THE INHIBITION OF HIV-1 ACTIVITY BY CRUDE MUCUS AND PURIFIED MUCIN (MUCOUS GLYCOPROTEINS) FROM SALIVA, BREAST MILK AND THE CERVICAL TRACT OF NORMAL SUBJECTS, HIV POSITIVE INDIVIDUALS AND PATIENTS WITH HIV-AIDS

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BSc (Hons), MSc

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Surgery

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

February 2007

PUBLICATIONS

Published articles related to this work are:

Habte, H.H., Mall, A.S., de Beer, C., Lotz, Z.E. and Kahn, D. (2006). The role of crude human saliva and purified salivary MUC5B and MUC7 mucins in the inhibition of Human Immunodeficiency Virus type 1 in an inhibition assay. *Virology Journal* **3,** 99.

Habte, H.H., Lotz, Z.E., Tyler, M.G., Abrahams, M., Rodriques, J., Kahn, D., Kotwal, G.J. and Mall, A.S. (2007). Anti viral activity of purified human breast milk mucin. *Neonatology* **92,** 96-104.

Habte, H.H., de Beer, C., Lotz, Z.E., Kahn, D. and Mall, A.S. (2007). Inhibition of Human Immunodeficiency Virus type 1 activity by purified human breast milk mucin (MUC1) in an inhibition assay. *Neonatology* "in press".

Manuscripts under review

Habte, H.H., de Beer, C., Lotz, Z.E., Tyler, M.G., Schoeman, L., Kahn, D and Mall, A.S. (2007). The inhibition of the Human Immunodeficiency Virus type 1 activity by crude and purified human pregnancy plug mucus and mucins in an inhibition assay. Journal of General Virology "under review"

Published abstracts

Habte, H., Mall, A., Lotz, Z. and Kahn, D. (2005). The characterisation of salivary mucins with a view to testing their anti-HIV activity. *South Africa Journal of Surgery* **43,** 120.

Habte, H., Mall, A., Kotwal, G., Lotz, Z., Tyler, M., Abrahams, M., Schoeman, L. and Kahn, D. (2006). Antiviral activity of human salivary, breast milk and cervical secretion mucins. *South Africa Journal of Surgery* **44,** 168.

Habte, H., Mall, A., de Beer, C., Lotz, Z., Tyler, G., Schoeman, L. and Kahn, D. (2006). Anti HIV activity of salivary, breast milk and cervical mucins. *South Africa Journal of Surgery* **44,** 168.

PREFACE

This study was carried out from February 2005 to February 2007 under the supervision of Professor Anwar Suleman Mall, in the Department of Surgery, Faculty of Health Sciences, University of Cape Town, South Africa.

These studies represent the original work by the author and have not been submitted in any other form to another University. Where use has been made of the work of others, it has been duly acknowledged in the text.

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ABSTRACT

Human saliva, breast milk and cervical secretions contain several non-immunological components including mucins (mucous glycoproteins), which protect the gastrointestinal and female reproductive tracts and breast fed infants from bacterial, viral and fungal infections. In addition to their well known function in lubrication, tissue coating and digestion, mucus and mucins have been used as pathological markers in diseases such as asthma, chronic bronchitis, cystic fibrosis, and carcinomas of the breast, lung and colon. Crude saliva is also known to inhibit the activity of human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS).

According to the joint United Nations Programme on HIV/AIDS (UNAIDS) worldwide an estimated 38.6 million people were living with HIV in 2005 with 4.1 million newly infected and 2.8 million deaths. It has been reported that an estimated 24.5 million of the HIV infected people of whom 60% females live in sub-Saharan Africa with the Southern African region having the highest prevalence in Africa. Furthermore the incidence of opportunistic diseases such as TB is also reported to increase with HIV prevalence. Thus far, despite the discovery of highly active antiretroviral therapies which contain both protease and reverse transcriptase inhibitors, HIV remains as a global threat especially to the third world countries. Therefore there is a need for the development of safe compounds to reduce viral loads in infected people and to prevent the transmission of the virus from one individual to another. The search for a suitable vaccine is ongoing.

This is a novel study that investigated the anti-HIV-1 activity of human saliva, breast milk and pregnancy plug mucus and mucins from HIV negative individuals and compared this to the anti-HIV-1 activity of the salivary mucins from patients with HIV infection or full blown AIDS in an *in vitro* inhibition assay. Furthermore this study investigated the role of salivary MUC7 in inhibiting HIV-1 infection of the peripheral blood mononuclear cells (PBMCs), reducing viral infection of the HIV-1 infected PBMCs and minimizing the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs. The role of salivary, breast milk and pregnancy plug mucins

in the inhibition of enveloped viruses specifically poxvirus activity was also investigated.

Following Sepharose CL-4B gel filtration and caesium chloride density-gradient ultra-centrifugation, the presence of MUC5B and MUC7 in saliva, MUC1 in breast milk and MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6 mucins in the pregnancy plug mucus was confirmed by Western blotting and immuno-histochemistry analysis. The amino acid analysis which showed high amounts of serine, threonine, glutamic acid, glycine, aspartic acid and proline was suggestive of a typical mucin amino acid profile of which serine, theronine and proline make up 20.1%, 26% and 28% of the MUC1, MUC5B and MUC7 mucins respectively.

Mucins of various sources were shown to be non-toxic to CEM SS cells. The anti-HIV-1 activity of crude saliva, breast milk and crude pregnancy plug mucus together with their purified components (MUC5B and MUC7 in saliva, MUC1 in breast milk and MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6 in pregnancy plug) from HIV negative individuals and salivary MUC5B and MUC7 from HIV positive patients (with different CD4 counts <200, 200-400 and >400) was determined by incubating them with HIV-1 prior to infection of the CEM SS cells. The crude saliva, salivary MUC5B and MUC7, milk mucin (MUC1) and pregnancy plug mucins inhibited HIV-1 activity by 97.5%. Crude breast milk and pregnancy plug mucus did not inhibit the virus. MUC5B and MUC7, purified from the crude saliva of HIV positive patients did not inhibit the virus either irrespective of their CD4 counts. Agarose gel electrophoresis showed these mucins, especially MUC7, to have varying mobilities compared to their normal counterparts suggesting altered glycosylation patterns that very likely cause poor entrapment of the virus.

Salivary MUC7 did not inhibit HIV-1 infection of the PBMCs when MUC7 was first incubated with these cells rather than with the virus. This suggested that mucin inhibition is by physical entrapment of the virus than by blocking putative viral binding sites on the surface of PBMCs. MUC7 had no effect on reduction of intracellular viral loads of infected PBMCs. There was very little viral exchange between infected PBMCs and non-infected PBMCs. Although the incubation of the

mucins with the infected cells prevented this transmission, the finding needs to be further explored.

Salivary MUC5B and MUC7, milk mucin (MUC1) and pregnancy plug mucins also inhibited the activity of vaccinia virus (vGK-5 strain) which is a family of the enveloped poxvirus in a dose dependent manner up to 100%. This suggests that mucins may also possess a strong activity against other enveloped viruses. This result could provide valuable information in the attempt to use mucins as therapeutic agents against HIV-1 and other enveloped viruses.

ACKNOWLEDGEMENTS

I would like to extend my appreciation to the following people for their substantial contributions to this thesis.

To my supervisor Professor Anwar Suleman Mall, for his guidance, availability including weekends, endless ideas, thorough proof reading and above all his willingness to accommodate my ideas which in turn gave me confidence. Without his supervision this study would not have been completed. I really thank you for your endless patience.

To Professor Delawir Kahn for his valuable contribution in designing and coordination of the study

To Dr Corena de Beer of the Discipline of Medical Virology, University of Stellenbosch and National Health Laboratory Service, Tygerberg Business Unit, for the HIV inhibition assays. I thank you for your support and patience.

I am grateful to our collaborators Dr Paul Roux of Paediatric Medicine, Dr Leann Schoeman of Obstetrics and Gynaecology and Dr Natasha Rhoda and Dr Pieper of the Neonatology, University of Cape Town for kindly providing salivary, blood, cervical secretion and breast milk from normals and HIV positive individuals.

To Professor Girish J Kotwal and Melissa Abrahams of the Department of Medical Virology, University of Cape Town, for the poxvirus inhibition assay.

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

To Professor Dallas Swallow of the Galton Laboratory University College London and Professor David Thornton and Dr Sara Kirkham of the University of Manchester Medical School for kindly providing antibodies.

I am very grateful to the MRC, NRF and the University of Cape Town Postgraduate Funding Office for the financial support.

To Marilyn Tyler and Zoe Lotz for their immense contribution to the histo-chemistry and biochemical aspect of the study. I can not thank you enough for your motherly care which was a blessing that allowed me to concentrate on my research.

To my sister Eden Tesfay and great friend Stefan Andersson for their moral and financial supports.

To my family and colleagues in the Department of Surgery, Gert, Lizette, Ntato (rabbit), Jason, Astrid, Erika, Dr Maren, Lutske, Ole, Marita and Karol. Thank you for your support and beautiful memories filled with fun and laughter.

To the secretaries in the Department of Surgery: Ayeesha, Micheele, Brenda, Yasheera, Mrs Sanger, Valarie and Ethel for their continuous support.

To Adri Winckler of the Postgraduate Admission Office for her support in my admission and financial issues.

To Yohannes, Aron, Ureshni, Ikechukw (Ike) and Gonzalo for being great friends and advisors, I thank you very much for the intellectual, social and sports life we had.

To my friends Robiel, Kefali, Henok, Hermon, Awet, Dogole, Kesete, Muller, Medhanie, Simon, Hadgut, Toronto, Prof, Mandela, Filmon, Lidia, Adiam, Yakini, Ariam, Kiflom, John, Eyob, Asier, Mamo, Bini, Ida, Heike, Vonee, Peter, Charlotte, Sam, Shanel,Marion, Anja, Ines, Jocelyn, Kim, Suraia, Line, Lisa, Machu, Ramadhan, Tanja, Thandi, Ulashnie, Prof Mario and Vegard for all the good times we had.

To my family Haileselassie Habte, Lemlem Belay, Zewditu Ghebregziabher, Mufugeta Haileselassie, Samson Haileselassie, Rgbe Belay and family, Mebrak Gebray and Ftwi Gedle for all the years of love and support. I would not be who I am today if it was not for your continuous love and support. Lastly, I thank the almighty God for this great opportunity

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ABBREVATIONS

AIDS	acquired immunodeficiency syndrome
BSA	bovine serum albumin
BSC-1	African green monkey epithelial cells
CD4	cluster of differentiation 4
CEM-SS	human T lymphoblastoid cell line
CHAPS	3-((3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate
CsCl	caesium chloride
DAB	3,3'-diaminobenzidine
DMSO	di-methylsulphoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ENF	enfuvirtide
ER	endoplasmic reticulum
ERBB	epidermal growth factor receptors
FCS	fetal calf serum
FEV_1	forced expiratory volume
gp	glycoprotein
GT	glycosyltransferases
GuHCl	guanidinium chloride
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HRPO	horse radish peroxidise
HSMSL	human submandibular-sublingual
HSV	herpes simplex virus
IL2	interleukin-2
kb	kilo-base
kDa	kilo-Dalton

mA	miliamper
mbar	millibar
MCV	molluscum contagiosum virus
MFGM	milk fat globule membrane
M_w	molecular weight marker
mRNA	messenger RNA
MUC	mucin
NA	not available
Na_2-EDTA	ethylenediaminetetra-acetic acid disodium salt
NEM	N-ethylmaleimide
OPA	O-phthalaldehyde
PAS	periodic acid schiff's
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plague forming units
PHA	phytohaemagglutinin
PMSF	Phenylmethylsulfonylfluoride
rev	revolution
RBC	red blood cells
RPMI	media developed by Moore et. al. at Roswell Park Memorial Institute, hence the acronym RPMI.
RT	room temperature
SARS	severe acute respiratory syndrome
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
sIgA	secretory immunoglobulin A
SLPI	secretory leukocyte protease inhibitors
SNF	supernatant fluid
TAE	tris-acetate buffer
TB	tuberculosis
TBS	tris buffered saline
TBST	tris buffered saline-Tween
$TCID_{50}$	50% tissue culture infective dose

TEMED	*N, N, N, N'*-tetramethylethylenediamine
TMB	3,3'5,5'-Tetramethylbenzidine
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultraviolet
V	voltage
VNTR	variable number of tandem repeats
WBC	white blood cells
ρ	density
A_{280}	absorbance at 280nm
7-AAD	7-Amino-actinomycin D

CHAPTER 1

LITERATURE REVIEW

1.1 Background

Mucus is a slimy viscoelastic or gelatinous blanket secretion that coats the surface of epithelial tissues of the gastrointestinal, respiratory and reproductive tracts as well as the skin of some amphibians (Olmsted *et al.*, 2001; Reid, 1978). Not only does mucus provide protection against microbial infections, dehydration, physical or chemical injuries, its contribution to the innate immune system is also well documented (Bolscher *et al.*, 1999; Bosch, *et al.*, 2000: Perez-Vilar and Hill, 1999; Rose and Voynow, 2006). In relationships of structure and function mucus plays an important role in growth, fetal development, epithelial renewal and differentiation, epithelial integrity, carcinogenesis and metastasis (Moniaux *et al.*, 2001). Its presence in different glands of the body and epithelia such as the mammary gland, salivary gland, oesophageal epithelium, stomach, pancreas, bile ducts, lung epithelium, kidney, bladder, liver, uterus, rete testis, prostate, cornea and conjunctiva make mucus the most widely distributed bodily secretion (Duwe and Ceriani 1989; Gipson *et al.*, 1995; Hollingsworth and Swanson, 2004; Idris and Carraway, 1999; Patton *et al.*, 1995; Taylor *et al.*, 1998).

In addition to its well known function in lubrication, tissue coating and digestion, human salivary mucus functions as an anti-viral, bacterial, toxin and fungal agent by aggregating and removing these pathogenic micro-organisms from the oral cavity (Gururaja *et al.*, 1999; Wei and Bobek, 2005). Some of the functions of mucus in the gastrointestinal tract include the aggregation and removal of agents such as cholera toxin in the stomach, parasitic nematodes in the intestine, regulating the movement of hydrogen, glucose and other low-molecular-weight ions through the gastroduodenal epithelium, protection against acid, pepsin, bile, alcohol, non-steroidal anti-inflammatory drugs and antioxidant agents. Furthermore mucus protects the soft luminal tissue from mechanical damage which can be caused by the passage of solids.

undigested food, and faeces through the gastrointestinal tract (Allen, 1981). Salivary mucus is also reported to play a significant role in minimizing the effect of or inhibition of the human immunodeficiency virus (HIV) infection through the oral cavity (Nagashunmugam *et al.*, 1997).

Human breast milk is known to contain a number of non-immunological components including mucin, which is biologically active and protects breast fed infants against infections from bacteria, viruses, fungi and toxins (Schroten *et al.*, 1992; Wiederschain and Newburg, 2001). Cervical mucus also plays a significant role in safeguarding the female reproductive tract physiology from internal and external factors (Carlstedt *et al.*, 1983; Carlstedt *et al.*, 1982). In addition to its protection against microbial colonization, infection and regulation of sperm transport to the upper reproductive tract (Eriksen *et al.*, 1998; Wolf *et al.*, 1980; Yurewicz and Moghissi, 1981), it provides lubrication, tissue hydration, cell attachment and anti-proteolytic activity (Gipson *et al.*, 1995; Idris and Carraway, 1999; Lagow *et al.*, 1999; Venegas *et al.*, 1995). According to Argüeso *et al.* (2002) abnormality in cervical mucus production affects fertility by blocking sperm transport to the upper reproductive tract where fertilization occurs.

The epithelial surface of the human respiratory tract is also covered by the slimy and viscoelastic mucus secretion (Hovenberg *et al.*, 1997), which provides protection against a number of external agents such as chemical and pathological insults (Thornton *et al.*, 1997). In addition to its function in the protection of the airway epithelium from pathogenic and lethal agents, it participates in the mucosal response to inflammation and infection (Rose *et al.*, 2001).

Mucus is a crude body secretion containing water, glycoproteins (mucins), lipids, nucleic acids, lactoferrin, lysozyme, immunoglobulins and ions (Creeth, 1978; Gipson *et al.*, 2001; Leikauf *et al.*, 2002; Tabak, 1995). The long and heavily *O*-glycosylated glycoproteins called mucins are the major components (~85% of the total macromolecules) of the crude mucus secretion (Argüeso *et al.*, 2002). According to Moniaux *et al.* (2001), mucins are reported to be synthesized and secreted by specialized cells of the epithelium and in some cases by the non mucin-secreting cells of the body. The focus of the present study was to purify and characterize these

mucins from human saliva, breast milk and cervical secretions of HIV negative individuals and compare their anti-HIV-1 activity to the salivary mucins from HIV positive individuals in an *in vitro* HIV inhibition assay.

1.2 Mucus and mucins as pathological markers

Human mucins have been used as pathological markers in diseases such as malignancies of the gastrointestinal and respiratory tracts (Bolscher *et al.*, 1995). The over-production or expression of these mucins is associated with pathological conditions (Louahed *et al.*, 2000). For instance, mucus over-production in the respiratory tract is associated with a number of diseases, such as asthma, chronic bronchitis, cystic fibrosis and lung carcinoma (Louahed *et al.*, 2000) which are known to induce obstruction in the airways (Figure 1.1) (Rose *et al.*, 2001).

In the female reproductive tract, less or over-production of mucus or mucins is associated with hormonal or reproductive status, infection, and pathology of the female reproductive tract (Gipson *et al.*, 1997). According to Yurewicz and Moghissi (1981) these abnormalities may cause infertility. Simultaneously the rate and level of mucus or mucin production in human breast milk is known to be an important marker in metastatic breast cancer progression (Sekine *et al.*, 1985). Hollingsworth and Swanson (2004) reported the area surrounding the tumour to be hypoxic, acidic, and rich in proteases and other biologically active materials. This could support their hypothesis that tumours may use mucins during invasion, metastasis and growth. One example of this is the shift in the nature of mucins from neutral to acidic in malignant progression of the prostate gland (Goldstein *et al.*, 1995).

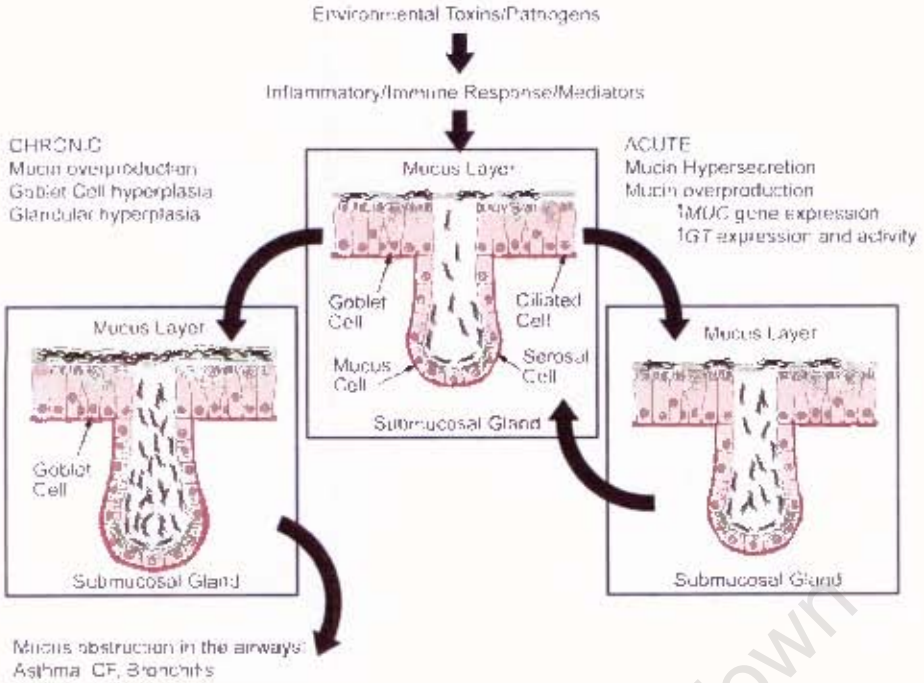


Figure 1.1 Over production of mucus and mucins induces airway obstruction. Exposure of human airway epithelium to environmental agents such as pathogens or toxins induces inflammation or an immune response. The inflammatory or immune response mediator activates secretion of mucins from surface goblet cells or glandular secretory cells in the acute condition and goblet cell hyperplasia and glandular hyperplasia/hypertrophy in the chronic condition. This results in over production of mucins in patients with asthma, chronic obstructive pulmonary disease, or cystic fibrosis (reproduced from Rose and Voynow, 2006).

1.3 Definition of mucins

Mucins which are the major glycoprotein components of the crude mucus secretion (Moniaux *et al.*, 2001; Thomsson *et al.*, 2002) are highly glycosylated and large in size with molecular weight ranging between 5×10^5 to 4×10^6 Da (Yurewicz *et al.*, 1987). Their carbohydrate content which constitutes 50 to 85% of the molecule is known to protect mucins from enzymatic degradation (Figure 1.4) (Leikauf *et al.*, 2002). The monosaccharide composition of mucins is comprised of five sugar residues namely: *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, fucose and sialic acid (Müller *et al.*, 1997; Peterson *et al.*, 1998).

The deficiency of uronic acid and the small amounts of mannose (<1%) are among the factors which distinguishes mucin glycoproteins from the glycoproteins of serum and connective tissues or proteoglycans (Reid, 1978). Furthermore their variable number of tandem repeats (VNTR) plus the high contents of threonine, serine, and proline

amino acids (which make up 80% of the total amino acid) are other distinguishing factors (Müller *et al.*, 1997; Perez-Vilar and Hill, 1999; Peterson *et al.*, 1998). Of the five sugar residues, the sulphate residues are known to bind to *N*-acetylglucosamine or galactose while the *N*-acetylgalactosamine, usually at the reducing ends of the chain is *O*-linked to serine or threonine through an oxygen atom (Allen, 1981). In *N*-linked sugars such as in proteoglycans the attachment of *N*-acetylglucosamine to asparagine is through a nitrogen atom and the link is an *N*-glycosidic one (Reid, 1978). The sugar residues at the non reducing termini are usually α-linked fucose or sialic acid, with their amount inversely proportional to each other, with the exception of the *N*-acetylgalactosamine, which is attached to the protein core, the terminal sugars are generally β-linked (Figure 1.2) (Gipson *et al.*, 1995; Wiggins *et al.*, 2001). According to Reid (1978) therefore mucins are described as polypeptide chain with the oligosaccharide units attached to every third amino acid and projected to give the mucins a lamp brush or a test-tube brush like structure.

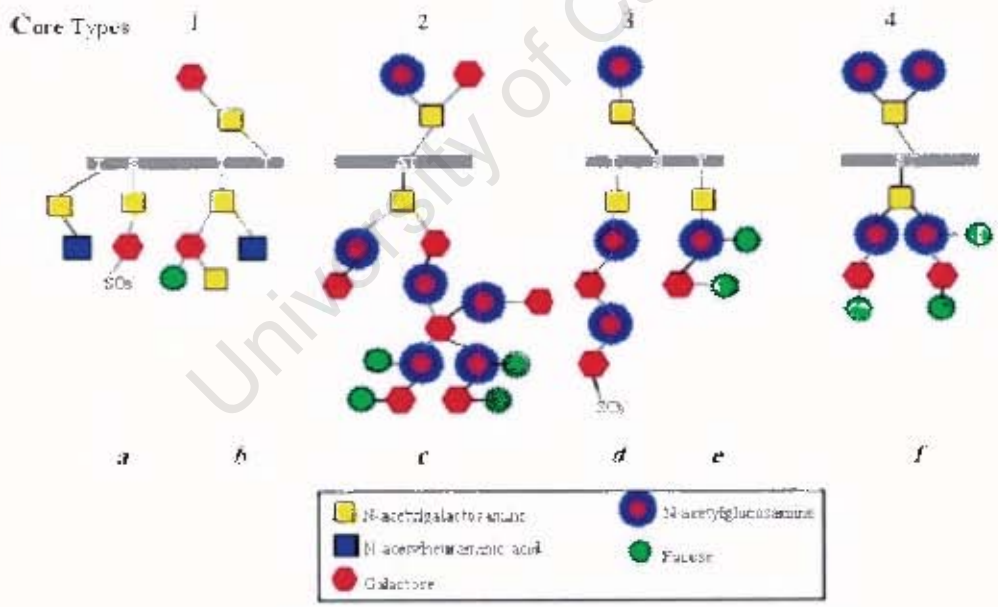


Figure 1.2 Diagrammatic representations of the five sugar units of a mucin. In mucins the *N*-acetylgalactosamine is usually attached or *O*-linked to serine or threonine which is elongated by the addition of galactose or *N*-acetylglucosamine. Further terminal glycosylation occurs by the addition of fucose, sialic acid or sulfate and blood-group determinants. Letters a and d represents lung mucin, b porcine submaxillary mucin, c ovarian cyst fluid mucin with two Le^b blood group determinants, and e and f; bronchiectasis lung mucin (reproduced from Rose and Voynow, 2006).

1.4 Biosynthesis of mucins

Mucins biosynthesis is reported to start in the nucleus where the mucin or MUC gene expression is activated by the transcription factors and translated to MUC proteins in the ribosomes (Phelps, 1978; Rose and Voynow, 2006). Subsequent to the *N*-linked glycosylation in the endoplasmic reticulum (Patton *et al.*, 1995), *O*- glycosylation of the MUC protein backbone occurs in the cis-Golgi apparatus (Lidell *et al.*, 2006; Zalewska *et al.*, 2000). *N*-acetylgalactosamine addition is step one in the synthesis of *O*-glycans and precedes elongation (Leikauf *et al.*, 2002; Lidell *et al.*, 2003). The transfer of *N*-acetylgalactosamine is reported to initiate the subsequent addition of hexoses and/or hexosamines to each glycosylation site (*O*-glycan) by a series of glycosyltransferases in a stepwise manner. Finally the fully glycosylated or matured mucin is stored in secretory granules until mucin secretion is triggered by either internal or external factors (Perez-Vilar and Hill, 1999; Rose and Voynow, 2006) or transported to the plasma membrane (Figure 1.3) (Patton *et al.*, 1995).

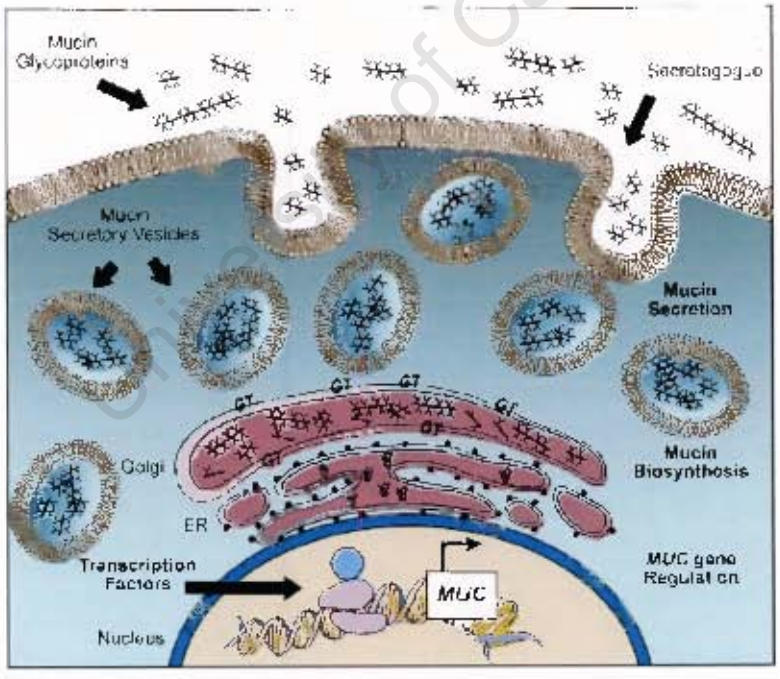


Figure 1.3 **Diagrammatic representation of mucin biosynthesis.** The mucin or MUC gene expression is initiated in the nucleus and translated to MUC proteins in ribosomes. Subsequent to *N*-linked glycosylation in the endoplasmic reticulum and *O*- glycosylation in the cis-Golgi apparatus, further glycosylation is initiated by glycosyltransferases. Ribosomes, endoplasmic reticulum and glycosyltransferases are represented by (•), (ER) and (GT) respectively (reproduced from Rose and Voynow, 2006). For details see mucin biosynthesis in the previous paragraph.

1.5 Physical properties of mucins

Based on its sensitivity to enzymatic digestion a mucin is divided into two regions namely, the glycosylated and nonglycosylated (naked) regions (Reid, 1978; Sellers *et al.*, 1988). While the glycosylated region is characterized by a number of tandem repeats rich in serine, threonine and proline, the naked region is rich in cysteine and contains low amounts of threonine, serine, and proline amino acids (Creeth, 1978; Wickstrom *et al.*, 1998). The amino acids or the protein backbone in the glycosylated region is protected from enzymatic digestion by the carbohydrate units whereas the naked region is very susceptible to a variety of enzymes such as pepsin, trypsin, papain, and pronase (Figure 1.4) (Creeth, 1978; Godl *et al.*, 2002; Lidell *et al.*, 2006; Tabak, 1995; Wickstrom *et al.*, 1998; Wiggins *et al.*, 2001). In the presence of reducing agents such as mercaptoethanol, dithiothreitol, and *N*-acetylcysteine the size of mucins is reported to decrease, which led to the suggestion that the disulfide bridges located between the naked regions of mucins may maintain mucins in multimeric form (Allen, 1981; Perez-Vilar and Hill, 1999; Tabak *et al.*, 1982).

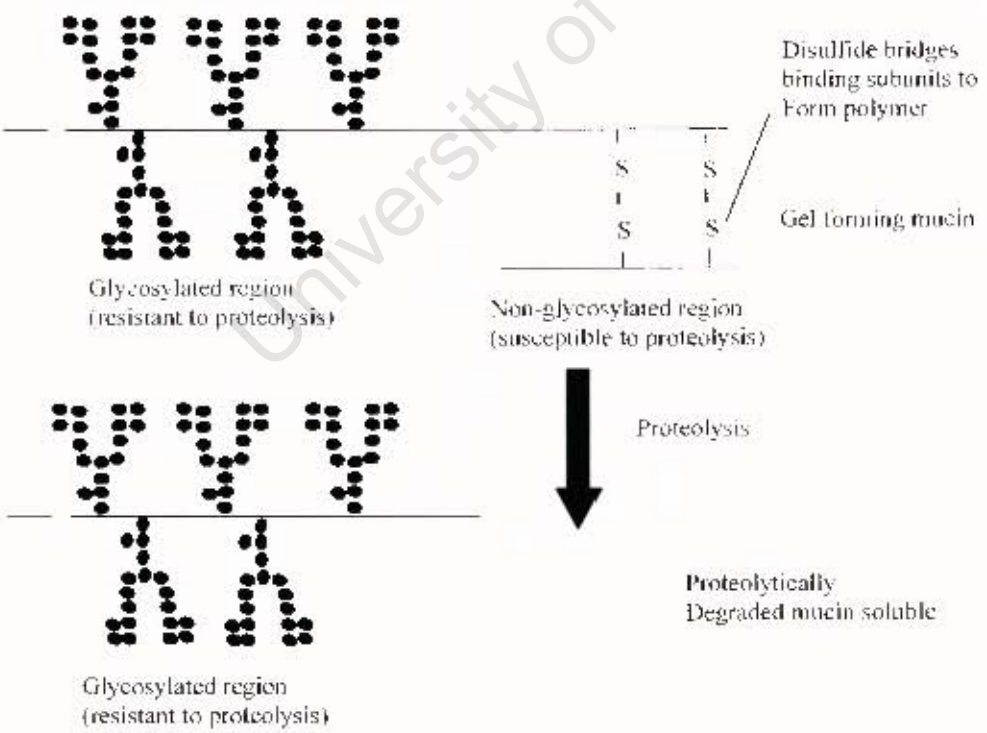


Figure 1.4 Diagrammatic representation of mucin proteolysis. The carbohydrate side chains in the glycosylated region protect mucins from proteolysis (reproduced from Allen, 1981).

