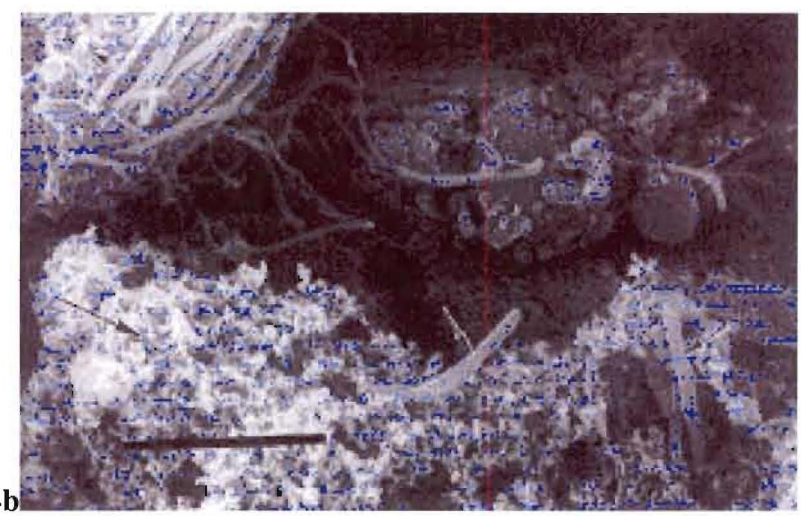
1.4b 

Figure 1.4 Scanning electron micrographs of *Mycobacterium tuberculosis* in respiratory mucosa. 1.4a illustrates the binding of the *mycobacterium* to healthy unciliated mucosa and 1.4b illustrates the adherence of the mycobacterium to damaged mucosa in the extracellular matrix. (Middleton *et al.*, 2002)

1.9.3 TB and Mucins

Despite their prevalence and the fact that diseases such as HIV and TB are considered to be one of the major causes of death in South Africa (Kirkbride *et al.*, 2002), the role of mucus and mucins in the pathogenesis of diseases such as TB has not been investigated. It is known that recurrent hypersecretion of mucus with increased amounts of mucin is characteristic of diseases such as TB (Bobek *et al.*, 1993). Furthermore it is hypothesized that the mechanism behind the development of endobronchial TB involves the colonisation of the respiratory mucosa by *Mycobacterium tuberculosis* (Middleton *et al.*, 2002, Middleton *et al.*, 2003) but knowledge concerning the type and amounts of specific mucins related to TB is minimal. This is the first known study concerning the role of mucus and mucins in TB. It is proposed that the findings of this study would contribute further to the understanding of the pathogenesis, treatment and management of TB as TB patients are known to produce vast quantities of mucus (Epstein *et al.*, 1987), which in general is known to aggravate a variety of respiratory diseases.

1.10 The biochemical objectives of this study

The biochemical aspect of this study aimed to determine the type of mucin/s secreted in children and adults with TB and to a) compare these with normal non-TB secretions, b) to determine the dominant mucins in children and adults with TB and c) to determine the roles of these mucins. The hypothesis is that besides the up-regulation of commonly expressed respiratory MUC5AC and MUC5B mucins in TB, a change in chemical composition and the expression of different glycoforms of these mucins are associated with disease, together with the possibility of the expression of other mucin(s), which would impact on the pathogenesis of TB.

1.10.1 The biochemical techniques

Mucus samples from adults and children with TB, along with controls were purified by caesium chloride (CsCl) density gradient centrifugation, and their purity determined by SDS-PAGE. Purified mucin was thereafter subjected to amino acid analysis to determine if the chemical composition of the mucins differed between the TB positive state and controls. Mucins were also subjected to agarose gel electrophoresis. MUC5AC,

MUC5B and MUC2 antibody detection was essential to the study as it served to illustrate the presence of the dominant mucin types, the presence of mucin glycoforms and novel mucin types; in doing so it was hypothesized that differences would be found in the TB and control groups.

1.11 Molecular study of TB and mucins

A brief molecular study was also conducted on children and adults with TB.

1.11.1 Respiratory mucin genes

The MUC5AC, MUC5B and MUC2 genes are gel forming mucins found on chromosome 11p15.5. MUC5AC and MUC5B gene products dominate the goblet cells and submucosal glands of the respiratory tract, respectively. The MUC2 gene products have also been described in the airways specifically during bacterial invasion. The central domains of MUC5AC and MUC2 contain tandem repeats rich in serine, threonine, glycine and alanine. They are flanked at either end by unique cysteine rich domains and appear similar to each other in amino acid sequence. These sequences are also similar to the human pro-von Willebrand factor (Desseyn *et al.*, 1998).

The MUC5B gene is comprised of 48 exons and 47 introns. It has an unusually large central exon (exon 30, 10713 bp) which houses a serine and threonine rich tandem repeat. MUC5B presents with a direct perfect repeat of 59 bp in intron 36 (previously known as intron G), which is identified by the following sequence: cctgtgcggt gagtgggggc ggccccgggc cccccagacc cctcggcctc tctgagtgt (Desseyn *et al.*, 1999).

MUC5B and MUC5AC appear similar at the peptide level. According to Desseyn *et al.*, (1999), MUC5AC, MUC5B and MUC2 may have evolved from the same ancestral line; this is supported by the fact that are classified as a subclass of mucins comprised of cysteine subdomains that occur several times between their heavily glycosylated regions (Figure1.2) (Desseyn *et al.*, 1998). The majority of mucin genes appear to have high levels of genetically determined polymorphisms (Vinall *et al.*, 2000). MUC5B in

particular presented with a polymorphism within intron 36 which is speculated to play a role in gene regulation (Desseyn *et al.*, 1999).

1.11.2 Mucin polymorphisms

The polymorphisms of the apomucin and the structural variation of the oligosaccharides has allowed for vast inter-individual differences in the structure of mucins. These differences have possibly allowed species to adapt to changing environments; this is observed in microbes and viruses that alter their properties through mutations. Polymorphisms of mucins may be advantageous to a population at large but are capable of making certain individuals more susceptible to bacterial colonisation or antigen attack (Davies *et al.*, 2002).

1.11.3 Mucin genes and Respiratory diseases

Mucin genes are highly polymorphic due to the presence of long stretches of variable number of tandem repeats (VNTRs) that are heavily glycosylated (Rose and Voynow, 2006). VNTRs are a result of the instability of the number of repetitions that occur from generation to generation (Moniaux *et al.*, 2001). They take up a large part of the coding region and are very similar but not identical to each other. Lengthwise, they range between 24 nucleotides in MUC5AC to 507 nucleotides in MUC6 (Vinall *et al.*, 2000).

In a number of human diseases, aberrant O-glycosylation is associated with variations in the properties of the membrane-associated and secreted mucins (Reid *et al.*, 1999). Several studies reported an association between disease, for example in asthma, and the length of the VNTR region (Vinall *et al.*, 2000, Kirkbride *et al.*, 2001, Rose and Voynow, 2006). This association was also made with polymorphisms at the level of a single nucleotide as in ulcerative colitis (Kyo *et al.*, 2001), and in allelic variation in subjects of different ancestry (Kirkbride *et al.*, 2001). The over-expression, followed by hyper-secretion, of mucins has been established secondary to inflammatory diseases (Vinall *et al.*, 2000, Voynow 2002). MUC1, MUC2, MUC4, MUC5B and MUC5AC have each been expressed in inflamed and noninflamed tissue at the mRNA and protein levels. With the exception of MUC5B they have also illustrated allelic length variation (Desseyn

et al., 1999, Vinall *et al.*, 2000). This length variation is observed in "longer" MUC2 alleles suspected of protecting predisposed individuals from the development of asthma. In contrast MUC5B is hypothesized to have rare variant length alleles present during a diseased state such as severe asthma. If so, this would explain the presence of large amounts of MUC5B that were found in an individual who died from status asthmaticus (Vinall *et al.*, 2000).

Investigations involving obstructive lung diseases generally focus on the expression of MUC5AC and MUC5B, as their mRNA is well expressed in normal airways and their gene products have been identified in increased concentrations in lung mucus of asthmatic patients (Rose *et al.*, 2001, Voynow 2002). In chronic obstructive pulmonary disorder (COPD) patients, MUC5AC is believed to be controlled by inflammatory mediators, such as cytokines. MUC5AC and MUC5B gene expression in normal and diseased airways is generally greater than that of MUC2 or MUC1 (Voynow *et al.*, 1998, Voynow, 2002). MUC2 produces a diffuse, weak signal in normal respiratory epithelium which increases in inflamed airways (Leikauf *et al.*, 2002). Various studies support the hypothesis that MUC2 is more actively involved in the pathogenesis of inflammatory airway diseases such as cystic fibrosis; during which the MUC2 gene is transcriptionally activated by *Pseudomonas aeruginosa* (Buisine *et al.*, 1999). MUC2 mRNA expression was shown to be further elevated in rat airways damaged by SO_2, endotoxins and viruses (Jany *et al.*, 1991, An *et al.*, 1994). In general, the overexpression of MUC5AC, MUC5B and MUC2 has been associated with asthma, cystic fibrosis and chronic bronchitis; diseases strongly associated with mucus obstruction and goblet cell hyperplasia and metaplasia. Each of these pathologies has via *in vitro* studies been linked to mucin expression (Voynow, 2002).

1.11.4 The genetic susceptibility to TB

TB has reached epidemic proportions and is suspected to be related to a genetic susceptibility within certain populations (Hoal *et al.*, 2004). The condition has been linked to various polymorphisms such as *SLC11A1* (previously known as *NRAMP1*) (Hoal *et al.*, 2004). *SLC11A1*, according to Hoal *et al* (2004) is associated with

protection against TB in most of the populations studied. Investigations involving restriction fragment length polymorphisms (RFLPs) have also made strong ties between ones lifestyle and a susceptibility to TB. This lifestyle is associated with prisons, bars, homeless shelters, and in general poverty (Munch *et al.*, 2003, Hoal *et al.*, 2004). A strong association was also made between the incidence of TB and unemployment and overcrowding (Munch *et al.*, 2003).

Furthermore it appears that the ethnicity of a population is involved in regulating genes that are associated with TB susceptibility. Generally within the world, the Asian and African populations have presented with high incidences of TB in comparison to the Caucasian population, which is usually found to have a low incidence of TB. Within South Africa terms such as Black, White, Coloured (mixed race) and Indian are used to identify the different races. The Coloured population in the Western Cape has presented with the highest incidence of TB in South Africa. Their gene pool has been influenced by Khoisan, Malaysian, Black and White populations and has therefore been the focus of various molecular studies regarding TB (Hoal *et al.*, 2004). South Africa, particularly the Western Cape, has presented with an ideal environment for various TB related studies. The demographic profile and differences in socio-economic conditions amongst various population groups provides the opportunity to study the impact of polymorphisms and TB susceptibility. The role of respiratory mucin gene polymorphisms has previously not been investigated in TB. An investigation into this association is of significance as respiratory mucin gene polymorphisms could make certain individuals more susceptible to TB than others.

1.11.5 The objectives of the molecular study

The molecular aspect was a pilot study that focused on genetic polymorphisms in mucin genes and their associated susceptibility to TB. The objectives included a) determining if the genetic polymorphisms for MUC2 and MUC5B reported in the literature were also present in the Western Cape population and b) and to gauge if a large scale investigation would be warranted.

CHAPTER TWO

MATERIALS AND METHODS

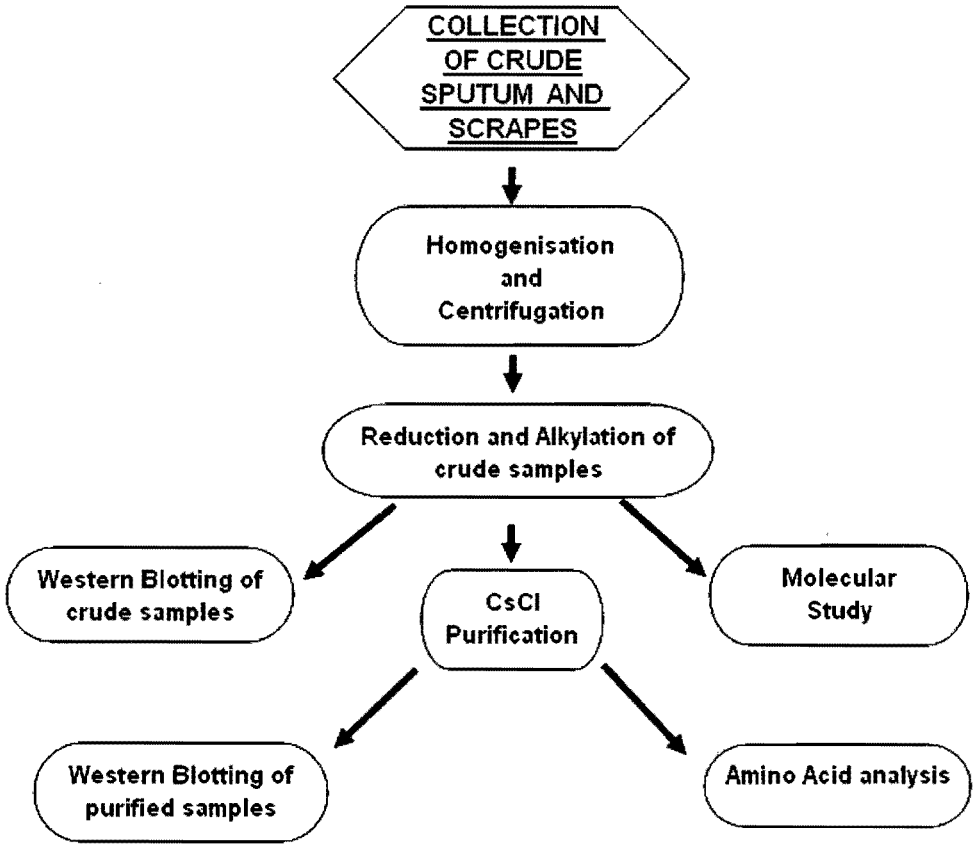


Figure 2.1 Illustrates a flowchart of the methods carried out in the study.

2.1 Materials

Poly-L-Lysine, resorcinol, guanidinium chloride (GuHCl), agarose (TYPE V), urea and bovine albumin were obtained from Sigma Company St Louis U.S.A. Sodium chloride, dialysis tubing, Super Fuji Medical X-ray film HR-GB (18-24cm) were purchased from Kimix, SA. Ethylenediaminetetra-acetic acid disodium salt (Na_2-EDTA), Tris (Hydroxymethyl) aminomethane, ammonium persulfate, iodoacetamide (IAA), Folins

Ciocalteu's phenol reagent acrylamide, *NN'*-methylenebisacrylamide (Bis), ammonium persulfate (AMPS), *N*-ethylmaleimide (NEM), sodium metabisulphate, sodium dodecyl sulphate (SDS), periodic acid and 2-mercaptoethanol were supplied by the British Drug House (BDH), Dorset, UK. ECL Western Blotting detection reagents were supplied by Amersham Biosciences. Nitrobind pure nitrocellulose (0.22μ) was supplied by Kimix, Cape. Sodium carbonate anhydrous, di-sodium hydrogen orthophosphate anhydrous and sodium dihydogen orthophosphate were from SAARCHEM (Merck). ULTRA-CLEAR™ centrifuge tubes (16x76mm) were supplied by Beckman International. Buccal swabs were purchased from Cliniscience. The Sepharose CL-2B column was supplied by Pharmacia Ltd, Buckinghamshire U.K.

2.2 Collection of crude sputum samples and mucus scrapes

Samples were obtained from 4 different groups:

1. sputum from healthy adult volunteers (V)
2. mucus scrapes from the tracheas of cadavers (PM)
3. sputum from diseased adults (J)
4. sputum from diseased HIV+ children (RX)

Groups 1 and 2 were the control groups. The volunteers were "healthy" in that they declared themselves as such at the time of the study. Sputum samples were induced with 0.9% sodium chloride for 15 minutes via a Nebysol nebuliser and collected on ice. Samples were obtained from post mortem cadavers 48-72 hours after death. Crude mucus scrapings were collected on ice by slitting the trachea longitudinally and scraping the epithelial surface with a metal spatula.

The adults at risk for TB were drained from the area known as the Cape Flats which is occupied predominantly by the population known as the Cape Coloured, so called because of its ethnic mixture (mostly Khoisan, Caucasian, Xhosa and Indonesian). Patients suspected of having tuberculosis were identified by medical staff of the intake wards at G.J. Jooste Hospital. Sputum was taken from these patients prior to any treatment. Patients rinsed their mouths with water before expectorating into a pre-weighed jar containing 6M GuHCl, on ice. A nursing sister was called upon to assist if

and when a patient required assistance to mobilise sputum. A medical intern also assisted in seeking out patients and in the collection of samples. A clinical report was compiled on the method of TB diagnosis and the presence of extraneous complicating factors related to mucus production (Chapter 3, Clinical data).

Children were intubated to obtain tracheal mucus at Red Cross Children's Hospital. All these children were HIV positive and diagnosis of TB was made as described above. Material for DNA analysis was taken by scraping the mucosal lining of the inner cheek with a buccal swab in groups 3 and 4. The samples obtained for the 4 groups were solubilised in 6M Guanidinium hydrochloride (GuHCl) made up in sodium phosphate buffer containing proteinase inhibitors, namely 10mM EDTA and 5mM NEM. Swabs and samples were stored at 4°C until further investigations.

2.3 Solubilisation of samples

The mucus scrapings from cadavers and sputum samples were homogenised with a Junkel and Kunkel Ultra-Turrax (40sec, 9500min^{-1}, RT) to disaggregate the sputum and increase its solubility in GuHCl. After sonication the samples were centrifuged in a HITACHI, HIMAC centrifuge (60min, 3000rpm, 4°C) to remove insoluble debris (Allen, 1977). The supernatant was removed and stored at -20°C.

2.4 Purification of mucins

Mucins were purified via the CsCl density gradient method. The samples were subjected to two 48hr spins at 40 000g, 4°C (Creeth and Denborough, 1970, Starkey *et al.*, 1974). The density of the samples was adjusted to values between 1.39-1.4g/ml using GuHCl and CsCl. After centrifugation the samples were fractionated in a top down method into 8 fractions. The fractions were dialysed to remove CsCl and a Lowry and PAS assay were carried out to determine the presence and level of protein and carbohydrate in each fraction. After the second spin the fractions that contained the purified mucin peaks (Starkey *et al.*, 1974, Pearson *et al.*, 1980) were pooled, dialysed against distilled water and freeze dried.

2.5 Reduction and alkylation

The samples were reduced in 6M GuHCl and10mM DTT for 5hrs at 37°C. DTT disrupts disulfide bonds in mucus and allows for a uniform dispersal of cells (Hargreave, 1999). The resulting sulfhydryl (–SH) groups were then treated with 25mM IAA, overnight in the dark, to avoid the re-formation of non-specific disulphide bonds.

2.6 Analytical procedures

2.6.1 Lowry assay

The Lowry assay was carried out according to the method of Lowry *et al* (1951). It makes use of the Folin-Ciocalteu reagent to either quantitatively or qualitatively determine the concentration of proteins. The quantitative assay requires the use of a standard curve. The assay is based on two vital steps whereby a) copper reacts in an alkaline solution and b) the Folin-Ciocalteu reagent is reduced. The advantages to the Lowry method include its sensitivity to detect low protein concentration (Oosta *et al.*, 1977), which is greater than the biuret and ninhydrin reactions (Lowry *et al.*, 1951). It is also more sensitive than UV detection of proteins at 280nm. It falls short with respect to constancy as the colour varies depending on the protein and is not necessarily proportional to the concentration (Lowry *et al.*, 1951). The standard was created via a doubling dilution using bovine serum albumin (BSA) as the standard protein. A solution containing alkaline and copper added to the samples. After 10mins diluted Folins reagent (200µl) was added to the samples which were mixed and incubated at 25°C for 30mins. The absorbance of the samples was read at 700nm.

2.6.2 PAS assay

The PAS assay was carried out to determine the presence and relative amount of mucin during CsCl purification. The principle involves the oxidation of vicinal hydroxyl groups by periodic acid and the coupling of a Schiff base (PAS) to identify glycoproteins (Mantle and Allen, 1978). Periodic acid oxidation results in the formation of two aldehyde groups which combine with the Schiff base to form a red solution. The PAS method is a reliable, simple and sensitive assay for the colorimetric detection of

carbohydrates. It has proven to be 20 times more sensitive than the orcinol assay, 10 times more sensitive than measuring at A_{280} and 3 times more sensitive than the anthrone assay with respect to the detection of gastric glycoproteins. It is important to note that detergents containing formaldehyde are extremely Schiff sensitive or PAS positive and could interfere with the results. In this study distilled water (950µl) and periodic acid solution (200µl) were added to the sample (50µl). This solution was incubated at 37°C in a water bath for an hour. Simultaneously Schiff's reagent was prepared and incubated at 37°C in a water bath until the solution turned colourless. The decolourized Schiff's reagent (200µl) was added to the samples. The samples were vortexed and left to stand at room temperature for 30mins. A graph of the absorbance of the mucins was read at 555nm.

2.7 Biochemical procedures

2.7.1 Dialysis

Dialysis was carried out during the purification procedure a) to remove GuHCl and CsCl as they interfere with the Lowry and PAS assay and b) to concentrate the purified mucin before freeze drying. It is common to small scale applications and is based on the principle of osmosis (Degerli *et al.*, 2001). One should note that detergents interfere with dialysis as they form high molecular weight micelles which also attempt to pass through the membrane pores (Stoscheck, 1990). Tubing was prepared for dialysis by boiling in distilled water with EDTA and sodium carbonate. The samples were dialysed at -20°C for 24hrs under constant stirring in a bucket of distilled water.

2.7.2 Freeze drying

After dialysis, purified samples were placed in plastic tubes with holes in the lids. They were then frozen by immersing the tubes in liquid nitrogen. The tubes were thereafter packed into vacuum flasks which were connected to a Christ ALPHA I-5 freeze drier from Lasec Laboratory and Scientific equipment. The samples were dried at -80°C for approximately 24hrs. The lyophilised mucin samples were stored at -20°C until further investigations.

2.7.3 Western blotting

Reduced and alkylated mucin subunits were separated by gel electrophoresis in 0.7% (w/v) agarose gels in 40mM Tris/acetate/1mM EDTA (1x TAE), pH 8.0, containing 0.1% (w/v) SDS. Loading buffer containing 1x TAE / 50% glycerol/ bromophenol blue was added to the samples which were applied to the gel in volume equivalents. After electrophoresis, proteins were transferred to a nitrocellulose membrane by vacuum blotting in 0.6M sodium chloride/60mM sodium citrate (transfer buffer) with a Pharmacia VacuGene XKL apparatus at a suction pressure varying between 50-60mbar. Membranes were then probed with polyclonal anti-mucin antibodies (1h or overnight), provided by Dr Sara Kirkham, Manchester. LUM5-1 is a rabbit anti-serum raised against a synthetic peptide RNQDQGPKFMC (Hovenberg *et al.*, 1996) corresponding to MUC5AC, LUM5B-2 recognises the epitope RNREQUGKFKMC (Wickstrom *et al.*, 1998) in the NH_2 terminus of the MUC5B apomucin, and LUM2-3 identifies the MUC2 epitope NGLQPVRVEDPDGC (Herrmann *et al.*, 1999) situated in the non-tandem repeat region of the C-terminus (Aksoy *et al.*, 1999). LUM5-1 and LUM2-3 recognise the fully glycosylated forms of MUC5AC and MUC2 respectively (Davies *et al.*, 1999) Immunoreactivity was detected by development with an Enhanced Chemiluminescence System (ECL) (Amersham, UK) and the films were processed in a standard x-ray developer with an exposure time of five minutes. Reactivity with all three antibodies was tested for the four groups.

2.7.4 SDS-PAGE

A 10 % SDS-PAGE was setup according too Laemmli (1970), to evaluate the purity of samples after CsCl purification. The samples were dialysed in distilled water to remove the CsCl and GuHCl. They were thereafter reduced in reducing sample application buffer containing 2-mercaptoethanol. The gel was run at 20 amps until the bromophenol blue dye reached the bottom of the gel. Once complete the gel was stained with 0.2% Coomassie blue in 50% ethanol and 10% acetic acid to determine the presence of protein bands. The gels were thereafter destained in 25% ethanol and 10% acetic acid to clear the background of the gel and to clearly view the protein bands.

2.8 Amino acid analysis

The amino acid content of purified freeze dried mucins were analysed using a high pressure liquid chromatography (HPLC) system. The analysis procedure was similar to that of Klapper (1982). Norleucine was used as a standard for the calibration of peaks. The samples were vacuum dried and placed in a hydrolysis vessel containing constant boiling HCl and 1% (v/v) phenol. The vessels was purged with nitrogen gas and sealed under vacuum. The samples were then hydrolysed in the gas phase at 110°C for 24hrs. Following hydrolysis, the vials were cooled and vacuum dried to remove the residual HCl. The dried samples were redissolved in citrate buffer pH 2.2 and injected into a HPLC column from Waters Associates, Medford, MA., packed with a cation exchange resin (sulfonated polystyrene crosslinked with divinylbenzene) and eluted with a series of buffers ranging from a low (0.25M trisodium citrate, pH 3.05) to high (0.25M sodium nitrate, pH 9.5) pH. Detection was carried out using post column derivatization with o-phthalaldehyde (OPA), a fluorescent reagent that reacts with all the amino acids except proline. Proline analysis was carried out separately and followed the same procedure with the exception of the use of OPA. 1% sodium hypochlorite was used to detect proline as OPA is unable to interact with its ring structure. The relative ratios of the individual amino acids for each sample was determined and compared to each other.

2.9 Molecular Study

2.9.1 DNA extraction

DNA was extracted from buccal swabs using the QIAmp DNA Mini kit (Qiagen, Germany). The Buccal Swab Spin protocol was performed as described in the manufacturer's manual. The swabs were washed in 1xPhosphate buffered saline (PBS) to get cells into solution. The cells were lysed with proteinase K (40mAU/mg protein) and lysis buffer at 56°C. Buffering conditions were optimised to facilitate binding of DNA to the silica gel membrane whereas protein and other contaminants were allowed to pass through. Ethanol was added to the solution which was mixed and transferred directly onto the filter of the spin column. After a series of washes using buffers

designed to remove contaminants buffers, AW1 and AW2, purified DNA was eluted by centrifugation in 100µl of sterile distilled water. DNA was stored -20°C until further use.

2.9.2 Polymerase Chain Reaction (PCR) primers

Exons containing known variants for MUC2 and MUC5B were amplified using standard PCR techniques Primers used for amplification of the intronic MUC5B polymorphism are the same as those described by Vinall *et al* (2000). The primers were complementary to the variable number of tandem repeat (VNTR) region (Table 2.1).

Table 2.1 Primers used for MUC2 and MUC5B PCR.

Gene	Sense Primer	Antisense Primer
MUC2	5'-CTGAGCAACCTCGGCCTT-3'	5'-ATCGTCCAACCAGTCCAGC-3'
MUC5B	5'AGTGTGCAGTGACTGGCGAG-3'	5'-CTAGAGTTGCAGGTGGCAGG-3'

2.9.3 Polymerase Chain Reaction (PCR)

PCR of the MUC5B and MUC2 alleles were carried out on purified DNA. The DNA was amplified in a PCR Sprint (Hybaid, UK) machine. Each MUC5B PCR contained a final concentration of (50 mM KCl, 10mM Tris-HCl, pH 9, 1.5 mM $MgCl_2$, 0.01% Triton-X), 0.25µM of deoxynucleotide triphosphates (dNTP's), 10pmol of Sense and Antisense primers, 7.5% glycerol, 3-5µl of purified DNA, 1 unit of D-aza for GTP, 1 unit Taq polymerase (SequiTherm, Hoffman-La Roche) with purified water making a final volume of 20µl. The cycling conditions were as follows: denaturation for 3mins at 95°C; 30 cycles consisting of 30sec denaturation at 95°C, 30sec annealing at 60°C and a 45sec extension at 72°C and a final extension of 7mins at 72°C. Loading buffer was added to the PCR products and the molecular weight marker (MWM, Superladder-Low 100 Ladder with ReddyRun™ from ABgene, UK) which were resolved by electrophoresis through a 2% agarose gel using the BIO-RAD MINI-SUB® CELL GT. The amplified VNTR region of intron 36 of MUC5B was visualized with ethidium bromide under ultraviolet light. Two samples were used as controls during electrophoresis of the MUC5B PCR products. These samples were included in all gels to control for variations

across gels that occur due to variant running conditions (Vinall *et al.*, 2000). To ensure correct sizing of alleles 0.25ug of the 1kb MWM was run on either side of the gel.

PCR of the MUC2 alleles followed a similar procedure to that of the MUC5B PCR except for minor changes. The PCR cocktail for MUC2 excluded glycerol, $MgCl_2$ and 7-deaza GTP, and the cycling conditions required annealing at 61°C and not 60°C.

2.9.4 Restriction enzyme digestion of MUC2

The MUC2 PCR products were digested with the restriction endonuclease, *Pvu*II. The PCR product was treated with *Pvu*II in a final volume of 25µl at 37°C for 2-4 hrs. Loading buffer was added to the digestion products and the MWM which were resolved by electrophoresis through a 2% agarose gel and thereafter visualized with ethidium bromide under ultraviolet light.

2.9.5 Sequencing of MUC5B

2.9.5.1 Gel extraction

The MUC5B PCR products were electrophoresed on a 2% agarose gel. Fragment sizes were approximated by comparison with the band sizes of the MWM. Whilst under UV light specific alleles selected for sequencing were excised from the gel using a scalpel and needle. The DNA was purified using the QIAquick® Spin gel extraction kit (Qiagen, Germany). The Qiaquick gel extraction protocol was carried out according to the manufacturer's manual. The gel slice was solubilized in a buffer containing (250ml Solubilization and Binding buffer, with a pH indicator) at 50°C to release the DNA. The addition of Isopropanol and a series of centrifugations facilitated the adsorption of the DNA to the membrane traces of agarose and other contaminants that could interfere with the sequencing procedure. The purified DNA was eluted by centrifugation in 30µl of sterile distilled water and its concentration determined against DNA of a known size.

2.9.5.2 Big dye chain termination for sequencing

Prior to sequencing the DNA, fluorescent dyes were incorporated into the purified DNA using the 2720 Thermal cycler from AB applied Biosystems. The reaction was carried

out according to the BigDye® terminator v3.1 cycle sequencing Ready Reaction kit (Applied Biosystems). A cocktail was prepared containing 1µl purified DNA (3-10ng), 1µl of the sense (10pmol) and the anti sense primer (10pmol), 4µl of TTRM, 2µl of the 5x buffer (provided by the manufacturer) and sterile water making up a final volume of 20µl. The cycling conditions were as follows: denaturation for 5mins at 96°C; 25 cycles consisting of 30sec denaturation at 96°C, 15sec annealing at 50°C and a final 4min extension at 60°C. The labelled samples were then analysed using the ABI PrismTM 377 DNA sequencer. The ChromasPro package (Technelysium Pty. Ltd. Version 1.33) was used to analyse the sequences.

CHAPTER THREE

CLINICAL DATA

3.1 Adult sample collection

Clinical data for adult patients with Tuberculosis (TB) (J) was obtained from G.F. Jooste hospital, a second tier hospital that drains patients from a huge community of poor socio-economic background in an area commonly known as the 'Cape Flats'. South Africa's health care system has been restructured in accordance with the principles of Primary Health Care in which day clinics form the first tier. Hospitals such as G.F. Jooste are considered second tier centers that are both teaching hospitals and can act as referral hospitals to tertiary hospitals such as a university teaching hospital, where highly specialized care is provided.

Initially a nursing sister accompanied us on our rounds to help mobilize patients to cough and produce sputum samples. Later a medical intern was recruited to facilitate access to newly admitted patients suspected of having TB. The intern ensured that fresh samples were collected from patients before they were placed on treatment and that samples were collected and stored in the cold as soon as possible and collected (on ice) and frozen at the earliest convenience. The sputum samples were collected in 6M guanidinium chloride (GuHCl) containing proteinase inhibitors, 10mM EDTA and 5mM NEM, and stored in a fridge at 4°C until we were able to collect them. The investigation was explained to the patients to gain their consent and a brief medical history was taken. The consent form is included as Appendix A. The TB diagnosis was made according to the criteria detailed in Appendix B. Sputa were collected on ice and a buccal swab taken from each patient after the mouth was rinsed with water. Procedures for extracting and analyzing the mucus were then followed as described in the Materials and Methods section (Chapter Two) whilst we waited upon the final diagnosis from the hospital.

3.1.1 TB diagnosis

The medical diagnosis for TB was made on the criteria of AFB stain, culture, chest X-rays (CXR) (Appendix B) for 21 subjects, 15 of whom were male and 6 female. The median age of subjects in total was 36.

3.1.2 Criteria for diagnosis

TB was diagnosed according to the criteria set out by the World Health Organization (WHO). Interfering variables that were thought to be responsible for exacerbating mucus production, were identified in study subjects. These included a history of cigarette smoking, alcohol consumption, substance abuse e.g. dagga and diabetes mellitus. The presence of other mucus - producing respiratory diseases such as bronchietasis, chronic obstructive pulmonary disease (COPD) and asthma were also recorded as these were expected to possibly interfere with mucus and mucin production.

3.1.3 Diagnostic definitions

The WHO diagnosis of TB is categorized as follows:

a. Possible: a patient is considered to be a possible TB subject when the clinical data and radiological results are in agreement with the consultant's diagnosis and the microscopy and culture results are negative.
b. Probable: this diagnosis is made when direct microscopy of sputum, lymph node aspirates and other samples are declared acid fast bacilli (AFB) positive.
c. Definite: a patient is diagnosed as TB positive when *Mycobacterium tuberculosis* is cultured from sputum or any other sample.

Of the 21 adults present in the study, seven were defined as possible, four as probable and ten as definite, according to the WHO criteria.

3.1.4 Other complicating variables

The definite TB patients varied in their HIV status; they were diagnosed as positive, not suspected or negative. The majority of the definite cases were complicated with bronchiectasis alone, two HIV patients presented individually with COPD, and COPD

complicated with bronchiectasis and diabetes (Table 3.1). The probable TB patients were HIV negative; with one of the patients featuring with bronchiectasis (Table 3.1). The possible TB patients were also diagnosed as HIV positive, not suspected or negative and appeared to present mainly with bronchiectasis followed by COPD and asthma. None of the TB patients presented with interfering variables such as smoking, drinking or substance abuse.

3.2 Children sample collection

Collection of sputum from children, between the ages of 3months-5yrs, was carried out at the Red Cross Children's Hospital. HIV was a complicating factor in all the babies but was not found to interfere with TB diagnosis. Of the 69 HIV positive children from whom sputum was collected, 9 children were diagnosed as TB positive, 3 of which were on treatment, a single child had been treated for TB previously and 58 children were TB negative. The procedure involved intubation of the babies for tracheal mucus which was carried out by a nursing sister. The investigators were provided with buccal swabs for the DNA. The swabs and mucus were stored at 4°C until collected for lab investigation. Once collected the tracheal mucus were solubilised in 2.5ml 6M GuHCl and stored at -20°C for further use. Except for the TB diagnosis and HIV status further clinical data could not be obtained for the children.

3.3 Discussion

The sample number for TB positive adults was small to begin with as sample collection presented great difficulty during that period of the study. The additional help from a medical intern resulted in success with respect to sample collection but other factors appeared to create further obstacles, the main obstacle being that DNA samples could not be obtained from discharged or deceased patients. Nevertheless clinical data was obtained for 21 subjects involved in the study; of the 21 subjects the patients in table 3.1 presented with significant results in the western blotting investigation (Chapter four). Sputum (especially induced sputum) was the sample of choice for cellular and biochemical analysis as it is found to be valid when monitoring airway inflammation (Gershman *et al.*, 1999). Bronchiectasis appeared to be the major complicating factor in

the TB positive adults, especially the definite TB patients. This could be explained by studies which state that bronchiectasis is a late complication to TB (Andreu *et al.*, 2004) hence a closer association with definite TB.

With respect to sputum collection from children, samples were taken from an investigation that was already underway at the Red Cross Childrens Hospital. Dr Heather Zar, the coordinator of the study, established the reliability of the TB diagnosis, irrespective of the HIV status of the children. Furthermore the study confirmed the clinical efficacy of sputum induction for TB diagnosis in children (Personal communication with Dr Zar).

Table 3.1 Adult Patients diagnosed with TB according to the WHO standard.

Case number	Gender and Age	TB status	HIV status	Respiratory diseases	Other variables
1	Male 34	possible	suspected	none	N/A
3	Male 23	possible	negative	bronchiectasis COPD	N/A
*4	Female 31	possible	positive	asthma	N/A
8	Female 30	definite	suspected	N/A	N/A
9	Male 51	definite	positive	COPD	N/A
11	Female 15	definite	Not suspected	bronchiectasis	N/A
*13	Male 35	probable	negative	bronchiectasis	N/A
*22	Female 15	definite	Not suspected	bronchiectasis	N/A
*25	Male 35	possible	positive	N/A	N/A
*26	Male 49	possible	Not suspected	bronchiectasis COPD	N/A
27	Female 66	definite	Not suspected	bronchiectasis COPD	diabetic
28	Male 33	probable	negative	N/A	N/A
*30	Male 50	possible	Not suspected	bronchiectasis	N/A

* The adult samples used for MUC2 detection during western blotting (Chapter 4.1) are highlighted in blue (definite and probable) and red ('possible' TB patients) and those used for MUC5AC and MUC5B detection are highlighted in black.

CHAPTER FOUR

AGAROSE GEL ELECTROPHORESIS AND WESTERN BLOTTING

4.1 Identification of mucins by Western Blotting

Crude (induced) sputa from 'healthy' (symptom free) volunteers (V), mucus scrapings from the tracheal tissue of cadavers (PM), and expectorated sputum samples from adults with TB (J) and from HIV positive children with TB (RC) were collected and solubilised in cold 4M GuHCl and proteolytic inhibitors. The disulphide bonds of the mucins were then reduced with 10mM DTT and alkylated with 25mM IAA, to obtain mucin subunits with epitopes exposed for detection (Kirkham *et al.*, 2002). The reduced mucin subunits were then loaded on a 0.7% agarose gel and electrophoresed to separate them on the basis of their charge. Equal volumes of samples from different patients and groups were loaded onto the gel with the aim of detecting mucins of specific charge and the variety of 'possible' glycoforms present for each mucin type. Following electrophoresis the samples were vacuum blotted onto nitrocellulose to affirm the presence of the dominant respiratory glycoproteins, MUC5AC and MUC5B (Kirkham *et al.*, 2002), and to investigate the possible presence of any novel mucins being secreted in TB. Patients with suspected TB (group J) were classified according to the World Health Organization (WHO) classification as having 'definite', 'probable' or 'possible' TB (Chapter three, Clinical chapter). This study regarded the 'definite' and 'probable' TB patients as TB positive and compared these with those considered as 'possible' TB patients. The RX group was treated similarly.