1.6 Mucin purification

According to Creeth (1978) mucins can be separated from non-mucin components of the crude mucus secretions either by degradative or non-degradative methods. While methods such as proteolysis, thiol reduction, sonication and extraction with chaotropic reagents are degradative ones, sol-gel separation and homogenization, gel filtration, ion-exchange chromatography, affinity chromatography and equilibrium density gradient ultra-centrifugation represent the non-degradative methods. Of these methods gel filtration, equilibrium density gradient ultra-centrifugation and ion-exchange chromatography are found to be the most suitable methods in mucin isolation and purification (Allen, 1981). These methods can separate mucins from non-mucin components such as proteins, lipids, nucleic acids and enzymes based on their size, density, and charge differences respectively (Allen, 1981).

According to Allen (1981) gel filtration or size exclusion chromatography can partially purify mucins by eluting the larger molecular weight mucins in the excluded volume and the smaller molecular weight components in the included volume. Caesium chloride density gradient centrifugation which is a widely used purification method is capable of separating mucins at a density of ρ = 1.45-1.55 from lower-density material (lipid and protein) and higher-density material (nucleic acids). Ion-exchange chromatography can also separate mucins according to charge. Furthermore sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), which separates high molecular weight mucins from smaller size proteins according to their electrophoretic mobility, is also a routinely used method in mucin purification (Patton *et al.*, 1995). However, their larger size, high glycosylation and viscoelastic nature of mucins make their purification very difficult (Rose and Voynow, 2006).

1.7 Mucin genes and classification

Based on their amino acid sequence, chromosomal localization and form, human mucins are classified into three classes namely: gel forming (MUC2, MUC5AC, MUC5B, MUC6, and MUC19), soluble (MUC7, MUC8, and MUC9) and membrane bound (MUC1, MUC3A, MUC3B, MUC4, MUC11 or MUC12, MUC13, MUC16,

MUC17, and MUC20). On the other hand mucins such as MUC14, MUC15 and MUC18 do not have tandem repeats and remain unclassified (Godl *et al.*, 2002; Lidell *et al.*, 2003; Moniaux *et al.*, 2001; Rose and Voynow, 2006). With the exception of MUC19, which is localized on chromosome 12q12, the gel forming mucins (MUC2, MUC5AC, MUC5B, and MUC6) are localized on chromosome 11p15.5. The soluble mucins MUC7, MUC8, and MUC9 are localized on chromosome 4q13.q21, 12q24.3 and1p13 respectively. Of the membrane bound mucins, MUC3A, MUC3B, MUC11 or MUC12 and MUC17 are localized on chromosome 7q22; MUC4 and MUC20 on chromosome 3q29, MUC1 on chromosome 1q21-q24; MUC13 on chromosome 3q13.3 and MUC16 on chromosome 19q13.2 (Rose and Voynow, 2006).

1.7.1 Secreted and gel forming mucins

The secreted and gel forming mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19) are the largest in size and have viscoelastic properties (Hollingsworth and Swanson, 2004; Lagow *et al.*, 1999; Van Seuningen *et al.*, 2000). Their protein backbone is characterized by a number of tandem repeats or *O*-glycosylation sites separated by cysteine-rich domains. As the number of tandem repeats and *O*-glycosylation sites are directly proportional, either of them may influence the size of these mucins (Rose and Voynow, 2006). The cysteines in the C-termini of these mucins are thought to induce polymerization via the disulfide bonds (Lagow *et al.*, 1999; Van Seuningen *et al.*, 2000), which contributes to their large size and gel-forming forms (Figure 1.5) (Leikauf *et al.*, 2002; Rose *et al.*, 2001). However, the size and the glycosylation pattern of these mucins is tissue specific or differ from tissue to tissue (Lidell *et al.*, 2003; Moniaux *et al.*, 2001).

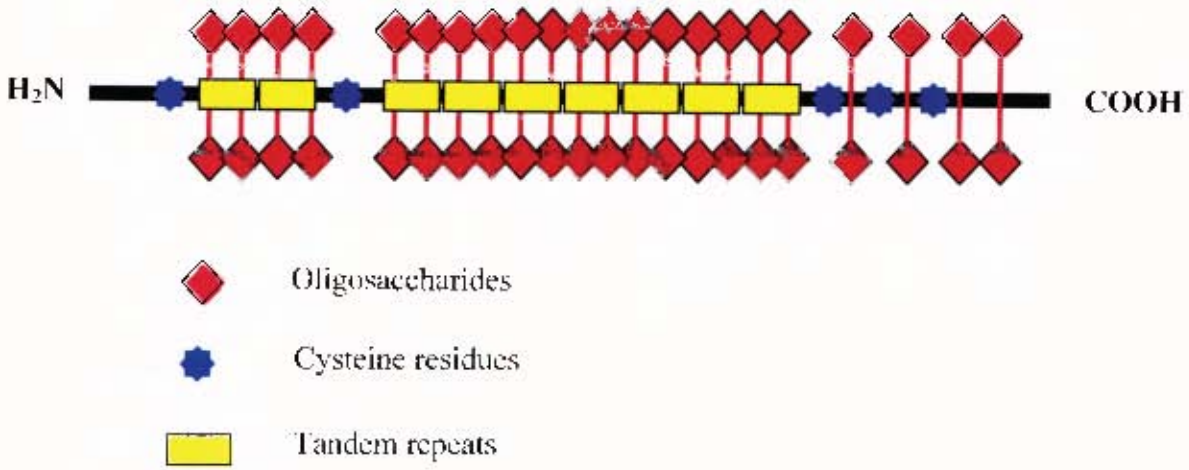


Figure 1.5 **Diagrammatic representation of a mucin structure (reproduced from Rose and Voynow, 2006).**

1.7.2 Membrane-bound mucins

The membrane-bound mucins (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC16, MUC17, and MUC20), which contain short cytoplasmic tails in association with the cytoskeletal and cytosolic adaptor proteins are bound to cells by an integral transmembrane domain (Hollingsworth and Swanson, 2004; Leikauf *et al.*, 2002; Rose and Voynow, 2006). They are normally present in four distinct forms namely; membrane-anchored, soluble, secreted and the tandem repeat array. While the soluble form is assumed to be the fragment of the membrane-bound form as a result of proteolysis, both the secreted and the tandem repeat array are reported to be the alternative splices or variants (Moniaux *et al.*, 2001). Variation in environmental factors such as pH, ionic concentration and hydration are reported to activate the release of the extracellular domains from the cell surface which might help in the clearance of surface-associated molecules under difficult environmental conditions (Hollingsworth and Swanson, 2004). Furthermore, like the epidermal growth factor (EGF), their juxta-membrane domain may play a significant role in regulating the mechanisms of growth, motility, differentiation, inflammation, tumour cell invasion and metastasis (Figure 1.6) (Hollingsworth and Swanson, 2004; Leikauf *et al.*, 2002).

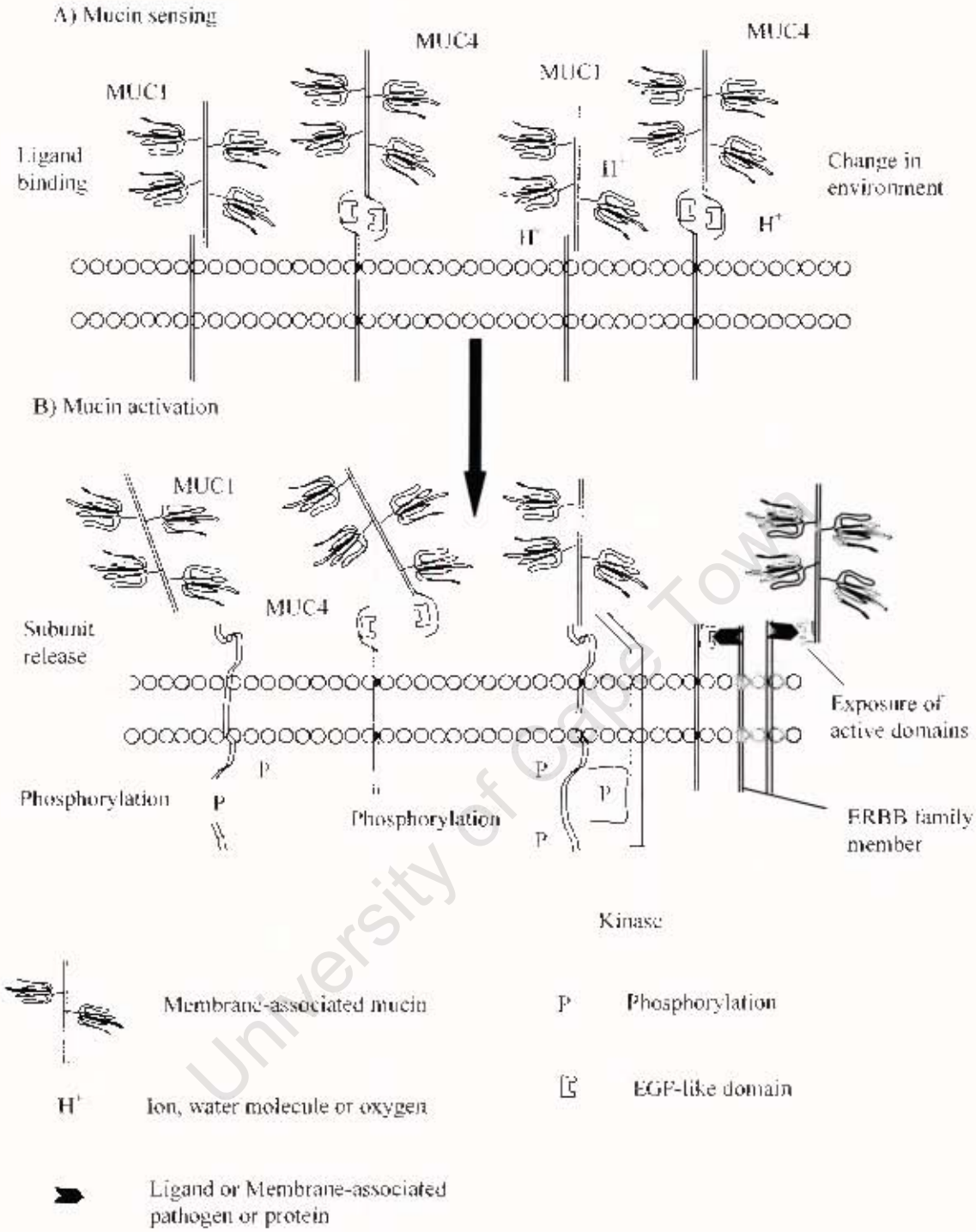


Figure 1.6 The role of membrane bound mucins in detecting and regulating environmental factors. A) In addition to their role as receptors for biological molecules such as, lectins, siglecs, selectins, adhesion molecules, and bacteria, membrane bound mucins can also detect changes in pH, hydration or oxygenation. B) These biological and environmental factors induce conformational change in the membrane-tethered subunit, phosphorylation of the cytoplasmic domain, exposure of active domains and recruitment of signal-transducing molecules. Epidermal growth factor and epidermal growth factor receptors are represented by EGF and ERBB respectively (reproduced from Hollingsworth and Swanson, 2004).

1.7.3 Secreted and non gel forming (soluble) mucins

Compared to the membrane bound and gel forming mucins, the secreted and non gel forming mucins (MUC7, MUC8, and MUC9) are smaller in size and possess simpler structural organization (Moniaux *et al.*, 2001; Rose and Voynow, 2006). Unlike the gel forming mucins, they also lack cysteine-rich domains. However, like the secreted and gel forming mucins, they are packed into mucous granules and secreted to the surface to form a mucus layer that protects the epithelium tissues from both physical and mechanical factors (Lin *et al.*, 2001).

1.8 Human saliva

Saliva is a dilute secretion of the oral cavity produced by three major glands, the parotid, submandibular, and sublingual, including the smaller glands such as the palatal and labial exocrines (Prakobphol *et al.*, 2005; Thornton *et al.*, 1999). Daily, up to 1500ml of human saliva (Zalewska *et al.*, 2000) containing cystatins, proline-rich proteins, proline-rich glycoproteins, carbonic anhydrases, peroxidases, statherins, histatins, lactoferrin, lysozyme, secretory immunoglobulin A (sIgA) and mucins is reported to be secreted by these glands (Prakobphol *et al.*, 2005; Ramasubbu *et al.*, 1991). The mucins, which constitute 26% of the total salivary secretion, are produced by mucous cells in the glands (Bolscher *et al.*, 1995; Klein *et al.*, 1992; Mehrotra *et al.*, 1998; Zalewska *et al.*, 2000). In addition to the high concentration of mucins, the submandibular and sublingual glands contain high levels of lysozyme and cystatin S. At the same time, the palatine, which is another source of mucin, is reported to contain high amounts of amylase. Whereas the parotid which contains high amounts of amylase, proline-rich protein bands, cystatins and lysozyme, it lacks mucins (Veerman *et al.*, 1996).

1.9 Salivary mucins

Human saliva is known to contain, amongst many other components, two carbohydrate rich mucin populations, namely MG1 and MG2 (Thornton *et al.*, 1999). MG1 the high molecular weight mucin (M_r >1,000,000) is encoded by the MUC5B

gene (Thornton *et al.*, 1999). This mucin is one of the most widely distributed mucins found in the saliva, respiratory and the female reproductive tracts (Argüeso *et al.*, 2002; Thomsson *et al.*, 2002). MG2, which is a low molecular weight mucin (M_r ~ 120, 000) is encoded by the MUC7 gene (Thornton *et al.*, 1999). Unlike MUC5B, it is found only in saliva (Thomsson *et al.*, 2002). Both mucins are reported to differ structurally and functionally (Thornton *et al.*, 1999; Troxler *et al.*, 1995) and are synthesized by different cells of the salivary glands (Zalewska *et al.*, 2000).

1.9.1 Salivary MUC5B

Salivary MUC5B is one of the secreted mucins with peptide subunits linked through the disulfide bonds to form a complex structure (Hollingsworth and Swanson, 2004). Secreted by all the salivary glands except the parotid, MUC5B is known to contain 19% protein and 81% carbohydrate (Thomsson *et al.*, 2002; Troxler *et al.*, 1995). The presence of more than one charged subpopulation is associated with the presence of MUC5B glycoforms (Bolscher *et al.*, 1995; Prakobphol *et al.*, 2005). The carbohydrate moieties of this mucin are reported to play a crucial role in the aggregation and clearance of bacterial, fungal and toxic pathogens from the oral cavity (Klein *et al.*, 1992; Thornton *et al.*, 1999; Veerman *et al.*, 1991). Furthermore MUC5B is also reported to aggregate or inactivate viruses such as HIV (Thomsson *et al.*, 2002).

1.9.2 Salivary MUC7

Salivary MUC7 which is the smallest of all human mucins is a non gel forming, single peptide chain and less heterogeneously glycosylated (Bolscher *et al.*, 1999; Lagow *et al.*, 1999; Situ and Bobek, 2000). Secreted by submandibular, sublingual and palatine salivary glands, MUC7 is reported to contain 30% protein and 68% carbohydrate (Thomsson *et al.*, 2002; Troxler *et al.*, 1995). Thus far two glycoforms of MUC7 namely MUC7a and MUC7b with similar amino acid but different sialic acid and fucose compositions are reported to exist in human saliva (Bolscher *et al.*, 1999; Liu *et al.*, 1999; Reddy *et al.*, 1992; Thomsson *et al.*, 2002).

Despite its smaller size and simple structure, MUC7 is reported to possess a strong activity against a number of bacterial, fungal and viral strains (Gururaja *et al.*, 1999; Soares *et al.*, 2003). Some of the examples include inhibition of *Streptococci, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus gordonii, Eikenella corrodens, Streptococcus mutants, Porphyromonas gingivalis, Candida albicans, Candida glabrata, Candida krusei, Actinobacillus actinomycetemcomitans, Saccharomyces cerevisiae,* and *Cryptococcus neoformans* (Bobek and Situ, 2003; Loomis *et al.*, 1987; Situ and Bobek, 2000; Wei and Bobek, 2005). It also plays a crucial role in the inhibition of HIV transmission through saliva (Moniaux *et al.*, 2001).

1.10 Anti-HIV activity of crude human saliva

Most HIV infected individuals are reported to be susceptible to oral infections such as mucosal and gingival lesions which might cause blood shedding into the oral cavity and opportunistic diseases such as gingivitis and candidiasis (Shine *et al.*, 1996). However, despite the possibility of blood shedding and viruses into the oral cavity, researchers are yet to provide credible evidence about oral transmission of HIV through kissing, dental treatment, biting, and aerosolization (Baron *et al.*, 1999; Bergey *et al.*, 1994; Fox *et al.*, 1988; Goto *et al.*, 1991; Malamud *et al.*, 1993; Nasar Qureshi *et al.*, 1995; Shine *et al.*, 1997; Yeh *et al.*, 1992a, 1992b; Yeung *et al.*, 1993). Furthermore, despite the detection of viral proteins or sequences by polymerase chain reaction (PCR) in saliva, several researchers have found the virus to be non infectious (Bolscher *et al.*, 2002; Nagashunmugam *et al.*, 1997; Phillips *et al.*, 1994; Wahl *et al.*, 1997; Yeh *et al.*, 1992b). These reports have led to the assumption that saliva may contain a factor or factors that inhibit HIV infectivity (Archibald and Cole, 1990; Shine *et al.*, 1996).

Several studies have continued the findings of Fultz (1986), which demonstrated the inhibition of HIV-1 activity by human crude saliva in an *in vitro* inhibition assay (Bergey *et al.*, 1994; Kennedy *et al.*, 1998; Moore *et al.*, 1993). In all these studies, HIV-1 was treated or incubated with human crude saliva at different time points. At the end of each incubation period, the mixture (Virus plus saliva) was filtered through

a 0.45μm pore size cellulose acetate filter paper (25mm diameter) and both the unfiltered and filtered samples were added to the $CD4^+$ cells and incubated for different lengths. Subsequent to washing three times with PBS, the cells were cultured in RPMI 1640 for several days and viral replication was measured or monitored by a number of assays. These assays include: transcriptase activity, p24 antigen levels and monitoring inhibition of syncytia formation via $TCID_{50}$ (Malamud *et al.*, 1997).

Thus far most of these studies have demonstrated the presence of highest anti-HIV-1 activity in submandibular and whole saliva compared to the saliva from the parotid gland (Archibald and Cole, 1990; Bolscher *et al.*, 2002; Malamud *et al.*, 1993; Nagashunmugam *et al.*, 1997; Wu *et al.*, 2003). However, the specificity of these anti-HIV-1 agents is among the debating points. According to Fox *et al.* (1988) and Nagashunmugam *et al.* (1997) human saliva possess very low activity against viruses such as herpes simplex virus (HSV), HIV-2, simian immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, hepatitis B virus and adenovirus. This has led to the suggestion that the anti-HIV-1 agents may possess some specificity against the virus (Crombie *et al.*, 1998; Kennedy *et al.*, 1998; Nagashunmugam *et al.*, 1998; Wu *et al.*, 2003).

While Bergey *et al.* (1994) demonstrated a maximum anti-HIV-1 activity of the human submandibular saliva specifically the mucin rich fractions, Robinovitch *et al.* (1993) and McNeely *et al.* (1995 and 1997) reported the anti-HIV-1 activities of proline-rich proteins and secretory leukocyte protease inhibitors (SLPI) respectively. In contrast, Turpin *et al.* (1996) demonstrated the failure of the secretory leukocyte protease inhibitor to inhibit or aggregate HIV-1 particles. Bolscher *et al.* (2002) also demonstrated the failure of HIV-1 inhibition or aggregation by the parotid secretion which lacks mucins. Meanwhile the presence of anti-HIV factors in saliva of HIV negative and positive individuals also exclude the possible role of anti-HIV antibodies (Malamud *et al.*, 1997). All these findings made the macromolecular components of the human saliva or mucins the most likely candidates to play a role in HIV inhibition.

1.11 Anti-HIV activity of salivary mucins

Human saliva is reported to play a significant role in the aggregation and clearance of pathogenic micro-organisms including HIV from the oral cavity by swallowing or expectoration, which may result in decreasing oral transmission of HIV (Malamud *et al.*, 1993; Yeh *et al.*, 1992a). Though the anti-HIV activity of saliva is highest in human submandibular-sublingual (HSMSL) secretions, their filtration prior to incubation with the virus is reported to reduce their activity significantly (Archibald and Cole, 1990; Crombie *et al.*, 1998; Malamud *et al.*, 1993; Nagashunmugam *et al.*, 1998; Shine *et al.*, 1996). This has led to the idea that the virus may bind to the high-molecular weight salivary components and form a macromolecular composite which is excludible by filtration through 0.45µm pore size filter paper (Archibald and Cole, 1990; Nagashunmugam *et al.*, 1997; Shugars and Wahl, 1998; Shugars *et al.*, 1999). Therefore one of the objectives of the present study is to purify salivary mucins from HIV negative and positive individuals and determine their anti-HIV-1 activity.

1.12 Hypotonic nature of saliva and lysis of HIV infected mononuclear leukocytes

As stated in the previous section (Section 1.10), despite the possible shedding of HIV infected blood into the oral cavity especially during mucosal and gingival lesions or dental treatment (Shine *et al.*, 1996), thus far the presence of infectious viruses is not confirmed (Bolscher *et al.*, 2002). According to Baron *et al.* (1999), unlike the vaginal and seminal fluids which contain >20% infectious viruses, saliva is deficient in or contains very low amount (<1%) of infectious viruses. Furthermore Baron *et al.* (1999), demonstrated the lysis of HIV infected cells and inhibition of viral activity by solutions which contain the same salt or ion concentration as saliva. This finding has led to the hypothesis that the hypotonic nature of the saliva may lyse or inactivate the infected mononuclear leukocytes which shed into the oral cavity (Wu *et al.*, 2003) and prevent HIV transmission (Baron *et al.*, 1999).

According to Baron *et al.* (1999), lysis of the HIV infected mononuclear leukocytes by saliva produces free viruses that are non infectious to the CD4 negative mucosal

epithelial cells. On the other hand, in the vaginal environment which is isotonic in nature, the HIV infected mononuclear leukocytes continues to be intact with the ability to attach to and infect the HIV resistant CD4 negative epithelial cells. This results in penetration of the epithelial layer and infection of the sub-epithelial HIV sensitive CD4 positive cells. Therefore viable HIV infected mononuclear leukocytes are required both for the attachment and penetration of the epithelial cells at the mucosal surfaces prior to infection of the sub epithelial CD4 positive cells.

Baron *et al.* (1999) also reported the ability of isotonic fluids such as blood, seminal fluid and breast milk, to change the hypotonic nature of saliva and prevent lysis of the HIV infected mononuclear leukocytes by saliva. For example seminal fluid and blood, at volumes >3.8ml and 4.5ml, are reported to be sufficient to modify the hypotonic nature of saliva and protect the possible lysis of the HIV infected mononuclear leukocytes. Consequently the HIV infected mononuclear leukocytes in the seminal fluid and blood may bind to and penetrate the epithelium of the mouth, pharynx, and oesophagus during oral sex and dental treatment. Furthermore the viscous nature or thickness of the seminal fluid may protect the infected mononuclear leukocytes from any possible contact with saliva which could result in hypotonic lysis. On the other hand, the small volume of the vaginal fluid is reported not to be enough to change the hypotonic nature of the saliva (Baron *et al.*, 1999).

Furthermore, tears and nasal secretions which are isotonic in nature are known to protect the HIV infected mononuclear leukocytes from lysis thus may cause HIV infectious (Baron *et al.*, 1999). At the same time a volume of approximately 2.25ml of breast milk is reported to change the hypotonic nature of the babies' saliva during breast feeding and cause transmission of HIV infected mononuclear leukocytes from HIV infected mothers to infants (Lifson and Rogers, 1986; Thiry *et al.*, 1985; van de Perre *et al.*, 1993; Ziegler *et al.*, 1985).

1.13 Human breast milk

Human breast milk is known to contain a number of non-immunological components such as glycolipids, glycoproteins, mucins, glycosaminoglycans (Schroten *et al.*,

1992; Wiederschain and Newburg, 2001), lactoferrin, α-lactalbumin, lysozyme, β-casein, secretory leukocyte protease inhibitors, lactodifucotetraose, lacto-N-fucopentaose I, II and III and monofucosyllacto-N-hexaose III (Naarding *et al.*, 2005). These components are biologically active and their ability to protect breast fed infants against microbes, viruses, and toxins is well documented (Schroten *et al.*, 1992; Wiederschain and Newburg, 2001).

The protective function of these components includes the inhibition of rotavirus and S-fimbriated *E. coli* by milk mucin, inhibition of cholera and labile toxins of *Escherichia coli* by milk glycolipids (Yolken *et al.*, 1992) and inhibition of *Streptococcus pneumoniae*, *Haemophilus influenzae* and enteropathogenic *E. coli* adherence by human milk oligosaccharides (Newburg *et al.*, 1994). Breast milk components are also reported to inhibit pathogens such as hepatitis C, Ebola, cytomegalovirus, Dengue virus, *Mycobacterium, Leishmania, Candida albicans*, and *Helicobacter pylori* (Naarding *et al.*, 2005). Furthermore, the milk mucin is also known to be used as pathological marker because of its involvement in metastatic progression (Duwe and Ceriani, 1989; Hanisch *et al.*, 1989).

1.14 Transmission of HIV through breast feeding

Despite its protective role against bacterial, fungal and viral pathogens, breast feeding is reported to be responsible for about 40% of the HIV transmission from mother-to-child (Naarding *et al.*, 2005). Large amount of free and cell-associated viruses, such as infected leukocytes, macrophages, mammary epithelial cells and $CD4^+$ T lymphocytes are reported to be present in breast milk of HIV infected mothers (Baron *et al.*, 1999; Lepage *et al.*, 1987; Naarding *et al.*, 2005; Thiry *et al.*, 1985; van de Perre *et al.*, 1993). As a result, children are most likely to be exposed to these free viruses or the infected cells which are capable of crossing the mucosal barrier during breast feeding (Baron *et al.*, 1999). According to Naarding *et al.* (2005), β-casein in the breast milk is known to facilitate the infection of $CD4^+$ T lymphocytes by HIV. Furthermore as HIV has been isolated from lymphocytes which can reach the fetus via transplacenta, HIV transmission from the mother to the child across the placenta could be an additional route of transmission (Ziegler *et al.*, 1985).

1.15 Breast milk mucin

Human breast milk is known to contain MUC1 which is the major component of the milk fat globule membrane (MFGM) (Imam *et al.*, 1982; Keenan *et al.*, 1970; Peterson *et al.*, 1998; Schroten *et al.*, 1992; Shimizu and Yamauchi, 1982). Unlike those secreted and gel forming or non gel forming mucins of the gastrointestinal, respiratory and reproductive tracts, the milk mucin (MUC1) is a membrane bound mucin (Patton *et al.*, 1995). In spite of the report that the milk mucin is present in the fat globule membrane fraction (cream fraction), some membrane associated mucins are also reported to be present in the skim fraction of the milk (Imam, *et al.*, 1981; Pallesen *et al.*, 2001; Patton, 1999; Shimizu *et al.*, 1986).

1.15.1 MUC1

MUC1 which is located on chromosome 1q21-q24 (Rose and Voynow, 2006), is the most widely distributed human mucin which is expressed in many organs or glands such as in the mammary gland, lungs, pancreas, gastrointestinal tract, kidney, bladder, endometrium, ovary and testes (Leikauf *et al.*, 2002; Peterson *et al.*, 1998). In spite of their identical protein compositions or back bone, MUC1 in different tissues possess different glycoforms with tandem repeats ranging between 25 to 125 and a molecular weight between 250-1000 kDa (Lagow *et al.*, 1999; Leikauf *et al.*, 2002; Moniaux *et al.*, 2001; Pallesen *et al.*, 2001). It is also known to contain 50% carbohydrate (Patton *et al.*, 1995).

1.15.2 Biochemical composition of MUC1

Like all the membrane associated mucins, after *N* and *O*-glycosylations in the endoplasmic reticulum and Golgi apparatus respectively, MUC1 is transported to the plasma membrane via the membranes of the secretory vesicles (Altschuler *et al.*, 2000; Patton *et al.*, 1995). MUC1 is known by its high polymorphic characteristics in which genetic variabilities are reported to occur on the number of tandem repeats. Subsequently, the two alleles inherited from each parent are reported to be responsible for the formation of different size proteins (Patton, 1999; Peterson *et al.*, 1998). Thus

more than one mucin band is reported to present on the SDS-PAGE (Patton *et al.*, 1995, Patton, 1999).

According to Peterson *et al.* (1998), threonine, serine, and proline are reported to constitute 80% of the tandem repeat units. While threonine and serine provide *O*-glycosylation sites, proline is thought to be responsible for the formation of an extended rod-like or filamentous structure which rises above the cell surface (Figure 1.7). This rod-like or filamentous structure is known to play a crucial role in the anti-adhesive property of MUC1 during morphogenesis, tumour progression or metastasis (Moniaux *et al.*, 2001). However, despite the presence of cysteine residues the size of MUC1 on SDS-PAGE remains to be the same both under reducing and non-reducing conditions. This excludes the possible binding of MUC1 to other proteins via disulfide bond (Patton *et al.*, 1995).

1.16 Human cervical mucus

Cervical mucus is known to play a crucial role in human reproductive physiology by providing protection against microbial colonization, fluid loss and controlling sperm survival and migration to the upper reproductive tract (Argüeso *et al.*, 2002; Carlstedt *et al.*, 1982; Carlstedt *et al.*, 1983; Lagow *et al.*, 1999). This is reported to be achieved by protecting the sperm from the acidic environment of the vagina and facilitating the transport of the normal and actively mobile sperm cells into the upper reproductive tract especially during the proliferative phase or under the influence of oestrogen by selectively screening them from the abnormal and poorly mobile sperm cells. Cervical mucus is also reported to protect the sperm cells from phagocytosis (Elstein, 1978). Furthermore its daily secretion of 20-60mg is also reported to provide lubrication, enhance wetness, prevents desiccation, inhibits and promotes cell attachment, and minimizes enzymatic degradation (Gipson *et al.*, 1995; Idris and Carraway, 1999; Lagow *et al.*, 1999; Venegas *et al.*, 1995). Despite the continuous change in mucus viscosity throughout the menstrual cycle which may allow pathogen entrance at times such as during the proliferative phase, millions of micro-organisms daily are reported to be cleared from the female reproductive tract by cervical secretion (Wiggins *et al.*, 2001).