MUC5AC is a known mucin component in gastric mucus and secretions (Pinto-de-Sousa *et al.*, 2004). Salivary mucus is a negative control for MUC5AC and the positive control for MUC5B (Veerman *et al.*, 1996, Thornton *et al.*, 1999). The specificity of the MUC5AC antibody LUM5-1, in the volunteer (V) blot (Figure 4.1), was confirmed against purified gastric cancer mucin (positive control, lane 1) and crude saliva (negative control, lane 2). There was very little or no MUC5AC in the induced sputum of the

volunteers (Figure 4.1, lanes 3-8). The trace amounts of crude material present in lanes 6 and 8 (Figure 4.1) were in a comparable position on the gel to the larger amounts of MUC5AC in the sputa of TB positive adult patients (Figure 4.3, lanes 3-10). MUC5B appeared as dark, broadly smeared material in group V (Figure 4.2, lanes 3, 4, 7 and 8) almost covering the entire gel. Sample H (lane 5) and F (lane 6) had no MUC5B. Interestingly sample H had no MUC5AC either (Figure 4.1, lane 5).

MUC5AC gave a single distinct band for sputa from each of the TB positive adults (J) (Figure 4.3) with a strong signal for purified gastric cancer mucin (positive control) (Figure 4.3, lane 1). The specificity of the MUC5AC antibody was supported not only by the positive control but also by the absence of this mucin in crude saliva (Figure 4.3, lane 11). The position and appearance on the gel of MUC5AC in TB (Figure 4.3) is similar to that described by Thornton *et al* (1996), for patients with asthma and chronic bronchitis, and in a local study of asthma patients attending the Respiratory Clinic at Groote Schuur Hospital (Mall, personal communication). The difference in the position of the gastric cancer mucin on the gel (Figure 4.3, lane 1) compared to that of the respiratory mucins (Figure 4.3, lanes 3-10) could be attributed to the difference in charge of this mucin in two separate secretions in the body.

MUC5B in the TB positive adults (J) (Figure 4.4, lanes 3-10) showed a similar electrophoretic mobility to that of the volunteer (V) group (Figure 4.2, lanes 1-6) with the range of charged species somewhat less than that of the V group (Figure 4.2). As in the V group, inter-individual variation appeared to exist within the TB positive adults (J). Once again the specificity of the MUC5B antibody showed in its reactivity to the positive control (crude salivary mucin, Figure 4.4, lane 11), and the absence of this reactivity to the negative control (purified gastric cancer mucin, Figure 4.4, lane 1). The TB positive adults (J) appeared to be positive for both MUC5AC and MUC5B (Figures 4.3 and 4.4, respectively) as reactivity to the antibodies were detected in the same 8 adult samples (Figures 4.3 and 4.4, lanes 3-10). The appearance of MUC5B on the gels was different to that of MUC5AC (Figures 4.4 and 4.3, respectively) in that the MUC5B displayed

variation in terms of wider range of charge. A single TB positive patient was negative for both MUC5AC and MUC5B (Figures 4.3 and 4.4, lanes 2).

The pattern of reactivity for MUC5AC (Figure 4.5) and MUC5B (Figure 4.6) in the RX group (HIV positive children with TB) were similar to that of the adult group with TB (J) (Figures 4.3 and 4.4). Again, MUC5B displayed a broad range of charge and MUC5AC appeared as a more distinct entity. Positive and negative controls (Figures 4.5 and 4.6, lanes 1 and 2) presented with a similar pattern to that seen in the V (Figure 4.1 and 4.2, lanes 1 and 2) and J (Figures 4.3 and 4.4, lanes 1 and 11) blots. MUC5AC in the RX group (Figure 4.5) appeared to travel slightly further into the gel in comparison to MUC5AC in group J, suggesting a slightly higher charged species in the children compared to the adults (Figure 4.3). MUC5AC was detected in 6 of the 10 TB positive children (Figure 4.5, lanes 3, 5, 7, 8, 9 and 11). MUC5B was detected in 8 of the 10 TB positive children (Figure 4.6, lanes 3-5 and 7-11). MUC5AC was not detected in two patients (Figure 4.5 lanes 6 and 12); the same patients (Figure 4.6, lanes 6 and 12) had very little MUC5B.

Western blots comparing the control and diseased groups were probed with the MUC2 antibody, LUM2-3 (Herrmann *et al*, 1999). Figure 4.7 illustrates the absence or in the case of one patient (lane 4) a small amount of MUC2 material in the volunteer group (Figure 4.7, lanes 4, 5 and 6). The positive control (purified colon mucin) appeared at the top and quite faintly in the middle of the gel (Figure 4.7, lane 1). MUC2 was detected in crude saliva from a healthy individual with severe sinusitis (Figure 4.7, lane 2). The MUC2 antibody detected purified mucin from a gastric cancer which appeared as a dark smear at the top of the gel (Figure 4.7, lane 3). In figure 4.7 (lanes 7-11) the TB positive adults and children show varying amounts of material at the bottom of the gel. A TB positive adult (Figure 4.7, lane 7) and a TB positive child (Figure 4.7 lane 9) presented as dark prominent smears indicative of a high charge form of MUC2 (illustrated by arrows). A TB positive adult (lane 8) and child (lane10) and an adult with 'possible' TB (lane 11) also presented as fainter high charged bands of MUC2 (Figure 4.7, indicated by an arrow) at similar positions to the darker smears of MUC2 (Figure 4.7, lanes 7 and 9). The two

TB positive children also presented with faint traces of a low charge band (lanes 9 and 10) (illustrated by circles).

The positive control for MUC2 in figure 4.8, purified colon mucin, along with the purified gastric cancer mucin and crude salivary mucin (lanes 2, 1 and 3 respectively) were detected at the same positions in figure 4.7 (lanes 1, 3 and 2). Patients classified as having 'possible' TB and also being diagnosed with other non-TB respiratory problems (Figure 4.8, lanes 4-12) gave two distinct charges of MUC2 which presented as a doublet of varying intensities. Although not clear in all samples, the doublet (illustrated by the red circle) appeared in each of the 'possible' TB samples (Figure 4.8, lanes 4-12) with the most distinct bands featuring in lanes 7 and 9 (Figure 4.8). The 'possible' TB subjects (adults) also presented with faint high charge bands in lanes 9, 10, 11 and 12 (Figure 4.8, indicated by arrows). It should be noted that the high charge form of MUC2, presented in the TB positive adults and children (Figure 4.7, lanes 7-10), was barely discernable in the blots for patients who were 'possible' TB subjects (Figure 4.8, lanes 9, 10, 11 and 12). The volunteers (controls) had trace amonts (Figure 4.7, lanes 4, 5) or no MUC2 (lane 6).

MUC5B appeared as broad bands compared to MUC5AC in all four groups (groups V, N, J and RX). Glycoforms of MUC5B were not easily identified within the broad smears. In addition it was interesting to note that the respiratory sputa in this investigation differed in electrophoretic movement in comparison to the positive controls for MUC5AC and MUC2. MUC5AC detected purified gastric cancer mucin was identified at the top of the gel while the respiratory mucins sat in the middle of the gel (Figures 4.3 and 4.5). Mucin from a purified colon and gastric cancer probed for by MUC2 sat the top of the gel whilst MUC2 detected respiratory mucins were found in the middle and bottom of the gel (Figures 4.7 and 4.8).

4.2 Discussion

Western blotting was the method of choice as it has proven to be a widely used analytical tool in mucin detection (Dunn, 1986). This investigation made use of SDS-agarose gels during western blotting and not SDS-PAGE gels, although initially SDS-PAGE gels were the forerunners in the analytical study of mucin biosynthesis. SDS-PAGE gels highlight dispersity within each homogenous mucin population (Berry *et al.*, 2000). Their intrinsic negative charge of mucins, imposed by sialic acid and sulphate residues at the terminus of O-glycans, interact with SDS to influence their mobility. Agarose gel electrophoresis, as described by Thornton *et al* (1997) was the method of choice in the separation of mucins in this study. SDS-PAGE gels are successful at separating lower molecular weight mucins such as MUC7. High molecular weight mucins (MUC5AC, MUC5B, MUC2), due to their degree of glycosylation and large size, are incapable of entering the polyacrylamide matrix. Agarose gels appear to optimally separate large charged and uncharged glycoproteins which can not penetrate SDS-PAGE gels (Tytgat *et al.*, 1995). This is clearly observed in Figures 4.1-4.8.

Reduction and alkylation was carried out on the sputum and scrapes prior to SDS-agarose gel analysis. This was a necessary preliminary step as respiratory secretions such as human bronchial mucus have a gel-like viscosity due primarily to mucin glycoproteins (Houdret *et al.*, 1981). Its mucoid nature makes it problematic to work with, hence the reduction of mucins to generate subunits. This allows for the mucin subunits to enter agarose gels during electrophoresis. Reduction is also essential for antibody detection of mucin epitopes. This was observed in MUC5B detection of cervical secretions. Whole cervical mucins yielded a low reactivity to the MUC5B in comparison to its reduced and alkylated subunits. Salivary and respiratory mucins behaved similarly (Wickstrom *et al.*, 1998). It is further mentioned that the protein core of the mucin subunit is representative of the primary gene product making it even more advantageous to work with (Thornton *et al.*, 1995). Proteolysis was excluded from this study preventing cleavage to the protein core hence the attempt to maintain the protein structure as close as 'possible' to its *in vivo* state (Getz *et al.*, 1999).

Personal experience and literature (Dunn, 1986, Gershoni *et al.*, 1983, Thornton *et al.*, 1994) have emphasized the importance involved in the stringency of the blotting procedure. The gels are incubated in transfer buffer to prevent swelling during the blotting procedure. If this step is omitted the gel swells during vacuum blotting resulting in distorted protein bands (Gershoni *et al.*, 1983). Poly-L-lysine aids greatly in the binding of the mucins to the nitrocellulose via an electrostatic interaction (Thornton *et al.*, 1989). Background staining was successfully avoided as is seen in Figures 4.1-4.8 by:

a) careful handling of the gel during blotting
b) the removal of bubbles between the membrane and gel during blotting
c) the use of milk powder (non-fat) and Tween-20 in the blocking solutions (Dunn, 1986) and antibody solutions
d) extensive washing between the antibody incubation periods
e) care taken during the ECL

Western blotting presented with contrasting results after MUC5AC and MUC5B detection of the volunteer group (control group). MUC5AC was barely visible in the six volunteer samples (Figure 4.1). This experience has been the experience of other researchers (Dr John Sheehan, personal communication during a visit to laboratory in November 2005). This was significant as literature states that both the MUC5AC and MUC5B mucins are clearly present in normal healthy controls (Henke *et al.*, 2004). It is suggested that MUC5AC exists in trace amounts in "healthy" individuals and that it was reduced even further during the stringent intermediate steps of the western blotting procedure previous to antibody detection. It is also hypothesized that antibody detection of these highly glycosylated mucins is increased during a diseased state due to a loss of certain sugar side chain residues and hence a greater exposure to, and antibody detection of the apomucin. This could explain why Rose and Voynow, (2006) found that MUC5AC and MUC5B "appear" at reduced levels in healthy airways compared to sputum from asthma, chronic bronchitis and cystic fibrosis (CF) patients. It is further suggested that an alteration in glycosylation could be a result of the TB mycobacterial invasion as in the case of *Helicobacter pylori* a bacterium reported to reversibly alter the

glycosylation patterns of gland mucus cell mucins (GMC) in the gastric mucosa (Matsuzwa *et al.*, 2003). Gastric MUC5AC is known to present with a primary receptor, a blood group Le^b oligosaccharide, for *Helicobacter pylori* in the stomach (Rose and Voynow, 2006). Bacteria are also known to adhere to specific oligosaccharide sequences on intestinal mucins (Brockhausen, 2004). In contrast to MUC5AC, MUC5B was observed as distinct broad smears in 4 of the 6 volunteer (Figure 4.2, lanes 3, 4, 7 and 8). Two of the six volunteers did not present with MUC5B (Figure 4.2, lanes 5 and 6). This could be explained by inter-individual variation within a 'normal' population although a greater number of samples would be required to support this line of argument.

Prior to this study, investigations on mucins have been carried out in respiratory diseases such as CF, chronic bronchitis (CB) and asthma (Kirkham *et al.*, 2002). The results from this study correlate well with past respiratory studies as the major respiratory mucins, MUC5AC and MUC5B have been detected in TB positive adults and children (Figures 4.3-4.6). The absence of MUC5AC and MUC5B in a single TB positive adult (Figures 4.3 and 4.4, lane 2) and two TB positive children (Figure 4.5 and 4.6, lanes 6 and 12) could possibly be, amongst other reasons, explained by the subjects having a dry cough, hence the lack in sputum production.

Past studies have emphasized the difficulty associated with the identification of MUC2 in normal and diseased respiratory tract secretions (Thornton *et al.*, 1997, Buisine *et al.*, 1999, Davies *et al.*, 1999). This study illustrated the absence of MUC2 in healthy volunteer samples (Figure 4.7, lanes 4-6) and its presence in TB positive and the 'possible' TB adults and children (Figures 4.7, lanes7-11 and 4.8, lanes 4-12). Takeuchi *et al* (1995) hypothesized that MUC2 mRNA is expressed in various nasal diseases and is absent in normal, healthy nasal mucosa. This was confirmed by their studies which illustrated the expression of MUC2 mRNA during bacterial infection and stimulation via allergens, and its absence in a normal state. This could explain the absence of MUC2 in the volunteers (V) and its presence in the TB positive adults and children (Figure 4.7). Furthermore, it was suggested that the amount of MUC2 in the healthy volunteers was too low to detect (personal communication with Dr Annkatrin Herrmann). Asthma

studies have also illustrated a higher percentage of MUC2 during the diseased state in comparison to the healthy individuals (Rose and Voynow, 2006). The presence of MUC2 in the TB positive samples could be strongly related to the influence of the TB bacilli. This idea is supported by findings such as Kim *et al* (2003) who discovered that MUC2 gene expression in the airways was stimulated by an endotoxin from the gram negative bacteria, *Pseudomonas aeroginosa.* The TB bacilli could be playing a similar role to that of *Pseudomonas aeroginosa* by stimulating the MUC2 gene expression during TB.

MUC5AC and MUC5B did not appear to present with glycoforms in both control and diseased groups. The MUC5AC results are consistent with literature as it is described as a homogenous species in comparison to the heterogeneous MUC5B (Kirkham *et al.*, 2002, Thornton and Sheehan, 2004). The MUC5B results are significant as they do not present with the well documented oligomeric glycoforms described by Thornton *et al* (1997), Sheehan *et al* (1999) and Kirkham *et al* (2002) as well as others. According to Thornton and Sheehan (2004), identification of individual mucin species requires the purification of reduced mucin preparations on a Mono Q anion exchange column. This is a well noted future consideration into the identification of glycoforms, especially respiratory MUC5B which is known to present with a low and high charge species (Thornton and Sheehan, 2004). MUC2 did exhibit varying bands depending on the TB diagnosis (Clinical Chapter, table 3.1).

MUC2 was detected in the saliva of an individual with sinusitis (Figure 4.7, lane 2). Takeuchi *et al* (1995) have illustrated the expression of the MUC2 gene in the nose and paranasal sinus which could explain its presence in the crude saliva. It also presented as a prominent high charged broad band in a TB positive adult and child (Figure 4.7, lanes 7 and 9). A similar, yet not as prominent, band was found in a TB positive adult and child and a 'possible' TB adult (Figure 4.7, lanes 8, 10 and 11 respectively). The two TB positive children also presented with a low charge form of MUC2 (Figure 4.7, lanes 9 and 10). MUC2 appeared as a pronounced doublet in adults and children categorized as 'possible' TB patients (Figure 4.8, lanes 4-12). The 'possible' TB adults also presented

with faint traces of a high charge form (Figure 4.8, lanes 9, 10, 11 and 12). It is postulated that MUC2 appears as different glycoforms as TB progresses from the 'possible' state to the probable and definite states. This theory is supported by the change in the intensity and presence of the high and low (doublet) charge bands. It is hypothesized that the high charge band appears as a weak signal during the 'possible' TB state and increases in intensity as definite TB is established; this is illustrated in the 'possible' TB positive adults (Figure 4.8, lanes 9, 10, 11 and 12). Furthermore it is suggested that the doublet that features prominently in the 'possible' TB adults and children weakens to a faint barely visible band observed in the two TB positive children. This is supported by the presence of the faint low charge band in the TB positive child that also presented with the distinct high charged band (Figure 4.7, lanes 7 and 9). If this theory proves true then the MUC2 doublet could not only aid in diagnosis of TB but may also be related to a susceptibility to the disease. This would require further molecular studies as the hypothesized changes in the doublet and high charged form of MUC2 suggests an alteration that is gene regulated. Literature has illustrated via molecular studies that longer MUC2 alleles in atopic non-asthmatics could be acting as a protective mechanism against the development of asthma (Vinall *et al.*, 2000).

The general broadened or smear like appearance of MUC5AC and MUC5B (Figures 4.1-4.8) can be explained by oligomerization (which results in their high molecular weight), by microheterogeneity which is imposed by heterogeneous carbohydrate side-chains (Carlsson, 1993) and by the high levels of glycosylation of mucin itself, which could be up to 70% (Allen, 1981). MUC2 was also detected as a broadened band in the controls and in a TB positive adult and child (Figure 4.7 and 4.8). The varying intensity of the mucin bands within each blot is probably a result of the samples being loaded onto the gel as volume equivalents although studies such as those by Henke (2004) on cystic fibrosis secretions found that samples that were loaded onto gels at either the same concentration or volume were similar in appearance after MUC5AC and MUC5B detection. This suggests that the intensity of the mucin smears is not directly related to the concentration of the sample loaded into a gel. It is therefore hypothesized that a

darker smear could be indicative of a greater number of epitopes on a single mucin subunit.

The electrophoretic differences observed between positive controls and samples are largely indicative of the differences in intrinsic negative charge. This similarity was observed by Thornton *et al* (1995). Human cervical, gastric, respiratory and salivary mucins were detected using a polyclonal antiserum raised to reduced mucin subunits. The mucins differed in terms of electrophoretic mobilities which were attributed to differences in their charge densities. This appears to be a similar case in this study. The MUC5AC detected purified human gastric cancer mucin has retarded mobility due to a lower intrinsic charge (Figures 4.3 and 4.5) (Tytgat *et al.*, 1995) in comparison to the respiratory mucins. The MUC5B detected saliva appears to share a similar intrinsic charge with the respiratory mucins (Figures 4.2, 4.4 and 4.6). The MUC2 detected purified colon and gastric cancer mucins, appeared to have lower intrinsic charges in comparison to the high charged saliva and respiratory secretions (Figures 4.7 and 4.8). Respiratory MUC2 yielded 2 low charged species in the 'possible' TB state (Figure 4.8) and a single high charge species in the TB positive state (Figure 4.7). Furthermore MUC5AC in group RX (Figure 4.5, TB positive children) appeared to have a slightly higher charge than group J (Figure 4.3, TB positive adults). This could be related to differences in charge density and/or glycosylation between adults and children, and would therefore require deeper investigation. Future studies involving children would be problematic considering the difficulty associated in obtaining controls for children.