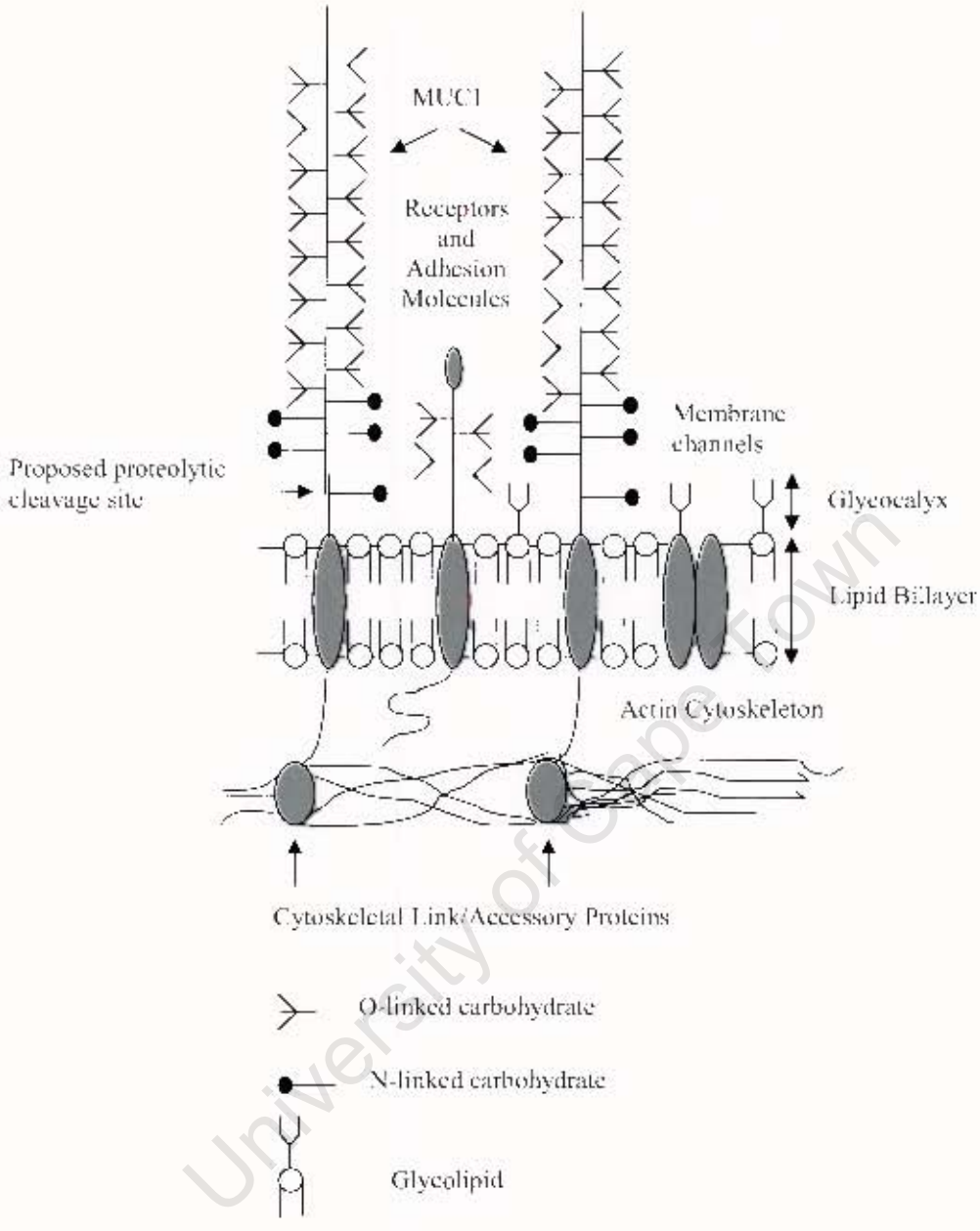


Figure 1.7 Diagrammatic representation of MUC1. This figure shows the long and highly glycosylated extracellular and intracellular segments of MUC1 (reproduced from Patton *et al.*, 1995).

1.17 Mucin expressions in the female reproductive tract

Thus far eight mucins such as MUC1, MUC4, MUC5AC, MUC5B, MUC6, MUC8, MUC9 (Lagow *et al.*, 1999), and MUC2 (Gipson *et al.*, 1997) are reported to be expressed by the female reproductive tract (Lagow *et al.*, 1999). Of these MUC1 is the most widely expressed mucin expressed by the ovary, oviduct/fallopian tube,

uterus, cervix, and vagina. In addition to MUC1, the human cervix expresses MUC4, MUC5AC, MUC5B, MUC6, MUC8 (Lagow *et al.*, 1999), and MUC2 (Gipson *et al.*, 1997). The oviduct/fallopian tube and vagina expresses MUC9 (Lagow *et al.*, 1999) and MUC4 (Wiggins *et al.*, 2001) respectively. In addition to MUC1 the uterus also expresses MUC6 and MUC8 (Lagow *et al.*, 1999).

1.18 The effect of menstrual cycle on the expression of mucins

The quality and quantity of the cervical mucins expressed by the female reproductive tract is reported to significantly change during the different phases of the menstrual cycle especially during the mid-proliferative (non-receptive) and mid-luteal (receptive) phases (Elstein, 1978; Idris and Carraway, 1999). During these different phases the physiology and mucin secretion capacity of the different regions of the female reproductive tract is known to be influenced by or respond to steroid hormones differently. As a result these different regions may express the same mucins differently (Lagow *et al.*, 1999). The glycosylation pattern, especially the addition of sugars such as L-fucose and sialic acid to cervical mucins is reported to be determined by the level of glycosyltransferase activity which is phase dependent. For instance the proliferative phase is characterized by the highest fucosyltransferase activity followed by the ovulatory phase and very low activity during the luteal phase. These changes may affect the composition and function of the cervical mucus during the menstrual cycle (Wiggins *et al.*, 2001).

1.19 The role of cervical mucus in birth control

According to Yurewicz and Moghissi (1981), cervical mucus is reported to regulate fertilization by controlling sperm transportation to the upper reproductive tract. As a result, abnormalities in the physicochemical compositions of this secretion throughout the menstrual cycle may cause infertility (Argüeso *et al.*, 2002). For example, in response to increased levels of estrogens prior to ovulation, the mucus changes from it's viscous or jelly nature to a watery fluid and this result in maximum sperm penetrability to the upper reproductive tract (Edwards, 1978; Gipson *et al.*, 1997; Parke, 1978). On the contrary, during the luteal phase, or under the influence of oral

contraceptive steroids, the higher secretion of progestogens induces very thick mucus which blocks sperm migration via the cervix completely (Eriksen *et al.*, 1998; Yurewicz and Moghissi, 1981; Yurewicz *et al.*, 1987).

The change in quality and quantity of the cervical mucus during the different stages of the menstrual cycle is very high. For instance the rate of cervical mucus secretion increases from the approximately 40mg/day in the early stage of the proliferative and late stage of the luteal phases to the approximately 600mg/day at mid-cycle (Parke, 1978). According to this author oral contraceptive drugs such as chlormadinone acetate, norgestrel, norethindrone and megestrol acetate of the synthetic progestogens are reported to induce the production of viscous or thick cervical mucus which is resistant to sperm penetration. Furthermore the luteal phase is characterized by the presence of a large mucus plug in the mouth of the cervix especially during pregnancy (Carlstedt *et al.*, 1983; Eriksen *et al.*, 1998).

Several reports have also revealed that cervical mucus, specifically mucins, may prevent ectopic pregnancy by covering the epithelial cells (Lagow *et al.*, 1999). According to the report of Lagow *et al.*, (1999), during the phase of embryo implantation the production of MUC1 in the luminal epithelial is known to be down regulated, suggesting MUC1 may inhibit the attachment of embryo.

1.20 The burden of HIV-AIDS in sub-Saharan Africa

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) (Si *et al.*, 2004; Wyatt and Sodroski, 1998) is reported to be responsible for the death of more than 20 million people worldwide (Ouellet *et al.*, 2005) of which the sub-Saharan Africa is the most affected region (Shaikh *et al.*, 2006), having about 25 million adults and children living with HIV/AIDS (Losina *et al.*, 2006). The Southern part of Africa is reported to have the highest prevalence compared to East and West Africa (Shaikh *et al.*, 2006). According to Stevens *et al.* (2006) this epidemic is expected to cost South Africa 17% of its GDP growth by 2010.

1.21 HIV Vaccine

Despite the discovery of highly active antiretroviral therapies which contain both protease and reverse transcriptase inhibitors to down regulate HIV infection (Reeves *et al.*, 2005), HIV remains a threat world wide specifically in third world countries. Thus far a number of antiretroviral drugs which target the virus entry have been developed, with most of them targeting either the HIV envelope glycoprotein gp120 or the CD4 and chemokine receptor family such as CCR5 or CXCR4 (Figure 1.8) (Moore and Doms, 2003). One example of an entry inhibitor drug is enfuvirtide (ENF) which is made up of the amino acid sequence of gp41 subunit of the HIV envelope protein to prevent membrane fusion and virus entry into the cytoplasm of the host cell (Reeves *et al.*, 2005). However, in addition to the highest variable nature of the viral envelope proteins, the speed and efficiency of the fusion rate significantly affects the activity of these drugs. For instance, the faster the rate of fusion and entry the less activity/potency of the drugs. Moreover, the potency of these drugs, including the neutralizing antibodies, can also be influenced by the density and glycosylation level of the viral envelope proteins (Moore and Doms, 2003; Reeves *et al.*, 2004; Ugolini *et al.*, 1997).

Whilst the search for a vaccine against HIV is ongoing in many centres of the world, the need to formulate new therapeutic agents that could act by reducing viral loads in infected individuals or transmission from infected to non-infected individuals is a priority. Whilst saliva is known to prevent oral transmission of the virus through its mucus component, the individual mucins that compose the mucus have not been tested against the virus. It is also intriguing that the two well known areas of transmission of the virus, namely the breast and cervix, have mucus and mucins, the broad structures of which are similar to those in saliva, and yet they seem to have no anti-HIV activity. Also any inhibitory effect of saliva in HIV positive individuals has also, to our knowledge, not been investigated.

This study is an attempt to explore these questions.

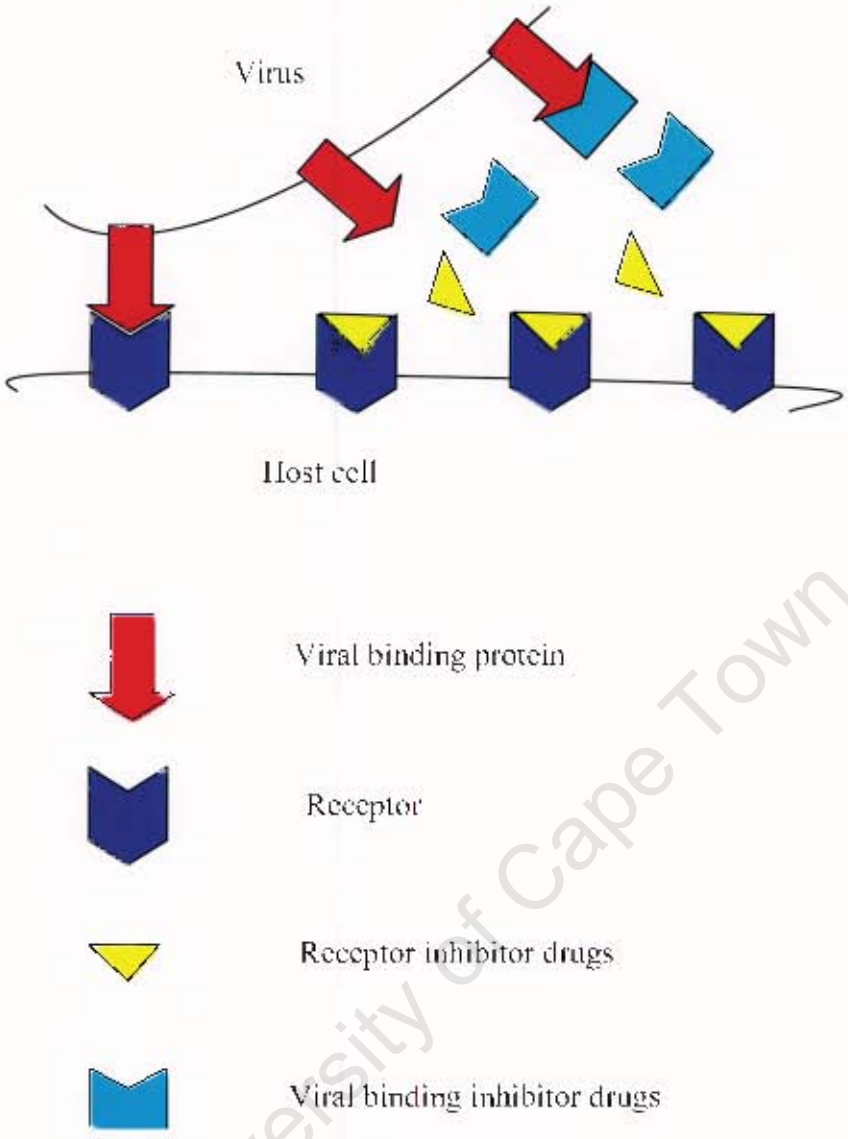


Figure 1.8 Diagrammatic presentations of the principles of viral entry inhibitor drugs in inhibition or blocking of viral infections. These drugs target either the viral binding proteins or receptors (reproduced from Lentz, 1990).

1.22 Poxvirus

In addition to HIV, enveloped viruses such as hepatitis B and influenza are among the major scares to human life with three of them reported to infect more than one billion of the world population (Kotwal *et al.*, 2005). Recently, due to their inclusion in the list of bioterrorism, poxviruses, specifically smallpox, is also attracting great attention globally (Jonczy *et al.*, 2000). According to Kotwal *et al.* (2005), the recent appearance of the monkey pox that causes infection in central African countries such

as Democratic Republic of Congo, the spread of the sever acute respiratory syndrome (SARS), together with the failure to provide effective and long lasting vaccines against these enveloped viruses, make the world very susceptible to the possible emergences of any new strains. Therefore the search for safe, effective and affordable anti-viral agents has to be among the priorities.

1.23 Objectives of this study

The main objectives of this study were to isolate and purify human salivary, breast milk and pregnancy plug mucins from HIV negative individuals and to determine their anti-HIV-1 activities in an *in vitro* HIV inhibition assay. We also aimed to purify salivary mucins from HIV patients and compare their anti-HIV-1 activities to the salivary mucins from HIV negative individuals. Furthermore we wished to isolate peripheral blood mononuclear cells (PBMCs) from HIV positive and negative individuals and determine the role of salivary mucins specifically MUC7 in inhibiting HIV-1 infection of the PBMCs, reducing viral infection of the HIV-1 positive PBMCs and minimizing the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs. Lastly to determine if these mucins posses the same potency against other enveloped viruses such as poxvirus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethics

The University of Cape Town Research and Ethics Committee approved this study (ethics approval number REC REF: 283/2004).

2.2 Materials

This Chapter describes all the materials and methods used during the course of this research project. These materials were purchased from Kimix, Santa Cruz, Novocastra, Sam Ho, Dakocytomation, AIDS Research and Reference Reagent Programme, Gibco, Roche, Biomérieux, Highveld Biological, Costar, Merck and Sigma and were of the highest purity available. The ECL™ Western blotting detection kit was from Amersham Biosciences (Amersham UK). Nitrocellulose membrane and dialysis tubing were from Kimix (Chemical and Laboratory Suppliers, SA). Polyclonal rabbit anti-MUC2, anti-MUC5AC, anti-MUC5B and goat anti-rabbit horse radish peroxidise (HRPO) linked secondary antibodies were kindly provided by Sara Kirkham (Manchester, UK). Polyclonal goat anti-MUC7 and rabbit anti-goat and goat anti-mouse HRPO linked secondary antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, California). Monoclonal mouse anti-MUC7 (EU-MUC7a) was kindly provided by Dallas Swallows (University College London, UK). Monoclonal mouse anti-MUC1 and anti-MUC6 antibodies were from Novocastra (Newcastle, UK). Envision-® system labelled polymer-HRP anti-mouse, anti-rabbit and 3,3'-diaminobenzidine (DAB) substrate chromogen were from Dakocytomation (USA). Rabbit anti-MUC4 polyclonal antibody was from (Sam Ho, USA). The human T lymphoblastoid cell line (CEM SS cells) was from AIDS Research and Reference Reagent Programme (Germantown, USA). RPMI 1640, L-Glutamine and heat-inactivated Fetal Calf Serum were from Gibco (Massachusetts, USA). IL-2 and p24 antigen kit were from Roche Diagnostics (Germany) and Vironostika HIV-1 Antigen

kit, Biomérieux (France) respectively. African green monkey kidney cells (BSC-1) and Earle's Base Minimum Essential Medium containing L-glutamine, non-essential amino acids, $NaHCO_3$ and Fetal Calf Serum were from Highveld Biological. Culture plates and Crystal Violet Dye were from Costar and Merck (Germany) respectively. Sepharose CL-4B, guanidinium chloride (GuHCl), caesium chloride (CsCl), Schiff's reagent, 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propane-sulfonate (CHAPS), agarose, N-ethylmaleimide (NEM), 2-mercaptoethanol, 7-Amino-actinomycin D (7AAD), Histopague®-1077 and *N, N'*-methylenebisacrylamide were from Sigma (UK). Phenylmethylsulfonylfluoride (PMSF), *N, N, N, N'*-tetramethylethylenediamine (TEMED), coomassie brilliant blue R-250, ethylenediaminetetra-acetic acid disodium salt (Na_2-EDTA), 3,3'5,5'-Tetramethylbenzidine (TMB), di-methylsulphoxide (DMSO) and polyoxyethylene sorbitan monolaurate (Tween 20) were from Merck.

NCL-MUC1 (201607) and NCL-MUC6 are mouse monoclonal antibodies raised against a carbohydrate epitope of the human MUC1-glycoprotein and synthetic peptide of the MUC6 tandem repeat sequence respectively (data sheet from Novocastra Laboratories Ltd). LUM2-3, LUM5-1 and LUM5B-2 are rabbit anti-serums raised against synthetic peptides of NGLQPVRVEDPDGC (Herrmann *et al.*, 1999), RNQDQGPKFMC (Hovenberg *et al.*, 1996) and RNREQGKFKMC (Wickstrom *et al.*, 1998) which corresponds to the tandem repeats of human MUC2, MUC5AC and MUC5B mucins respectively. Rabbit polyclonal anti-MUC4 antibody was raised against a synthetic peptide TSSASTGHATPLPVTD corresponding to the tandem repeat sequence of MUC4 (Lopez-Ferrer *et al.*, 2000). EU-MUC7a, is a mouse anti-serum raised against a synthetic peptide E G R E R D H E L R H R R H H H Q located in the N-terminal part of the MUC7 protein within the Histatin-like domain (kindly provided by Dallas Swallows, University College London, UK) and sc-16918, a goat anti-serum was raised against amino acids in the rage of 100-150 of Mucin 7 of human origin (data sheet from Santa Cruz Biotechnology).

2.3 Sample collection

2.3.1 Collection of saliva from symptom free group

Human saliva was collected from twenty healthy 'symptom free' female and male volunteers (who declared a risk-free lifestyle) who abstained from eating and drinking for at least 2h prior to collection. The production of saliva was stimulated by chewing on parafilm and collected into 5ml of 6M GuHCl containing a cocktail of protease inhibitors such as 10mM EDTA, 5mM NEM and 1mM PMSF pH 6.5. Samples were collected into cooled containers on ice and stored at -20°C until purification.

To be used as a crude sample for HIV inhibition assay, whole saliva was collected into 5ml of cold 0.1M Tris-HCl, 2% (w/v) EDTA and 5mM PMSF pH 7.5. The sample was centrifuged at 10 000*g* for 10min at 4°C to remove the insoluble debris, dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

2.3.2 Collection of saliva from HIV patients

Saliva was collected from HIV positive female volunteers from the clinic of infectious diseases in Groote Schuur Hospital (Cape Town, South Africa). The production of saliva was stimulated by chewing on parafilm and collected into 10ml of 6M GuHCl containing a cocktail of protease inhibitors such as 10mM EDTA, 5mM NEM, and 1mM PMSF pH 6.5. Samples were collected into cooled containers on ice and stored at -20°C. Samples were grouped into three categories according to the CD4 counts of the patients, less than 200, between 200 and 400 and greater than 400. The patients with CD4 count <200 have full blown AIDS.

Table 2.1 Samples from HIV patients of different CD4 counts and related infections

Case number	Age	CD4 count	Related infection
1	28	121	TB
2	26	146	TB
3	32	159	TB
4	26	170	Diarrhoea & skin rash
5	31	185	Gastritis & respiratory infection
6	38	211	Oral infection
7	28	244	TB
8	28	252	TB
9	23	266	Genital infection
10	29	288	NA
11	30	303	TB
12	28	307	TB
13	30	323	Conjunctivitis
14	25	356	TB
15	18	388	Oedema
16	25	399	NA
17	27	426	Oral infection
18	27	433	Acute diarrhoea
19	33	476	NA
20	32	573	TB
21	41	618	TB
22	32	707	NA
23	24	935	TB
24	41	1070	NA

2.3.3 Pregnancy plug mucus and cervical scrapings

Cervical secretions were collected from twenty HIV negative female volunteers from the Department of Gynaecology and pregnancy plug mucus from Maternity Divisions of Groote Schuur Hospital. Vaginal mucus specimens were collected by gently scraping the vaginal mucosa with a wooden paddle and the pregnancy plugs were extracted prior to delivery by a gynaecologist. Samples were collected into 6M GuHCl containing a cocktail of protease inhibitors such as 10mM EDTA, 5mM NEM and 1mM PMSF pH 6.5 and stored at -20°C until further analysis.

To be used as a crude sample for HIV inhibition assay, pregnancy plug mucus and cervical secretions were collected into 5ml of cold 0.1M Tris-HCl, 2% (w/v) EDTA and 5mM PMSF pH 7.5. Samples were solubilised by gentle stirring for 15h at 4°C and the insoluble material was removed by high-speed centrifugation at 9 000g for 2h

at 4°C. The supernatant was dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

2.3.4 Collection of human breast milk

Human breast milk samples were collected from twenty HIV negative lactating mothers from the Virology and Microbiology laboratory of the Maternity Division in Groote Schuur Hospital. After collection, milk samples were immediately frozen and stored at -20°C. To be used as a crude sample, fresh milk in 0.2M NaCl, 0.02%NaN_3 containing a cocktail of protease inhibitors, 10mM EDTA, 5mM PMSF and 5mM NEM, was dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

Human breast milk samples from HIV positive lactating mothers were also collected from the Division of the Neonate Maternity Hospital in Groote Schuur.

2.3.5 Collection of human blood

Blood samples from HIV positive patients were collected from the Divisions of the Neonate and Paediatric Medicine in Groote Schuur Hospital and HIV negative blood samples from volunteers in the laboratory (Department of Surgery, Research Laboratory, University of Cape Town, South Africa). Viral load of both the HIV positive and negative blood samples was determined prior to any analysis (see Table 8.1 in Chapter 8).

2.4 Preparation of salivary and pregnancy plug samples prior to purification of mucins

Salivary samples were solubilised by overnight mixing at 4°C on a revolving rotor and the insoluble debris was separated from the soluble mucus by centrifugation at 4400g for 10min at 4°C. In the case of pregnancy plugs and cervical scrapes, samples were solubilised by gentle stirring for 15h at 4°C and the insoluble material was removed by high-speed centrifugation at 9 000g for 2h at 4°C.

2.5 Preparation of milk sample prior to mucin purification

Sample preparation was performed according to the method of Schroten *et al.* (1992). Frozen milk samples were thawed at room temperature (RT) in the presence of 1mM PMSF and defatted by centrifugation at 3 000*g* for 1h at 4°C. The cream fraction on the top (see Figure 4.1 in Chapter 4) was collected and suspended in a suitable volume of 0.2M NaCl, 0.02%NaN_3 containing a cocktail of protease inhibitors, 10mM EDTA and 5mM NEM, and kept at 4°C for 1h. The suspension was shaken on a laboratory shaker at RT until butter was formed and the membrane was released by incubation of this mixture at 40°C for 30min. Following centrifugation at 35 000*g* for 30min at 4°C the membranes were recovered as a yellow pellet. The pellet was then suspended in 0.2M NaCl, 0.02%NaN_3 containing a cocktail of protease inhibitors and subjected to homogenization with a Junkel and Kunkel Ultra-Turrax (1min, 9500rev/min at RT) to disaggregate and increase its solubility. After sonication insoluble materials were removed by centrifugation at 4 400*g* for 10min at 4°C.

2.6 Size or molecular exclusion chromatography

Size exclusion chromatography which separates biological materials according to their size between a continuous phase and the interior pores of the gel (Brooks *et al.*, 2000; Porath and Flodin, 1959; Whitaker, 1950) was used to separate human salivary and breast milk mucins from the non-mucin components of these secretions such as proteins.

In this study aliquots of the supernatant (20ml) were chromatographed on a Sepharose CL-4B gel filtration column equilibrated and eluted with 4M GuHCl containing a cocktail of protease inhibitors, 10mM EDTA and 5mM NEM, and 0.05%CHAPS pH 6.5 at a flow rate of 48ml/h at RT. Following a periodic acid schiff's (PAS) and protein (A_{280}) assays, the void volume (V_0) fractions and included volume (V_t) fractions were pooled separately, dialysed against three changes of distilled water overnight at 4°C and freeze-dried. In the case of milk the column was equilibrated and eluted with 0.2M NaCl, 0.02%NaN_3 containing a cocktail of protease inhibitors.

2.7 Dialysis of samples

Prior to any analysis or assay, GuHCl and caesium chloride were removed from samples by dialysis against three changes of distilled water overnight at 4°C by constant stirring.

2.8 Freeze drying

To determine the amount (concentration) of samples used in each analysis or assay, dialysed samples were freeze-dried by a Christ ALPHA I-5 freeze drier from Lasec Laboratory and Scientific equipment.

2.9 Analytical determinations

Glycoprotein was estimated by the PAS procedure of Mantle and Allen (1978) and protein according to the method of Lowry *et al.* (1951).

2.10 Mucin purification

Salivary, breast milk and pregnancy plug mucins were purified according to the method of Creeth and Denborough, (1970). Briefly mucins were prepared in 4M GuHCl containing a cocktail of protease inhibitors, 10mM EDTA and 5mM NEM, and 0.05% CHAPS at pH 6.5 and adjusted to a density of 1.39 to 1.40g/ml with solid caesium chloride. Density gradient centrifugation was performed in a Beckman L45 ultra-centrifuge for 48h twice, at a 105 000g at 4°C. Mucin positive fractions with some contaminating protein were pooled and prepared for the second centrifugation step. Finally mucin rich fractions were pooled, dialysed against three changes of distilled water at 4°C and freeze-dried.

2.11 Sodium dodecyl sulfate polyacrylamide-gel electrophoresis

To determine their purity and size salivary, breast milk and pregnancy plug mucins (30µg each) were prepared in reducing gel loading buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and boiled for 2min prior to loading. Electrophoresis was performed according to the method of Laemmli (1970) in a 10% (w/v) running gel and a 4% (w/v) stacking gel using the Hoeffer Mighty Small mini-electrophoresis system. The gel was run at 20amp until the bromophenol blue dye reached the bottom of the gel.

2.12 Gradient gel

For better resolution milk mucin (MUC1) and salivary MUC5B and MUC7 from HIV-1 positive individuals with different CD4 counts (<200, 200-400 and >400) (30µg each) were prepared in reducing gel loading buffer as above and subjected to a 3-5% and 4-20% gradient gels respectively. This gel is prepared by mixing two solutions of different acrylamide concentrations to form a gradient with decreasing concentrations of acrylamide (Pierce Proteomics) (www.piercenet.com/Proteomics).

2.13 Coomassie brilliant blue R-250 gel staining

Gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol and 10% acetic acid to view the protein bands.

2.14 Periodic acid schiff's gel staining

Gels were fixed in 7.5% acetic acid, then stained with PAS and destained in 25% methanol and 10% acetic acid according to the method of Dubray and Bezard (1982).

2.15 Agarose gel electrophoresis

Salivary, breast milk and pregnancy plug mucins (40µg each) were prepared in a sample loading buffer containing 40% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol in 1 × Tris-acetate buffer (TAE) and boiled for 2min prior to loading. Electrophoresis was carried out according to the method of Thornton *et al.* (1999), in a 1% (w/v) agarose gel (15 × 15cm) prepared in running buffer containing 40mM TAE, 1mM EDTA, and 0.1% SDS pH 8.0. Briefly, agarose (1.6g in 160ml of running buffer) was boiled in a microwave until completely dissolved and cooled down to approximately 50°C before pouring into the Bio-Rad DNA sub cell gel apparatus. Upon polymerization the apparatus was filled with running buffer and electrophoresis was performed at 100V for 2.5h at room temperature.

2.16 Western blotting

After SDS-PAGE and agarose gel electrophoresis MUC1, MUC2, MUC5AC, MUC5B and MUC7 from HIV negative individuals and salivary MUC5B and MUC7 from HIV patients with CD4 counts (<200, 200-400 and >400) were transferred onto nitrocellulose membranes (Nitrocellulose, 0.22Micron) using a semi-dry electro-blotting unit at 0.8mA/cm^2 or by vacuum blotting for 1h at a suction pressure of 40mbar according to the method of Thornton *et al.* (1999). In the case of SDS-PAGE the transfer buffer used contained 192mM glycine, 25mM Tris, 1.3mM SDS and 20% (v/v) methanol whereas in agarose gel the transfer buffer used was 4 × SSC (0.6M NaCl, 60mM tri-sodium citrate, pH 7.0). After electro-blotting, non-specific bindings were blocked by incubating the membranes overnight in 5% (m/v) low fat milk powder in TBS, 0.05% Tween-20 (TBST) at 4°C. The membranes were then washed with TBST 3 × 5min and incubated for 2h with mouse anti-MUC1 monoclonal, rabbit anti-MUC2, rabbit anti-MUC5AC, rabbit anti-MUC5B polyclonal and mouse anti-MUC7 monoclonal antibodies diluted in 5% (m/v) low fat milk powder in TBST at 1 in 100 (mouse anti-MUC1), 1 in 5000 (rabbit anti-MUC2 and anti-MUC5AC), 1 in 2000 (rabbit anti-MUC5B) and 1 in 1000 (mouse anti-MUC7). The membranes were washed 3 × 5min with TBST and incubated for 1h with HRPO linked secondary

antibodies (goat anti-mouse and goat anti-rabbit) diluted in 5% (m/v) low fat milk powder in TBST at dilutions of 1 in 1500 (goat anti-mouse) and 1 in 5000 (goat anti-rabbit). After another TBST wash (3 × 5min), bands were detected using an ECL detection kit.

2.17 Enzyme linked immunosorbent assay.

Salivary MUC5B and MUC7 mucins from HIV negative and HIV positive individuals with different CD4 counts (<200, 200-400 and >400) were coated (10µg/ml) in PBS (150µl per well, overnight at 4°C). Non-specific binding of the antibodies was prevented by blocking the wells with 0.5% BSA-PBS (200µl, 1h at 37°C) and the plates were washed three times with PBS-Tween. Serial two-fold dilutions of primary antibodies starting from 8µg/ml (goat anti-MUC5B) and 12µg/ml (goat anti-MUC7) were added to the plate in 0.5% BSA-PBS and incubated (100µl, 2h at 37°C). The plates were washed three times with PBS-Tween and rabbit anti-goat HRPO-linked secondary antibody (1 in 5000 in 0.5% BSA-PBS) was added to each well and incubated (120µl, 1h at 37°C). Following three washes with PBS-Tween, 150µl of the substrate solution (TMB in 0.15M citrate-phosphate buffer, pH 5.0) was added to each well and the colour was allowed to develop in the dark against the background of the controls (10-15min) and the A_{405} of each well was measured in a Titertek ELISA plate reader. As a control, wells were coated with PBS.

2.18 Immuno-histochemistry

Immuno-histochemical analysis was used to identify mucins expressed by the female reproductive tract. Human endometrium and cervix tissues were fixed in buffered formalin processed in alcohols and xylol and embedded in paraffin wax using the standard methods before being dewaxed in xylol for 15min and rehydrated by absolute, 96% and 70% alcohols for 1min each. After a water rinse, tissue sections were incubated with fresh 3% hydrogen peroxide in methanol for 10min and then washed in running tap water for 15min followed by PBS. Antigen retrieval was carried out by incubation of the specimens in citrate buffer pH 6.0 using pressure cooker for 2min followed by cooling in running water for 15min. Subsequent to 2 ×

5min PBS washes, sections were blocked with normal goat serum at 1 in 20 in PBS for 20min. After removal of serum (drain), sections were incubated with mouse anti-MUC1 and anti-MUC6 monoclonal antibodies at 1 in100 for 30min and rabbit anti-MUC4 polyclonal antibody at 1 in 400 dilutions in PBS for 1h. After 2 × 5min PBS wash sections were incubated with Envision anti-mouse (Dakocytomation Envision® system labelled polymer-HRP anti-mouse) and Envision anti-rabbit (Envision® system labelled polymer-HRP anti-rabbit) for 30min. Subsequent to 2 × 5min PBS washes, sections were developed using chromogen (1 drop of DAB in 1ml of cytomation substrate buffer) for 10min. Prior to colour enhancement with 1% Copper Sulphate for 10min sections were rinsed with PBS and water. The sections were counter stained with hematoxylin and Scott's water for 1-2min and dehydrated by 70%, 96% and absolute alcohols for 5sec each before being left in xylol. Finally slides were cover slipped using entellan and viewed under the light microscope.