Figure 4.1 0.7% Agarose gel electrophoresis of MUC5AC detected crude sputa from volunteers (V), used as controls in this study. Samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with 25mM IAA overnight. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. Samples were run at 200A and 90V. They were then vacuum blotted onto nitrocellulose at a pressure of 40-50bars. The primary antibody used is a MUC5AC, LUM5-1 (Hovenberg *et al.*, 1996) rabbit polyclonal and the secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, purified gastric cancer mucin (positive control); 2, crude saliva (negative control); 3, Mari; 4, Z; 5, H; 6, F; 7, U ; 8, Mini

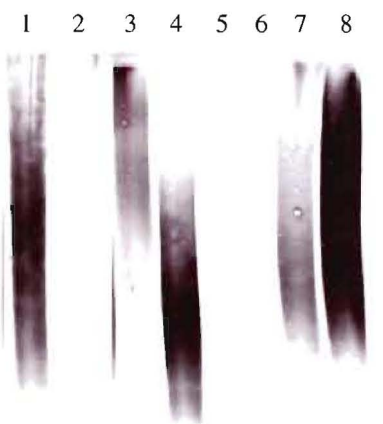


Figure 4.2 0.7% Agarose gel electrophoresis of MUC5B detected crude sputa obtained from volunteers (V), used as controls in this study. The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 200A and 90V. They were then vacuum blotted onto nitrocellulose at a pressure of 40-50bars. The primary antibody used is a MUC5B, LUM5B-2 (Wickstrom *et al.*, 1998) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, crude saliva (positive control); 2, purified gastric cancer mucin (negative control); 3, Mari; 4, Z; 5, H; 6, F; 7, U; 8, Mini

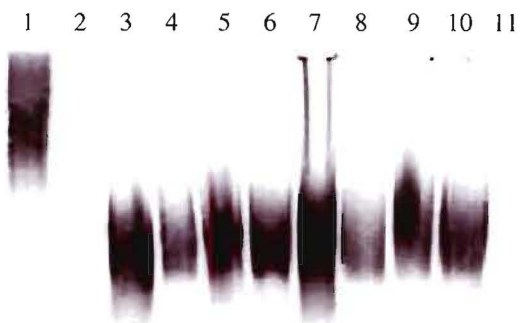


Figure 4.3 0.7% Agarose of MUC5AC detected crude adult sputa (J). The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 200A and 90V. They were then vacuum blotted onto nitrocellulose at a pressure of 40-50bars. The primary antibody used is a MUC5AC, LUM5-1 (Hovenberg *et al.*, 1996) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, purified gastric cancer mucin (positive control); 2-10, TB positive adult samples; 11, crude saliva (negative control)

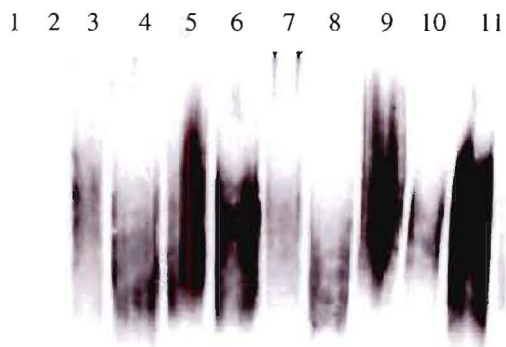


Figure 4.4 0.7% Agarose of MUC5B detected crude adult sputa (J). The MUC5AC blot (Figure 3) was stripped with mercaptoethanol and reprobed with a MUC5B, LUM5B-2 (Wickstrom *et al.*, 1998) rabbit polyclonal. The secondary antibody was a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, purified gastric cancer mucin (negative control); 2-10, TB positive adult samples; 11, crude saliva (positive control)

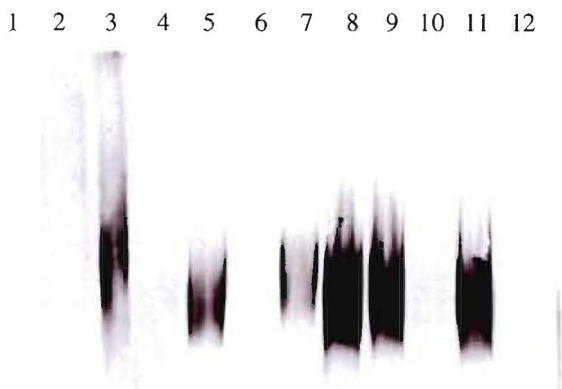


Figure 4.5 0.7% Agarose of MUC5AC detected crude children sputa (RX). The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 200A and 90V. They were then vacuum blotted onto nitrocellulose at a pressure of 40-50bars. The primary antibody used is a MUC5AC, LUM 5-1 (Hovenberg *et al.*, 1996) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, crude saliva (negative control); 2, purified gastric cancer mucin (positive control); 3-12, TB positive children (RX)

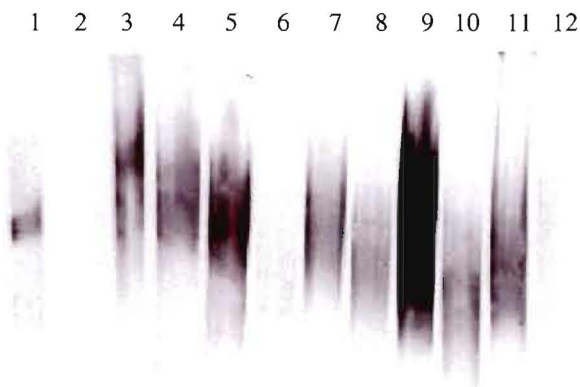


Figure 4.6 0.7% Agarose of MUC5B detected crude children sputa (RX). The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 200A and 90V. They were then vacuum blotted onto nitrocellulose at a pressure of 40-50bars. The primary antibody used is a MUC5B, LUM5B-2 (Wickstrom *et al.*, 1998) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1: crude saliva (positive control); 2, purified gastric mucin (negative control); 3-12, TB positive children (RX)

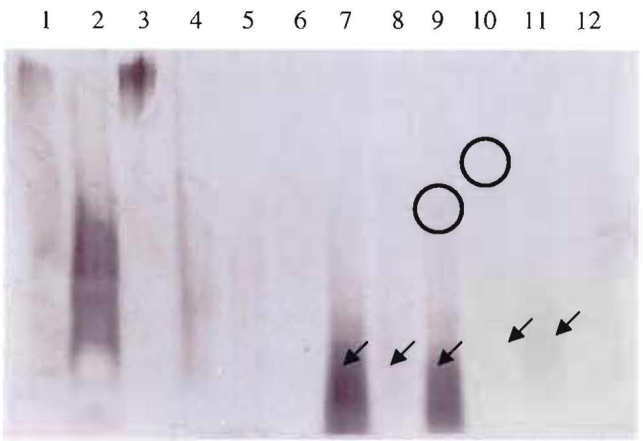


Figure 4.7 07% Agarose of MUC2 detected sputa. The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 400A and 65V. They were then vacuum blotted onto nitrocellulose at a pressure of 50-60bars. The primary antibody used is a MUC2, LUM2-3 (Herrmann *et al.*, 1999) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands. The black circles indicate the presence of a faint band present in lanes 9 and 10 and the arrows point to the high charge form of MUC2.

Lanes 1; purified colon mucin (positive control); 2, saliva; 3, purified gastric cancer mucin; 4-6, volunteer sputa (V), control; 7 and 8, TB positive adults (J); 9 and 10, TB positive children (RX); 11, 'possible' TB adult (J); 12, 'possible' TB child (RX)

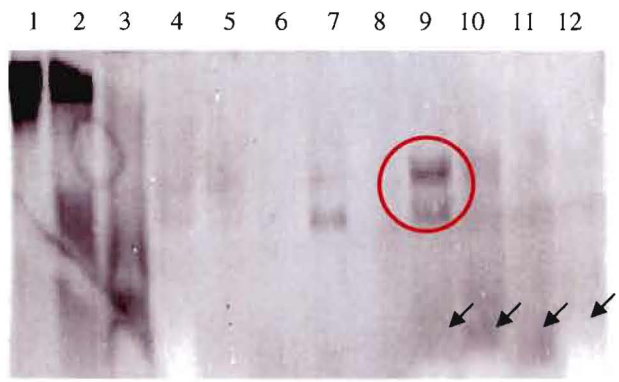


Figure 4.8 0.7% Agarose gel of MUC2 detected sputa. The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 400A and 65V. They were then vacuum blotted onto nitrocellulose at a pressure of 50-60bars. The primary antibody used is a MUC2, LUM2-3 (Herrmann *et al.*, 1999) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands. The red circle highlights the MUC2 doublet and the arrows indicate faint high charge bands in lanes 9, 10 and 11).

Lanes 1; purified gastric cancer mucin (positive control); 2, purified colon mucin (positive control); 3, saliva; 4-8, 'possible' TB positive children (RX); 9-12, 'possible' TB positive adult (J)

CHAPTER FIVE

MUCIN EXTRACTION AND PURIFICATION

5.1 Solubilisation and purification of mucin

Sputum samples and crude mucus scrapings were suspended in 6M GuHCl containing a mixture of proteinase inhibitors namely NEM and EDTA, in phosphate buffer, pH 6.5. Mucus scrapings were solubilised by brief homogenization and centrifuged at 1000 rpm (6000g) for 1 hour at 4°C, to remove insoluble debris. The supernatants were fractionated by two successive equilibrium density gradient centrifugations in CsCl, for 48h (starting density 1.420g/ml), at 105 000g (40 000rpm) in a Beckman centrifuge. Following ultracentrifugation the isolated mucin was subjected to SDS-PAGE analysis to confirm that it was free of contaminant protein, lipid and nucleic acids.

Figure 5.1 illustrates the PAS, protein and density profile of the repiratory mucins after the first CsCl centrifugation. The profile presented with traces of protein contaminants, as can be observed from the protein peak at fraction 6 (indicated by the blue square), hence the need for a second CsCl purification. The mucins that peaked at approximately 1.3-1.4g/ml (fractions 4, 5 and 6) were pooled and prepared for a second purification.

Figure 5.2 shows that the mucins are free of contaminant protein (the protein profile did not present with peaks) after two successive spins in a CsCl density gradient centrifugation. Mucin fractionated at a density of approximately 1.3-1.4g/ml in fractions 4, 5 and 6.

An SDS-PAGE analysis of the purified and isolated mucins is illustrated by Figure 5.3. The running gel was stained with Brilliant Coomassie Blue (BCB) to determine the presence of protein contaminants such as serum proteins and secretory glycoproteins (Bhaskar *et al.*, 1981). After staining and destaining the High MWM was clearly detected in lane 1. The lanes containing the purified respiratory mucin samples from

groups V, J and RX groups (lanes 2-12) appeared blank. This indicated a lack of detectable protein in the isolated mucins.

Figure 5.4 and 5.5 represent a TB positive post mortem (PM) scrape (lanes 3 and 4). Post-mortem samples (PM) were obtained early in this study due to the difficulty experienced in obtaining samples from 'normal' volunteers. The scrape was purified via a CsCl density gradient and was compared to its crude equivalent. Antibody detection for MUC5AC (Figure 5.4) and MUC5B (Figure 5.5) revealed clear, broad bands for both the crude (Figures 5.4 and 5.5, lane 3) and purified TB positive scrape (Figures 5.4 and 5.5, lane 4) with a broader background to the unpurified material in each case.

5.2 Discussion

Mucins were extracted in a mixture of GuHCl and proteolytic inhibitors in order to minimize degradation through proteolysis, according to the method of Carlstedt and Sheehan (1984). EDTA was required to inhibit metalloproteinases (Carlstedt and Sheehan, 1984) and NEM served to prevent disulfide exchange (Sheehan *et al.*, 1981) and inhibit degradation via thiol proteinases (Carlstedt and Sheehan, 1984). Mucins were initially identified in crude sputum specimens by Western Blotting analysis. Some purified mucin was also analyzed by this method to determine whether association of mucin with protein and other components had any effect on separation by agarose gel electrophoresis. Purification and isolation of mucin was also necessary for amino acid analysis.

Isolation and purification of mucins plays a pivotal role towards the understanding of their structure-function relationship. Various isolation techniques namely exclusion chromatography and ion-exchange chromatography have been employed in the past. The chosen purification technique of this study has also proved its worth in the isolation of mucins. The CsCl density gradient method has been well documented in studies involving the isolation of bronchial mucins, blood group substances, pig gastric and intestinal mucus and human gastric mucus (Woodward *et al.*, 1982). CsCl has also

played a crucial role in the isolation of plant ribosomal DNA via the actinomycin-CsCl method (Hemleben *et al.*, 1977).

Mucins are well resolved in CsCl although better separated in caesium bromide (CsBr) (Bhaskar *et al.*, 1981). CsCl is more often used due to its high diffusion constant, less viscous solutions and ability to create steeper gradients (Polson *et al.*, 1963). The principle behind this method is that the mucins will find their isodensity as the sedimentation equilibrium is established. According to the Figures 5.1 and 5.2 the tracheal mucins sedimented around fractions 4, 5 and 6 at an approximate density of 1.4g/ml. The isolation procedure was similar to that of Bhaskar *et al* (1981) hence agreeing with their statement that "mucus glycoprotein should be in the bottom half of the preparative tubes while all the protein should be the upper half". This was demonstrated as the mucins were well separated from proteins, which appeared at greater concentrations in the higher fractions, and DNA which bands at approximately 1.63g/ml (Creeth, 1978). Separation from the protein fractions can be explained in terms of caesium's high ionic strength which is capable of breaking the strong non-covalent bonds that exist between protein-glycoprotein interactions (Allen, 1981).

According to Allen, (1981) SDS-PAGE analysis is a valid method in the determination of purity of mucins. The excellent resolution power of the SDS-PAGE highlights the purity of the second spin mucins (Figure 5.3). The MWM is well observed whereas all the lanes (except lane 3) containing the purified material appear blank. Lane 3 presented with a single band toward the bottom of the gel. This could be explained the presence of contaminating non-covalently bound, low molecular weight proteins which were absent in the other lanes (Allen, 1981).

CsCl purification involves lengthy intermediate steps, requires the use of large quantities of reagents and involves two and even three 48-65hr ultracentrifugation spins. In this case we showed that CsCl purification was unnecessary to detect the relevant antigens for MUC5AC and MUC5B. The electrophoretic behaviour of the crude material, in terms of its broader mobility compared to purified material, could be explained by the 'age' of the

PM samples as cadavers were available only 48-72hr after death. Therefore in this case protein contamination and degradation are significant factors in explaining the slightly different electrophoretic behaviour the crude and pure sample. This resulted in the use of crude sputa throughout the western blotting investigation (Chapter 4) which was greatly beneficial to the study as both time and expense were reduced. A project of this nature could yield hundreds of samples and if purification of mucin was required there would be time, labour, financial and logistical factors to consider.

The preparative mode of CsCl isolation is advantageous in that fractions can be quantitatively analysed (Bhaskar *et al.*, 1981). This study produced satisfactory amounts of purified mucin for small scale analysis such as the amino acid analysis. The volunteer (V) and children group (RX) had the lowest mucin yield followed by the adult group (J). Studies on "normal" tracheal secretions such as group (V) are problematic due to the difficulty associated with sample collection (Woodward *et al.*, 1982). The post mortem cadavers (PM) resulted in a greater number of samples with the highest yield of purified mucin. This could be explained by the fact that mucus was scraped directly from the epithelial lining of the trachea. The amount of scrapable material was also larger due to the increase of tissue degradation with time after death. With respect to the other groups the method by which mucus was collected allowed for greater contamination.

CsCl is also known to operate in an analytical mode which allows for the identification of lipids and serum glycoproteins. Lipid and DNA analysis were not carried out on the purified samples and should be of future consideration. According to Woodward *et al.*, (1982), "the contribution of lipid analysis to the overall architecture of mucus secretions cannot be overlooked". Lipid has been documented in the sputum of patients who suffer from varying bronchial diseases, and at elevated levels in the secretion of cystic fibrosis patients (Slayter, 1987). DNA analysis as observed from McLeod's (1992) study aids in the analysis of the step-by-step purification procedure and should be included in future investigations.

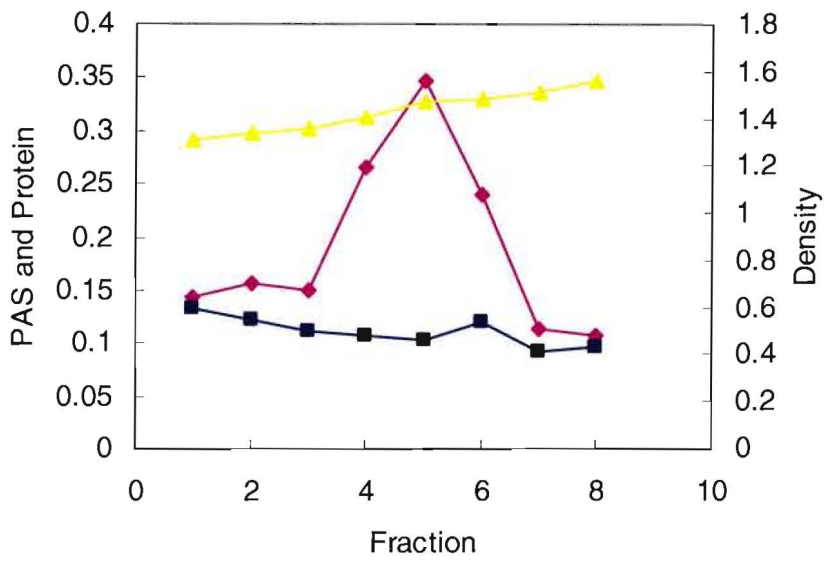


Figure 5.1 A PAS, protein and density profile of the first CsCl purification of respiratory mucus. Mucus was adjusted to a density of 1.39-1.4g/ml by the addition of solid caesium chloride and centrifuged at 40000rpm for 48hr at 4°C. The resultant gradient was fractionated into 8 equal parts. The density of each fraction was measured (▲), and following exhaustive dialysis of an aliquot against distilled water, each fraction analyzed for glycoprotein (◆) (PAS) and protein (■) (Lowry). The results are expressed as absorbance values and in g/ml for each fraction.

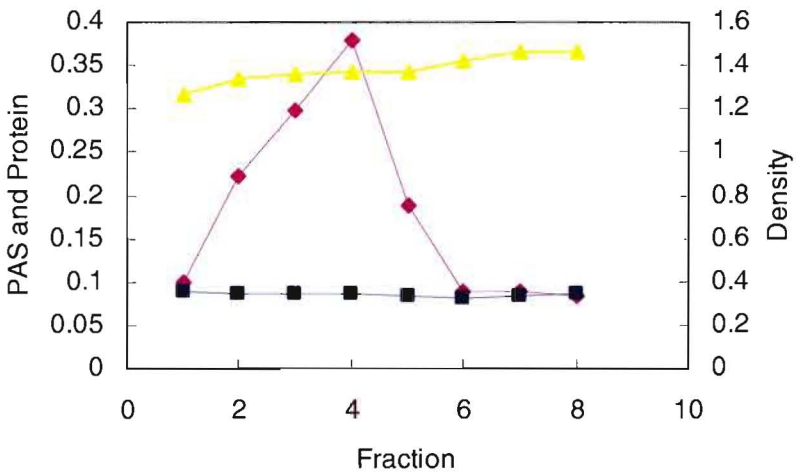


Figure 5.2 A PAS, protein and density profile of the second 48hr CsCl purification of respiratory mucus. Mucus was adjusted to a density of 1.39-1.4g/ml by the addition of solid caesium chloride and centrifuged at 40000rpm for 48hr at 4°C. The resultant gradient was fractionated into 8 equal parts. The density of each fraction was measured (▲), and following exhaustive dialysis of an aliquot against distilled water, each fraction analyzed for glycoprotein (◆) (PAS) and protein (■) (Lowry). The results are expressed as absorbance values and in g/ml for each fraction.

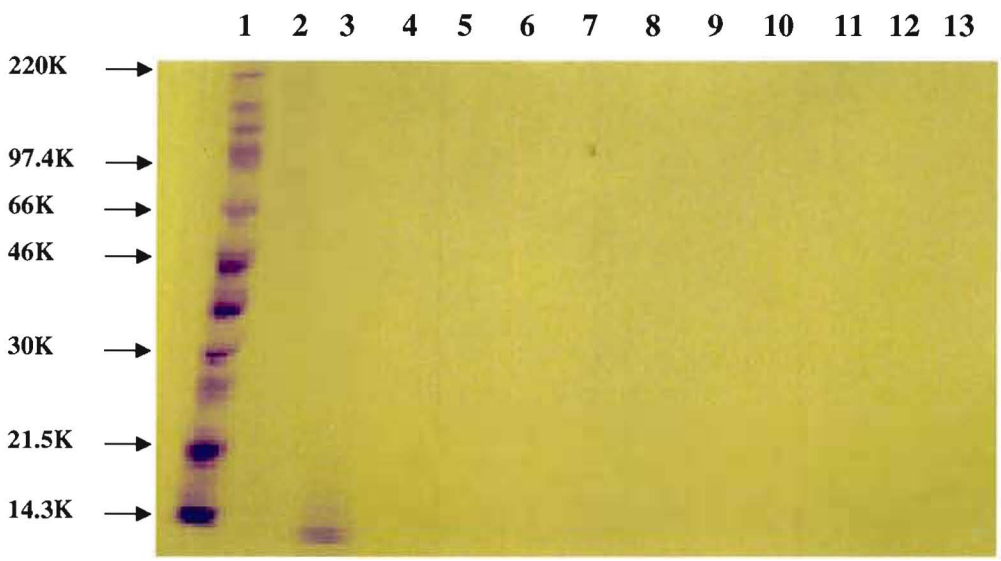


Figure 5.3 A 10% SDS-PAGE gel of CsCl purified samples. The samples underwent density gradient centrifugation twice, were dialysed and reduced in mercaptoethanol prior to gel electrophoresis. The gel was stained with Brilliant Coomassie Blue.

Lane 1, MWM: the High molecular weight markers indicated by arrows in descending order are myosin (220K), phosphorylase b (97.4K), bovine serum albumin (66K), ovalbumin (46K), carbonic anhydrase (30K), trypsin inhibitor (21.5K), lysozyme (14.3K); Lane 2-13: purified respiratory mucin samples from V, J and RX groups

Figure 5.4 0.7% Agarose of MUC5AC detected crude post mortem scrapes (N). The samples were reduced in 6M GuHCl, 10mM DTT for 5hrs and alkylated with IAA. They were combined with agarose loading buffer (1xTAE, 50% glycerol, bromophenol blue) and boiled for 90sec. The samples were run at 200A and 90V. They were then vacuum blotted onto nitrocellulose at a pressure of 40-50bars. The primary antibody used is a MUC5AC, LUM5-1 (Hovenberg *et al.*, 1996) rabbit polyclonal. The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, purified gastric cancer mucin (positive control); 2, crude saliva (negative control); 3, crude TB positive scrape; 4, purified TB positive scrape

Figure 5.5 0.7% Agarose of MUC5B detected crude post mortem sputa (N). The MUC5AC blot (Figure 9) was stripped and reprobed for MUC5B, LUM5B-2 (Wickstrom *et al.*, 1998). The secondary antibody is a goat anti-rabbit HRP. A 5min ECL was carried out for detection of protein bands.

Lanes 1, purified gastric cancer mucin (negative control); 2, crude saliva (positive control); 3, crude TB positive scrape; 4, purified TB positive scrape

CHAPTER SIX

ACID AMINO ANALYSIS

6.1 High pressure liquid chromatography (HPLC) analysis of amino acids

Purified unreduced mucins were selected from the control groups (V and N) and TB positive groups (J and RX) for HPLC analysis. Altogether, two samples were selected from the volunteer group (V), three from the post mortem group (PM), two from the TB positive adult group (J) and two from the TB positive children group. The samples were hydrolysed to remove the carbohydrate chains from the mucin peptide and were placed on a HPLC column to elute the primary amino acids.

Table 6.1 represents an average of the amino acid values for samples within each group. Glycine, threonine, serine, proline and alanine constituted 54.99 mol% of the volunteer group (V), 43.35 mol% of the PM group, 46.58 mol% of the TB positive adults (J) and 48.94 mol% of the children (RX).

Serine and threonine combined appeared at 22.38 mol% in the V group and at 16.01 mol% in the PM group. Although the TB positive adults and children (18.37 mol% and 17.17 mol% respectively) had slightly lower serine and threonine values compared to the volunteer group, they compared closely to each other.

Serine, threonine and proline (PTS) accounted for 34.67 mol% in the V group, 22.88 mol% in the PM group, 29.55 mol% in the J group and 29.31 mol% in the RX group. The PTS mol% was approximately 5% higher in the healthy volunteers (V) compared to the TB positive adults (J) and children (RX), who presented with similar values.

6.2 Discussion

Amino acid analysis was carried out to determine the composition of the respiratory apomucins from the control (V and N) and TB positive groups (J and RX). This was expected to lend insight into similarities or differences between the amino acid compositions from each group. According to Woodward *et al* (1982), a high amino acid combination of glycine, alanine, threonine, serine and proline is characteristic of the typical mucin composition. This combination in the V group (54.99 mol%) compared well with the findings of Woodward *et al* (1982) who presented with a 54 mol% combination in bronchial aspirates of patients without pulmonary diseases. This is also similar to the findings of Bhattacharyya *et al* (1990) who claimed that the glycine, threonine, serine, alanine and proline accounted for 50-60 mol% of the total bronchial amino acid composition. In contrast to the volunteer group, the PM, J and RX group presented with much lower values of the amino acid combination (43.35 mol%, 46.58 mol% and 48.94 mol% respectively). Respiratory diseases such as CF and asthma have presented with higher percentages (63 mol% and 57 mol% respectively) (Chace *et al.*, 1985) of the amino acid combination compared to the TB diseased groups. Chace *et al* (1989), like Bhattacharyya *et al* (1990), found that the glycine, threonine, serine, alanine and proline represented 71 mol% of CF mucins compared to the 61 mol% in normal mucins. In contrast this study found that the V group (normal healthy controls) presented with an amino acid combination which was approximately 10% higher than the TB positive groups (group J and RX). Similarly the PTS, and serine and threonine combination for the V group appeared higher compared to the J and RX groups. The differences observed in the PM group could be accounted for by proteolytic degradation. As suggested by Chace *et al* (1985), the differences observed in mol% between the TB groups and the control groups, and the TB groups and the CF and asthma studies could be related to "differences in the proportion of the different types of mucins present in the respiratory secretions". It is hypothesized that the level of secretion of respiratory mucins from different cell types (such as the goblet cells or submucosal glands) differs between healthy and diseased individuals. This hypothesis is extended to differences in the proportion of varying mucin types between respiratory diseases.

The hydroxyamino acids serine and threonine are of particular importance as they represent the sites at which O-glycosylation takes place (Hovenberg *et al.*, 1997). A combination of serine and threonine constituted approximately 20% of the total amino acid composition in each group (Table 6.1). This value compares closely to the serine and threonine range in previous studies (Mall *et al.*, 1999). The TB positive adults and children appeared to share similar serine and threonine values (18.37 mol% and 17.17 mol% respectively). Although not drastically different, these values are slightly lower in comparison to the volunteer group (22.38 mol%). It is postulated that the minor decrease in the serine and threonine composition of the TB positive groups is due to bacterial invasion. Based on previous studies it is hypothesized that the TB bacterium has a greater affinity for the sugar side chains of respiratory mucins compared to the amino acids of the protein backbone. According to literature certain carbohydrates present with epitopes that are possible sites of attachment for microbes and viruses (Lamblin *et al.*, 2001, Voynow, 2002). Literature has also associated glycosylation changes with bacterial invasion; *Helicobacter pylori* has been reported to reversibly alter the glycosylation of gastric mucins (Matsuzwa *et al.*, 2003). The change in amino acid composition could be secondary to the TB bacterium's interaction with the sugar side chains. The high proline content of the V, J and RX group is suggestive of a closely packed protein backbone. According to Allen (1981) this enables "the close packing of large carbohydrate side chains" hence their protection from proteolytic enzymes.

It is observed from past studies (Chace *et al* (1989), Bhattacharyya *et al* (1990) that serine, threonine and proline are present at high percentages in comparison to the other amino acids that constitute the protein backbone (Thornton *et al.*, 1996). Table 6.1 reflects higher percentages of glycine and glutamic acid compared to serine, threonine and proline. It is suggested that the purified mucins were not fully deglycosylated as was discovered by Perini *et al* (1989), hence the greater mol percentage of glycine and glutamic acid. From their study it was illustrated that trifluoromethane sulfonic acid (TFMS) alone led to incomplete deglycosylation of human bronchial mucins hence the use of TFMS/anisole mixture and solvolysis in hydrogen fluoride (HF). The

deglycosylation process of the purified control and TB positive mucin samples using HCl, phenol and nitrogen gas was probably inefficient.

Future studies should focus on isolating and purifying respiratory MUC5AC, MUC5B and MUC2 from control and diseased groups and thereafter carrying out amino acid analyses. This would better illustrate direct amino acid changes to specific mucin types within control and TB groups. Furthermore, a comparison of the amino acid and sugar analysis of a specific mucin type might lend insight into the presence and differences between mucin glycoforms. This would determine if it is the peptide backbone or sugar side chain that undergoes changes during the diseased state. One might also identify simultaneous alterations to both the protein backbone and sugar side chain hence revealing varying and unique amino acid sequences and O-glycosylation patterns explaining the heterogeneity between mucins (Voynow, 2002). It is also suggested that cysteine analysis be carried out as this would lend insight into cysteine rich domains which are major features of the tandem repeat regions of respiratory MUC5AC and MUC5B (Rose and Voynow, 2006).

Table 6.1 The mean amino acid composition (mol %) of deglycosylated respiratory mucin peptides.

Amino acid	Group V (mol%)	Group PM (mol%)	Group J (mol%)	Group RX (mol%)
Asp	6.89	8.47	7.83	9.20
Thr	11.25	8.69	10.34	8.49
Ser	11.13	7.32	8.03	8.68
Pro	12.29	6.87	11.18	12.14
Glu	12.01	10.66	13.02	13.82
Gly	13.45	9.90	10.99	12.11
Ala	6.87	10.57	6.04	7.52
Val	5.07	7.27	5.58	5.47
Met	0.30	1.38	1.22	1.15
Ile	2.41	2.54	2.67	2.33
Leu	5.43	9.33	6.29	6.58
Tyr	2.27	2.12	2.18	3.03
Phe	1.91	4.09	2.36	3.08
Lys	3.97	6.57	4.72	4.13
Arg	4.74	4.23	7.55	2.26
Total	**100**	**100**	**100**	**100**

CHAPTER SEVEN

THE MOLECULAR STUDY

7.1 The study group

Of a total of 55 adults and children who were recruited for this study, thirty three were investigated for the MUC2 polymorphism and all were screened for the MUC5B polymorphism. The adults ranged between 14 and 66 years of age and the children were between the ages of 4 months and 6 years. The study group was further categorized into 21 TB positive, 16 TB negative and 18 undetermined diagnoses. The TB positive group comprised 'possible', probable and definite TB individuals, as classified by the World Health Organisation (WHO). Regions of the MUC2 and MUC5B genes were amplified and investigated for known polymorphisms.

7.2 Identification of the MUC2 polymorphism

The MUC2 PCR products were digested with *Pvu*II which recognizes a polymorphism at the 5' end of exon 11. The *Pvu*II digestion of MUC2 is represented schematically in figure 7.1. In the absence of the polymorphism, *Pvu*II cleaves the uncut 748bp PCR product into two fragments of 299bp and 449bp and represents the G allele. If the polymorphism at exon 11 is present then the 449bp fragment is further cleaved into a 383bp and 66bp fragment which represents the C allele.

Both the C and G alleles were seen in the study sample (Table 7.1). Of the 33 individuals investigated 12 (36%) were homozygous for the G allele compared to only 6 (18%) who were homozygous for the C allele. Fifteen (45%) of the 33 were heterozygous for the MUC2/*Pvu*II polymorphism.

Categorisation of the sample on the basis of TB status showed that 8 of the 20 (40%) TB positive individuals were homozygous for the G allele, 6 (30%) were homozygous for the C allele and the remaining 6 (30%) were heterozygous for the two alleles. In contrast

none of the 12 TB negative individuals were homozygous for the C allele, only a third was homozygous for the G allele and the majority (66.7%) was heterozygous for both alleles (Table 7.1).

In the total study sample the G allele appeared at a frequency of 59.1% and the C allele at a frequency 40.9% (Table 7.2). Categorisation according to TB status showed similar frequencies for the G (55%) and C (45%) alleles in the TB positive individuals. In contrast, the TB negative group had a considerable lower frequency of the C allele (33%) compare to the G allele (67%).

7.3 Identification of the MUC5B polymorphism

The primers for MUC5B were designed to amplify across the VNTR region of intron 36 which contains variable number of tandem 59bp repeats. Electrophoresis of the amplified VNTR region revealed seven different fragment sizes and eleven different genotype combinations, nine of which are observed in figure 7.3. Allele sizes were estimated by comparison against the band sizes of a 1kb MWM. Accurate sizing of fragments was undertaken by direct sequencing of the fragments to determine the number of repeats present within each allele. The 59bp repeats reported by Desseyn *et al* (1999) were illustrated in the sequenced MUC5B electrophoretic fragments (Figure7.4 chromatogram).

The MUC5B alleles appeared at 360, 420, 480, 540, 600, 660 and 720bp containing 4, 5, 6, 7, 8, 9 and 10 59bp repeats, respectively (Table 7.3). The 8 repeating unit repeat had a frequency of 59.5% within the TB positive group and 50% within the TB negative group (Table??). Numerous allele combinations were seen in this study (Table 7.4) with the 8/8 and 8/6 combinations of repeat units appearing at the highest frequencies in both the TB positive and negative groups. The genotypes observed in the TB positive group were 8/8, 8/6, 8/7, 6/6, 7/6, 9/8 and 8/5, with the 8/8 presenting at the highest frequency in 8 of 21(38.1%) individuals. The allele combinations present within the TB negative individuals were 8/8, 8/6, 7/6, 9/6, 10/6 and 6/5bp, with the 8/6 combination presenting at the highest frequency in 8 of 16 (50%) individuals (Table 4.5).

7.4 Discussion

On the basis of previous European studies (Desseyn *et al.*, 1999, Vinall *et al.*, 2000) a small scale investigation on MUC2 and MUC5B polymorphisms was carried out in a TB suspected community of the Western Cape. Although the sample size was too small to make definite correlations between TB and allele frequencies and allele proportions of the groups in question, it did illustrate a difference between the TB positive and negative groups for the MUC2/PvuII polymorphism, with 30% of the 20 positive cases being homozygous for the polymorphism in contrast to its absence in the 12 TB negative individuals. As a result, the C allele appeared at a higher frequency (45%) in the TB positive cases than in the other group. The findings of the pilot study suggest that the C allele may result in a genetic predisposition to TB. According to Vinall *et al* (1998), analyses of the MUC2, MUC6, and MUC5AC genes presented with high levels of heterozygous VNTR polymorphisms. This is of interest as most mucin genes have high levels of genetically determined polymorphisms (Vinall *et al.*, 2000, Rose and Vinall, 2006) which have been associated with respiratory diseases. This was illustrated by length variations of MUC2 in asthma were "longer" MUC2 alleles were suspected of protecting predisposed individuals from the development of asthma (Vinall *et al.*, 2000). Cystic fibrosis studies support the upregulation of the transcription of MUC2 gene in response to pathogens such as *Pseudomonas aeruginosa* (Buisine *et al.*, 1999). It is suggested by Vinall *et al* (2000), that allelic differences are associated with the amount of MUC2 secreted during inflammatory diseases such as cystic fibrosis. They further claim that the allelic difference could be related to a linked polymorphism within the MUC2 gene or the length of the MUC2 RNA transcript. This study therefore warrants further investigation on a larger study sample to investigate the allele frequencies in the general South African population and in larger TB-prone populations in the country.

This Western Cape study revealed similar repeat sizes to that found in Europe (Desseyn *et al.*, 1999) with variations in the number of repeat sizes and the number of allele combinations. Compared to their results of five alleles and seven genotypes that were found in 172 individuals, our study presented with 7 alleles (Table 7.3) and 11 genotypes

(Table 7.5) in only 55 individuals. The 7 repeating unit dominated in Desseyn's European population, whereas the 8 repeating unit dominated within the sample group of the Western Cape, especially within the TB positive individuals. Although the 8/8 combination was common to the entire group it appeared more frequently in the TB positive group compared to the TB negative group.

The 10 repeating unit is also of interest as it was not reported by Buisine *et al* (1998) (who reported a maximum of 9 repeats of the 59bp) and Desseyn *et al* (1999) with a maximum of 5 repeats. This is of interest as the 10 repeating unit appeared in a single TB negative individual and not in the TB positive group. Furthermore Desseyn *et al* (1999) presented with a 3 repeating unit of the polymorphism which was absent in this study. Conversely this study illustrated the presence of 4 repeating units of the polymorphism which was absent in the Desseyn *et al* (1999) study. Based on these findings the TB study could be expanded to determine if the 8 repeating unit and its homozygous state remain at a higher frequency within the TB positive group of the Western Cape population and to investigate the frequency of the 10 repeating unit.

Desseyn *et al* (1999) discovered that intron 36 of MUC5B repeat is structurally similar to intron 6 of human interleukin-1α, which also houses several repeats of 46bp. The human interleukin-1α polymorphism is associated with gene function as each repeat has three potential binding sites for transcriptional factors. It was suggested that this polymorphism is linked to the complex regulation of IL-1α gene transcription and, based on their similarities, that the MUC5B polymorphism could also play a similar role in gene regulation. Gene regulation studies could also lend insight into the cytokine mediated upregulation of the MUC5B gene in the TB positive and negative samples as the upregulation of MUC5AC and MUC5B by IL-17 is suggested to be specific to inflammatory or immune mediators (Rose and Voynow, 2006).