2.19 Amino acid analysis

The amino acid contents of salivary (MUC5B and MUC7) and milk (MUC1) mucins were analysed using a high pressure liquid chromatography (HPLC) system according to the methods of Cohen and Strydom (1988) and Klapper (1982). In this method samples were vacuum-dried and placed in a hydrolysis vessel containing some constant boiling HCl and 1% (v/v) phenol before hydrolysing in the gas phase at 110°C for 24h. The vessel was cleaned with nitrogen gas and sealed under vacuum before use. Subsequent to cooling and vacuum drying to remove the residual HCl, samples were redissolved in citrate buffer pH 2.2 and introduced into a HPLC column from Waters Associates, Medford, MA., filled with a cation exchange resin (sulfonated polystyrene cross-linked with divinylbenzene) and eluted with a number of buffers ranging from a low (0.25M trisodium citrate, pH 3.05) to high (0.25M sodium nitrate, pH 9.5) pH. Detection of amino acids was performed using post column derivatization with O-phthalaldehyde (OPA), a fluorescent reagent which interacts with all but not proline amino acid. For proline detection treatment of samples with sodium hypochlorite ahead of post column derivatization with OPA was required. The relative ratios of the individual amino acids for each sample was determined and compared to each other.

2.20 Toxicity of human salivary, breast milk and pregnancy plug mucus and mucins from HIV negative and salivary mucins from HIV positive individuals to the human T lymphoblastoid cell line.

Prior to the HIV inhibition assay the toxicity of crude saliva, breast milk, pregnancy plug mucus, purified MUC5B and MUC7 from saliva, MUC1 from milk and pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) from HIV negative individuals and purified salivary MUC5B and MUC7 from HIV positive individuals with different CD4 counts (<200, 200-400, and >400) to the phytohemagglutinin (PHA) stimulated human T lymphoblastoid cell line (CEM SS) was determined by the toxicity assay. Briefly 500µl of the CEM SS cells in RPMI complete containing 10% Fetal Calf Serum, 1% Penicillin/Streptomycin antibiotic, 10 µmol Fungin and 50µmol 2-mercaptoethanol (final concentration 2.5×10^6 cells/ml) were incubated with 250µl of IL-2 and 250µl (0.9mg) of each sample in a CO_2 incubator for 24h. As controls, cells incubated with IL-2 only or IL-2 without cells (blank) were used. After spinning at 1000g for 5min cells were re-suspended in 500µl of RPMI and live and dead cells were counted using Trypan blue exclusion criteria. The percentage of viable cells was calculated as live cells/total cells × 100.

2.21 Toxicity of MUC7 to white blood cells

The toxicity of salivary MUC7 to white blood cells (WBC) was determined by the cyto-toxicity assay. Blood was taken from two volunteers (20ml each) in EDTA tube and diluted to 35ml with PBS. To separate WBC from red blood cells (RBC) and plasma, the blood was layered over Histopague®-1077 solution by inclining the tube. After centrifugation (300g, 10min, 4°C) with a slow break to avoid disruption of the layers, the WBC forming a white band at the interface were collected. After PBS wash by centrifugation (100g, 10min, 4°C) cells were recovered as precipitate and resuspended in RPMI containing 10% Fetal Calf Serum and 1% Penicillin/Streptomycin antibiotic. Cells (1.5ml or 6.4 - 16.2 × 10^6 cells) were then mixed with salivary MUC7 at different concentration (1mg, 0.5mg and 0.25mg) and incubated in a CO_2 incubator for 48h. As control, cells without MUC7 were incubated in a CO_2 incubator for 48h. At the end of the incubation period cells were washed

with PBS by centrifugation (100g, 5min, 4°C) and recovered as precipitate. After resuspending in 250µl of PBS, cells were incubated with 7-Amino-actinomycin D (7AAD) and cell viability was monitored by flow cytometry. 7AAD which intercalates into double-stranded nucleic acids and can penetrate cell membranes of dead cells is used to distinguish between live and dead cells.

2.22 Toxicity of MUC7 to red blood cells

The toxicity of salivary MUC7 to RBC was also determined prior to inhibition assay. Blood was taken from a volunteer (10ml) in EDTA tube and placed into four EDTA tubes (2.5ml or 10.5 - 14.7 × 10^9 cells each). The first three tubes were incubated with 1mg, 0.5mg and 0.25mg of MUC7 for 60min at room temperature respectively. As a control the fourth tube was incubated with PBS. At the end of the incubation period the blood was centrifuged (900g, 15min, 4°C) and cell lysis (haemolysis) was determined by observation.

2.23 Preparation of peripheral blood mononuclear cells

Human blood (10ml) within 6h of collection was diluted in 10ml of sterile 1 × PBS with 3 or 4 times gentle inversion of the tube. The diluted blood (9ml) was then slowly layered onto 3ml of Ficoll and centrifuged (300g, 30 min, RT). After discarding the supernatant fluid (SNF), peripheral blood mononuclear cells (PBMCs) fractions pooled from different tubes (20ml) was resuspended in 30ml of PBS and centrifuged (100g, 10 min, RT). The pellet was then resuspended in 20ml of PBS. To confirm cells viability 20µl of the cell suspension was mixed with 20µl of 0.4% trypan blue and incubated for 3 to 5min at RT prior to cell counting using a binocular microscope. The suspension was centrifuged (100g, 10 min, RT) and the pellet was resuspended in Freezing medium/DMSO at 1 ×10^6cells/ml by addition of Freezing medium/DMSO drop wise, with constant mixing, over 1 to 2min with samples placed on ice. Aliquots (1.5ml) in ampoules were stored in cryopreservation units in the UDF P3 Revco in R8031 for 24h, before transferring to the liquid nitrogen units (R8120) for long-term storage.

2.24 Phytohaemagglutinin stimulation of peripheral blood mononuclear cells

An ampoule (1.5ml) of PBMCs was removed from liquid nitrogen unit (R8120 Unit 2) and transferred to the P3 laboratory on ice. The sample was diluted in 10ml of sterile PBS drop wise by gently mixing the tube and centrifuged (100g, 10min, RT). The pellet was resuspended in about 7ml of pre-warmed RPMI media (without IL-2) and transferred to a 25cm^2 flask prior to the addition of the phytohaemagglutinin (PHA) to a final concentration of 0.5µg/ml (100µl/10ml). The cells were incubated at 37°C in a CO_2 incubator (5% CO_2 and 95% humidity) for 36h (to promote blast formation and replication of T-cells) with the flask positioned upright and the cap slightly loosened. Cells in a 15ml centrifuge tube was centrifuged (100g, 10min, RT) and the pellet resuspended in pre-warmed RPMI complete medium (containing 2% IL-2) and transferred to the 25cm^2 flask.

2.25 HIV inhibition assay

The anti-HIV-1 activities of crude saliva, breast milk, pregnancy plug mucus, purified MUC5B and MUC7 from saliva, MUC1 from milk and pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) from HIV negative individuals and purified salivary MUC5B and MUC7 from HIV positive individuals with different CD4 counts (<200, 200-400, and >400) was tested in an inhibition assay according to the method of Nagashunmugam *et al.* (1997). Each sample was dissolved in 0.25% PBS and (500µl or 0.9mg each) was mixed with 4ml of the Subtype D HIV-1 supernatant fluid (SNF) and incubated for 60min at 37°C. As controls, heat inactivated HIV-1 and HIV-1 plus media (RPMI 1640 with 10% fetal calf serum and IL-2) were used. The virus was first isolated from an AIDS patient by the Department of Medical Virology, Tygerberg Hospital (Cape Town, South Africa), in February 1988, and it was fully characterised and sequenced (Treurnicht *et al.*, 2002). At the end of the incubation period the mixtures (HIV-1 plus crude saliva), (HIV-1 plus breast milk), (HIV-1 plus pregnancy plug mucus), (HIV-1 plus MUC5B), (HIV-1 plus MUC7), (HIV-1 plus MUC1), (HIV-1 plus pregnancy plug mucins), (HIV-1 plus MUC5B from HIV patients with CD4 count <200), (HIV-1 plus MUC5B from HIV patients with CD4 count between 200 and 400), (HIV-1 plus MUC5B from

HIV patients with CD4 count >400), (HIV-1 plus MUC7 from HIV patients with CD4 count <200), (HIV-1 plus MUC7 from HIV patients with CD4 count between 200 and 400), (HIV-1 plus MUC7 from HIV patients with CD4 count >400) and the controls (HIV-1 plus media) and heat inactivated HIV-1 were filtered through 0.45μm pore size cellulose acetate filter (25mm diameter) and both the unfiltered and filtered samples were incubated with the PHA stimulated CEM SS cells at 37°C at a concentration of 0.5×10^6 cells/ml for 30min, 1h and 3h. Cells were then washed three times with PBS to remove free virus and cultured. Supernatant fluid was harvested on Day 4 and viral replication was measured by a qualitative p24 antigen assay. Endpoints were calculated by the Reed-Muench formula and the 50% tissue culture infective dose ($TCID_{50}$) was expressed as the highest dilution that produced a positive qualitative P24 antigen result. All samples were done in triplicate and the anti-HIV-1 activity of each mucin was tested in a serial tenfold dilution (10^{-1} to 10^{-4}).

To determine if MUC7 can inhibit HIV-1 infection by blocking the putative viral binding sites (receptors), the PHA stimulated CEM SS cells and PBMCs from HIV negative individuals were incubated with different concentrations of salivary MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) for 60min at 37°C. As a positive control the cells were incubated with PBS and as a negative control untreated or cells only were used. At the end of the incubation period HIV-1 Subtype D and Subtype C were added to the mixtures (CEM SS cells plus MUC7) and (PBMCs plus MUC7) respectively except to the negative control. To check if MUC7 could reduce viral infection of the HIV-1 infected PBMCs, HIV-1 positive PBMCs were incubated with different concentrations of MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) and cultured. Inhibition assay was performed as above

The role of MUC7 in minimizing the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs was also determined by the inhibition assay. Briefly the PHA stimulated HIV-1 positive PBMCs were incubated with different concentrations of MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) for 60min and then added to PBMCs from HIV-1 negative individuals. As control the HIV-1 positive PBMCs were incubated with the PBMCs from HIV-1 negative individuals. Cells were then cultured and inhibition assay was performed as above.

To check if there are differences in the degree of infectivity or virulence of the viruses in the saliva, breast milk, cervical secretion and plasma of HIV-1 positive individuals these secretions were incubated with CEM SS cells. As a control the CEM SS cells were incubated with HIV-1 Subtype D. Cells were then cultured and viral infection was determined as above.

2.26 Toxicity of human salivary, breast milk and cervical mucins to the African green monkey kidney cells.

Prior to the inhibition assay the toxicity of the purified salivary MUC5B and MUC7, breast milk mucin (MUC1) and pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) to African green monkey kidney cells (BSC-1) was determined by the toxicity assay. Briefly African green monkey kidney cells in a monolayer on 96-well confluent plates were treated with 50µl (0.1mg) of each mucin and then stained with crystal violet to determine cell cytotoxicity as judged by the integrity of the monolayer.

2.27 Vaccinia virus inhibition assay

The anti-vGK-5 (vaccinia virus strain, which is a family of the poxvirus) activity of purified salivary, breast milk and pregnancy plug mucins was determined by an inhibition assay. In this assay African green monkey kidney epithelial cells (BSC-1), maintained in Earle's Base Minimum Essential Medium containing L-glutamine, non-essential amino acids, $NaHCO_3$ and Fetal Calf Serum at a 10% final concentration were grown to 100% confluence in wells of a 96-well culture plate. Wells were inoculated with serial dilutions of untreated vGK-5 or vGK-5 treated with 50µl (0.1gm) of purified salivary MUC5B and MUC7, breast milk mucin (MUC1) and pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) and then incubated at 37°C in a CO_2 incubator (5% CO_2) for 48h to allow for the formation of viral plaques which are circular 'breaks' in the cell monolayer caused by infectious virus particles entering cells and subsequently killing them. Each plaque represents a single virus particle.

Media was removed from each well and cell monolayers were stained with a Crystal Violet Dye solution (10% Crystal Violet, 20% ethanol, 70% dH_2O) in order to distinguish viral plaques and cell death. Plaques in each well were counted and the anti-vGK-5 activity of the mucins was quantified by calculating the percentage reduction in plaques following treatment.

CHAPTER 3

PURIFICATION AND CHARACTERISATION OF HUMAN SALIVARY MUCINS

3.1 Introduction

The objective of the study reported in this chapter was to purify and characterize human salivary mucins and to later examine their anti-HIV-1 activity (see Chapter 6). Human crude saliva was separated by Sepharose CL-4B gel filtration and purified by a two-step isopycnic density gradient centrifugation in caesium chloride. The purity and identity of these mucins was assessed by SDS-PAGE and Western blotting respectively. The amino acid composition of the mucins was also determined by HPLC analysis.

3.2 Results

3.2.1 Sepharose CL-4B gel filtration of human salivary mucins

Sepharose CL-4B gel filtration chromatography was performed to separate human salivary mucin components according to size. As shown in Figure 3.1 the elution profile demonstrated the presence of two mucin rich macromolecules in human saliva which are detectable by the PAS assay and distinguishable by their difference in size. The MG1 eluted in the void volume (V_0) whilst the MG2 in the included volume (V_i) of the column. Protein detection was by absorbance at 280nm. The MG1 containing fraction has low 280nm absorbance, unlike the MG2 containing fraction which is associated with a considerable amount of protein.

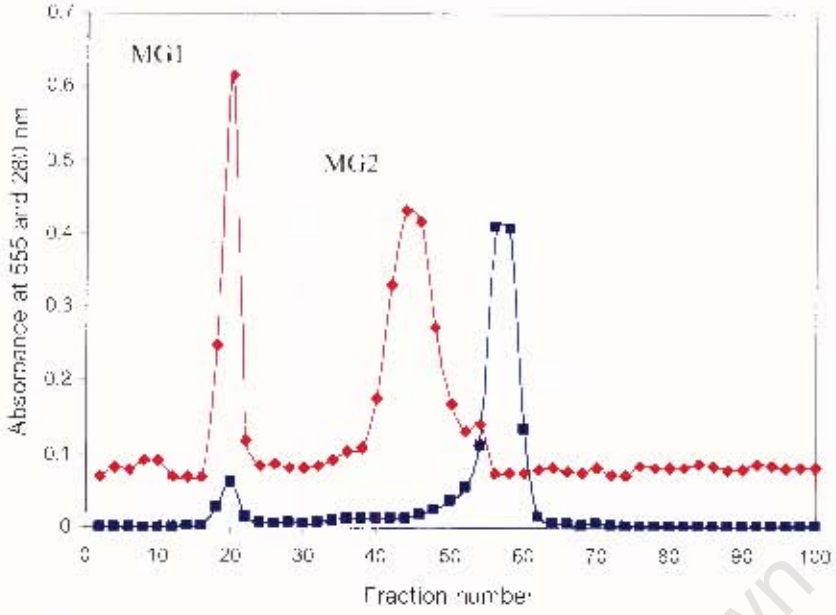


Figure 3.1 **Sepharose CL-4B gel filtration chromatography of human crude saliva.** Soluble (10ml) crude human saliva extracted in 6M GuHCl containing 10mM EDTA, 5mM NEM, 1mM PMSF and 0.1% CHAPS pH 6.5 was chromatographed on a Sepharose CL-4B column (100 × 1.6cm, volume 200ml). The column was eluted with 4M GuHCl containing 10mM EDTA, 5mM NEM and 0.05% CHAPS, pH 6.5 at a flow rate of 48ml/h at room temperature. Fractions (2.0ml) were analysed for carbohydrate with PAS at 555nm (♦) and for protein by A_{280} (■). MG1 and MG2 containing fractions were pooled separately and dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

3.2.2 SDS-PAGE analysis of the MG1 containing fractions

To determine the efficiency of the separation by Sepharose CL-4B gel filtration and the extent of purity of the material that eluted under the MG1 peak (V_0), freeze-dried MG1 material under the V_0 peak was suspended in gel loading buffer containing 5% 2-mercaptoethanol and subjected to 10% SDS-PAGE (Figure 3.2). Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (Figure 3.2A) and PAS (Figure 3.2B) to determine the presence of protein contaminants and mucins respectively.

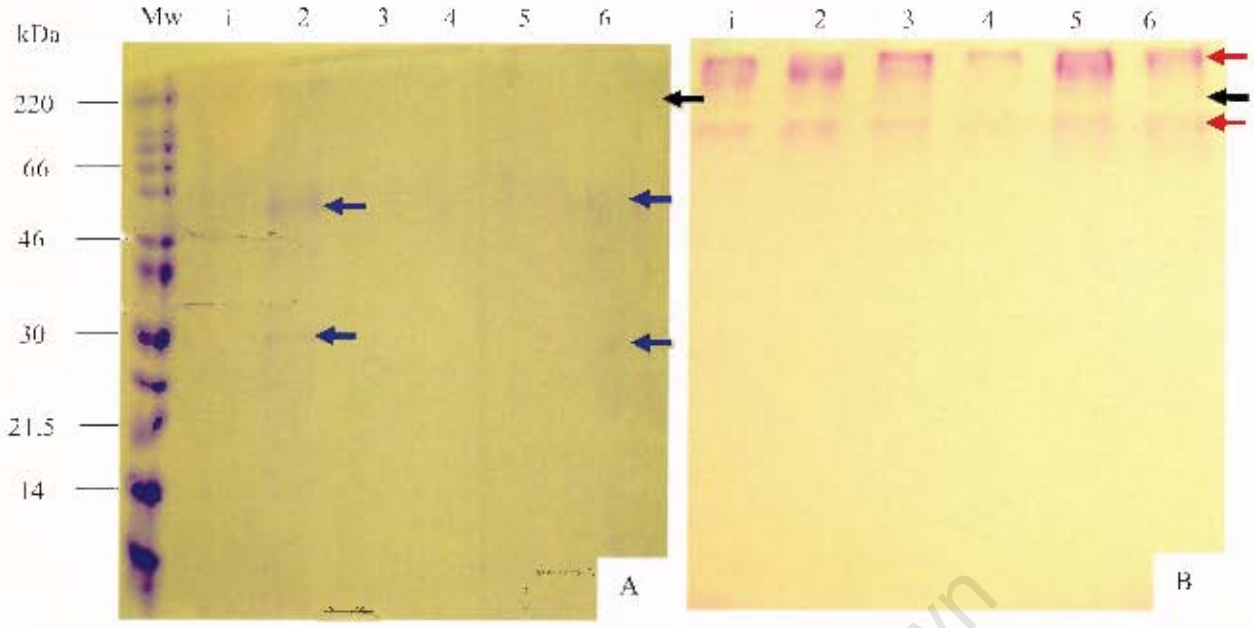


Figure 3.2 SDS-PAGE analysis of the MG1 material. Freeze-dried samples (30µg) of the MG1 material from six donors were prepared in reducing gel loading buffer and separated on 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (A) and PAS (B). Lanes are numbered on the top of each gel and the molecular weight markers indicated by Mw. The arrows in blue indicates the faint protein bands of the MG1 material (A), and those in red indicate the mucins on top of the stacking and on top of the running gel (B). The black arrows are at the start of running gels.

The Coomassie Brilliant Blue R-250 stained gel (Figure 3.2A) demonstrated the presence of very faint protein bands ranging in size 30-60kDa (blue arrows) suggesting that further purification of mucin was not required for void volume material. When the same gel was stained with PAS (Figure 3.2B) high molecular weight mucin bands with a size greater than 220kDa were seen on top of the stacking and running gel (red arrows).

3.2.3 SDS-PAGE analysis of the MG2 containing fractions

To determine the extent of purity of the fractions eluted under the MG2 peak or included volume (V_i), freeze-dried samples of the MG2 containing fractions prepared in a reducing gel loading buffer were also subjected to 10% SDS-PAGE (Figure 3.3). Gels were stained for proteins with Coomassie Brilliant Blue R-250 (Figure 3.3A) and for carbohydrate with PAS (Figure 3.3B).

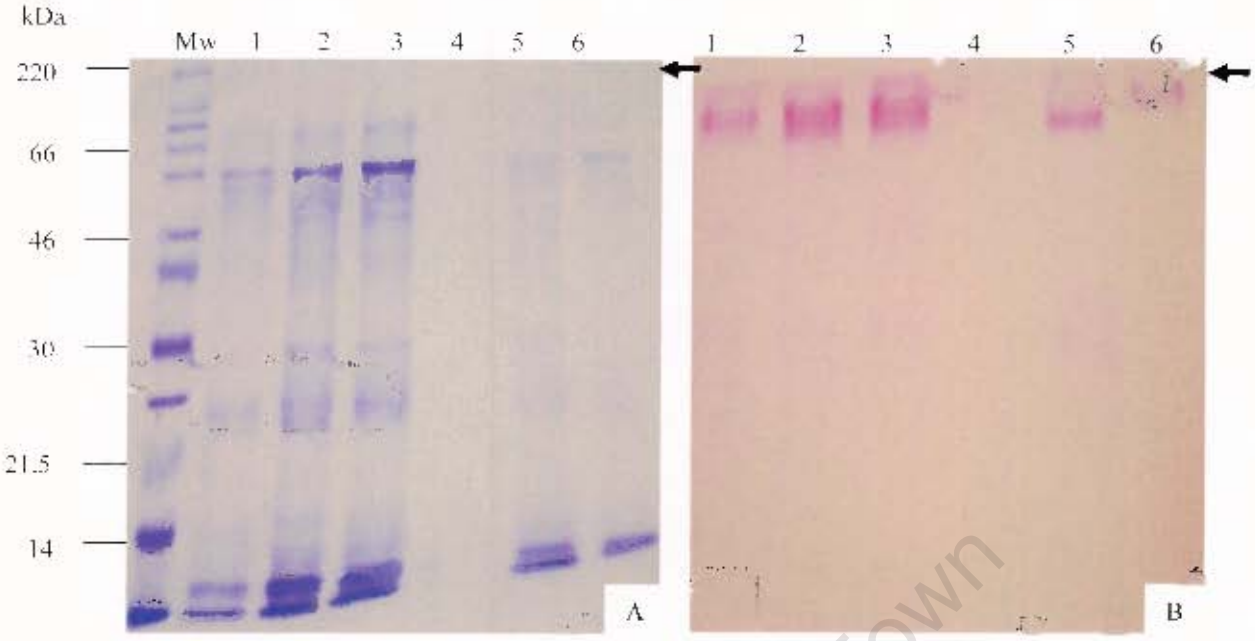


Figure 3.3 SDS-PAGE analysis of the MG2 material. Freeze-dried samples (30µg) of the MG2 material from six donors were prepared in reducing gel loading buffer and separated on a 7.0% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (A) and PAS (B). Lanes are numbered on the top of each gel and the molecular weight markers indicated by (Mw). The black arrows are at the start of running gels.

In contrast to the MG1 containing fractions (Figure 3.2A), the Coomassie Brilliant Blue R-250 stained gel (Figure 3.3A) showed more smaller molecular weight contaminant proteins associated with the MG2 mucin indicating that further purification of the MG2 containing fractions was required. When the same gel was stained with PAS (Figure 3.3B) the material that eluted under the MG2 peak or included volume showed a relatively smaller size mucin that partially entered the running gel.

3.2.4 Caesium chloride isopycnic density gradient centrifugation

The MG2 material eluting in the V_i of the gel filtration column was purified by a two-step caesium chloride isopycnic density gradient centrifugation with a buoyant density between 1.39 and 1.40g/ml to remove the protein contaminants. As shown in Figure 3.4, there was clear separation between the proteins (positive for Lowry) that

fractionated at a lower density and mucins (positive for PAS) that fractionated at a higher density.

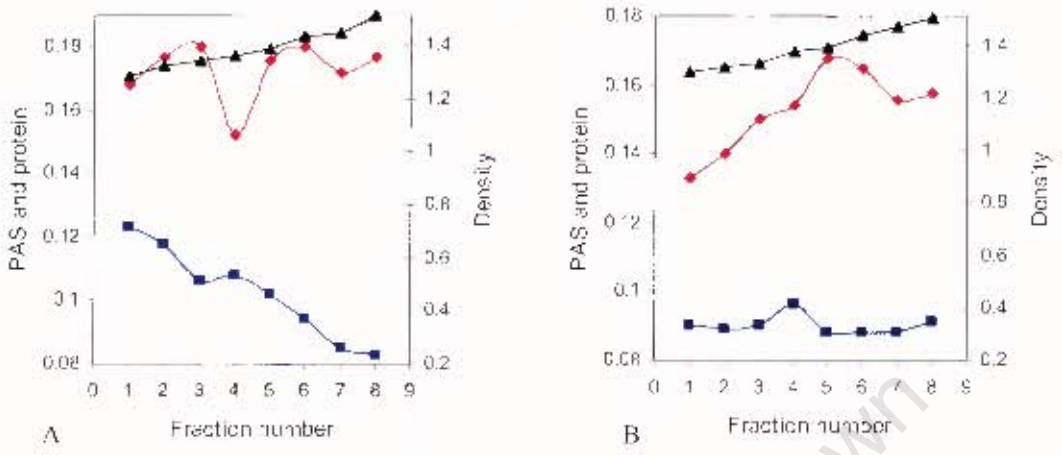


Figure 3.4 Caesium chloride isopycnic density gradient purification of MG2 material. Freeze-dried MG2 material was dissolved in 4M GuHCl containing 10mM EDTA, 5mM NEM and 0.05% CHAPS pH 6.5 and adjusted to a density of 1.39 to 1.40g/ml with solid caesium chloride. Density gradient centrifugation was performed in a Beckman L45 ultra-centrifuge for 48h at a 105 000g at 4°C. Mucin positive fractions (♦) at a density (▲) between 1.37-1.42 and still associated with some protein (■) (A) were pooled and prepared for the second centrifugation step (B). Finally fractions (fraction number 4, 5 and 6) were pooled, dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

Subsequent to the two-step caesium chloride isopycnic density gradient purification the purity of the MG2 fraction was assessed by SDS-PAGE (Figure 3.5). Two very clear but diffuse PAS positive bands (Figure 3.5 lane 3 arrows) for MG2a and MG2b (MG2 glycoforms) were seen in the running gel. The MG2b band was more diffuse than the MG2a band. Two protein bands (Figure 3.5 lane 2) were seen in the same position as the PAS positive MG2a band (Figure 3.5 lane 3 arrows). There was no equivalent band to MG2b on the Coomassie gel. Unlike the Coomassie stained gel of the un-purified MG2 material (Figure 3.3A), only faint protein bands with molecular weight of ≤30kDa were observed in the Coomassie stained gel of the purified MG2 material (Figure 3.5 lane 2). These low molecular weight materials of <30kDa were also seen on the PAS stained gel.

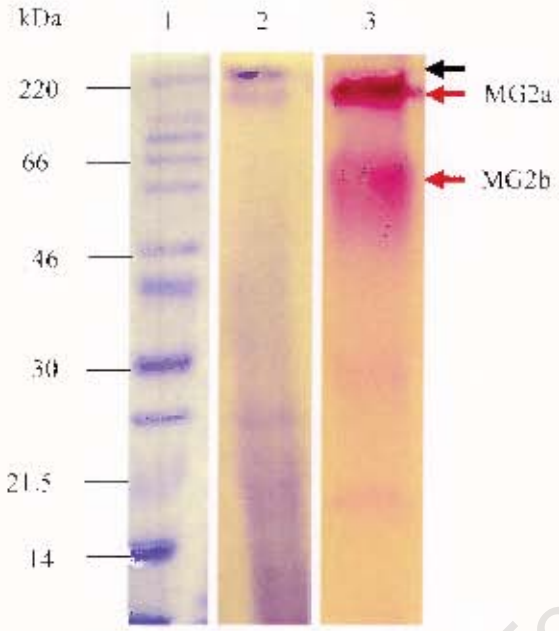


Figure 3.5 SDS-PAGE analysis of the purified MG2 material. After caesium chloride isopycnic density gradient purification freeze-dried (30µg) of the MG2 containing material was prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) and PAS (lane 3). Lane 1 is the molecular weight marker. The arrows in red indicate the MG2 mucin bands (MG2a and MG2b) and the black arrow is at the start of running gel.

3.2.5 Western blotting analysis of the MG1 mucin

To determine its identity, MG1 mucin was separated by SDS-PAGE and transferred to a nitrocellulose membrane. After probing with rabbit anti MUC5B polyclonal antibody, the MG1 mucin was found to be MUC5B (Figure 3.6 lanes 2 and 3). Respiratory mucin was used as a positive control, and was positive for MUC5B (Figure 3.6, lane 4) (see Thornton *et al.*, 1999). MG2 mucin did not react with the antibody (Figure 3.6, lane 1).

Figure 3.6 Western blotting analysis of the MG1 mucin using rabbit anti-MUC5B polyclonal antibody. Lane 1, MG2 mucin (negative control), lanes 2 and 3, MG1 mucins from two donors and lane 4, respiratory mucus (positive control) were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Following overnight blocking, the membrane was incubated for 2h with rabbit anti-MUC5B polyclonal antibody diluted in 5% (m/v) low fat milk powder in TBST at 1 in 500. The membrane was then washed 3 × 5min with TBST and incubated for 1h with HRPO linked goat anti-rabbit secondary antibody diluted in 5% (m/v) low fat milk powder in TBST at a dilution of 1 in 5000. After another TBST wash (3 × 5min), bands were detected using an ECL detection kit.

3.2.6 Western blotting analysis of the MG2 mucin

The MG2 mucin was also subjected to 10% SDS-PAGE and then transferred to nitrocellulose membrane and probed with goat anti-MUC7 polyclonal antibody. The two bands of the MG2 mucin that slightly entered the running gel were found to strongly interact with the anti-MUC7 polyclonal antibody. These could be the two glycoforms of MUC7 (Figure 3.7, lane 3). Again whilst the positive control, crude saliva (Figure 3.7, lane 1) reacted with the anti-MUC7 antibody, the negative control, gastric mucus (Figure 3.7, lane 2) did not react with the antibody.

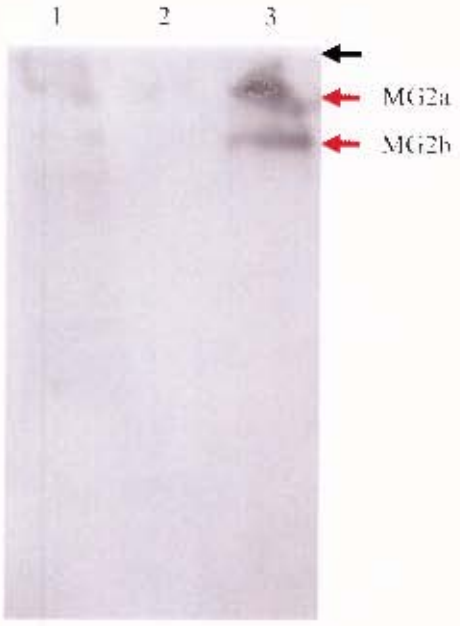


Figure 3.7 Western blotting analysis of the MG2 mucin using goat anti-MUC7 polyclonal antibody. Lane 1, crude saliva (positive control), lane 2, gastric mucus (negative control) and lane 3, MG2 mucin were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Following overnight blocking, the membrane was incubated for 2h with goat anti-MUC7 polyclonal antibody diluted in 5% (m/v) low fat milk powder in TBST at 1 in 100. The membrane was then washed 3 × 5min with TBST and incubated for 1h with HRPO linked rabbit anti-goat secondary antibody diluted in 5% (m/v) low fat milk powder in TBST at a dilution of 1 in 5000. After another TBST wash (3 × 5min), bands were detected using an ECL detection kit. White arrows in red indicate the MG2 mucin bands (MG2a and MG2b), the black arrow is at the start of running gel.