An investigation into the expression of the MUC5B polymorphism in TB is warranted by past studies which identified MUC5B mRNA gene products at increased concentrations in lung mucus of asthmatic patients (Rose *et al.*, 2001, Voynow, 2002). According to

Voynow *et al* (2002), a variation in the number of 59bp repeats at intron 36 could be related to MUC5B expression. MUC5B has also been expressed in respiratory diseases such as COPD and Diffuse Panbronchiolitis (DPB). An insertion/deletion polymorphism within the MUC5B promoter region was closely associated with DPB, a genetic disorder that afflicts mainly the Asian population (Kamio *et al.*, 2005). As in the DPB study a deeper investigation into MUC5B polymorphisms at large could also lead to information regarding the genetic susceptibility and pathogenesis of TB within the Western Cape.

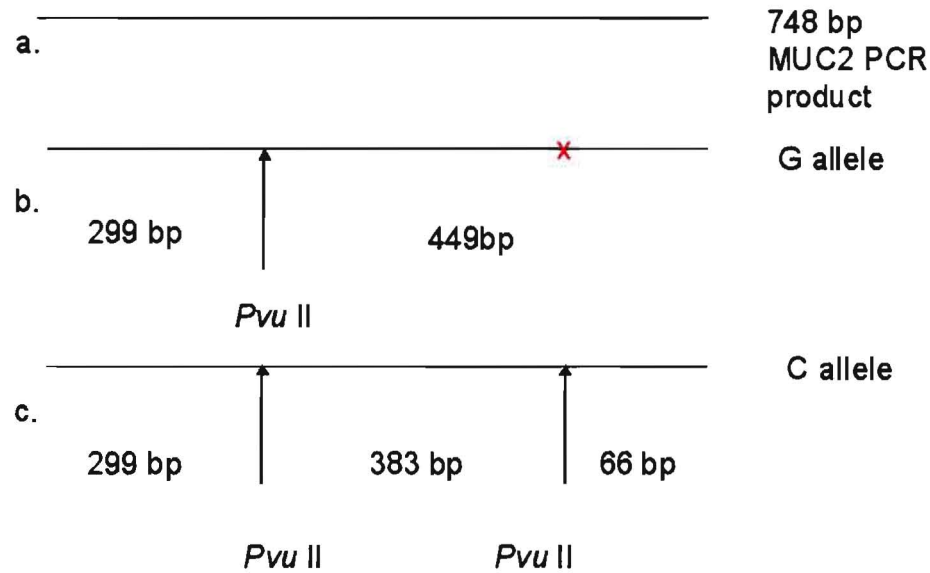


Figure 7.1 Illustrates the results of *Pvu*II enzyme digestion of the MUC2 PCR products. a. represents the uncut MUC2 PCR product at 748bp b. if the RFLP is absent *Pvu*II cleaves the G allele once to produce a 299bp product and 449bp product and c. *Pvu*II cleaves the C allele twice to produce the 294bp, 383bp and 66bp products.

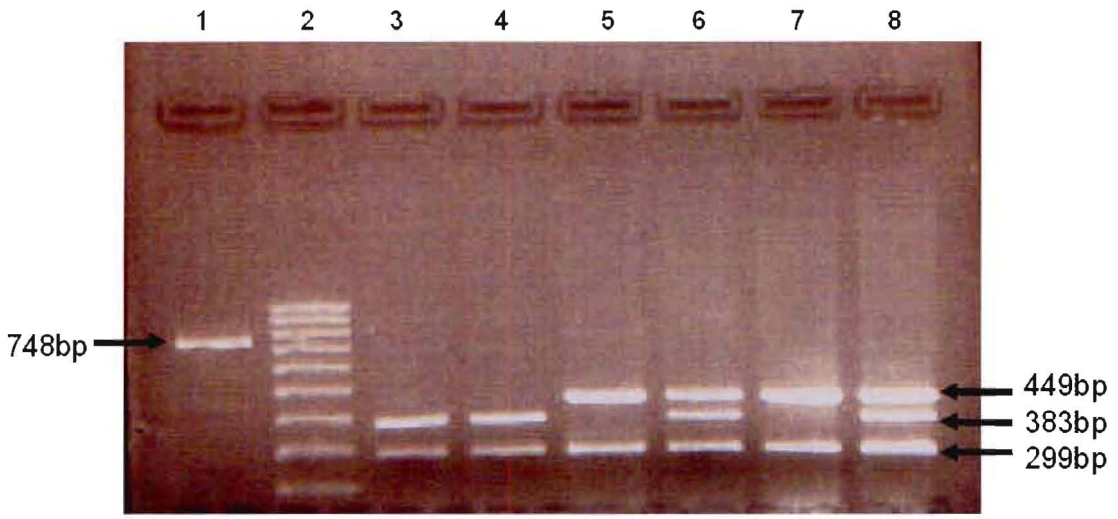


Figure 7.2 A 2% agarose gel illustrating the results of *Pvu*II enzyme digestion of the MUC2 PCR products. a. represents the uncut MUC2 PCR product at 748bp b. if the RFLP is absent *Pvu* II cleaves the PCR product once to produce a 294bp product and 449bp product and c. *Pvu* II cleaves the C allele twice to produce the 294bp, 383bp and 66bp products.

Lanes 1, uncut MUC2 PCR product (control); 2, MWM; 3, C/C; 4, C/C; 5, G/G; 6, C/G; 7, G/G; 8, C/G

Table 7.1 MUC2 C and G allele proportions in the TB positive, negative and undetermined groups.

TB Status	C Allele (%)	G Allele (%)	CG Allele	Total Number of samples
TB positive	6(30%)	8(40%)	6(30%)	**20**
TB negative	-	4(33%)	8(66.7%)	**12**
Status undetermined	-	-	1(100%)	**1**
Total	**6(18%)**	**12(36%)**	**15(45%)**	**33**

Table 7.2 MUC2 C and G allele frequencies in the TB positive, negative and undetermined groups.

TB Status	C Allele (%)	G Allele (%)	Total Number of chromosomes
TB positive	18(45)	22(55)	**40**
TB negative	8(33)	16(67)	**24**
Status undetermined	1(50)	1(50)	**2**
Total	**27(40.9)**	**39(59.1)**	**66**

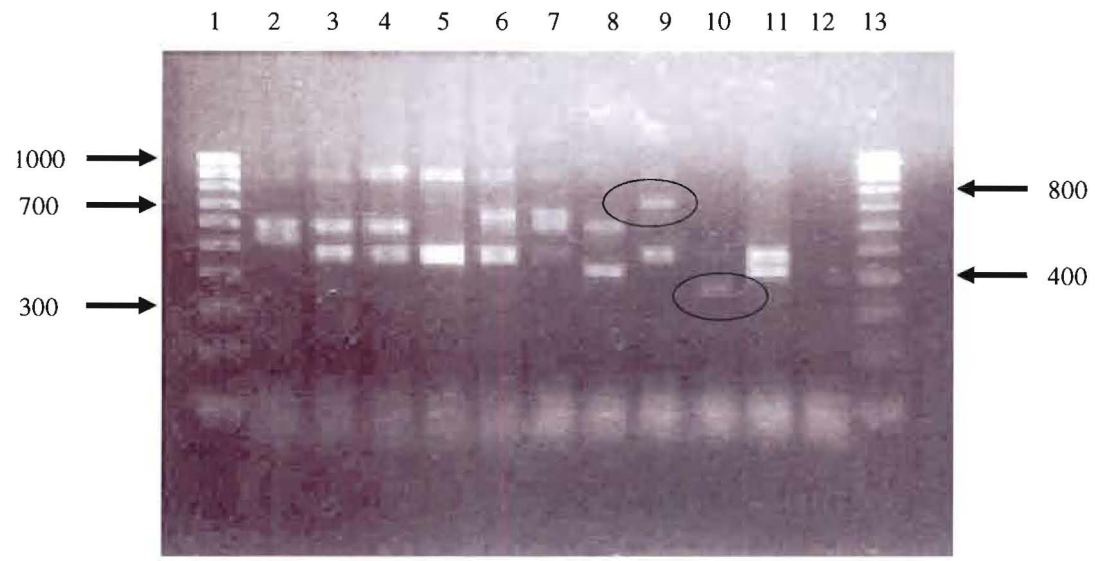


Figure 7.3 A 2% agarose gel of the amplified VNTR region within MUC5B illustrating 9 genotypes along with the 8 alleles. The PCR products containing 4, 5, 6, 7, 8, 9 and 10 59bp repeats are 360, 420, 480, 540, 600, 660, 720bp respectively. The circles illustrate the first allele at 360bp (4 repeats) and the seventh allele at 720bp (10 repeats). The positions of the 1000, 800, 700, 400 and 300bp bands comprising the MWM are indicated by arrows.

Lanes 1, MWM; 2, has 8 and 7 repeat; 3, has 6 and 8 repeats; 4, has 6 and 8 repeats; 5, the two alleles have 6 repeats; 6, has 6 and 9 repeats; 7, has 6 and 8 repeats; 8, 5 and 8 repeats; 9, 3 and 10 repeats; 10, 4 and 6 repeats; 11, 12, blank; 13, MWM

Table 7.3 Allelic sizes and frequencies at intron 36 of the MUC5B gene in 55 individuals.

Desseyn's study				Our Study			
Number of repeats	size (bp)	N (total = 172)	Frequency	Number of Repeats	size (bp)	N (total = 110)	Frequency
3	280	8	0.047	-	-	-	-
-	-		-	4	360	2	0.018
5	400	10	0.058	5	420	3	0.027
6	450	2	0.012	6	480	34	0.309
7	500	141	0.820	7	540	7	0.063
8	550	11	0.064	8	600	59	0.536
-	-	-	-	9	660	4	0.036
-	-	-	-	10	720	1	0.009

Table 7.4 The frequencies of the repeating units within the TB positive, negative and undetermined groups in the present study.

Repeat	Size (bp)	TB positive (%)	TB negative (%)	Undetermined
4	360	0	0	2
5	420	1(2.4)	1(3.12)	1
6	480	12(28.6)	12(37.5)	9
7	540	3(7.14)	1(3.12)	3
8	600	25(59.5)	16(50)	18
9	660	1(2.4)	1(3.12)	3
10	720	0	1(3.12)	0
Total		**42**	**32**	**36**

Table 7.5 The MUC5B genotypes for the TB positive, negative and undetermined individuals.

Individual	Genotype	TB status	Individual	Genotype	TB status
1	8/6	Positive	**29**	8/8	Negative
2	8/8	Positive	**30**	8/6	Positive
3	8/7	Positive	**31**	8/6	Negative
4	8/6	Positive	**32**	7/6	Positive
5	8/6	Negative	**33**	8/6	Negative
6	8/6	Positive	**34**	8/8	Negative
7	6/6	Positive	**35**	8/6	Positive
8	8/8	Positive	**36**	8/6	Negative
9	6/6	Positive	**37**	8/6	Negative
10	7/6	Positive	**38**	8/8	Negative
11	8/8	Positive	**39**	10/6	Negative
12	8/8	Positive	**40**	9/8	Undetermined
13	8/8	Negative	**41**	6/4	Undetermined
14	8/8	Positive	**42**	8/6	Undetermined
15	9/6	Negative	**43**	8/6	Undetermined
16	8/6	Negative	**44**	8/6	Negative
17	9/8	Positive	**45**	8/6	Negative
18	8/5	Positive	**46**	6/4	Undetermined
19	8/8	Positive	**47**	7/6	Negative
20	8/6	Positive	**48**	7/6	Undetermined
21	8/8	Positive	**49**	6/5	Negative
22	8/8	Undetermined	**50**	6/6	Undetermined
23	8/5	Undetermined	**51**	8/7	Undetermined
24	8/8	Undetermined	**52**	8/8	Undetermined
25	9/8	Undetermined	**53**	8/6	Undetermined
26	8/8	Positive	**54**	8/6	Undetermined

Individual	Genotype	TB status	Individual	Genotype	TB status
27	9/8	Undetermined	**55**	8/8	Undetermined
28	8/7	Undetermined			

Table 7.6 The MUC5B allele combinations within the TB positive, negative and undetermined groups.

Genotype	TB Positive (%)	TB negative (%)	Undetermined
8/8	8(38.1)	4(25.0)	4(22.2)
8/6	6(28.57)	8(50)	4(22.2)
8/7	1(4.76)	0	2(11.5)
6/6	2(9.52)	0	1(5.56)
7/6	2(9.52)	1(6.25)	1(5.56)
9/8	1(4.76)	0	3(16.67)
8/5	1(4.76)	0	1(5.56)
9/6	0	1(6.25)	0
10/6	0	1(6.25)	0
6/5	0	1(6.25)	0
6/3	0	0	2(11.1)
Total	**21**	**16**	**18**

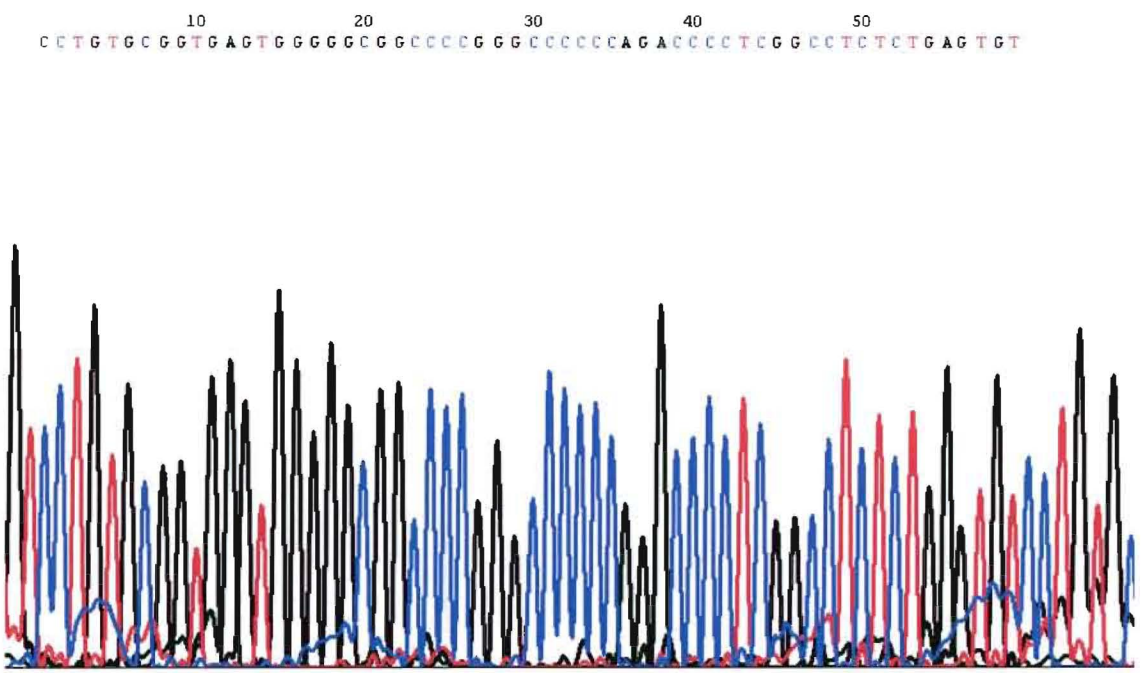


Figure 7.4 the Chromatogram of the 59bp polymorphism within intron 36 of the MUC5B gene.

CHAPTER 8

CONCLUSION

8.1 The biochemical study

Mucus is the body's first line of immune defence against various pathogens and irritants. It lines the epithelial layer and is in direct contact with bacteria, viruses, fungi, inhaled tobacco smoke and air pollution. Mucins, glycoproteins within mucus confer upon mucus their rheological, physicochemical and biological properties. It is in response to stimuli that mucin genes are upregulated resulting in the hypersecretion of mucus and mucin glycoproteins (Basbaum *et al.*, 2002). Although hypersecretion is probably a defence mechanism against the pathogen, it does result in pathological changes such as goblet cell hyperplasia and metaplasia due to an excessive production of mucus. Mucus hypersecretion is of great concern due to the severe morbidity and mortality rates associated with respiratory diseases such as cystic fibrosis, chronic obstructive pulmonary disease and asthma. Antibody detection of the prominent respiratory mucins MUC5AC and MUC5B have illustrated their presence in healthy individuals. They have also been detected in various respiratory diseases but contrasted in appearance or concentration. From literature it is known that their sugar side chains behave as receptors for various bacteria, such as *Streptoccoci*.

TB is a respiratory disease that spreads via the bacterium *Mycobacterium tuberculosis*. Its growth rate in Africa (6%) is currently the highest globally (Walzl, 2004). TB is rife within South Africa, specifically the Western Cape. It is a disease that preys on the impoverished communities (Munch *et al.*, 2003) due to the lack of medical resources and the growth of HIV-AIDS (Bradshaw *et al.*, 2002). Respiratory mucins have been associated with various respiratory diseases but not TB.

The biochemical study focused on the characterization of respiratory mucins in adults and children with TB. MUC5AC and MUC2 were barely visible in the volunteer group

(controls) compared to MUC5B. The presence of MUC5AC and MUC5B was confirmed in the TB positive groups of this study. The results compared well with literature as respiratory MUC5AC and MUC5B have been observed at varying intensities, in terms of their appearance in western blots, in both healthy and diseased states. Besides the presence of the dominant respiratory mucins, MUC5AC and MUC5B, MUC2 was also detected in the TB adults and children. The LUM2-3 antibody kindly donated to us by Dr Sara Kirkham of Manchester, UK was used to identify the MUC2 glycoforms in TB and non TB chest disease patients. Upon presentation of the MUC2 results at the 8th International Workshop on Carcinoma-associated Mucins in 2005 by Professor Mall (Govender *et al.*, 2005) a question was raised by Professor Ingemar Carlstedt and Dr Annkatrin Herrmann about the nature of the band at the end of the gels for MUC2 in TB patients (Figure 4.7 and 4.8, lanes 7-11 and 9-12 respectively). Dr Herrmann subsequently supplied us with an affinity purified LUM2-3 against MUC2 (Herrmann *et al.*, 1999) to confirm the presence of the above mentioned band. This did not work in our hands. In November of 2005, Dr John Sheehan visited our labs and suggested that the agarose gel, after electrophoresis, be incubated with 10mM DTT prior to blotting. This also failed to produce any results. The presence of MUC2 in TB will be further investigated as part of a PhD project as it could yield a wealth of information concerning diagnosis and susceptibility to TB.

Unlike MUC2, MUC5AC and MUC5B did not appear as different glycoforms. Past literature has reported the presence of two MUC5B glycoforms in respiratory studies (Thornton *et al.*, 1997, Sheehan *et al.*, 1999 and Kirkham *et al.*, 2002). Antibody detection of MUC5B in the controls and TB samples presented as smears that did not reveal different glycoforms. Identification of the glycoforms would probably require purification of reduced mucin preparations on a Mono Q anion exchange column as suggested by Thornton and Sheehan (2004). This might lead to similar findings as by Kirkham *et al* (2002) who discovered that the more acidic form of MUC5B was found in healthy airways in comparison to CF and COPD airways. We have just procured funding for an HPLC system.