3.2.7 Amino acid analysis of MUC5B and MUC7 mucins

To determine the amino acid composition of MUC5B and MUC7 mucins, both MUC5B and MUC7 were subjected to amino acid analysis. As presented in Table 3.1, both mucins were found to contain more serine, glutamic acid, glycine and aspartic acid than the other amino acids. The MUC5B contained high amounts of threonine but the amount of threonine in the MUC7 was low. Of these amino acids serine, threonine, and proline comprises 26% and 28% of the MUC5B and MUC7 mucins respectively.

Table 3.1 Amino acid composition (mol %) of MUC5B and MUC7

Amino acids	MUC5B (mole %)	MUC5B[a] (predicted; mole %)	MUC7 (mole %)	MUC7[b] (predicted; mole %)
Aspartic acid	8.9	4.9	9.1	8.5
Threonine	10.0	20.7	4.0	7.7
Serine	7.4	11.1	5.5	9.1
Glutamic acid	9.0	6.3	12.4	9.1
Proline	8.5	9.9	18.6	14.0
Glycine	9.0	7.0	14.4	3.6
Alanine	6.6	8.4	3.9	4.8
Valine	6.4	5.3	4.2	4.8
Methionine	1.6	1.2	1.7	1.2
Isoleucine	4.0	2.2	2.9	2.4
Leucine	7.6	5.8	4.7	6.6
Tyrosine	3.2	1.7	1.7	1.2
Phenylalanine	3.8	2.3	1.9	3.0
Lysine	4.6	1.8	4.7	6.1
Histidine	4.4	2.2	3.9	10.3
Arginine	4.0	3.4	5.6	6.1

[a]Based on DNA sequence data from Thomsson *et al.* (2002) and [b]Liu *et al.* (2000)

3.3 Discussion

One of the ultimate objectives of this study was to test the hypothesis that it was the mucus and purified mucin component of crude saliva that prevented the transmission of the human immunodeficiency virus through the exchange of saliva. The first step towards the achievement of this goal was to extract crude saliva in a suitable buffer that minimized the risk of endogenous proteolysis and to isolate, purify and identify the mucin component of the crude secretion.

According to Allen (1981), mucins are susceptible to degradation by endogenous proteinases during isolation and purification, which could result in a collapse of their mechanical properties. This was confirmed by Sellers *et al.* (1988) who showed that sheep submaxillary and pig submaxillary mucins, which were isolated in the presence of proteinase inhibitors, were clearly different in structure from those extracted in the absence of the inhibitors. As judged by gel filtration on Sepharose CL-2B, in the absence of inhibitors mucins were eluted in the included volume rather than in the excluded volume as the digested mucins did. Therefore, throughout this study (starting from sample collection), GuHCl buffer which contained a cocktail of protease inhibitors such as EDTA, NEM, and PMSF was used to minimize any

enzymatic degradation which could occur during the course of this experiment. While PMSF and EDTA are reported to inhibit serine and metallo-proteases respectively by inhibiting thiol proteases, NEM is believed to block any disulfide bond formation between mucins and other molecules. Furthermore, the addition of GuHCl, a denaturant (Allen, 1981) is also thought to enhance solubility of these mucins (Carlstedt *et al.*, 1983).

One of the difficulties in mucin purification is their association with other molecules. According to Ge *et al.* (2004), Situ and Bobek. (2000) and Tabak, (1995), salivary mucins are known to bind with non-mucinous salivary components such as amylase, lysozyme, proline-rich proteins, statherin, histatins, secretory IgA (sIgA) and lactoferrin to form a concentrated protective barrier in the oral cavity. However, this complex formation may change the physicochemical properties of the salivary mucins and subsequently their function in the oral cavity (Tabak, 1995). The hydrophobic regions of these mucins are the parts reported to be responsible for these complex formations and their sticky characteristics (Mehrotra *et al.*, 1998). During this experiment, especially during gel filtration and dialysis, special care was needed to minimize or, if possible, to avoid any loss of mucin which could stick to the filtration gel and dialysis tubing. Thus CHAPS, a detergent was included in our chromatography buffers to minimize the sticky characteristic of mucins and prevent their loss (Bolscher *et al.*, 1999).

As mentioned in the preceding paragraph, although some salivary components may adhere to and elute with mucins, large-pore size gel filtration columns are reported to separate mucins in the excluded volume from the lower molecular weights proteins in the included volumes (Allen, 1981). As a result, in the present study, Sepharose CL-4B gel filtration, which separated human crude saliva into MG1 and MG2 mucin-rich populations in the V_0 and V_i respectively, was used. This finding agreed with that of Mehrotra *et al.* (1998), Tabak (1995) and Thornton *et al.* (1999) that gel filtration on a Sepharose CL-4B, separated human crude saliva into the larger (MG1) and smaller (MG2) mucin populations. Subsequently caesium chloride isopycnic density-gradient centrifugation was also employed to remove the non-mucinous salivary components or proteins which were detected in the Coomassie Brilliant Blue R-250 stained gel of the MG2 containing fraction, from the MG2 mucin. As reported by Allen, (1981) this

method is known to separate mucins from lower density lipid and protein and higher density nucleic acids successfully. As it is conducted in the presence of high concentrations of GuHCl, this method not only removed protein contaminants, but also suppressed the activity of the degradative enzymes (Carlstedt *et al.*, 1983).

In this experiment the saliva samples from different donors behaved similarly upon gel filtration, SDS-PAGE and Western blotting. However, a different elution profile of the Sepharose 4B gel filtration especially with the MG2 peak was seen with few salivary samples which were from donors who were suffering from chronic sinusitis. Although the reason is not clear why these elution profiles should differ, there could be a mixing of secretions from the nasal cavity and the saliva which could be the reason for the different peak patterns of the elution profiles. Thus these samples were excluded from the study.

According to the report of Creeth (1978), while the separation of lipids can be achieved with little difficulty, the separation of the non-covalently bounded proteins, and nucleic acids from mucins by non-degradative methods was found to be very difficult. However, Liu *et al.* (1999) reported that salivary mucins, which were purified by reductive methylation and alkylation procedures and which resulted in covalently modified mucins, caused mucins to lose their ability to promote bacterial adherence and aggregation. Since the objective of this part of the experiment was to isolate and purify salivary mucins and determine their anti-HIV-1 activities using *in vitro* inhibition assays, the use of this purification method was avoided. Instead GuHCl was used to reduce the viscosity of the crude saliva and to dissociate possible complex formation between the salivary mucins and other salivary proteins (Creeth, 1978).

Subsequent to Sepharose CL-4B gel filtration and caesium chloride isopycnic density-gradient purification, the purity of the samples was assessed by SDS-PAGE stained for protein with Coomassie Brilliant Blue R-250. The removal of contaminant protein after caesium chloride isopycnic density-gradient purification was evident. However, the higher cost of the GuHCl, long time of ultra-centrifugation and fractionation made this method expensive and labour intensive.

Once the purity of the MG1 and MG2 salivary mucins was confirmed, their identity was determined by Western blotting. As a result the MG1 and MG2 mucins were found to be MUC5B and MUC7 mucins respectively. This was in agreement with that of Thornton *et al.* (1999), who indicated that MG1 and MG2 mucins are encoded by the MUC5B and MUC7 genes respectively.

Thornton *et al.* (1999) reported the presence of two isoforms of MUC5B mucin in human saliva which differ in their charge density. Although the SDS-PAGE analysis demonstrated the presence of two mucin bands closely sitting on the top of the gel, it is too early to conclude that they are the two isoforms of MUC5B. According to Gipson *et al.* (2001), the identification and characterization of these glycoforms requires immuno analysis on the mucin fractions by a wide range of mucin-specific antibodies, in addition to the extensive caesium chloride isopycnic density-gradient purification. However, this is very expensive and requires large amounts of sample. Furthermore, as reported by Thornton *et al.* (1995), the difficulty of identifying these glycoforms by monoclonal antibodies is that, since the monoclonal antibodies are directed at sequences present in the highly *O*-glycosylated domains of these molecules, separation and deglycosylation of these glycoforms is required.

MUC7 mucin gave two bands both in the SDS-PAGE and Western blotting membrane. As both are detected by the same antibody, it is possible that these two mucins may contain the same protein backbone. This finding agreed with that of Bolscher *et al.* (1999), Mehrotra *et al.* (1998) and Reddy *et al.* (1992), which demonstrated the presence of two isoforms of MUC7, designated MUC7a and MUC7b in human saliva. According to these authors, these two isoforms contain the same amino acids but different terminal sugars such as, sialic acids and fucose. Therefore separation of these bands and determination of their amino acid and sugar composition is required.

The amino acid analysis results agreed with the findings of Tabak *et al.* (1982) who indicated the presence of large amounts of serine, threonine, proline, glutamic acid, glycine and alanine in the peptide portions of the salivary mucins. However, the amount of threonine in MUC7 was very small, which is far less than the expected

value. According to Mehrotra *et al.* (1998) this could be caused by the level of oligosaccharide substitution of this residue.

All the above biochemical methods confirmed that the salivary mucin which was eluted in the void volume of the Sepharose 4B gel filtration column was MUC5B and the mucin which was eluted in the included volume was MUC7. As human salivary mucins are known to inhibit a number of micro-organisms including bacteria, viruses, yeasts and toxins in the oral cavity (Bobek and Situ, 2003; Bosch *et al.*, 2000), the anti-HIV-1 activity of these mucins will be examined in Chapter 6.

CHAPTER 4

THE PURIFICATION AND CHARACTERIZATION OF HUMAN BREAST MILK MUCIN

4.1 Introduction

The objective of the study reported in this Chapter was to purify and characterize the human breast milk mucin and to later determine its anti-HIV-1 activity (see Chapter 6). Milk fat globule membrane (MFGM) was prepared from the human breast milk and chromatographed on a Sepharose CL-4B gel filtration column. Upon purification by caesium chloride isopycnic density gradient centrifugation, the purity and identity of the mucin was assessed by SDS-PAGE and Western blotting respectively. Furthermore the amino acid composition of the mucin was determined by HPLC analysis.

4.2 Results

4.2.1 Preparation of milk fat globule membrane

To prepare the milk fat globule membrane (MFGM), human breast milk was defatted by centrifugation at 3 000g for 1h at 4°C. The cream fraction indicated by the arrow in Figure 4.1 was collected and processed as described in the Materials and Methods section (Chapter 2) which resulted in the preparation of MFGM.

Figure 4.1 Preparation of milk cream. Human breast milk was defatted by centrifugation at 3 000*g* for 1h at 4°C. The cream fraction (red arrow) was collected and processed for the preparation of MFGM (for details see the Materials and Methods section in chapter 2).

4.2.2 Sepharose CL-4B gel filtration of the milk fat globule membrane

The milk fat globule membrane (MFGM) was subjected to Sepharose CL-4B gel filtration chromatography to isolate the milk mucin. The elution profile (Figure 4.2) indicated that MFGM contains two populations, a mucin rich population (MFGM-A) eluting as sharp PAS positive peak in the V_0 (void volume) of the column and another (MFGM-B) that eluted in the V_t (included volume) of the column, separated on the basis of size. The protein profile at an absorbance of A_{280} showed a large peak eluting in the V_0 of the column associated with the MFGM-A fractions, coinciding with but broader than the PAS positive peak, whilst a small protein positive peak, indicating a far less amount of protein, eluted in the V_t associated with the MFGM-B containing fractions.

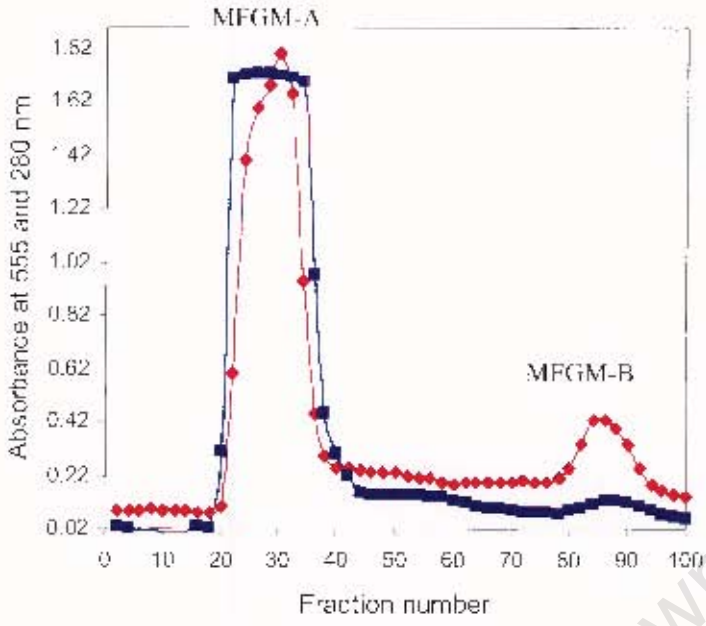


Figure 4.2 Sepharose CL-4B gel filtration of the milk fat globule membrane. Soluble (20ml) MFGM was chromatographed on a Sepharose CL-4B column (100 × 1.6cm, volume 200ml). The column was eluted with 0.2M NaCl, 0.02% NaN_3 containing 10mM EDTA and 5mM NEM, at a flow rate of 48ml/h at room temperature. Fractions (2.0ml) were analysed for carbohydrate with PAS at 555nm (♦) and protein A_{280} (■). The V_0, MFGM-A and V_i, MFGM-B containing fractions were pooled separately and dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

4.2.3 SDS-PAGE analysis of the milk fat globule membranes

To determine the presence of mucin/s in the milk fat globule membranes, freeze-dried V_0 (MFGM-A) and V_i (MFGM-B) materials were dissolved in sample loading buffer. Samples were then subjected to 10% SDS-PAGE and stained for protein with Coomassie Brilliant Blue R-250 and for carbohydrate with PAS (Figure 4.3). Protein staining showed a large number of bands ranging in size from >220kDa to below 14kDa for the V_0 material (Figure 4.3 lane 2) whilst less protein eluted in the V_i with clear bands at around 60kDa, 25kDa and 20kDa (Figure 4.3 lane 3). Upon staining with PAS the V_0 material showed high molecular weight mucin bands sitting on the stacking and at the top of the running gel (>220kDa) with an additional three smaller bands that entered the running gel ranging in size 50-30kDa (Figure 4.3 lane 4). No mucin band was detected on the MFGM-B containing fractions (Figure 4.3 lane 5).

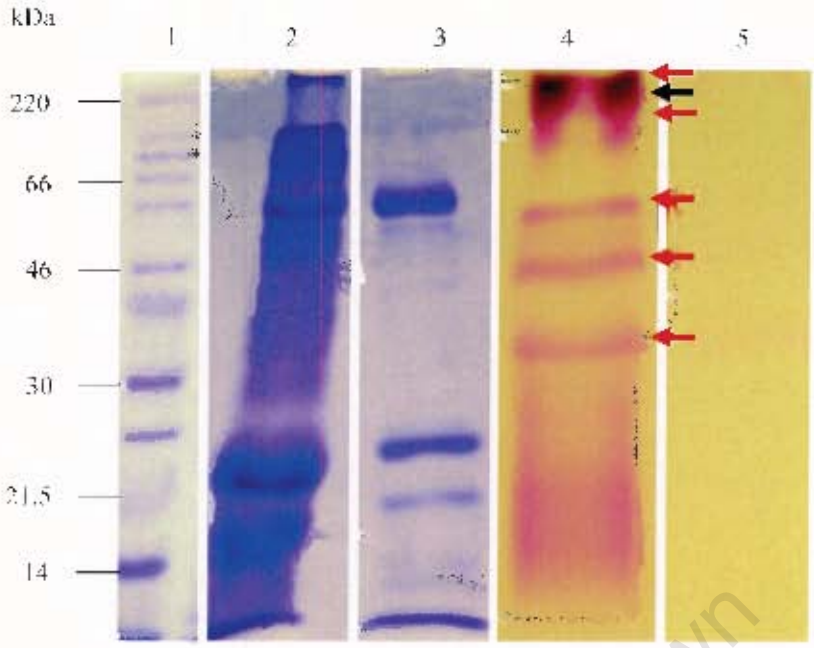


Figure 4.3 SDS-PAGE analysis of the milk fat globule membranes. Freeze-dried samples (30µg) of MFGM-A (lanes 2 and 4) and MFGM-B (lanes 3 and 5) were prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R 250 (lanes 1, 2 and 3) and PAS (lanes 4 and 5). The arrows in red indicate the mucins on the stacking, on top of the running and slightly entering the running gel and the arrow in black is at the start of running gel. Lane 1 is a molecular weight marker.

4.2.4 Caesium chloride isopycnic density gradient centrifugation

The V_0 material (MFGM-A) of the Sepharose CL-4B gel filtration column was subjected to caesium chloride isopycnic density gradient centrifugation to purify the mucin by removing the associated contaminating protein. The purification profile (Figure 4.4) demonstrated that the PAS positive mucin was clearly separated from the protein (Lowry) by this method.

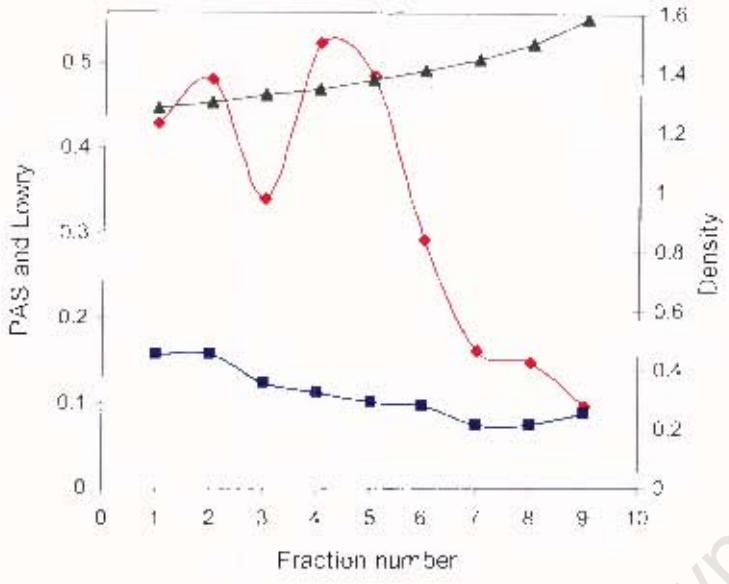


Figure 4.4 Caesium chloride isopycnic density gradient purification of MFGM-A containing fractions. Freeze-dried MFGM-A containing fractions were dissolved in 4M GuHCl containing 10mM EDTA, 5mM NEM and 0.05% CHAPS pH 6.5 and adjusted to a density of 1.39 to 1.40g/ml with solid caesium chloride. Density gradient centrifugation was performed in a Beckman L45 ultra-centrifuge for 48h at a 105 000g at 4°C. Fractions (fraction number 4, 5 and 6) positive for PAS (♦), and with hardly any protein (Lowry) (■) and of density (▲) between 1.37-1.42 were pooled, dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

Following caesium chloride isopycnic density gradient purification, the purity of the MFGM-A material was determined by SDS-PAGE (Figure 4.5). Staining for protein showed that despite the removal of the protein contaminants ranging in size from 220kDa to 30kDa (Figure 4.3 lane 2) bands at the stacking gel (>220kDa), 30kDa and 21.5kDa were clearly seen in the gel (Figure 4.5 lane 2). The PAS stained gel of the purified V_a material showed similar band patterns to that of the un-purified MFGM-A material (Figure 4.3 lane 4) with high molecular weight mucin bands sitting at the stacking and at the top of the running gel (≥220kDa) with an additional three smaller bands that entered the running gel ranging in size from 50kDa to 30kDa (Figure 4.5 lane 3). A degraded or faint mucin and protein bands ranging in size from 30kDa to 14kDa were also seen.

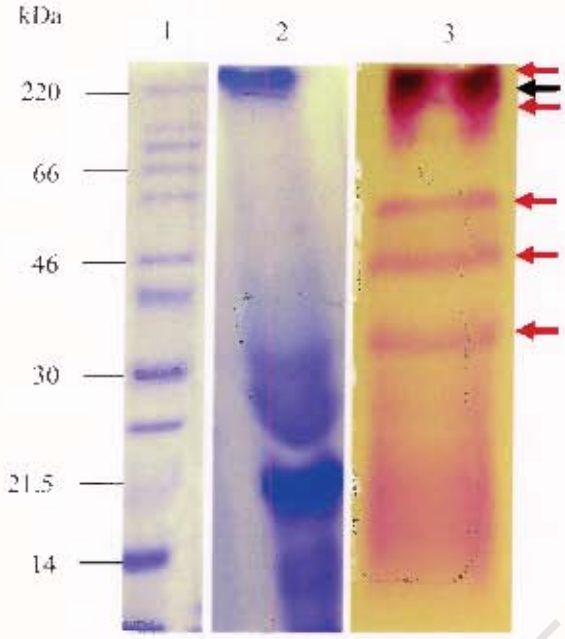


Figure 4.5 SDS-PAGE analysis of the purified MFGM-A material. After caesium chloride isopycnic density gradient purification freeze-dried MFGM-A material (30µg) was prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) and PAS (lane 3). The arrows in red indicate the mucins on the stacking, on top of the running and slightly entering the running gel and the black arrow is at the start of running gel. Lane 1 is a molecular weight marker.

4.2.5 Gradient gel

For purposes of better resolution the purified V_0 material (Figure 4.2) was subjected to a 3-5% gradient gel (Figure 4.6). As indicated by the red arrows prominent mucin band on top of the running gel (≥220kDa) with an additional broad and diffuse band slightly entered the running gel (ranging in size from 95kDa to 60kDa) were clearly seen (Figure 4.6 lane 2). Unlike in the 10% SDS-PAGE (Figure 4.5 lane 3) the smaller bands that entered the running gel appeared in a compacted manner at around 21.5kDa (Figure 4.6 lane 2). Once more, unlike in the 10% SDS-PAGE (Figure 4.5 lane 3) no degraded or faint mucin bands ranging in size from 30kDa to 14kDa were seen (Figure 4.6 lane 2).

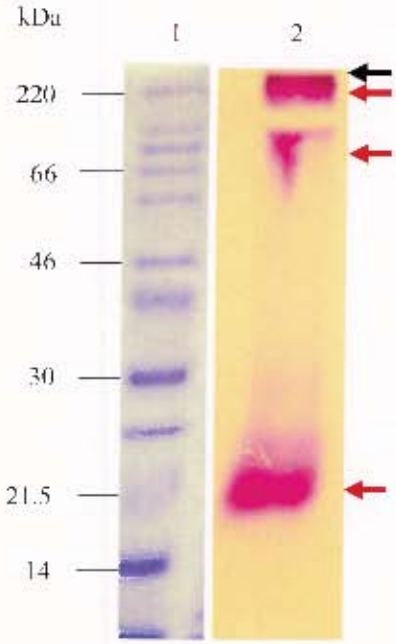


Figure 4.6 Gradient gel analysis of the purified MFGM-A material. After caesium chloride isopycnic density gradient purification freeze-dried MFGM-A material (30µg) was prepared in reducing gel loading buffer and separated on a 3-5% gradient gel. Gels were stained with Coomassie Brilliant Blue R-250 (lane 1) and PAS (lane 2). The arrows in red indicate the mucins on top of the running gel, slightly entering the gel and near the bottom of the gel and the black arrow is at the start of running gel. Lane 1 is a molecular weight marker.

4.2.6 Western blotting analysis of the milk fat globule membrane A

To determine the identity of the human breast milk mucin, the MFGM-A material was separated in a 1% agarose gel and transferred on to a nitrocellulose membrane. The blot was incubated with the mouse anti-MUC1 monoclonal antibody showing the human breast milk mucin (MFGM-A) to be MUC1 (Figure 4.7 lane 1). Whilst the positive controls, cervical and gastric mucus (Figure 4.7, lanes 3 and 4) reacted with the anti-MUC1 antibody as expected, the negative control, purified salivary MUC5B (Figure 4.7, lane 2) did not react with the antibody.

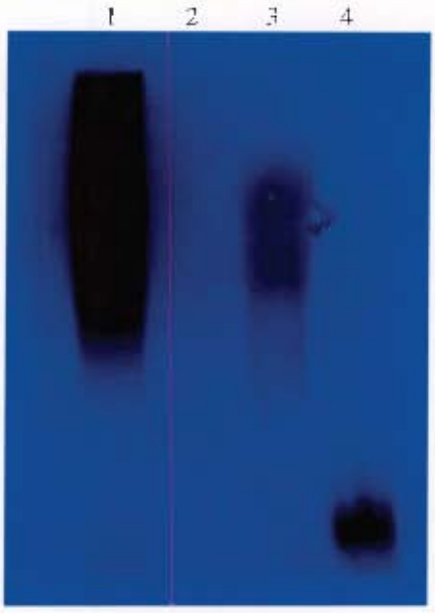


Figure 4.7 Western blotting analysis of the milk mucin using mouse anti-MUC1 monoclonal antibody. Lane 1, purified milk mucin (MFGM-A material), lane 2, purified MUC5B (negative control), and lanes 3 and 4, cervical and gastric mucus (positive controls) were separated by a 1% agarose gel and transferred to nitrocellulose membrane. Following overnight blocking, the membrane was incubated for 2h with mouse anti-MUC1 monoclonal antibody diluted in 5% (m/v) low fat milk powder in TBST at 1 in 100. The membrane was then washed 3 × 5min with TBST and incubated for 1h with HRPO linked goat anti mouse secondary antibody diluted in 5% (m/v) low fat milk powder in TBST at a dilution of 1 in 1500. After another TBST wash (3 × 5min), bands were detected using an ECL detection kit.

To determine whether the prominent mucin bands that entered the running gel of the 10% SDS-PAGE (Figure 4.3 lane 4) are parts of the MUC1 mucin or non-mucin glycoproteins (impurities), the MFGM-A (V_0) material was subjected to a 10% SDS-PAGE and then transferred to nitrocellulose membrane and probed with mouse anti-MUC1 monoclonal antibody. The result showed that the bands that entered the running gel were found to be positive to the mouse anti-MUC1 antibody along with the bands on the stacking and top of the running gel (Figure 4.8 lane 3). As indicated by the red arrows the anti-MUC1 antibody also detected a faint band of roughly 20kDa. Once again, whilst the positive control (cervical mucus) (Figure 4.8, lane 1) reacted with the anti-MUC1 antibody, the negative control (purified salivary MUC5B) (Figure 4.8, lane 2) did not react with the antibody.

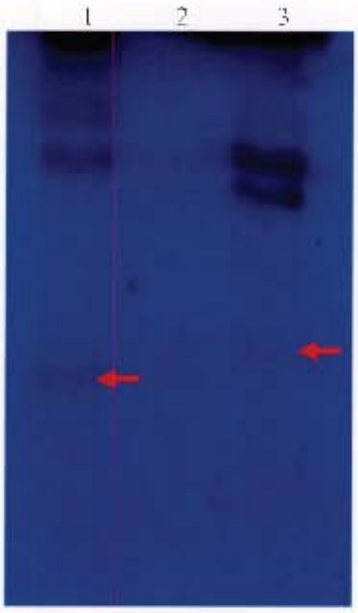


Figure 4.8 Western blotting analysis of the milk mucin using mouse anti-MUC1 monoclonal antibody. Lane 1, cervical mucus (positive control), lane 2, purified MUC5B (negative control) and lane 3 purified milk mucin (MFGM-A material) were separated by a 10% SDS-PAGE and transferred to nitrocellulose membrane. Following overnight blocking, the membrane was incubated for 2h with mouse anti-MUC1 monoclonal antibody diluted in 5% (m/v) low fat milk powder in TBST at 1 in 100. The membrane was then washed 3 × 5min with TBST and incubated for 1h with HRPO linked goat anti-mouse secondary antibody diluted in 5% (m/v) low fat milk powder in TBST at a dilution of 1 in 1500. After another TBST wash (3 × 5min), bands were detected using an ECL detection kit.

4.2.7 Amino acid analysis of the milk fat globule membrane A

Once the MFGM-A mucin glycoprotein was isolated and purified, its amino acid composition was determined by amino acid analysis. As shown in Table 4.1, the milk mucin was found to contain higher amounts of threonine, serine, glutamic acid, aspartic acid, histidine and leucine. However, the amount of proline was relatively smaller. As shown in Table 4.1, the signature amino acids for mucin which are serine, theronine and proline make up 20.1% of the mucin.

Table 4.1 Amino acid composition (mol %) of the breast milk mucin (MFGM-A)

Amino acids	MUC1 (mole %)	Total MFGM protein
Aspartic acid	9.0	8.7
Threonine	7.8	6.7
Serine	7.0	8.1
Glutamic acid	14.3	11.0
Proline	5.5	6.1
Glycine	4.0	8.1
Alanine	6.1	8.1
Valine	7.2	6.7
Methionine	2.0	2.1
Isoleucine	4.8	4.4
Leucine	8.9	9.1
Tyrosine	3.5	2.8
Phenylalanine	2.6	3.5
Lysine	4.8	5.4
Histidine	8.2	2.5
Arginine	3.6	4.9

Total MFGM protein based on data from Shimizu and Yamauchi (1982)

4.3 Discussion

Among the ultimate objectives of this study was to determine the role of breast milk and milk mucin in the inhibition of HIV-1 activity. The first step towards the achievement of this objective was to isolate, purify and characterize breast milk mucin using suitable biochemical methods.

After defatting, the milk fat globule membrane (MFGM) was prepared from the cream fraction of the breast milk. The MFGM was then separated into a mucin rich fraction, the milk fat globule membrane-A (MFGM-A) in the V_0 and non-mucin fraction, the milk fat globule membrane-B (MFGM-B) in the V_t of a Sepharose CL-4B gel filtration column. The MFGM-A material had to be purified by caesium chloride isopycnic density gradient centrifugation prior to Western blotting and amino acid analysis.

As GuHCl, which is a denaturing agent (Francis and Bradford, 1976), is too expensive to be used routinely as an elution buffer in column chromatography, 0.2M NaCl, 0.02%NaN_3 buffer containing cocktail of protease inhibitors such as 10mM EDTA, 5mM NEM and 1mM PMSF was used (Younan *et al.*, 1982). Proteolytic inhibitors

such as PMSF and EDTA were used as serine and metallo-protease inhibitors respectively and NEM was used as thiol protease inhibitor (Carlstedt *et al.*, 1982).

As in the case of saliva (Chapter 3) and pregnancy plug mucins (Chapter 5), the milk mucin (MFGM-A) was also purified by caesium chloride isopycnic density gradient centrifugation, a method which separates mucins from the lower density proteins and higher density nucleic acids (Allen, 1981). However, huge reduction of the contaminant protein was shown after one step caesium chloride isopycnic density gradient centrifugation, thus saving time and cost compared to the saliva and pregnancy plug mucin purifications. As no protein was detected by the Lowry assay, the low molecular weight bands, which were revealed by the Coomassie Brilliant Blue R-250 stained gel, could be mucin fragments.

According to Mall (1988) interference by protein can exaggerate a PAS positive peak. It is also unusual for a protein peak to be as large in the V_0 as the one described here, coinciding with the PAS peak (Mall *et al.*, 1997; Mall *et al.*, 1999). However, most previously reported gel filtration data have been for gastrointestinal (Allen, 1981) and respiratory (Thornton *et al.*, 1999) mucus and mucins. Thus the PAS positive V_t peak is most likely as a result of interference from protein.

As shown in the result section high molecular weight mucin bands on the stacking and top of the running gel together with three smaller molecular weight mucin bands that slightly entered the running gel were seen on the PAS stained SDS-PAGE. While the mucin on the stacking gel is most likely to be MUCX (Patton 1999), the band on top of the running gel together with the bands which slightly entered the running gel is most likely to be MUC1. This finding also agreed with the findings of (Patton *et al.*, 1995; Patton, 1999; Peterson *et al.*, 1998) that one or two bands of MUC1 from the same sample may be detected by SDS-PAGE. These bands could be the result of the two alleles inherited from each parent. As reported by Patton *et al.* (1995), since both MUCX and MUC1 share the same carbohydrate structure and elute in void volumes of different pore size gel filtration columns, MUCX could simply be a highly glycosylated form of MUC1.