The biochemical study focused on the characterization of respiratory mucins in adults and children with TB. MUC5AC and MUC2 were barely visible in the volunteer group (controls) compared to MUC5B. The presence of MUC5AC and MUC5B was confirmed in the TB positive groups of this study. The results compared well with literature as respiratory MUC5AC and MUC5B have been observed at varying intensities in healthy and diseased states. Besides the presence of the dominant respiratory mucins, MUC5AC and MUC5B, MUC2 was also detected in the TB adults and children. The LUM2-3 antibody kindly donated to us by Dr Sara Kirkham of Manchester, UK was used to identify the MUC2 glycoforms in TB and non TB chest disease patients. Upon presentation of the MUC2 results at the 8th International Workshop on Carcinoma-associated Mucins in 2005 by Professor Mall (Govender *et al.*, 2005) a question was raised by Professor Ingemar Carlstedt and Dr Annkatrin Herrmann about the nature of the band at the end of the gels for MUC2 in TB patients (Figure 4.7 and 4.8, lanes 7-11 and 9-12 respectively). Dr Herrmann subsequently supplied us with an affinity purified LUM2-3 against MUC2 (Herrmann *et al.*, 1999) to confirm the presence of the above mentioned band. This did not work in our hands. In November of 2005, Dr John Sheehan visited our labs and suggested that the agarose gel, after electrophoresis, be incubated with 10mM DTT prior to blotting. This also failed to produce any results. The presence of MUC2 in TB will be further investigated as part of a PhD project as it could yield a wealth of information concerning diagnosis and susceptibility to TB.

Unlike MUC2, MUC5AC and MUC5B did not appear as different glycoforms. Past literature has reported the presence of two MUC5B glycoforms in respiratory studies (Thornton *et al.*, 1997, Sheehan *et al.*, 1999 and Kirkham *et al.*, 2002). Antibody detection of MUC5B in the controls and TB samples presented as dark smears that did not reveal different glycoforms. Identification of the glycoforms would probably require purification of reduced mucin preparations on a Mono Q anion exchange column as suggested by Thornton and Sheehan (2004). This might lead to similar findings as by Kirkham *et al* (2002) who discovered that the more acidic form of MUC5B was found in healthy airways in comparison to CF and COPD airways. We have just procured funding for an HPLC system.

The presence of the dominant gel forming mucins, MUC5AC and MUC5B has been established in control and TB groups. Furthermore the presence of MUC2 in TB adults and children suggests the influence of a third gel forming mucin in TB. The implications of this are unknown but it is postulated that these mucins add to the increased viscosity and gel-like appearance of the TB sputum samples. TB is a recognized cause of bronchorrhea (Zar *et al.*, 1998) whereby patients produce up to a 100ml of mucus per day. It is conceivable that the increased mucus production is related not only to the hypersecretion of MUC5AC and MUC5B, MUC2 as well. The nature of the interaction between the three gel forming mucins is unknown and requires further investigation.

CsCl purification of crude samples was not required prior to western blotting as the MUC5AC and MUC5B antibody detection of purified and crude samples presented with similar results. The use of crude material led to a substantial reduction in expense and time as CsCl purification is a lengthy, expensive procedure. CsCl purification was required prior to amino acid analysis.

The amino acid analysis presented with a high combined value of glycine, threonine, serine, proline and alanine in the healthy control. This was indicative of a typical mucin amino acid profile. The glycine, threonine, serine, proline and alanine profile was lower in the PM and TB groups than in the volunteers. This was explained by differences in the secretion levels of varying mucin types depending on ones state of health. The individual serine, threonine and proline values did not dominate the amino acid composition; likely due to incomplete deglycosylation process prior to analysis. The PTS content and the combined serine and threonine content was slightly lower in the TB positive adults and children compared to the healthy volunteers. It is hypothesized that the TB bacterium has a greater affinity for the sugar side chains and that changes to the amino acids of the protein backbone would be secondary to that of the sugar side chains. Future studies should include the isolation and purification of individual mucins (MUC5AC, MUC5B and MUC2) from control and TB positive groups followed by amino acid analysis. This might lend insight into amino acid changes that are specific to the different mucin types. Sugar analysis was carried out but not reported due to incomplete data. It is planned for

the future to carry out an amino acid and sugar analysis of MUC5AC, MUC5B and MUC2 in the control and TB positive states. Amino acid sequencing would illustrate the sequence of the protein backbone and sugar analysis of the sialic acid and sulfate groups would provide information on the presence and difference between mucin glycoforms. Kirkham *et al* (2002) found that healthy airways house a more acidic form of MUC5B in comparison to CF and COPD.

Future analytical studies on lipid and DNA should be considered. The analysis of DNA during CsCl purification aids in determining the purity of the mucins hence the efficiency of the technique. Lipids are present in the sputum of patients who suffer from varying bronchial diseases; they exist at elevated levels in the secretion of cystic fibrosis patients (Slayter, 1987) and according to Woodward *et al.*, (1982), "the contribution of lipid analysis to the overall architecture of mucus secretions cannot be overlooked".

Another future consideration would be quantitative western blotting as studies based on equivalent weight or concentration analyses would better illustrate the difference in mucin levels during the control and disease states. Such studies are challenging due to the difficulty in solubilising mucins but have been fruitful. MUC5AC and MUC5B, which were normalized on weight basis, were found at lower levels in CF sputum in comparison to normal airways (Rose and Voynow, 2006). Immunohistochemistry would also be a helpful tool in determining the presence and location of mucins in the TB positive tissue compared to controls. This might lend insight into the presence and possible role of nonsecreted or membranous mucins in TB.

8.2 The molecular study

Mucus and mucin hypersecretion are a consequence of various acute and chronic respiratory disorders. The MUC genes that encode most mucins are known to house a great degree of length or sequence polymorphisms. Molecular studies have attempted to make a link between polymorphisms and susceptibility to the disease. A pilot study was carried out to determine if MUC2 and MUC5B polymorphisms present in European studies were found individuals in the Western Cape.

The MUC2 polymorphism for the homozygote C allele appeared in 6 of 20 (30%) TB positive individuals but did not feature in the TB negative individuals. Infact the TB positive group presented with a higher C allele frequency compared to the TB negative group. Although the sample size was too small to make inferences it does appear that the homozygote C allele could be associated with the TB positive group. If a correlation is made between the MUC2 polymorphism and the TB positive group within the Western Cape, it should be asked if the polymorphism suggests a susceptibility to TB and if and RFLP marker could be identified for TB. This would lead to screening for the marker, which would have great consequences not only for the individual but the country at large, as TB is a crippling disease within South Africa specifically in the Western Cape.

Like Desseyn *et al* (1999) the intronic MUC5B polymorphism was identified in the Western Cape population but the TB study group presented with a larger allele frequency and a greater number of repeating units in comparison to that of Desseyn *et al* (1999). The 10 repeating unit of the 59bp was absent in the European study yet appeared separately in a TB negative individual and the 8/8 allele combination appeared most frequently in the TB positive group compared to the TB negative group. Literature has illustrated the importance in the number of repeats of human MUC1 and rat Muc4 with respect to their anti-adhesive properties as it has been suggested that the number of repeats could be related to the physicochemical and rheological properties of mucus influencing bacterial adhesion to the cell surface (Escande *et al.*, 2001). The number of 59bp repeats could similarly influence the binding of the TB bacterium to hosts cell surface. This warrants further investigation within the Western Cape population.

Escande *et al* (2001) illustrated, after RT-PCR sequencing of the 5' region of tracheal MUC5AC, that MUC5AC, MUC5B, MUC2 and the von Willebrand factor shared common features. Infact, together with MUC6, they are located on the same chromosome, 11p15.5 (Rose and Voynow, 2006). Based on this similarity it would be advantageous to investigate polymorphisms within MUC5AC, MUC5B and MUC2, and their combined expression during TB, as pathogen by-products, immune mediators and

growth factors have been documented to upregulate mucin gene expression. By products of bacteria associated with cystic fibrosis (*Pseudomonas aeruginosa*) and COPD (*Staphylococcus aureus*) upregulate pathways that lead to the upregulation of MUC5AC and MUC2 (Rose and Voynow, 2006).

Plans for a larger study should also consider relating the expression of the MUC protein glycoforms to the genetic polymorphisms. The biochemical study illustrated the presence of MUC2 glycoforms in the TB positive states ('possible', probable and definite TB) and its absence in the healthy volunteers. It would be interesting to determine if the presence of MUC2 genetic polymorphism is associated with the varying glycoforms and if the level of gene expression is related to depending on the glycoform expression.

advantageous to investigate polymorphisms within MUC5AC, MUC5B and MUC2, and their combined expression during TB, as pathogen by-products, immune mediators and growth factors have been documented to upregulate mucin gene expression. By products of bacteria associated with cystic fibrosis (*Pseudomonas aeroginosa*) and COPD (*Staphylococcus aureus*) upregulate pathways that lead to the upregulation of MUC5AC and MUC2 (Rose and Voynow, 2006).

Plans for a larger study should also consider relating the expression of the MUC protein glycoforms to the genetic polymorphisms. The biochemical study illustrated the presence of MUC2 glycoforms in the TB positive states ('possible', probable and definite TB) and its absence in the healthy volunteers. It would be interesting to determine if the presence of MUC2 genetic polymorphism is associated with the varying glycoforms and if the level of gene expression is related to depending on the glycoform expression.

APPENDIX A

UNIVERSITY OF CAPE TOWN

DEPARTMENT OF SURGERY

MUCUS (SLIME) IN TUBERCULOSIS (TB) PROJECT: Information Leaflet

Dear patient,

We are doing a study on slime produced in your lungs that causes you to cough phlegm. We wish to find out what the slime is made of and whether it is different from the people who do not have a chest problem. The Nursing sister who has approached you will ask you to cough and 'spit' out the phlegm you produce into the jar she provides.

We would also appreciate you allowing us to take a sample from the inside of your mouth. This will not be painful. We will use the sample to tell us about the genes you have for producing slime that you cough up. All samples will be coded and only the researcher will have access to your personal details.

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 the samples for any other purpose than that we have described.

2. You have a right to say that you do not wish to take part in this study and can be assured that it will not affect the treatment you receive in hospital.

3. If you agree to participate in this 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

4. We will NOT test for HIV/AIDS.

5. The tests we mentioned cannot determine your complete genetic make-up.

6. It is possible that we will publish the findings of this study but your name will not be mentioned.

Any questions, please ask the Nursing Sister who is taking the sample from you. If there are any questions you would like answered at a later date, please contact me at the number below.

Professor A Mall
Tel: 021 4066186/6227

REQUEST FOR MOLECULAR STUDIES (DNA)

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

Tel: (021) 4066168/6227 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:

DNA number .....
Date Received: YY DD: Computer Index No.:

CONSENT FOR DNA ANALYSIS AND STORAGE

1. I, _____ give permission that a mouth swab is taken for research purposes in the investigation of mucus (slime) in tuberculosis (TB).

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

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

4. I have been informed that findings made in this study could help in the management of tuberculosis (TB) in the future.

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

_____

Please note that your HIV status is **not** recorded on this form.

APPENDIX B

TB Mucin Study- GFJH

Case Number _____
Folder Number _____
Initials _____
DOB ___/____/_____
1. Sex O Male O Female
2. Age _____

(for data below: yes= tick = 1 , no = no tick = 2)

3. WHO TB Diagnosis
   - 3.a O Definite
   - 3.b O Probable
   - 3.c O Possible

4. TB diagnostic criteria
   - 4.a O Culture
   - 4.b O AFB+ (microscopy)
   - 4.c O ADA raised
   - 4.d O CXR
   - 4.e O Clinical improvement on TB Rx
   - 4.f O Nodes on Abd U/S
   - 4.g O Splenic lesions on Abd U/S

5. Smoking History
   - 5.a O Never smoker
   - 5.b O Ex-smoker
   - 5.c O Current smoker

6. Smoking Consumption
   - 6.a O Never smoker
   - 6.b O <10 pack yrs
   - 6.c O 10 – 20 pack yrs
   - 6.d O >20 pack yrs

7. Alcohol History
   - 7.a O Never drinker
   - 7.b O Current regular alcohol use
   - 7.c O Previous regular alcohol use

8. Substance Abuse
   - 8.a O Mandrax
   - 8.b O Dagga
   - 8.c O Cocaine
   - 8.d O Tic

9. Diabetic:
   - 9.a O Known diabetic
   - 9.b O Requiring insulin
   - 9.c O Requiring oral medication
   - 9.d O Previous admission for poor control
   - 9.e Duration of admission : ____ days

10. HIV Status
    - 10.a O Negative
    - 10.b O Not suspected
    - 10.c O Suspected
    - 10.d O Positive
    - 10.e O WHO I/ II
    - 10.f O WHO III/ IV
    - 10.g O On HAART
    - 10.h Most recent CD4 count (if available)

      _____

11 Other Respiratory Disease

11.a O Bronchiectasis

11.b O Mouth crackles

11.c O Clubbing

11.d O Chronic sputum

11.e O CXR features

11.f O COAD

11.g O Chronic cough productive of cough >3/12 for 2 years

11.h O Smoking history > 20 pack years

11.i O Reduced FEV1/ FVC

11.j O Reversibility on bronchodilator therapy < 15 % (PEFR)

11.k O CXR features

11.l O Asthma

11.m O Convincing history of continuous episodic SOB, cough, tight chest

11.n O Known allergen/ event precipitating bronchospasm

11.o O Hx of atopy (allergic rhinitis/ urticaria/ eczema)

11.p O Reversibility spontaneous/ removal of known precipitating allergen

11.q O Reversibility on bronchodilator therapy > 15 % (PEFR)

REFERENCES

Adler KB, Li Y (2001): Airway epithelium and mucus: intracellular signalling pathways for gene expression and secretion. Am J Respir Cell Mol Biol. 25: 397-400.

Aksoy N, Thornton DJ, Anthony C, Paraskeva C, Sheehan J (1999): A study of the intracellular and secreted forms of the MUC2 mucin from the PC/AA intestinal cell line. Glycobiology. 9(7): 739-746.

Allen, A (1977): Structure and function in gastric mucus. In: Mucus in health and disease. (Elstein M and Parke DV, eds), Plenum Press, New York pp 275-299.

Allen A (1978): Structure of gastrointestinal mucus glycoproteins and the viscous and gel-forming properties of mucus. Br Med Bull. 34(1): 28-33.

Allen A (1981): Structure and function of gastrointestinal mucus. Physiology of the gastrointestinal tract. (Johnson LR, ed.), Raven Press, New York pp 617-639.

Allen A, Hutton DA, Pearson JP, Sellers LA (1990): The colonic mucus gel barrier: structure, gel formation and degradation. In: The Cell Biology of inflammation in the gastrointestinal tract (Peters TJ, ed), Corners Publication, United Kingdom pp 113-125.

Allen A, Flemstrom G (2005): Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. Am J Physiol Cell Physiol 288: 1-19.

An G, Luo G, Wu R (1994): Expression of MUC2 gene is down-regulated by vitamin A at the transcriptional level *In Vitro* in tracheobronchial epithelial cells. Am J Respir Cell Mol Biol. 10: 546-551.

Andreu J, Caceres J, Martinez-Rodriguez M (2004): Radiological manifestations of pulmonary tuberculosis. Eur J Radiol. 51(2): 139-49.

Argueso P, Spurr-Michaud S, Tisdale A, Gipson IK (2002): Variation in the amount of T antigen and *N*-acetyllactosamine oligosaccharides in human cervical mucus secretions with the menstrual cycle. J Clin Endocrinol Metab. 87(12): 5641-5648.

Baeckstrom D, Karlsson N, Hansson GC (1994): Purification and characterisation of Sialyl-Le-carrying mucuins of human bile; evidence for the presence of MUC1 and MUC3 apoproteins. J Biol Chem. 269(20): 14430-14437.

Bai XD, Liu XH, Tong QY (2004): Intestinal colonisation with *Candida albicans* and mucosal immunity. World J Gastroenterol. 10(14): 2124-2126.

Basbaum C, Li D, Gensch E, Gallup M, Lemjabber H (2002): Mechanisms by which gram-positive bacteria and tobacco smoke stimulate mucin induction through the epidermal growth factor receptor (EGFR). In: Mucus hypersecretion in respiratory disease (Chadwick DJ and Goode JA, eds), John Wiley and Sons, Chichester pp171-180.

Berry M, Ellingham RB, Corfield AP (2000): Membrane –associated mucins in normal human conjunctiva. IOVS 41(2): 398-403.

Bhaskar RK, Reid L (1981): Application of density gradient methods for the study of mucus glycoprotein and other macromolecular components of the sol and gel phases of asthmatic sputa. JBC 256(14): 7583-7589.

Bhattacharyya SN, Veit BC, Manna B, Enriquez JI, Walker MP, Khorrami AM, Kaufman B (1990): Neutral and acidic human tracheobronchial mucin. Inflammation 14(4): 355-373.

Bobek LA, Tsai H, Biesbrock AR and Levine MJ (1993): Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin. J Biol Chem. 268 (27): 20563-20569.

Bobek LA, Situ H (2003): MUC7 20-Mer: investigation of antimicrobial activity, secondary structure, and possible mechanism of antifungal action. Antimicrob Agents Chemother. 47(2): 643-652.

Bradshaw D, Schneider M, Dorrington R, Bourne DE and Laubscher R (2002): South African Cause-of-Death profile in transition-1996 and future trends. S Afr Med J. 92(8): 618-623.

Brockhausen I (2004): Commentary: Intestinal candyfloss. Biochem J. 384: e3-e5.

Buisine MP, Desseyn JL, Porchet N, Degand P, Laine A, Aubert JP (1998): Genomic organization of the 3' region of the human MUC5AC mucin gene: additional evidence for a common ancestral gene for the 11p15.5 mucin gene family. Biochem J. 332: 729-738.

Buisine MP, Devisme L, Copin MC, Reville MD, Gosselin B, Aubert JP, Porchet N (1999): Developmental mucin gene expression in the human respiratory tract. Am J Respir Cell Mol Biol. 20: 209-218.

Carlstedt I, Sheehan, JK (1984): Is the macromolecular architecture of cervical, respiratory and gastric mucins the same? Bioch Soc Trans. 12(4): 615-617.

Carlstedt I, Sheehan JK (1984): Macromolecular properties and polymeric structure of mucin glycoprotein. Ciba Found Symp. 109: 157-172.

Carlson DM (1977): Chemistry and biosynthesis of mucin glycoprotein. Adv Exp Med Biol. 89: 251-73.

Degerli N, Akpinar MA (2001): A novel concentration method for concentrating solutions of protein extracts based on dialysis techniques. Anal Bioch 297:192-194.

Dunn SD (1986): Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal Biochem. 157: 144-153.

Epstein L, Sayed AR, Bourne DE and Benatar SR (1987): Variations in mortality of the Coloured, White and Asian population groups in the RSA, 1978-1982. Respiratory Diseases. S Afr Med J. 72: 559-563.

Escande F, Aubert JP, Porchet N, Buisine MP (2001): Human mucin gene MUC5AC: organization of its 5'-region and central repetitive region. Biochem J. 358: 763-772.

Fenhalls G (2004): The use of modern molecular techniques to study tuberculosis immunology at the site of the disease. Curr Allergy and Clin Immunol. 17(4): 167-172.

Fitzgerald JE, Green GGR, Stafford FW, Birchall JP, Pearson JP (1987): Characterisation of human middle ear mucus glycoprotein in chronic secretory otitis media (CSOM). Clin Chim Acta. 169: 281-298.