When the MFGM-A material was subjected to 3-5% gradient gel the prominent mucin bands on top of the gel appeared in similar fashion to that of the 10% SDS-PAGE. However, the smaller bands that entered the gel were found to be very compacted and showed a higher electrophoretic mobility compared to those in the 10% SDS-PAGE. As gradient gels are prepared by mixing two solutions of different acrylamide concentration to form a gradient with decreasing acrylamide concentrations (Pierce Proteomics) (www.piercenet.com/Proteomics), this could affect the band pattern and mobility of the same bands in gradient gels compared to the discontinuous Laemmli gels. The higher concentration of acrylamide is at the bottom of the gel gradient, explaining why the smaller bands at the bottom of the gel are condensed compared to the same bands in the Laemmli gel.

The identity of the human breast milk mucin was confirmed by Western blotting. As shown in the results section the milk mucin was found to be MUC1. To confirm whether the bands that entered the running gel are non-mucin glycoproteins (impurities) or together with the bands on the stacking and on top of the running gel are alleles inherited from each parents (Patton *et al.*, 1995; Patton, 1999; Peterson *et al.*, 1998), the milk mucin was separated by SDS-PAGE instead of agarose which gave distinct bands and probed with the mouse anti-MUC1 antibody. As the bands reacted with the antibody strongly, they are more likely to be polymorphic forms of MUC1 or MUC1 fragments than impurities. This confirms the reports of these authors that as a result of the inheritance of two alleles, MUC1 may contain more than one band on SDS-PAGE.

Amino acid analysis showed the presence of relatively higher amounts of serine, threonine, glutamic acid and aspartic acid in the purified milk mucin. This result agreed with the findings of Shimizu and Yamauchi (1982) which demonstrated the presence of higher amounts of serine, threonine, glutamic acid and aspartic acid in human and bovine milk fat globule membranes (milk mucin). Surprisingly proline, the amino acid which is responsible for the formation of an extended rode like or filamentous structure rising above the cell surface (Peterson *et al.*, 1998), presented in relatively smaller amounts. Though the reason is not clear, its exposed structure may facilitate its destruction during purification or hydrolysis. No tryptophan was found which was possibly destroyed during hydrolysis (Imam *et al.*, 1981; 1982).

In summary, based on its size, appearance of the bands and its electrophoretic mobility on SDS-PAGE, Western blot results and amino acid composition, the human breast milk mucin is MUC1.

Human breast milk is reported to be the first line of defence against microbes, viruses and toxins for the new-borns, minimizing the incidence of diarrhoea, respiratory diseases, and otitis media in breast fed infants (Wiederschain and Newburg, 2001). Together with the other milk components such as secretory IgA (sIgA), lysozyme, lactoferrin, histatins, macrophages and lymphocytes, milk mucin plays a significant role in infant protection (Schroten *et al.*, 1992). Amongst the numerous benefits of milk mucin is its ability to inhibit rotavirus infection which causes gastroenteritis in infants of both developed and developing countries (Yolken *et al.*, 1992). The anti-HIV-1 activity of the purified milk mucin (MUC1) will be determined and presented in Chapter 6.

CHAPTER 5

PURIFICATION AND CHARACTERISATION OF HUMAN PREGNANCY PLUG MUCINS

5.1 Introduction

The objective of the study reported in this Chapter was to purify and characterize human pregnancy plug mucins and ultimately their anti-HIV-1 activity (see Chapter 6). Human pregnancy plug mucus was subjected to a two-step isopycnic density gradient centrifugation in caesium chloride. The purity and identity of the mucins expressed by the female reproductive tract were determined by SDS-PAGE, Western blotting and immuno-histochemical analysis.

5.2 Results

5.2.1 SDS-PAGE analysis of the pregnancy plug mucus

Prior to the purification and characterization methods, the presence of mucins in the pregnancy plug mucus was determined by SDS-PAGE. Freeze-dried crude pregnancy plug mucus which had been extracted in 6M GuHCl and protease inhibitors was dissolved in sample loading buffer and subjected to 10% SDS-PAGE. Gels were stained for carbohydrate with PAS and with Coomassie Brilliant Blue R-250 for protein (Figure 5.1). The PAS stained gel (Figure 5.1 lane 3) revealed the presence of a high molecular weight mucin band (≥220kDa) at the top of the running gel with another smaller mucin band (~ 97.4kDa) entering the running gel. When the same gel was stained with the Coomassie Brilliant Blue R-250 (Figure 5.1 lane 2) a protein band larger than 220kDa at the top of the running gel with additional lower molecular weight protein bands (between 220kDa and 14kDa) in the running gel were observed. This meant that the crude pregnancy plug mucus needed to be purified.

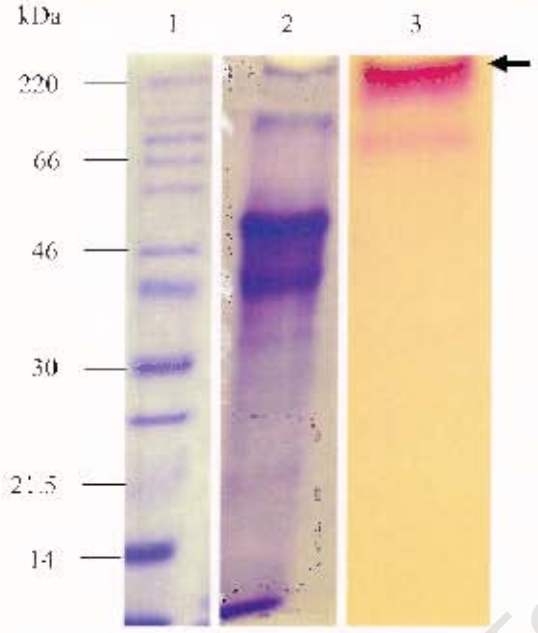


Figure 5.1 SDS-PAGE analysis of pregnancy plug mucus. Freeze-dried crude pregnancy plug mucus (30µg) was prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) and PAS (lane 3). Lane 1 is a molecular weight marker and the black arrow is at the start of running gel.

5.2.2 Caesium chloride isopycnic density gradient centrifugation

To remove the protein contaminants which were evident on the Coomassie Brilliant Blue G-250 stained gel (Figure 5.1 lane 2), the pregnancy plug mucus was purified by a two step caesium chloride isopycnic density gradient centrifugation (Figure 5.2A and 5.2B) at a buoyant density of 1.39-1.40g/ml. The purification profiles showed clear separation between the proteins (positive for Lowry) that fractionated at a lower density and mucins (positive for PAS) that fractionated at a higher density.

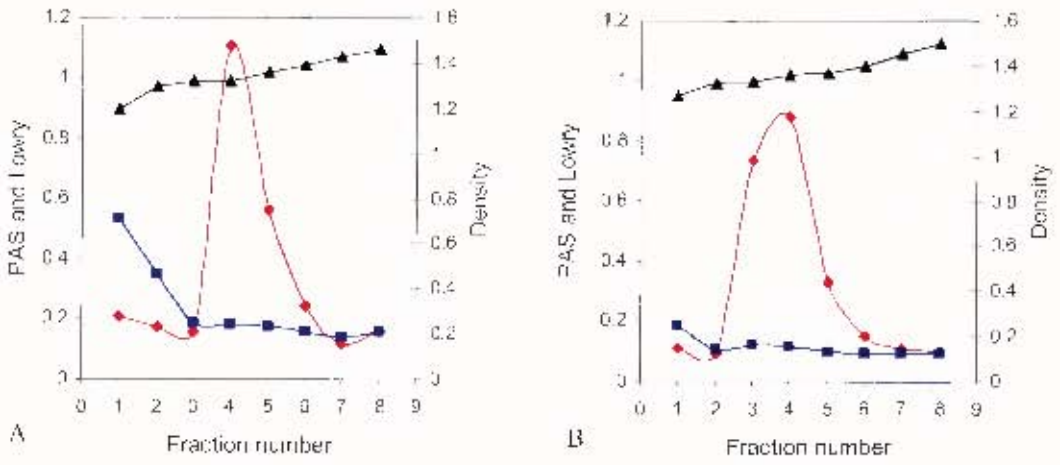


Figure 5.2 Caesium chloride isopycnic density gradient purification of pregnancy plug mucus. Freeze-dried pregnancy plug mucus was dissolved in 4M GuHCl containing 10mM EDTA, 5mM NEM and 0.05% CHAPS pH 6.5 and adjusted to a density of 1.39 to 1.40g/ml with solid caesium chloride. Density gradient centrifugation was performed in a Beckman L45 ultra-centrifuge for 48h at a 105 000g at 4°C. Mucin positive fractions (♦) at a density (▲) between 1.37-1.42 and still associated with some protein (■) (A), were pooled and prepared for the second centrifugation step (B). Finally fractions (fraction number 3, 4 and 5) were pooled, dialysed against three changes of distilled water overnight at 4°C and freeze-dried.

Subsequent to the caesium chloride isopycnic density gradient purification, the efficiency of the method and removal of the protein contaminants was assessed by SDS-PAGE. Gels were stained for carbohydrate with PAS and for protein with Coomassie Brilliant Blue R-250 (Figure 5.3). The Coomassie Brilliant Blue R-250 stained gel (Figure 5.3 lane 2) illustrated the removal of most of these protein contaminants which were visible in the Coomassie Brilliant Blue R-250 stained gel (Figure 5.1 lane 2) of the crude pregnancy plug mucus. Faint protein positive bands were still visible approximately from below 220kDa to 40kDa. The >220kDa band was also visible (Figure 5.3 lane 2). Two large diffuse bands, one at the top of the running gel and another with greater electrophoretic mobility were seen in the PAS stained gel (Figure 5.3 lane 3 red arrows).

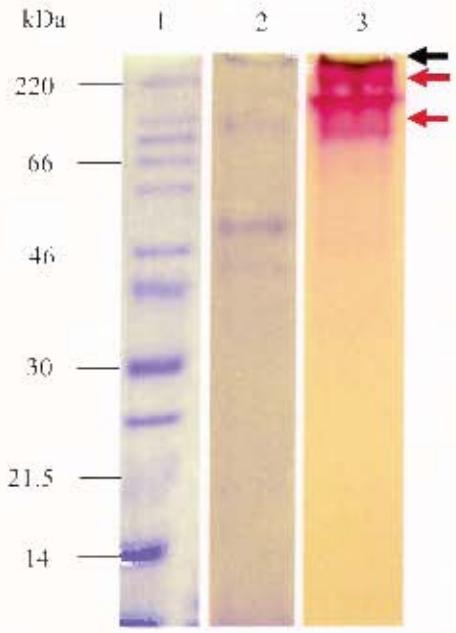


Figure 5.3 SDS-PAGE analysis of the purified pregnancy plug mucins. After caesium chloride isopycnic density gradient purification, freeze-dried pregnancy plug mucins (30µg) were prepared in reducing gel loading buffer and separated on a 10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) and PAS (lane 3). The arrows in red indicate the mucins on top of the running gel and slightly entering the running gel and the black arrow is at the start of running gel. Lane 1 is a molecular weight marker.

5.2.3 Western blotting analysis of pregnancy plug mucins

Western blot analysis was performed to determine the identity of the mucins present in the pregnancy plug mucus. Samples were loaded on a 1% agarose gel and subjected to electrophoresis. Mucins were then transferred from the gel to a nitrocellulose membrane and probed with mouse anti-MUC1 monoclonal (Figure 5.4 lanes 1, 2 and 3) and rabbit anti-MUC2 (Figure 5.4 lanes 4, 5 and 6), rabbit anti-MUC5AC (Figure 5.4 lanes 7, 8 and 9) and rabbit anti-MUC5B (Figure 5.4 lanes 10, 11 and 12) polyclonal antibodies. Western blotting confirmed the presence of MUC1, MUC2, MUC5AC and MUC5B mucins in the pregnancy plug mucus (Figure 5.4 lanes 3, 6, 9 and 12 respectively). While MUC5AC was strongly expressed (Figure 5.4 lane 9) MUC2 appeared in relatively smaller amounts and as a doublet (see the red arrows in Figure 5.4 lane 6). As shown in Figure 5.4, while the positive controls purified MUC1 (lane 1), colonic mucus (lane 4) and pseudomyxoma peritonei (lanes 7 and 10) (Chirwa *et al.*, 2007) reacted with the anti-MUC1, anti-MUC2, anti-MUC5AC and

anti-MUC5B antibodies respectively, the negative controls namely the purified salivary MUC5B (lane 2), tracheal sputum (lane 5), purified salivary MUC7 (lane 8) and gastric mucus (lane 11) did not react with the anti-MUC1, anti-MUC2, anti-MUC5AC and anti-MUC5B antibodies respectively.

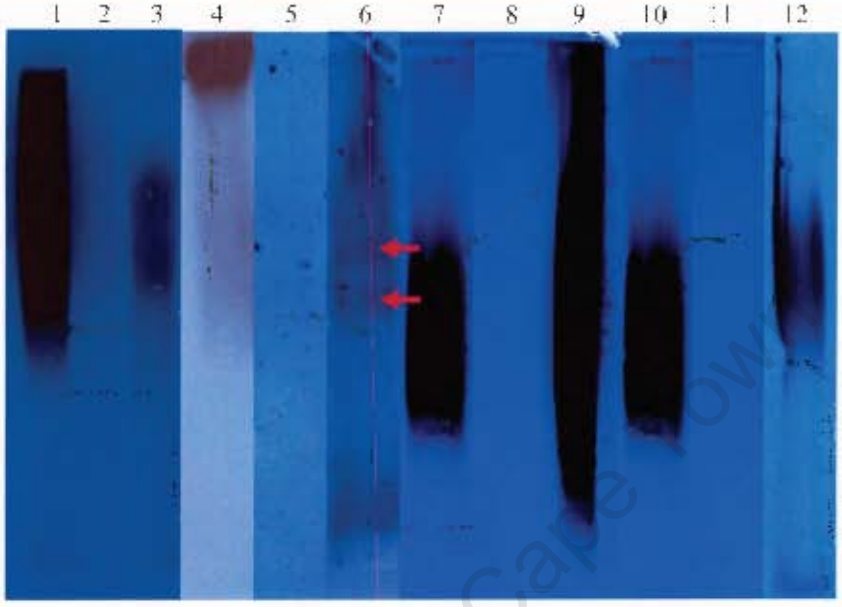
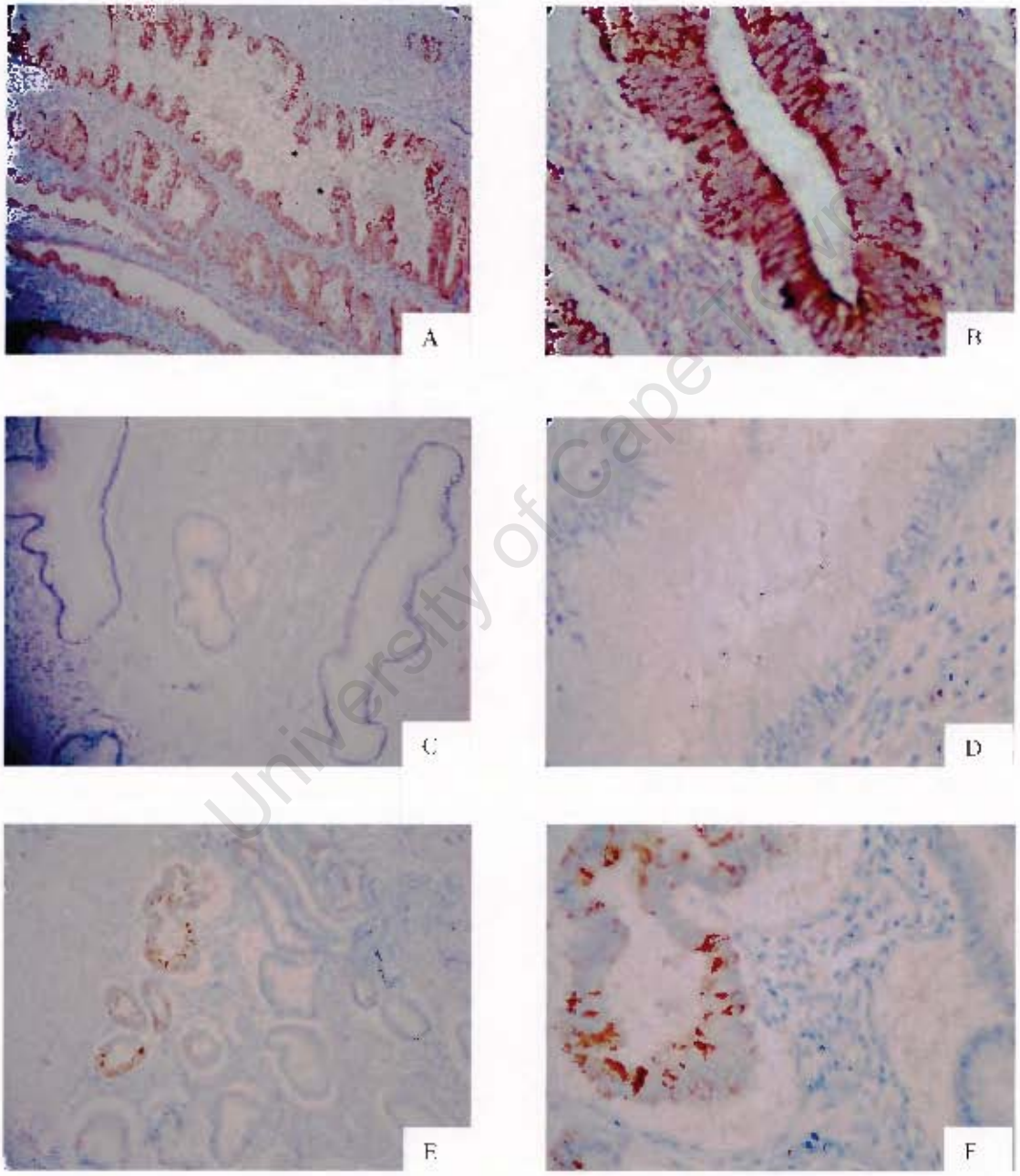


Figure 5.4 Western blotting analysis of the purified pregnancy plug mucins. Lane 1, purified MUC1 (positive control), lane 2, purified salivary MUC5B (negative control), lane 4, colonic mucus (positive control), lane 5, tracheal sputum (negative control), lane 7, pseudomyxoma peritonei (positive control), lane 8, purified salivary MUC7 (negative control), lane 10, pseudomyxoma peritonei (positive control), lane 11, gastric mucus (negative control) and lanes 3, 6, 9 and 12 purified pregnancy plug mucins were separated by a 1% agarose gel and transferred to nitrocellulose membrane. Following overnight blocking, membranes were incubated for 2h with mouse anti-MUC1 monoclonal (lanes 1, 2 and 3) and rabbit anti-MUC2 (lanes 4, 5 and 6), rabbit anti-MUC5AC (lanes 7, 8 and 9) and rabbit anti-MUC5B (lanes 10, 11 and 12) polyclonal antibodies diluted in 5% (m/v) low fat milk powder in TBST at 1 in 100 (mouse anti-MUC1), 1 in 5000 (rabbit anti-MUC2 and anti-MUC5AC) and 1 in 2000 (rabbit anti-MUC5B). The membranes were then washed 3 × 5min with TBST and incubated for 1h with HRPO linked goat anti-mouse and goat anti-rabbit secondary antibodies diluted in 5% (m/v) low fat milk powder in TBST at dilutions of 1 in 1500 and 1 in 2000 respectively. After another TBST wash (3 × 5min), bands were detected using an ECL detection kit. The red arrows in lane 6 indicate the doublet of MUC2.

5.2.4 Immuno-histochemical analysis of the mucins expression by the female reproductive tract tissues

Due to the lack of commercially and privately available antibodies for Western blotting, mucin expression was also studied in cervical and endometrial tissue by immuno-histochemical methods. Human endometrium and cervical tissues were fixed

and incubated with rabbit anti-MUC4 polyclonal and mouse anti-MUC6 monoclonal antibodies (Figure 5.5). The result demonstrated strong expression of MUC4 (Figure 5.5A and 5.5B) and low amounts of MUC6 (Figure 5.5E and 5.5F) by the endometrium epithelium. However, no expression of MUC4 (Figure 5.5C and 5.5D) and MUC6 (Figure 5.5G and 5.5H) was detected in the cervical tissues.



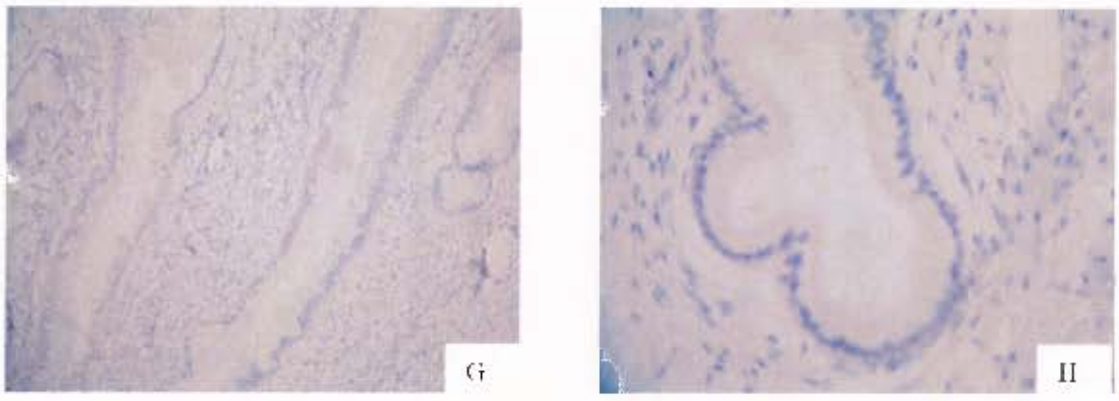


Figure 5.5 Immuno-histochemical expressions of MUC4 and MUC6 in the female reproductive tract. Human endometrium (A, B, E and F) and cervical (C, D, G and H) tissue was fixed in formalin and embedded in paraffin. Slides were incubated with rabbit anti MUC4 (A, B, C and D) and anti-MUC6 (E, F, G and H) antibodies. Tissue was counter stained as described under Materials and Methods. Slides A, C, E and G are magnified × 10 and slides B, D, F and H × 40.

5.3 Discussion

This Chapter described the isolation, purification and characterization of mucins from human pregnancy plug mucus using appropriate biochemical and immuno-histochemical techniques, with the ultimate aim of comparing their anti HIV-1 activity to that of crude mucous.

Cervical scrapings yielded very little material for biochemical analysis. It was decided to find an alternative source of mucus and mucin from the female reproductive tract that could yield sufficient material for analysis. We found the human pregnancy plug mucus, that sits on the mouth of the uterus throughout the period of pregnancy and which is very thick and large in size (Eriksen *et al.*, 1998), to be suitable for the purpose of this study. We wanted to determine whether cervical mucus and mucin behaved similarly to salivary mucus and mucin in the inhibition of the HI virus in an inhibition assay. The answer to this question would take us closer to an investigation of why the cervical route of transmission of the virus during sexual intercourse is so common, considering that mucins in the saliva have broad structural similarities to those in the cervix. It would also throw more light on the implication that cervical mucus and mucins are unable to prevent the transmission of the human immunodeficiency virus via this route.

Endogenous proteolysis was minimized by extracting mucus in 6M GuHCl and a cocktail of proteolytic inhibitors such as 10mM EDTA, 5mM NEM and 1mM PMSF (Carlstedt *et al.*, 1983). While PMSF and EDTA were added to inhibit serine and metallo-protease activity respectively, NEM was included to inhibit thiol protease activity thus preventing any possible thiol-disulfide exchange between mucins (Carlstedt *et al.*, 1982) and other proteins of the crude mucus or even non-covalent interactions with lipids, nucleic acids, lactoferrin, lysozyme, immunoglobulins and ions (Wiggins *et al.*, 2001). At the same time GuHCl, which is known to be a denaturing agent (Francis and Bradford, 1976), was used to increase the solubility of mucins (Carlstedt *et al.*, 1983).

There are strong non-covalent interactions between mucins and the other known protein and non protein components of crude mucus secretions (Tabak, 1995). In addition, mucus plug material during labour has considerable amounts of blood, making the purification of mucin by caesium chloride isopycnic density gradient centrifugation essential. The removal of these non-mucinous components is reported to be by dissociative conditions (Carlstedt *et al.*, 1982), which are capable of destroying the tertiary structure of mucins (Eriksen *et al.* 1998).

Western blotting confirmed the presence of MUC1, MUC2, MUC5AC, and MUC5B mucins in the pregnancy plug mucus. While MUC5AC appeared to be the dominant mucin as in the case of pseudomyxoma peritonei (Chirwa *et al.*, 2007), the amount of MUC2 was very low and appeared as a doublet (Govender, 2005). As the doublet was recognized by the same antibody this could suggest a similarity in their amino acid content (Bolscher *et al.*, 1999; Thornton *et al.*, 1997), but it is not clear whether this doublet is a glycoform of MUC2 or is it the result of the disulfide bond cleavage. Therefore further analysis including the amino acids, charge density and carbohydrate compositions of this doublet is required. It is important to remember that caesium chloride isopycnic density gradient centrifugation for the purification and isolation of mucins enabled us to purify MUC1, MUC2, MUC5AC and MUC5B together as these mucins appeared as a single group of purified material at a density of 1.40g/ml (Figure 5.2). We did not attempt to separate these mucins any further into their individual components, in this case. In the case of cervical mucin (pregnancy plug mucin), this purified mucin made up of MUC1, MUC2, MUC5AC and MUC5B was

used collectively in the HIV inhibition assay. Separation of individual mucin and its use in the HIV inhibition assay was more successful with saliva (MUC5B and MUC7) and breast milk (MUC1).

Due to the shortage of Western blotting antibodies against all the cloned human mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6 and MUC7), (Gipson *et al.*, 2001) the search for the other mucins expressed by the female reproductive tract had to be done by immuno-histochemical analysis. The endometrium expressed both MUC4 and MUC6 although the expression of MUC6 was very low. Cervical tissue on the other hand did not express MUC4 and MUC6. The endometrium appeared to express more mucins than the cervix, a finding which agreed with that of Gipson *et al.* (1997) in that the endocervix is a major mucin-producing gland of the female reproductive tract expressing multiple mucins.

In summary the presence of MUC1, MUC2, MUC5AC and MUC5B in the pregnancy plug mucus was confirmed by Western blotting with MUC2 expressed as a doublet and in a smaller amount compared to the other mucins. Immuno-histochemistry confirmed the expression of MUC4 and MUC6 by the endometrial tissue, but their presence in the mucus plug could not be confirmed due to the lack of antibodies to these mucins for Western blotting. This result agreed with that of Gipson *et al.* (1997), Gipson *et al.* (2001), Wickstrom *et al.* (1998) and Wiggins *et al.* (2001) studies which reported the expression of MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6 by the female reproductive tract. However, because of the close similarity in size it has been very difficult to isolate each mucin using the traditional biochemical techniques such as gel filtration, SDS-PAGE and caesium chloride isopycnic density gradient centrifugation.

The quality (glycosylation pattern) and quantity of cervical mucins during the different phases of the menstrual cycle are reported to vary either through the influence of oestrogen (proliferative phase) or of progesterone (luteal phase). For example the production of MUC5B was reported to increase at the mid-cycle and decrease during the secretory phase of the menstrual cycle whilst MUC4 increases during the luteal phase of the menstrual cycle (Argüeso *et al.*, 2002; Gipson *et al.*, 2001). The glycosylation pattern of salivary mucins specifically MUC5B is also

known to be influenced by the menstrual cycle which is hormonally regulated (see discussion part in Chapter 6). These cyclical variations, together with the fact that cervical scrapings, which yielded very small amounts of crude material, made it difficult to investigate the anti-HIV-1 activity of these mucins per se. Therefore pregnancy plug mucus which occludes the cervical canal throughout the pregnancy period (Carlstedt *et al.,* 1983; Eriksen *et al.,* 1998) was used. This large mucus plug which is more like the mucus of the luteal phase than the mucus of the mid-cycle (Carlstedt *et al.,* 1983) was obtained during labour and just prior to delivery.

As cervical mucins regulate the movement of sperm and micro-organisms to the upper reproductive tract (Argüeso *et al.,* 2002), the anti-HIV-1 activity of these mucins will be examined and compared with the salivary and breast milk mucins in Chapter 6.

CHAPTER 6

THE ANTI-HIV-1 ACTIVITIES OF CRUDE HUMAN SALIVA, BREAST MILK AND PREGNANCY PLUG MUCUS AND PURIFIED SALIVARY MUC5B AND MUC7, BREAST MILK MUC1 AND PREGNANCY PLUG MUCINS IN AN *IN VITRO* INHIBITION ASSAY

6.1 Introduction

The objective of this part of the study was to determine the anti-HIV-1 activities of crude saliva, breast milk and pregnancy plug mucus, together with purified mucins from these secretions namely, MUC5B and MUC7 from saliva, MUC1 from breast milk and the entire spectrum of pregnancy plug mucins, in an *in vitro* inhibition assay. The crude and purified material from each of these secretions was then incubated with HIV-1 and the mixture subsequently added to phytohemagglutinin (PHA) stimulated human T lymphoblastoid cell line (CEM SS cells). The cells were then cultured and viral replication was measured by a qualitative p24 antigen assay as described in the Materials and Methods section (Chapter 2).

6.2 Results

6.2.1 Toxicity assay

Prior to the HIV inhibition assay, the toxicity of the crude and purified material to the PHA stimulated CEM SS cells was determined using the toxicity assay. As shown in Table 6.1, none of the material was toxic except for MUC5B and crude breast milk, which caused only 5% cell death.

Table 6.1 Toxicity of crude saliva, MUC5B, MUC7, breast milk, MUC1, crude pregnancy plug mucus and purified pregnancy plug mucins to CEM SS cells.

Sample	Con	CEM SS cells	% of dead cells	% of live cells
Crude saliva	0.9mg	2.5×10^6/ml	0	100
MUC5B	0.9mg	2.5×10^6/ml	5	95
MUC7	0.9mg	2.5×10^6/ml	0	100
Breast milk	0.9mg	2.5×10^6/ml	4	96
MUC1	0.9mg	2.5×10^6/ml	0	100
Pregnancy plug mucus	0.9mg	2.5×10^6/ml	0	100
Pregnancy plug mucins	0.9mg	2.5×10^6/ml	0	100

The percentage of live cells was calculated as the number of live cells over total cells (live plus dead) × 100.

6.2.2 The anti-HIV-1 activities of human crude saliva and purified salivary MUC5B and MUC7 mucins

To determine the anti-HIV-1 activities of human crude saliva and purified salivary MUC5B and MUC7 mucins, HIV-1 was incubated with these secreted substances for 60min prior to addition to the CEM SS cells. It was shown that when HIV-1 was separately mixed with each component namely, crude saliva, MUC5B or MUC7 and then each mixture incubated with CEM SS cells for 30min, crude saliva and purified salivary MUC5B and MUC7 mucins inhibited HIV-1 activity by approximately 97.6%. Only 2.4% of the cells were infected as measured by the p24 antigen assay (Figure 6.1). To determine the effect of time or the length of the incubation period on the inhibition of the HIV-1 activity, the CEM SS cells were incubated with HIV-1 plus crude saliva, or MUC5B or MUC7 for longer time periods (1h and 3h). There was no difference on the rate of inhibition of HIV-1 or infection of CEM SS cells for different lengths of time (Figure 6.1).

In the controls, when HIV-1 was treated with the media instead of mucins prior to addition to the CEM SS cells, 100% HIV-1 replication or infection of the CEM SS cells was detected (Figure 6.1). The heat inactivated HIV-1 was also shown to cause approximately 30% infection of the CEM SS cells (Figure 6.1).

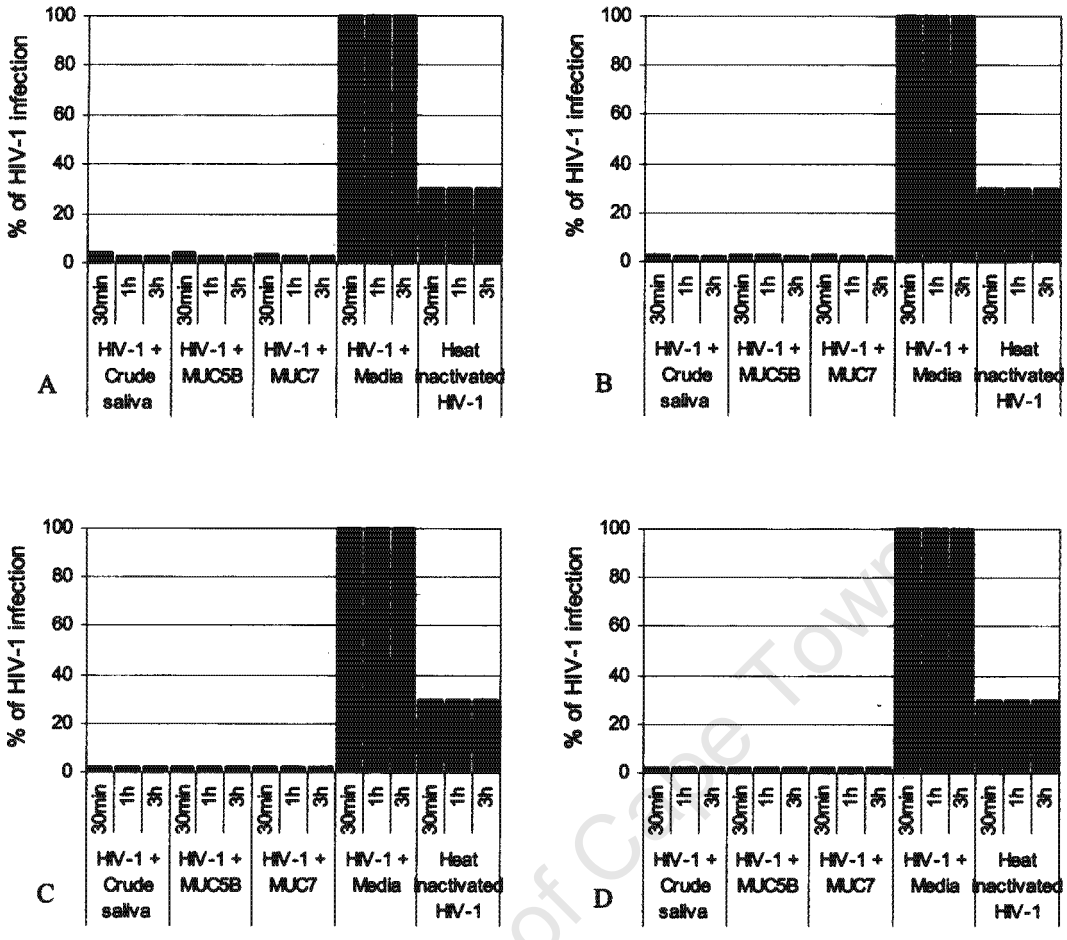


Figure 6.1 Inhibition of HIV-1 activity by human crude saliva and purified salivary MUC5B and MUC7 mucins in an *in vitro* assay. Crude saliva and purified salivary MUC5B and MUC7 mucins (500µl or 0.9mg each) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The unfiltered samples were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 3h. After PBS wash, cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B, C and D indicate the anti-HIV-1 activity of each sample in a serial tenfold dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively.

To determine the lowest concentration at which mucins inhibited viral activity, serial tenfold dilutions (10^{-1} to 10^{-4}) of the crude saliva and salivary MUC5B and MUC7 mucins were done in triplicate. No difference in the anti-HIV-1 activity of the samples was detected down to a dilution of the mucin to 10^{-4} (Figure 6.1A, B, C and D).

To determine the mechanism of the anti-HIV-1 activity of these mucins, mixtures of HIV-1 with either crude saliva, MUC5B or MUC7, or with media only (control), incubated for an hour, were filtered through a 0.45µm pore size cellulose acetate filter (25mm diameter). The filtrates were subsequently incubated with the CEM SS cells

for 30min. While the filtrate from the control caused 100% infection of the CEM SS cells (Figure 6.2), that of the other groups in which mucus or mucins were present gave only 2.4% infection of the CEM SS cells (Figure 6.2). Incubation of these filtrates with the CEM SS cells for longer time periods of 1h and 3h gave no difference in inhibition (Figure 6.2). The results suggest that HIV-1 inhibition is by a physical aggregation of the virus by the mucin.

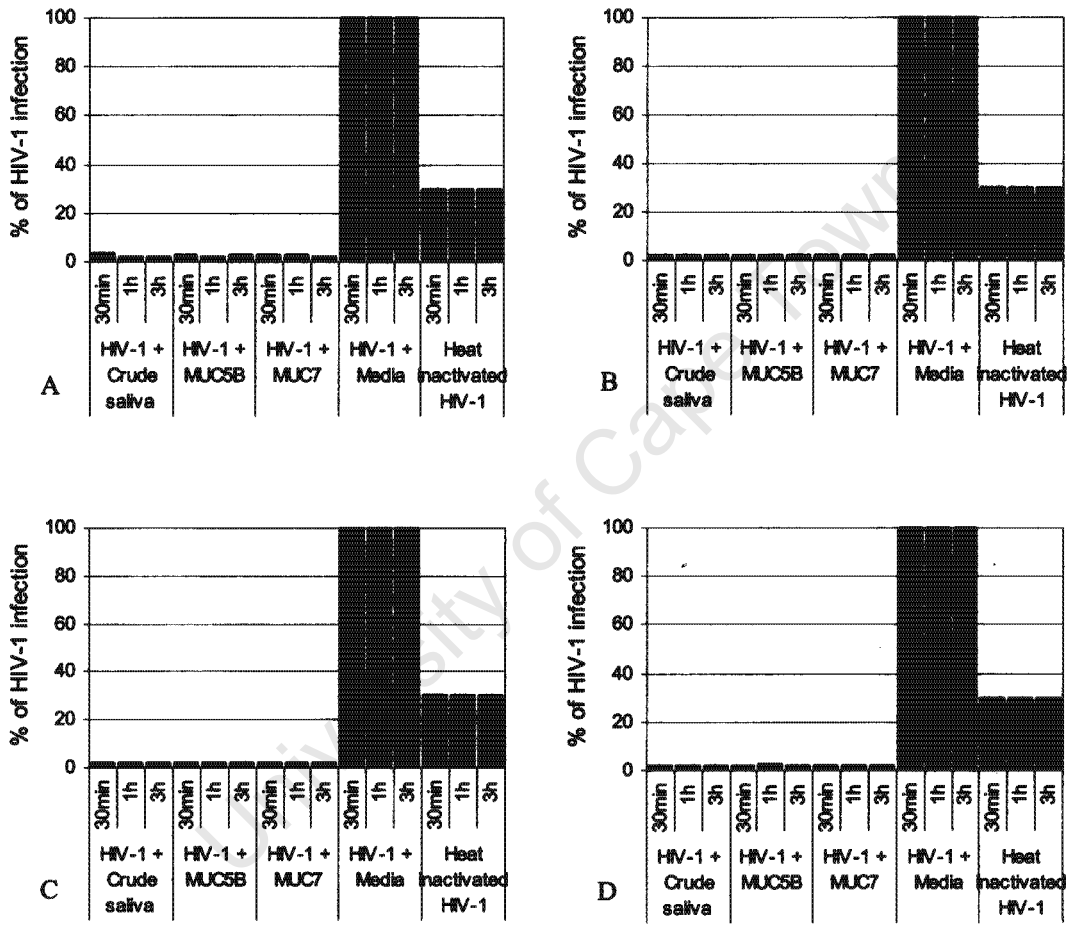


Figure 6.2 Inhibition of HIV-1 activity by human crude saliva and purified salivary MUC5B and MUC7 mucins in an *in vitro* assay. Crude saliva and purified salivary MUC5B and MUC7 mucins (500µl or 0.9mg each) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The filtrates of the mixtures were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 3h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B, C and D indicate the anti-HIV-1 activity of each sample in a serial tenfold dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively.

6.2.3 The anti-HIV-1 activities of human crude breast milk and purified milk mucin (MUC1)

An experiment similar to the one above for saliva and its components (Section 6.2.2), was performed with human breast milk and the MUC1 purified from milk. The HIV-1 virus was incubated with human breast milk and purified milk mucin (MUC1) prior to addition to the CEM SS cells for 30min. As controls HIV-1 plus media and heat inactivated HIV-1 were used. While the purified milk mucin (MUC1) inhibited HIV-1 activity by an approximately 97.2%, the crude breast milk unlike the crude saliva (Figure 6.1) did not inhibit HIV-1 infection; instead 100% HIV-1 replication was detected (Figure 6.3). In the controls, when media was used instead of MUC1 there was 100% infection of the CEM SS cells (Figure 6.3). When the CEM SS cells were infected with the heat inactivated HIV-1, an approximately 30% infection of the CEM SS cells was also detected (Figure 6.3).

To determine the effect of time on the infection of the CEM SS cells or viral inhibition, mixtures of HIV-1 with either crude breast milk or purified MUC1, were incubated with the CEM SS cells for 1h and 3h. No difference in the rate of infection of the CEM SS cells or inhibition of viral activity was detected (Figure 6.3).

Serial tenfold fold dilutions (i.e. 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}), giving different concentration of mucin also showed no difference in the anti-HIV-1 activity of MUC1, down to a dilution of 10^{-4} of the original 0.9mg of mucin (Figure 6.3A, B, C and D).

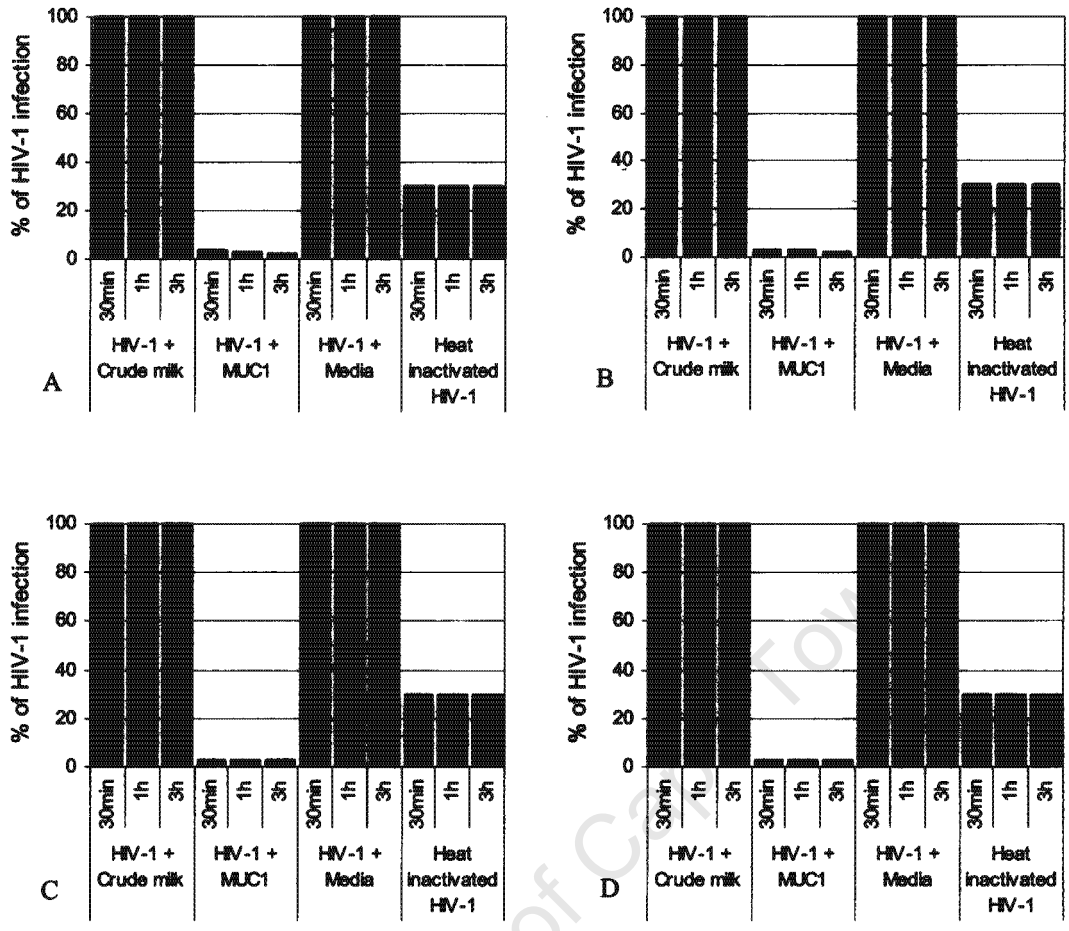


Figure 6.3 Inhibition of HIV-1 activity by human breast milk and purified milk mucin (MUC1) in an *in vitro* assay. Crude breast milk and purified MUC1 (500µl or 0.9mg each) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The unfiltered samples were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 3h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B, C and D indicate the anti-HIV-1 activity of each sample in a serial tenfold dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively.

To determine the mechanism of the anti-HIV-1 activity of milk and milk MUC1, mixtures of HIV-1 with either crude breast milk or MUC1, or with media only (control), incubated for an hour, were filtered through a 0.45µm pore size cellulose acetate filter (25mm diameter). The filtrates were subsequently incubated with the CEM SS cells for 30min. While the filtrate from the HIV-1 plus MUC1 mixture caused very low or an approximately 2.8% viral replication or infection of the CEM SS cells (Figure 6.4), 100% HIV-1 replication or infection of the CEM SS cells was detected when the filtrates from the mixtures, HIV-1 plus breast milk and the control,

HIV-1 plus media were used (Figure 6.4). It is very likely that once again the MUC1 inhibited the virus by physical aggregation.

Again, no difference in the rate of inhibition or viral infection due to the length of the incubation period or as a result of serial dilutions ($10^{-1}$to 10^{-4}) of mucin was observed (Figure 6.4A, B, C and D).

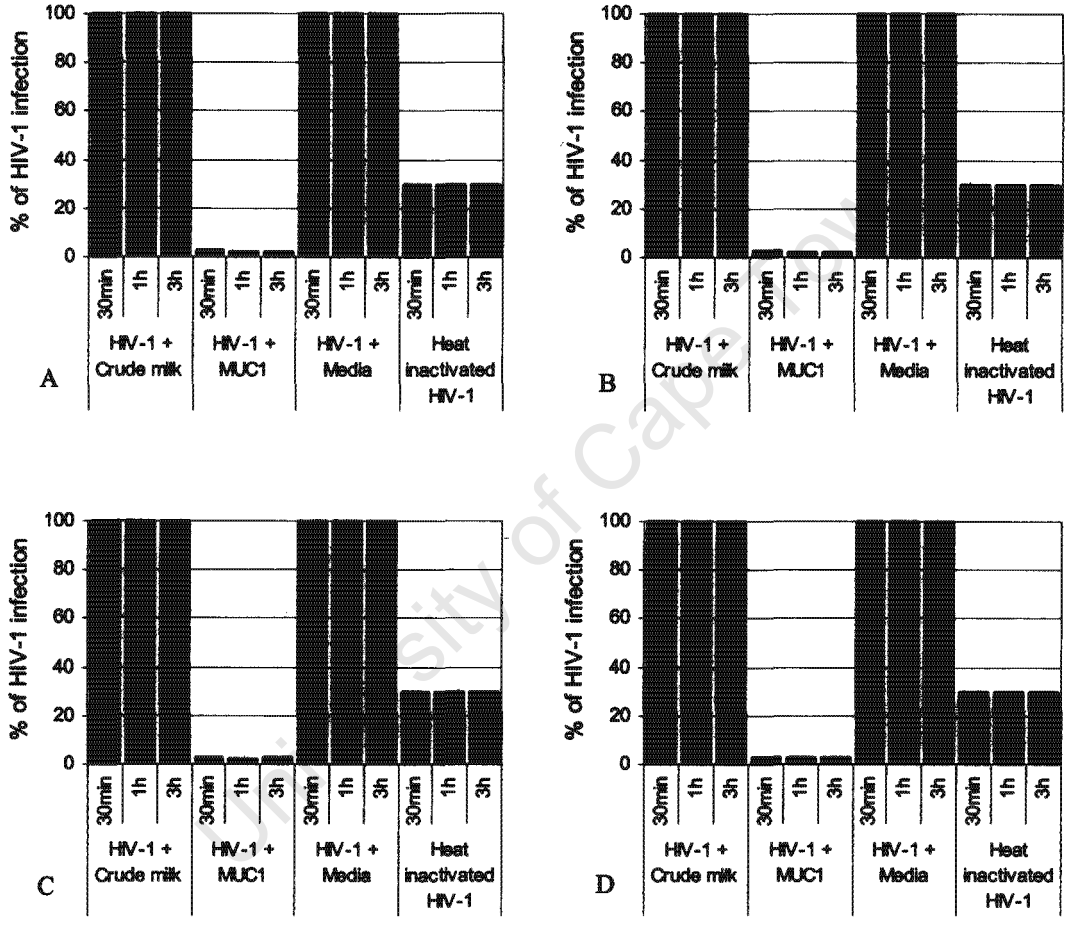


Figure 6.4 Inhibition of HIV-1 activity by human breast milk and purified milk mucin (MUC1) in an *in vitro* assay. Crude breast milk and purified MUC1 (500µl or 0.9mg each) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The filtrates of the mixtures were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 3h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B, C and D indicate the anti-HIV-1 activity of each sample in a serial tenfold dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively.

6.2.4 The anti-HIV-1 activities of human crude pregnancy plug mucus and purified pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6)

Like the above two assays (Sections 6.2.2 and 6.2.3) the anti-HIV-1 activities of crude pregnancy plug mucus and purified pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) was determined by incubating HIV-1 with these substances for 60min prior to addition to the CEM SS cells for 30min. As controls HIV-1 plus media and heat inactivated HIV-1 were used. It was shown that while the purified pregnancy plug mucins inhibited HIV-1 activity by about 97.5% (Figure 6.5), the crude pregnancy plug mucus unlike the crude saliva (Section 6.2.2) but like the crude breast milk (Section 6.2.3) did not inhibit HIV-1 activity. Instead 100% viral infection of the CEM SS cells was measured by the p24 antigen assay (Figure 6.5). The same result as in Sections 6.2.2 and 6.2.3 was obtained with both controls (i.e. media and heat inactivated HIV-1).

As in Sections 6.2.2 and 6.2.3, the effect of time (incubation period) on the rate of inhibition of the HIV-1 activity or infection of the CEM SS cells as well as the highest dilution (lowest concentration) of these mucins which can cause HIV-1 inhibition was also determined. The results were similar to those previously obtained and there was no effect of time or length of incubation or the dilution of crude and purified samples down to 10^{-4} (Figure 6.5A, B, C and D).

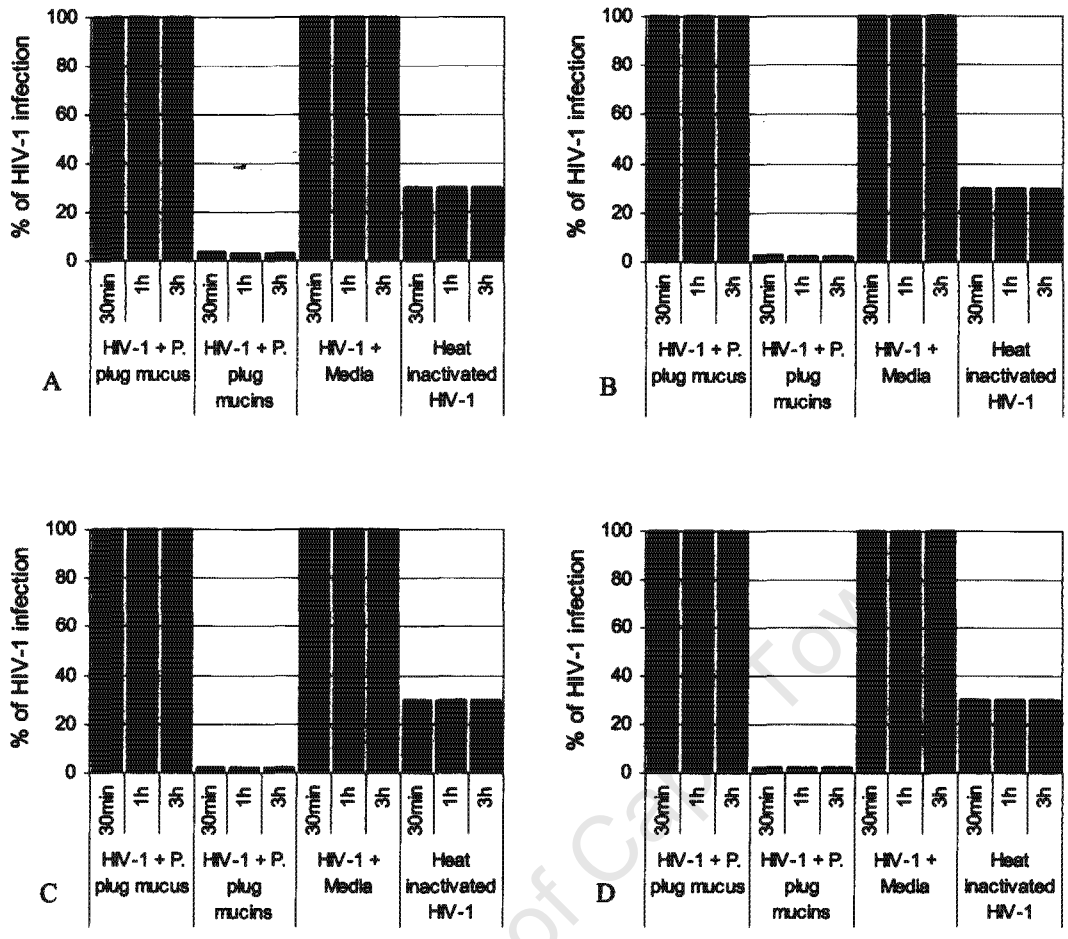


Figure 6.5 Inhibition of HIV-1 activity by human pregnancy plug mucus and purified pregnancy plug mucins in an *in vitro* assay. Crude pregnancy plug mucus and purified pregnancy plug mucins (500µl or 0.9mg each) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The unfiltered samples were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 3h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B, C and D indicate the anti-HIV-1 activity of each sample in a serial tenfold dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively. P. plug represents pregnancy plug.

To determine if the anti-HIV-1 activity of pregnancy plug mucins is by viral aggregation, the mixtures of HIV-1 with either crude pregnancy plug mucus or purified pregnancy plug mucins or with media only (control), incubating for an hour, were filtered through 0.45µm pore size cellulose acetate filter. The filtrates were subsequently incubated with the CEM SS cells for 30min. While the filtrate from the HIV-1 plus purified pregnancy plug mucins mixture caused approximately 2.5% viral replication or infection of the CEM SS cells (Figure 6.6), 100% HIV-1 replication or infection of the CEM SS cells was detected when the filtrates from the mixtures, HIV-

1 plus crude pregnancy plug mucus and the control, HIV-1 plus media were used (Figure 6.6). Once more there was no effect of time or length of incubation or the dilution of crude and purified samples down to 10^{-4} (Figure 6.6A, B, C and D).

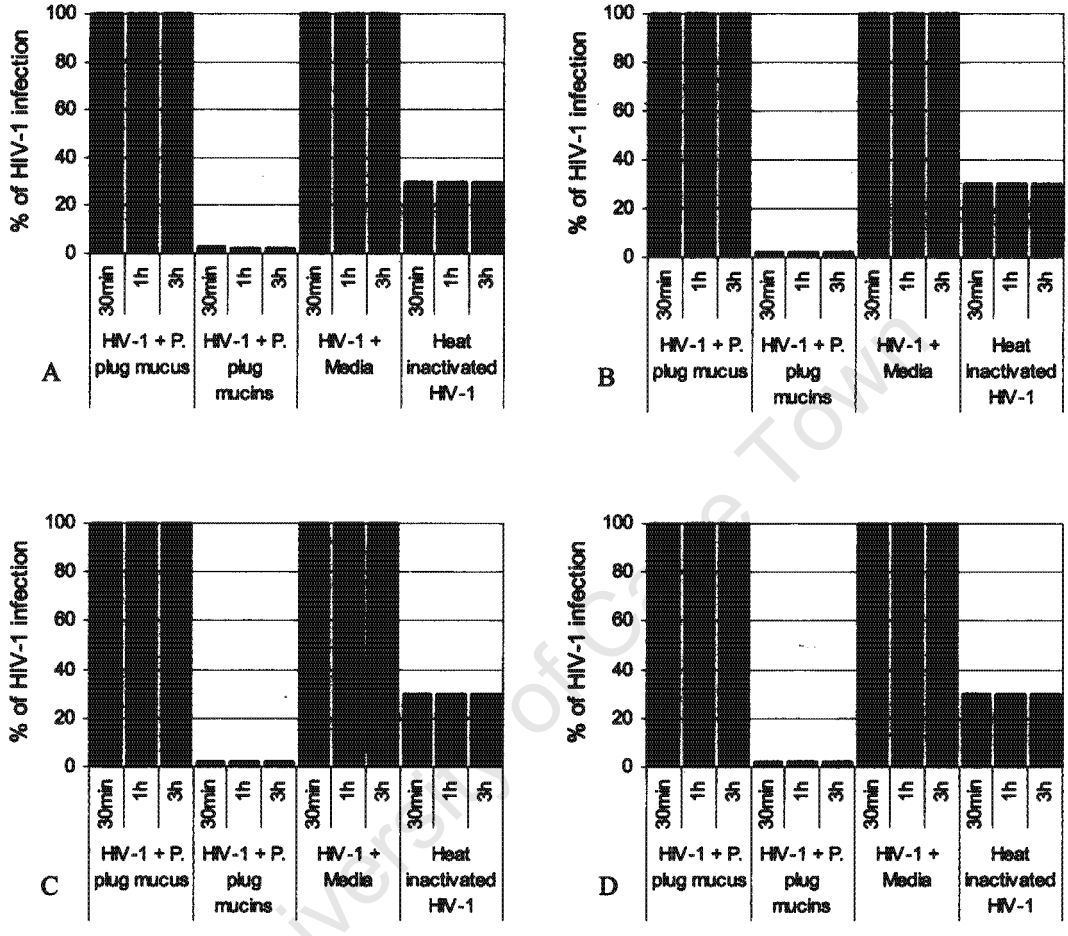


Figure 6.6 Inhibition of HIV-1 activity by human pregnancy plug mucus and purified pregnancy plug mucins in an *in vitro* assay. Crude pregnancy plug mucus and purified pregnancy plug mucins (500µl or 0.9mg each) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The filtrates of the mixtures were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 3h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B, C and D indicate the anti-HIV-1 activity of each sample in a serial tenfold dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively. P. plug represents pregnancy plug.

6.2.5 Inhibition of HIV-1 activity by blocking the putative viral binding sites (receptors) of the CEM SS cells using MUC7

To determine whether MUC7 can inhibit HIV-1 infection by blocking the putative viral binding sites on the surface of the CEM SS cells, the cells were incubated with

different concentrations of purified salivary MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) prior to addition of the HIV-1. The result revealed that even at the highest concentration (1mg), MUC7 did not inhibit HIV-1 infection of the CEM SS cells (Figure 6.7). The results were very similar to that in the positive control where the CEM SS cells were incubated with PBS prior to addition of the HIV-1 and 100% viral infection was detected. However, no viral infection was detected on the CEM SS cells alone which were used as a negative control (Figure 6.7).

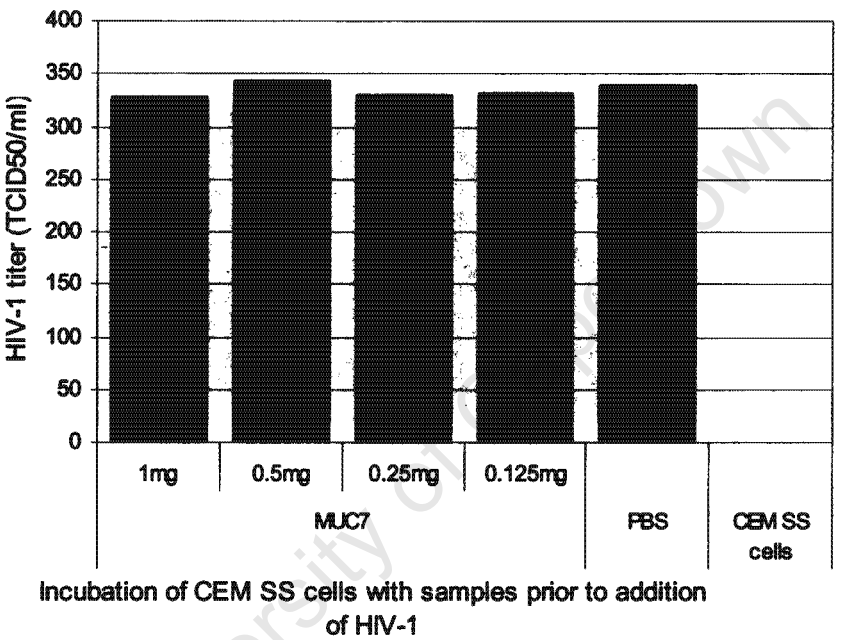


Figure 6.7 Inhibition of HIV-1 activity by blocking the putative viral binding sites (receptors) of the CEM SS cells using MUC7 in an *in vitro* assay. The CEM SS cells were incubated with different concentrations of salivary MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) for 60min. As controls CEM SS cells treated with PBS (positive) and CEM SS cells only (negative) were used. At the end of the incubation period HIV-1 was added to the mixtures except to the negative control. Cells were then cultured and viral replication was measured by a qualitative p24 antigen assay.

6.3 Discussion

In this Chapter the anti-HIV-1 activity of crude human secretions in the form of saliva, breast milk and pregnancy plug mucus, together with purified mucin from saliva (MUC5B and MUC7), breast milk (MUC1) and from pregnancy plug mucus (a combination of MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) was determined in an *in vitro* inhibition assay. These mucins were incubated with HIV-1

and subsequently added to PHA stimulated CEM SS cells. The inhibition of HIV-1 activity or infection of the CEM SS cells was then measured using the p24 antigen assay.

The HIV-1 Subtype C virus is the most widely prevalent strain in the Southern African region whereas the Subtype D virus which was used in this study was found during the early HIV epidemic in South Africa and used to establish an *in vitro* inhibition assay. It is still prevalent in this region, albeit to a lesser extent. The Subtype D strain is perhaps the only available laboratory adapted strain used in an HIV assay in the entire country. As described in the Materials and Methods section (Chapter 2), the virus was first isolated from an AIDS patient by the Department of Medical Virology, Tygerberg Hospital, University of Stellenbosch, South Africa, in February 1988, and was fully characterised and sequenced (Treurnicht *et al.,* 2002). The human T lymphoblastoid cell line (CEM-SS cells) which are used in this assay are known to produce a distinct and quantifiable syncytia formation in four to six days when infected with HIV-1 (Nara *et al.*, 1987). This cell line is reported to express CD4, CXCR4, ICAM-3 and MHC class II molecules (Lallos *et al.,* 1999), thus making them suitable as host cells for use in an HIV infection assay.