Fontenot DJ, Gatewood JM, Santhana Mariappan SV, Pau C-P, Parekh BS, George JR and Goupta G (1995): Human immunodeficiency virus (HIV) antigens: Structure and serology of multivalent human mucin MUC1-HIV V3 chimeric proteins. Prc Natl Acad Sci. 92: 315-319.

Fontenot JD, Zacharopolous VR and Phillips DM (1996): Proline-rich tandem repeats of antibody complementarity-determining regions bind and neutralize human immunodeficiency virus type 1 particles. J Virol 70(10): 6557-6562.

Gerard C, Eddy RL, Shows TB (1990): The core polypeptide of cystic fibrosis tracheal mucin contains a tandem repeat structure: evidence for a common mucin in airway and gastrointestinal tissue. J Clin Invest. (6): 1921-1927.

Gershman NH, Liu H, Wong HH, Liu JT, Fahy JV (1999): Fractional analysis of sequential induced sputum samples during sputum induction: Evidence that different lung compartments are sampled at different time points. J Allergy Clin Immunol. 104(2): 322-328.

Gershoni JM, Palade GE (1983): Protein blotting: Principles and applications. Anal Biochem. 131: 1-15.

Getz EB, Xiao M, Chakrabarty T, Cooke R, Selvin PR (1999): A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. Anal Bioch. 273: 73-80.

Gevers W (1987): Mucus and mucins. SAMJ 72: 39-42.

Govender U, Mall A, Ballo R, Lotz Z, Pepper D, Zar H, Lourens D, Rousseau K, Swallow DM, Hunter A, Kahn D (2005): The biochemical and genetic characterization of respiratory mucins from patients with tuberculosis (TB): A report of preliminary findings. Mucins in Health and Disease 8th International Workshop on Carcinoma-associated mucins, Cambridge UK, abstract p121.

Hargreave FE (1999): Induced sputum for the investigation of airway inflammation: Evidence for its clinical application. Can Respir J 6(2): 169-174.

Hayase T, Sheykhanazari M, Bhavanandan VP, Savage AV, Lee YC (1993): Separation and identification of O-linked oligosaccharides derived from glycoproteins by high-pH anion-exchange chromatography. Anal Biochem. 211: 72-80.

Hemleben V, Grierson D, Dertmann H (1977): The use of equilibrium centrifugation in actinomycin-caesium chloride for the purification of ribosomal DNA. Plant Sci Lett. 9: 129-135.

Henke MO, Renner A, Huber RM, Seeds MC, Rubin BK (2004): MUC5AC and MUC5B mucins are decreased in cystic fibrosis airway secretions. Am J Respir Cell Mol Biol. 31: 86-91.

Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, Carlstedt I (1999): Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. J Biol Chem. 274(22): 15828-15836.

Ho SB, Niehans GA, Lyftogt C, Yan P, Cherwitz DL, Gum ET, Dahiya R and Kim YS (1993): Heterogeneity of Mucin Gene Expression in Normal and Neoplastic Tissues. Cancer Res 53: 641-651.

Hoal EG, Lewis LA, Jamieson SE, Tanzer F, Roussouw M, Victor T, Hillerman R, Beyers N, Blackwell JM, Van Helden PD (2004): *SLC11A1(NRAMP1)* but not *SLC11A2(NRAMP2)* polymorphisms with susceptibility to tuberculosis in a high-incidence community in South Africa. Int J Tuberc Lung Dis. 8(12): 1464-1471.

Holden KG, Yim NCF, Griggs LJ, Weisbach JA (1971): Gel electrophoresis of mucous glycoproteins. II. Effect of physical deaggregation and disulfide-bond cleavage. Biochemistry 10(16): 3110-3113.

Hoskins LC (1978): Degradation of mucus glycoproteins in the gastrointestinal tract. In: The glycoconjugates (Horowitz MW and Pigman W, eds.), Academic Press, New York. pp 235-250.

Hovenberg HW, Davies JR, Carlstedt IC (1996): Different mucins are produced by the

surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from goblet cells. Biochem J. 318: 319-324.

Hovenberg HW, Carlstedt I, Davies JR (1997): Mucus glycoproteins in bovine trachea: identification of the major mucin populations in respiratory secretions and investigation of their tissue origins. Biochem J. 321: 117-123.

Jany B, Gallup M, Tsuda T, Basbaum C (1991): Mucin gene expression in rat airways following infection and irritation. Biochem Biophys Res Commun. 181(1): 1-8.

Jeena PM, Pillay P, Coovadia HM (2002): Impact of HIV-1 co-infection on presentation and hospital-related mortality in children with culture proven pulmonary tuberculosis in Durban, South Africa. Int J Tub Lung Dis. 6: 672-678.

Jeffrey PK and Li D (1997): Airway mucosa: secretory cells, mucus and mucin genes. Eur Resp Jou. 10: 1655-1662.

Jung A, Ruckert S, Frank P, Brabletz T, Kirshner T (2002): 7-Deaza-2'-deoxyguanosine allows PCR and sequencing reactions from CpG rich islands. Mol pathol. 55: 55-57.

Kaliner M, Shelhamer JH, Borson B, Nadel J, Patow C, Marom Z (1986): Human respiratory mucus. Am Rev Respir Dis. 134(3): 612-621.

Kamio K, Matsushita I, Hijikata M, Kobashi Y, Tanaka G, Nakata K, Ishida T, Tokunaga K, Taguchi Y, Homma S, Nakata K, Azuma A, Kudoh S, Keicho N (2005): Promoter analysis and aberrant expression of the MUC5B gene in diffuse panbronchiolitis. Amer J Respir Crit Care Med. 171: 949-957.

Kashket S, Donaldson CG (1972): Saliva-induced aggregation of oral streptococci. J Bacteriol. 112(3): 1127-1133.

Kanduma E, McHugh TD, Gillespie SH (2003): Molecular methods for *Mycobacterium tuberculosis* strain typing: a users guide. J Applied Microbiol. 94: 781-791.

Kim WD (1997): Lung mucus: a clinician's view. Eur Respir J. 10: 1914-1917.

Kim CK, Hisatsune A, Kim DJ, Miyata T (2003): Pharmacology of airway goblet cell mucin release. J Pharmacol Sci. 92: 301-307.

Kirkbride HJ, Bolscher JG, Nazmi K, Vinall LE, Nash MW, Moss FM, Mitchell DM and Swallow DM. (2001): Genetic polymorphism of MUC7: Allele frequencies and association with asthma. Eur J Hum Gen. 9 (5): 347-354.

Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ (2002): Heterogeneity of airway mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. Biochem J. 361: 537-546.

Klapper DG (1982): A new low cost, fully automated amino acid analyzer. In: Methods in protein sequence analysis. (Elzinga M. ed.), Humana press, Clifton New Jersey pp 509-517.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680-685.

Lamblin G, Degroote S, Perini JM, Delmotte P, Scharfman A, Davril M, Lo-Guidice JM, Houdret N, Dumur V, Klein A, Roussel P (2001): Human airway mucin glycosylation: A combinatory of carbohydrate determinants which vary in cystic fibrosis. Glycoconj J. 18(9): 661-684.

Leikauf GD, Borchers MT, Prows DR, Simpson LG (2002): Mucin apoprotein expression in COPD. Chest. 121: 166-182.

Lowry OH, Rosebrough NJ, Farr L, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem. 193(1): 265-275.

Madhi SA, Cutland C, Ismail K, O'Reilly C, Mancha A and Klugman KP (2002): Ineffectiveness of trimethropim-sulfamethoxazole prophylaxsis and the importance of bacterial and viral co-infections in African children with *Pneumocystis carinii* pneumonia. Clin Infect Dis. 35(9): 1120-1126.

Mall AS, Mcleod HA, Hickman R, Kahn D, Dent D (1999): Fragmentation patterns of mucins in normal and diseased gastric mucosae: a glycoprotein fractionates with gastric mucins purified from mucosal scrapings of cancer and peptic ulcer patients. Digestion. 60: 216-226.

Mantle M, Allen A (1978): A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain. Biochem Soc Trans. 6(3): 607-609.

Matsuzwa M, Ota H, Hayama M, Zhang MX, Sano K, Honda T, Ueno I, Akamatsu T, Nakayama J (2003): Helicobacter pylori infection up-regulates gland mucous cell-type mucins in gastric pyloric mucosa. Helicobacter. 8(6): 594-600.

Mcleod HA (1992): Biochemical characterisation of human gastric mucin in normal and diseased states. MSc. Thesis, University of Cape Town.

Middleton AM, Chadwick MV, Nicholson AG, Dewar A, Groger RK, Brown EJ, Ratliff TL, Wilson R (2002): Interaction of Mycobacterium tuberculosis with human respiratory mucosa. Tuberculosis. 82(2/3): 69-78.

Middleton AM, Chadwick MV, Nicholson AG, Dewar A, Feldman C, Wilson R (2003): Investigation of mycobacterial colonisation and invasion of the respiratory mucosa. Thorax. 58: 246-251.

Moniaux N, Escande F, Porchet N, Aubert JP, Batra SK (2001): Structural organisation and classification of the human mucin genes. Front Biosci. 6: 1192-1206.

Munch Z, Van Lill SWP, Booysen CN, Zietsman HL, Enarson DA, Beyers N (2003): Tuberculosis transmission patterns in a high-incidence area: a spatial analysis. Int J Tuberc Lung Dis. 7(3): 271-277.

Negus VE (1963): The function of mucus. Acta Otolaryngol. 56:204-214.

Negus VE (1967): The function of mucus: a hypothesis. Proc Roy Soc Med. 60: 75-84.

Neutra MR, Forstner JF (1987): Gastrointestinal mucus: synthesis, secretion and function. In: Physiology of the gastrointestinal tract 2nd ed. (Johnson LR, ed.), Raven Press, New York pp 975-1009.

Oosta GM, Mathewson NS, Catravas GN (1977): Optimization of folin-ciocalteu reagent concentration in an automated lowry protein assay. Anal Bioch. 89(1): 31-34.

Pearson JP, Allen A, Venables CW (1980): Gastric mucus: Isolation and polymeric structure of the undegraded glycoprotein: Its breakdown by pepsin. Gastroenterology. 78(4): 709-715.

Pearson JP, Kaura R, Taylor W, Allen A (1982): The composition and polymeric structure of mucus glycoprotein from human gallbladder bile. Biochim Biophys Acta 706: 221-228.

Perini JM, Marianne T, Lafitte JJ, Lamblin G, Roussel P, Mazzuca M (1989): Use of an antiserum against deglycosylated human mucins for cellular localization of their peptide precursors: antigenic similarities between bronchial and intestinal mucins. J Histochem Cytochem. 37(6): 869-875.

Piller F, Piller V (1993): Structural characterization of mucin-type O-linked oligosaccharides. In: Glycobiology, A practical approach. (Fukuda M and Kobata A eds.), Oxford University press, New York pp 291-328.

Pinto-de-Sousa J, Reis CA, David L, Pimenta A, Cardosa-de-Oliviera (2004): MUC5B expression in gastric carcinoma: relationship with clinico-pathological parameters and with expression of mucins MUC1, MUC2, MUC5AC and MUC6. Virchows Arch. 444: 224-230.

Polson A, Levitt J (1963): Density determination in a preformed gradient of caesium chloride. Biochim Biophy Acta. 75: 88-95.

Raja A (2004): Immunology of tuberculosis. Indian J Med Res. 120: 213-232.

Rogers D (2004): Airway mucus hypersecretion in asthma: an undervalued pathology. Curr Opin Pharmacol. 4(3): 241-250.

Rose MC (1992): Mucins: structure, function, and role in pulmonary diseases. Am J Physiol. 263: 413-429.

Rose MC, Nickola TJ, Voynow JA (2001): Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. Am J Respir Cell Mol Biol. 25: 533-537.

Rose MC, Voynow JA (2006): Respiratory tract mucin genes and mucin glycoproteins in health and disease. Physiol Rev. 86: 245-278.

Sheehan JK, Carlstedt, I, Coster L, Malmstrom, Fransson LA (1981): Isopynic-centrifugation studies in caesium chloride and in caesium sulphate on dermatan sulphate proteogylcans from bovine sclera. Biochem J. 199: 581-589.

Sheehan JK, Thornton DJ, Howard M, Carlstedt I, Corfield AP, Paraskeva C (1996): Biosynthesis of the MUC2 mucin: evidence for a slow assembly of fully glycosylated units. Biochem J. 315(3): 1055-1060.

Sheehan JK, Howard M, Richardson PS, Longwill T, Thornton DJ (1999): Physical characterization of a low-charge glycoform of the MUC5B mucin comprising the gel-phase of an asthmatic respiratory mucous plug. Biochem J. 338: 507-513.

Situ H, Wei G, Smith CJ, Mashhoon S, Bobek LA (2003): Human salivary MUC7 mucin peptides: effect of size, charge and cysteine residues on antifungal activity. Biochem J. 375: 175-182.

Silberberg A, Meyer FA, Gilboa A, Gelman RA (1977): Function and properties of epithelial mucus. Adv Exp Med Biol. 89:171-80.

Slayter HS, Lamblin G, Le Treut A, Galabert C, Houdret N, Degand P, Roussel P (1984): Complex structure of human bronchial mucus glycoprotein. Eur J Biochem. 142: 209-218.

Spyridaki MHE, Siskos PA (1996): An improved spectrophotometric method for the determination of free, bound and total N-acetylneuraminic acid in biological fluids. Anal Chim Acta. 327: 277-285.

Starkey BJ, Snary D, Allen A (1974): Characterisation of gastric mucoproteins isolated by equilibrium density-gradient centrifugation in caesium chloride. Biochem J. 141: 63-639.

Stoscheck CM (1990): Quantitation of protein. Methods Enzymol 182: 50-68.

Takeuchi K, Yuta A, Sakakura Y (1995): MUC2 mucin gene expression in the nose and maxillary sinus. Am J Otolaryngol. 16(6): 391-395.

Taylor KL, Mall AS, Barnard RA, Ho SB, Cruse JP (1998): Immunohistochemical detection of gastric mucin in normal and disease states. Oncol Res. 10(9): 465-473.

Taylor C, Draget KI, Pearson JP, Smidsrod O (2005): Mucous systems show a novel mechanical response to applied deformation. Biomacromolecules. 6(3): 1524-1530.

Thornton DJ, Holmes DF, Sheehan JK, Carlstedt I (1989): Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. Anal. Biochem. 182: 160-164.

Thornton DJ, Carlstedt I, Sheehan JK (1994): Identification of glycoproteins on nitrocellulose membranes and gels. Methods Mol Biol. 32: 119-28.

Thornton DJ, Howard M, Devine PL, Sheehan JK (1995): Methods for separation and deglycosylation of mucin subunits. Anal Biochem. 227: 162-167.

Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK (1996): Respiratory mucins: identification of core proteins and glycoforms. Biochem J. 316(3): 967-975.

Thornton DJ, Howard M, Khan N, Sheehan JK (1997): Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. J Biol Chem. 272(14): 9561-9566.

Thornton DJ, Khan N, Mehrotra R, Howard M, Veerman E, Packer NH, Sheehan JK (1999): Salivary mucin MG1 is comprised almost antirely of different glycosylated forms of the MUC5B gene product. Glycobiology. 9(3): 293-302.

Thornton DJ, Sheehan JK (2004): From mucins to mucus: toward a more coherent understanding of this essential barrier. Proc Am Thorac Soc. 1(1): 54-61.

Tytgat KMAJ, Bovelander FJ, Opdam FJM, Einerhand AWC, Buller HA, Dekker J (1995): Biosynthesis of rat MUC2 in colon and its analogy with human MUC2. Biochem J. 309: 221-229.

Tytgat KMA, Swallow DS, Van Klinken BJW, Buller HA, Einerhand AWC, Dekker J (1995): Unpredictable behaviour of mucins in SDS/polyacylamide-gel electrophoresis. Biochem J. 310:1053-1054.

Uplekar MW, Rangan S, Weiss MG, Ogden J, Borgdorff MW, Hudelson P (2001): Attention to gender issues in tuberculosis control. Int J Tuberc Lung Dis. 5(3): 220-224.

van Kooij RJ, Roelofs HJM, Kathman GAM, Kramer MF (1980): Human cervical mucus and its mucous glycoprotein during the menstrual cycle. Fertil Steril. 34: 226-233.

Veerman ECI, Van den Keybus PAM, Vissink A, Nieuw Amerogen AV (1996): Human glandular salivas: their separate collection and analysis. Eur J Oral Sci. 104: 346-352.

Venables CW (1986): Mucus, pepsin and peptic ulcer. Gut. 27: 233-238.

Vinall LE, Hill As, Pigny P, Pratt WS, Toribara N, Gum JR, Kim YS, Porchet N, Aubert JP, Swallow DM (1998): Variable number of tandem repeat polymorphism of the mucin genes located in the complex on 11p15.5. Hum Genet. 102: 357-366.

Vinall LE, Fowler JC, Jones AL, Kirkbride HJ, de Bolos C, Laine A, Porchet N, Gum JR, Kim YS, Moss FM, Mitchell DM, Swallow DM (2000): Polymorphism of human mucin genes in chest disease, possible significance of MUC2. Am J Respir Cell Mol Biol. 23: 678-686.

Vilar JP, Sheehan JK, Randell SH (2003): Making more MUCs. Am J Respir Cell Mol Biol. 28: 267-270.

Voynow JA, Selby DM, Rose MC (1998): Mucin gene expression (MUC1, MUC2, and MUC5/MUC5AC) in nasal epithelial cells of cystic fibrosis, allergic rhinitis, and normal individuals. Lung.176: 345-354.

Voynow JA (2002): What does mucin have to do with lung disease. Paediatr Respir Rev. 3(2): 98-103.

Walzl G (2004): Addressing the tuberculosis and infectious diseases burden. Curr Allergy and Clin Immunol. 17(4): 158.

Wei GX, Bobek L (2005): Human salivary mucin MUC7 12-Mer-L and 12-Mer-D peptides: antifungal activity in saliva, enhancement of activity with protease inhibitor cocktail or EDTA, and cytotoxicity to human cells. Antimicrob Agents Chemother. 49(6): 2336-2342.

Wickstrom C, Davies JR, Eriksen GV, Veerman ECI, Carlstedt I (1998): MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem J. 334: 685-693.

Wiggins R, Hicks SJ, Soothill PW, Millar MR, Corfield AP (2001): Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. Sex Transm Infect. 77(6): 4022-408.

Woodward H, Horsey B, Bhavanandan VP, Davidson A (1982): Isolation, purification and properties of respiratory mucus glycoproteins. Biochemistry. 21(4): 694-701.

Wolf DP, BlascoL, Khan MA, Litt M (1977): Human cervical mucus. Rheological characteristics. Fertil Steril. 28: 41-46.

Yeager H (1971): Tracheobronchial secretions. Am J Med. 50(4): 493-509.

Yurewicz EC, Matsuura F, Moghissi KS (1987): Structural studies of sialylated oligosaccharides of human midcycle cervical mucin. J Biol. Chem. 262: 4733-4739.

Zar H, Bateman E, Ramsay M (1998): New advances in cystic fibrosis –implications for developing countries. S Afr Med J. 88(8): 967-968.

Zar H, Tannebaum E, Apolles P, Roux P, Hanslo D and Hussey G (2000): Sputum induction for the diagnosis of pulmonary tubeculosis in infants and young children in an urban setting in South Africa. Arch Dis Child. 82: 305-308.