Guanidinium chloride (GuHCl) is a known denaturant (Francis and Bradford, 1976) and is commonly used, at a molar concentration of 6M, to prevent endogenous proteolysis in the extraction of mucin from a variety of tissue sources (Carlstedt *et al.,* 1983; 1982). This chaotropic agent is also (expectedly) known to denature viral protein and has been reported to kill viruses such as molluscum contagiosum virus (MCV) which is a member of the poxvirus group by more than 99.9% (Francis and Bradford, 1976). It was therefore imperative that the GuHCl in the buffers used for sample collection, chromatography and caesium chloride isopycnic density gradient centrifugation was later removed from the salivary, milk and pregnancy plug mucins by extensive dialysis against distilled water. Mucins were then freeze dried and re-suspended in 25% PBS buffer for use in the *in vitro* inhibition assay. In the case of crude salivary, breast milk and cervical secretion (pregnancy plug mucus) in which purification or dissociation of mucins from non-mucinous components was not required, samples were collected in cold 0.1M Tris-HCl, 2% (w/v) EDTA and 5mM

PMSF pH 7.5. This buffer was also used as a control and found not to be toxic to the virus.

Kennedy *et al.* (1998) and Malamud *et al.* (1993; 1997) have shown that human whole and submandibular saliva inhibit HIV activity in an inhibition assay. However, the anti-HIV activity of the whole and submandibular saliva was reported to be considerably reduced after filtration. It has been suggested that the macromolecular components, specifically the mucus components of the oral secretions (Bergey *et al.*, 1994; Bolscher *et al.*, 2002) are the most likely candidates in the oral defence against HIV infection. These observations coincide with the idea of Malamud *et al.* (1993) that saliva induces viral aggregation to clear viruses from the oral cavity and subsequently reduces transmission of HIV through saliva. Although these researchers speculated that crude saliva (Fox *et al.*, 1988; Fultz, 1986), MUC5B and MUC7 (Bolscher *et al.*, 2002), the two secreted salivary mucins (Thornton *et al.*, 1999), are potentially involved in inhibition of HIV infection by aggregating the viral particles, a definitive attempt to purify, identify and test these and other purified mucins from sites where infection is known to occur, has not been done. Therefore the objective of this study was to purify, identify and determine the anti-HIV-1 activities of salivary MUC5B and MUC7 mucins in an *in vitro* inhibition assays separately, and compare these with the anti-HIV-1 activities of the breast milk and cervical mucus secretions from regions that are known routes of transmission of the virus.

As shown in the results section crude saliva and purified salivary MUC5B and MUC7 mucins inhibited HIV-1 activity by approximately 97.6%. Though the mechanism of inhibition is not clear, it is possible that carbohydrate moieties or side-chains of mucins, that constitute 50 to 85% of the glycoprotein (Klein *et al.*, 1992, Leikauf *et al.*, 2002) aggregate the virus particles physically upon incubation with them, a purely physical phenomenon, thus inhibiting the virus from entering the host cells (CEM SS cells). This hypothesis is in keeping with the findings of Bosch *et al.* (2000) and Prakobphol *et al.* (1999) that the carbohydrate moieties of salivary mucins serve as an attachment site for a range of bacteria and viruses and inhibit bacterial or viral entry by aggregating these pathogenic micro-organisms, (Klein *et al.*, 1992, Prakobphol *et al.*, 1998, Prakobphol *et al.*, 2005; Yeh *et al.*, 1992a). This was further supported by the finding that the terminal sialic acid residues of the salivary mucins interact with

influenza virus and block the attachment of the virus to host cells (Levine *et al.,* 1987; Tabak *et al.,* 1982; Tabak, 1995). This phenomenon is diagrammatically represented in Figure 6.8.

Despite contradictory reports that proline-rich proteins (Robinovitch *et al.*, 1993) and secretory leukocyte protease inhibitors (SLPI) (McNeely *et al.*, 1995 and 1997) inhibit HIV activity, Turpin *et al.* (1996) demonstrated the failure of the secretory leukocyte protease inhibitor to inhibit or aggregate HIV-1 particles. Bolscher *et al,* (2002) also demonstrated the failure of HIV-1 inhibition or aggregation by parotid secretion which is the source of proline-rich proteins, but which lack mucin. These findings made salivary mucins the ideal candidates to investigate in the inhibition of HIV through saliva. Thus the focus of this study was only on crude material and purified mucins and their role in the inhibition of this virus.

To confirm the hypothesis that mucins inhibit viral activity by aggregating the virus, the CEM SS cells were infected or incubated with filtrates from the mixtures, HIV-1 plus crude saliva, HIV-1 plus MUC5B and HIV-1 plus MUC7. However, very low or only 2.4% HIV-1 infection of the CEM SS cells was detected. This suggested that HIV-1 may bind to the high-molecular weight salivary mucins which results in a macromolecular complex which is removable during filtration through 0.45μm pore filter paper (Archibald and Cole, 1990; Ge *et al.,* 2004; Nagashunmugam *et al.,* 1997; Shugars and Wahl, 1998; Shugars *et al.,* 1999). Thus there may not be enough viruses left un-aggregated by the mucins to pass through the filter paper and be present in the filtrates to bind and cause viral infection of the CEM SS cells. This finding agreed with that of Bobek and Situ (2003) that salivary mucins agglutinate or trap micro-organisms, thus clearing them from the oral cavity. This hypothesis also strengthens the report by Situ *et al.* (2003) that salivary mucins do not directly kill micro-organisms but instead aggregate the pathogens and clear them from the oral cavity.

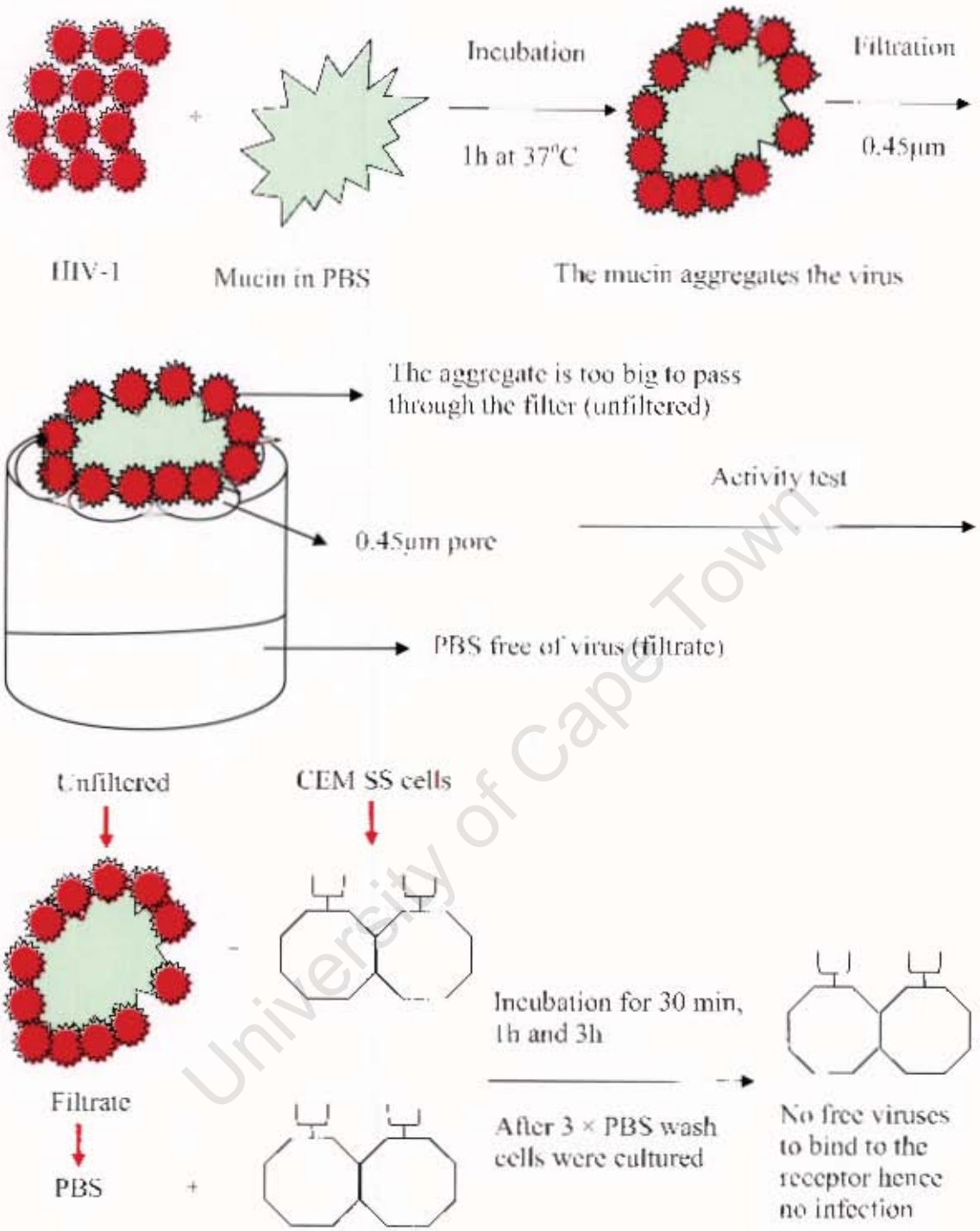


Figure 6.8 A sketch to explain our hypothesis of infection and its inhibition. After incubation of HIV-1 with mucins for 60min, the mixtures were filtered through 0.45µm pore size cellulose acetate filter. Both the unfiltered and filtrate samples were incubated with CEM SS cells for 30min, 1h and 3h. After PBS wash cells were cultured in media (RPMI 1640 with 10% fetal calf serum and IL-2) and viral replication was measured by a qualitative p24 antigen assay.

Further support for the above hypothesis was that when HIV-1 was incubated with media instead of the mucins, 100% HIV-1 infection of the CEM SS cells was detected. Incubation of CEM SS cells with the filtrate from the mixture, HIV-1 plus media also resulted in 100% viral infection of the CEM SS cells (Figure 6.9). This suggested that the media could not aggregate the virus as the high-molecular weight salivary mucins did; hence there are free viruses that could bind or enter the CEM SS cells and cause infection. As the media did not aggregate the virus which is small enough to pass through a 0.45μm pore size cellulose acetate filter, the viruses are expected to be present in the filtrate and cause infection upon addition to the host cells as was the case here.

Another important question was whether CEM SS cells would be infected if they were treated or incubated with mucins prior to the addition of the HIV-1. This study showed that when the CEM SS cells were treated or incubated with different concentrations of MUC7 prior to the addition of the HIV-1, no inhibition of infection of the cells was detected (i.e. 100% infection occurred). This suggested that there may be no receptors for mucins on the CEM SS cells and so there could not possibly be a competition between the virus and mucins for viral binding sites on the cells. However, electron microscopy is required to further clarify this finding.

As salivary mucins are thought to inhibit HIV-1 activity by physically aggregating the viruses and preventing host cell entry (Bergey *et al.*, 1994; Malamud *et al.*, 1993; Malamud *et al.*, 1997; Nagashunmugam *et al.*, 1997), a major debating point is whether this aggregation is specific or non-specific. However, the presence of infectious microbial agents such as herpes simplex virus (HSV), HIV-2, simian immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, hepatitis B virus, *Mycobacterium avium* and adenovirus in the oral cavity or saliva (Bosch *et al.*, 2000; Crombie *et al.*, 1998; Fox *et al.*, 1988; Malamud *et al.*, 1993; Nagashunmugam *et al.*, 1997; Tabak, 1995; Wagner *et al.*, 1996; Wu *et al.*, 2003) suggests that the aggregation of HIV-1 by crude saliva or purified salivary mucins could require some specificity. This suggestion is supported by the report of Bobek and Situ (2003) that salivary mucins selectively bind to pathogenic micro-organisms and prevent their binding to the host cell receptors and subsequent infections. Furthermore Prakobphol

et al. (2005) reported the ability of saliva to selectively bind and clear pathogenic micro-organisms from the oral cavity.

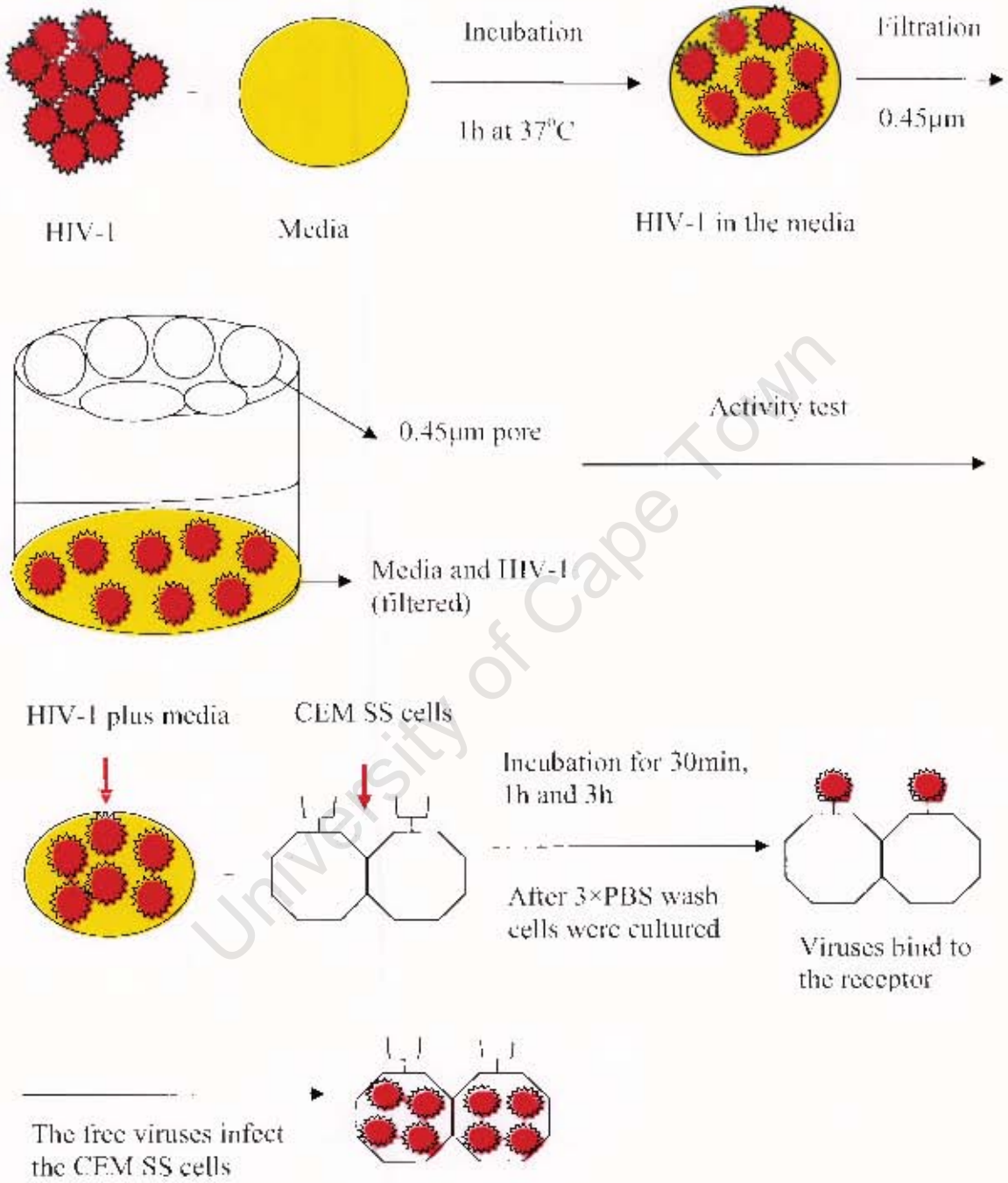


Figure 6.9 A sketch to explain our hypothesis of infection and its inhibition. After incubation of HIV-1 with media (control) for 60min, the mixture was filtered through 0.45µm pore size cellulose acetate filter. The filtrate was incubated with CEM SS cells for 30min, 1h and 3h. After PBS wash cells were cultured in media (RPMI 1640 with 10% fetal calf serum and IL-2) and viral replication was measured by a qualitative p24 antigen assay.

As the carbohydrate moieties of salivary mucins serve as an attachment sites for bacteria and viruses and inhibit host tissue entry by aggregating these pathogenic micro-organisms (Bosch *et al.*, 2000; Prakobphol *et al.*, 1999), changes on the pattern of glycosylation may affect their activity against these pathogens. Prakobphol *et al.* (2005) demonstrated that in females the glycosylation pattern of salivary mucins specifically MUC5B is strongly influenced by the menstrual cycle which is hormonally regulated. According to these authors the glycosylation pattern or expression of MUC5B was shown to be very low on the day first after menstruation but increases with the cycle until a few days after ovulation. The rate of glycosylation is highest during pregnancy and lactation; however it is constant in males and post menopausal women. According to Nagashunmugam *et al.* (1997), the antiviral activity of saliva also varies among individuals. As the effect of menstrual cycle on the anti-HIV activities of saliva and salivary mucins is not known, collection of saliva at different phases of the menstrual cycle and determining the anti-HIV-1 activities of the salivary mucins especially MUC5B, is required.

The amount or concentration of human salivary mucins is reported to decline with age (Bolscher *et al.*, 1999). Denny *et al.* (1991) demonstrated the concentrations of salivary MUC5B and MUC7 mucins in both unstimulated and stimulated saliva samples to decline significantly in the aged group compared to a younger group. Tabak *et al.* (1982) also reported the difference in sugar content of the salivary mucins in relation to age with the adult group possessing twice the amount compared with the younger group. Although the frequent use of medication in aged individuals was reported to be responsible for decline in the potency, amount and function of saliva, Denny *et al.* (1991) report that the loss of submandibular parenchyma with age should be the prime reason. With this in mind, the test for anti-HIV-1 activities of the crude saliva and salivary MUC5B and MUC7 mucins of different age groups is essential.

Human breast milk is known to protect the breast fed infants against bacteria, viruses and toxins thus minimizing diseases such as diarrhea, respiratory diseases, and otitis media (Wiederschain and Newburg, 2001). Examples include inhibition of cholera toxin and *Escherichia coli* by human breast milk glycolipid and inhibition of rotavirus and S-fimbriated *E. coli* activities by human breast milk mucin both *in vitro* and *in*

vivo (Peterson *et al.*, 1998; Yolken *et al.*, 1992). The cervical secretion which clears millions of micro-organisms a day is also considered to be the first line of defence in the female reproductive tract (Wiggins *et al.*, 2001). However, despite these defensive mechanisms and the presence of mucus and mucins, breast milk and the cervical tract still remain significant routes of transmission for the HI virus.

One of the important questions raised by this study was the role of mucus in breast milk and the cervix (pregnancy plug), which clearly seemed not to be effective against the virus, unlike crude saliva, a worthwhile consideration especially because of the known high incidence of mother to child transmission via breast feeding and transmission by sexual intercourse through the vaginal/cervical routes. Thus it was decided to purify the breast milk mucin and cervical mucins from breast milk and pregnancy plug mucus and to determine and compare their anti-HIV-1 activity to that of salivary mucins in an HIV inhibition assay.

As described in the results section, while the purified milk mucin (MUC1) inhibited HIV-1 activity by about 97.2%, the crude breast milk did not inhibit HIV-1 activity. Though the mechanism of inhibition is not clear, the carbohydrate moieties of the MUC1 mucin may play a role in aggregation of the virus and inhibit host cells entry. This hypothesis is in agreement with that of Newburg (1999) that the human milk mucin carbohydrate moieties, which act as host cell receptors, may aggregate the pathogenic micro-organisms and inhibit them from entering and infecting the host cells. According to Newburg (1999) and Peterson *et al.* (1998) the removal of sialic acid from the milk mucin hinders the anti-rotaviral activity of the mucin, indicating that the carbohydrate moieties of mucins plays a crucial role in the inhibition of viral activities. This hypothesis is represented in a schematic form in Figure 6.10.

The ability of MUC1 to inhibit HIV-1 activity by aggregating the virus was confirmed as the filtrate from its mixture with HIV-1 caused very low (2.8%) infection of the CEM SS cells compared to the 100% infection by filtrates from the mixtures, HIV-1 plus crude breast milk and HIV-1 plus media. As explained in the case of saliva and salivary mucins, the milk MUC1 may aggregate the viruses and thus there may not be enough free viruses in the filtrates to cause infection of the CEM SS cells. Since the crude breast milk and media failed to aggregate the virus, their presence would be

expected in the filtrates of these materials, which then would be able to infect the CEM SS cells.

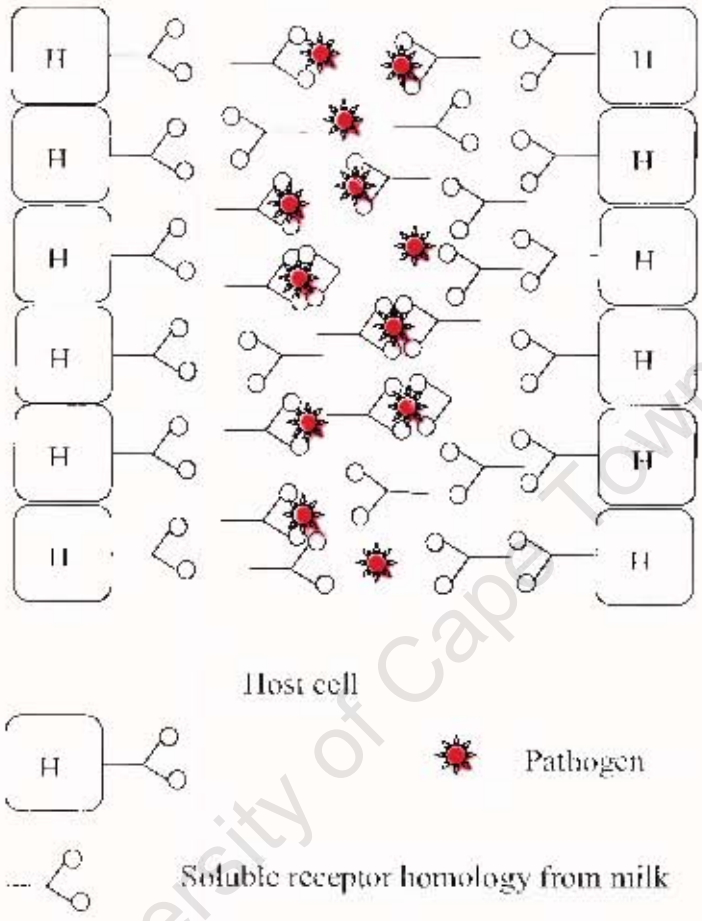


Figure 6.10 Schematic representation of inhibition of pathogen binding to their host cells by the milk mucin carbohydrates. In breast fed infants the carbohydrate complexes of the milk mucin aggregate the pathogens non-covalently and inhibit them from binding and entering the host cells, thus inhibiting infections (reproduced from Newburg, 1999).

Newburg *et al.* (1992) and Newburg (1999) show the ability of the milk mucin to bind or block host cell receptors and subsequently inhibit the binding of the pathogenic micro-organisms to the host cell receptors and cause infection. However in this study no such mucin-cell receptor activity was detected as a means of preventing HIV entry into host cells/ CEM SS cells. We suggested that mucins physically aggregate the virus through an interaction with carbohydrate side-chains. The interaction could be purely a physical entanglement through a charge interaction considering that mucins have a high density of negative charges through sialic acid and sulphate residues (Allen, 1978; Jones, 1978; Reid, 1978). This was supported by the finding that the

filtrate from the mixture, HIV-1 plus MUC1, failed to cause HIV-1 infection of the CEM SS cells.

As shown in the results section the purified pregnancy plug mucins inhibited HIV-1 activity by approximately 97.5%. However, like the crude breast milk, the crude pregnancy plug mucus failed to inhibit HIV-1 activity. The ability of the pregnancy plug mucins to inhibit HIV-1 activity by aggregating the virus was also confirmed as the filtrate from the mixture of HIV-1 plus pregnancy plug mucins infect 2.5% of the host cells. The filtrate from the mixture of HIV-1 plus crude pregnancy plug mucus, on the other hand caused a 100% infection of the host cells. Again, the reason for this is not clear and the explanation given for salivary and breast milk mucins should be applicable here.

The inability of crude breast milk and crude pregnancy plug mucous to inhibit HIV-1 is not known. However, it would be worthwhile to speculate why this was so. In the case of breast milk, MUC1 was enclosed by fat globules (Schroten *et al.*, 1992), and had to be extracted and isolated prior to testing for its anti-HIV-1 activity. There may not be any physical contact between the mucin and HIV-1 *in vitro* or between the mucin and the free viruses and infected mononuclear leukocytes present in the breast milk of HIV infected mothers (Lepage *et al.*, 1987; Naarding *et al.*, 2005; Thiry *et al.*, 1985; Van de Perre *et al.*, 1993). As a result no aggregation of the virus by MUC1 could occur in crude breast milk.

Freezing and heating of HIV-infected human breast milk prior to feeding the child has been reported to reduce HIV transmission from HIV positive mothers to infants (Baron *et al.*, 1999). This phenomenon might facilitate the release of the milk mucin (MUC1) which is enclosed by the milk fat globules in breast milk (Schroten *et al.*, 1992), as it was done in our purification method. This would enable the mucin to trap or aggregate the virus in the breast milk and reduce HIV transmission. Thus it may be worthwhile to heat crude breast milk before testing its anti-HIV-1 activity. Perhaps the dissociation of MUC1 from fat in breast milk is a necessary step to make crude milk effective against the virus.

It is also possible that the concentration of mucins within the crude pregnancy plug mucus and crude breast milk (which consists of water, mucins, lipids, nucleic acids, lactoferrin, lysozyme, secretary leukocyte protease inhibitors, β-casein, oligosaccharides, immunoglobulins and ions (Naarding *et al.*, 2005; Wiggins *et al.*, 2001), as found in the physiological situation, are maybe too low for to aggregate or be effective against the virus. For instance mucins constitute about 0.5-1% of the total mucus (Creeth, 1978; Gipson *et al.*, 2001; Morales *et al.*, 1993). Therefore compared to the our starting concentration of 0.9mg purified mucin, the amount of mucin in the 0.9mg crude or total mucus (which is equivalent to approximately 0.009mg) could be very small to cause viral inhibition or aggregation. However, quantification of the amount of mucins in the crude mucus prior to any assay should be considered for future studies (for example see Raynal *et al.*, 2002).

This however does not explain why crude saliva is effective as a total secretion against the virus. It is possible that (1) the mucin concentration is higher than in relation to the total volume of secretion (2) the hypotonic nature of saliva or (3) other components of saliva, as in the case of mucin, may also have anti-viral activity or, all three of the above.

The tonicity of saliva, breast milk and cervical secretion could also play a role in HIV transmission or its inhibition. Baron *et al.* (1999) demonstrated that human saliva is hypotonic in nature and can therefore burst HIV infected mononuclear leukocytes which are continually shed into the oral cavity during mucosal and gingival lesions or during dental treatment of HIV-infected patients. Free viruses are then trapped by the salivary mucins. Since viral infection requires crossing of the mucosal barrier (Naarding *et al.*, 2005), aggregation by mucus prevents viral multiplication and cell to cell transmission of HIV, or the attachment of HIV to the epithelial cells at the mucosal surfaces which is necessary for penetration and transportation to the sub-epithelial $CD4^+$ cells (Phillips *et al.*, 1995). In contrast breast milk, seminal fluid and cervical secretions are isotonic in nature and cannot disrupt HIV infected mononuclear leukocytes, as saliva does (Baron *et al.*, 1999). Therefore, unlike in the oral cavity, the viruses in breast milk and the female reproductive tract are protected inside the mononuclear leukocytes to which mucins have no access. Lastly a large

volume of breast milk and seminal fluid can disrupt the hypotonic nature of saliva (Baron *et al.*, 1999) during breast feeding and oral sex respectively, thereby overcoming the protective properties of saliva. Transmission of the virus through breast feeding maybe well documented (Naarding *et al.*, 2005) and oral sex may also be associated with a certain level of risk.

According to Parke (1978) the main difference between the diluted cervical mucus produced under the influence of oestrogens during the proliferative phase and the thick viscous cervical mucus produced under the influence of progestogens during the luteal phase or under the influence of contraceptive drugs is mainly a difference in sialic acid content. The diluted cervical mucus of the oestrogens is characterized by the presence of a much lower amount of the sialic acid than the viscous mucus. As the terminal sialic acid residues of mucins are reported to interact with viruses such as the influenza virus and prevent the host cell receptor attachment (Levine *et al.*, 1987; Tabak *et al.*, 1982; Tabak, 1995), the accessibilty of the HI virus and its transmission could differ based on the stage of the menstrual cycle. Thus collection of cervical mucus at different stages of the menstrual cycle and determination of their anti-HIV-1 activity is a question to be addressed.

No difference in the rate of inhibition or viral infection due to time difference was observed. This suggests that mucins may aggregate the virus immediately and permanently. Serial tenfold fold dilutions (i.e. 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of the samples were also done to determine their anti-HIV-1 activity at the highest dilution. Once more, no difference in the anti-HIV-1 activity of the mucins was detected down to 10^{-4}. Maybe the starting concentration which was 0.9mg was very high thus a lower concentration than 0.9mg is advisable to be used as a staring material.

In all the experiments heat inactivated HIV-1 was used as a negative control. As shown in the result section an approximately 30% infection of the CEM SS cells was observed. Heat inactivation did not completely destroy the virus.

In summary crude saliva and purified MUC5B and MUC7 inhibit the human immunodeficiency virus in an *in vitro* assay. Crude milk and crude pregnancy plug mucus do not inhibit the virus but their purified components do.

CHAPTER 7

ANTI-HIV-1 ACTIVITY OF SALIVARY MUC5B AND MUC7 MUCINS FROM HIV PATIENTS WITH DIFFERENT CD4 COUNTS (<200, 200-400 and >400)

7.1 Introduction

The objective of this part of the study was to determine whether salivary MUC5B and MUC7 mucins from HIV positive patients or patients with HIV-AIDS inhibit HIV-1 activity in an *in vitro* assay. Salivary MUC5B and MUC7 mucins from HIV patients with different CD4 counts (<200, 200-400 and >400) were incubated with HIV-1 as previously described and subsequently added to the CEM SS cells. Cells were then cultured and viral replication was measured by a qualitative p24 antigen assay. The size, charge and immunoreactivity of mucins from HIV negative and positive individuals or mucins from HIV patients of different CD4 counts, was analysed by SDS-PAGE, Western blot and ELISA respectively. The patients with CD4 count <200 have full blown AIDS. Difference in the degree of infectivity or virulence of the virus in the saliva, breast milk, cervical secretion and plasma of HIV positive individuals was also determined by incubating these secretions with CEM SS cells.

7.2 Results

7.2.1 Toxicity assay

Prior to the inhibition assay, the toxicity of salivary MUC5B and MUC7 mucins from HIV patients with CD4 count (<200, 200-400 and >400) to the CEM SS cells was determined by toxicity assay. As shown in Table 7.1, there was death of 5% of the cells when incubated with MUC5B from patients with CD4 count 200-400. Other than that, there was no toxic effect of any mucin to the CEM SS cells.

Table 7.1 Toxicity of salivary MUC5B and MUC7 mucins from HIV patients with CD4 count (<200, 200-400 and >400) to the CEM SS cells.

Sample	Con	CEM SS cells	% of dead cells	% of live cells
MUC5B CD4<200	0.9mg	2.5×10^6/ml	0	100
MUC5B CD4 200-400	0.9mg	2.5×10^6/ml	5	95
MUC5B CD4>400	0.9mg	2.5×10^6/ml	0	100
MUC7 CD4<200	0.9mg	2.5×10^6/ml	0	100
MUC7 CD4 200-400	0.9mg	2.5×10^6/ml	0	100
MUC7 CD4>400	0.9mg	2.5×10^6/ml	0	100

The percentage of live cells was calculated as the number of live cells over total cells (live plus dead) × 100.

7.2.2 The anti-HIV-1 activities of salivary MUC5B and MUC7 mucins from HIV patients with different CD4 counts (<200, 200-400 and >400)

To check whether the salivary MUC5B and MUC7 mucins from HIV patients possess the same inhibitory activity as those from HIV negative individuals (Chapter 6), the anti-HIV-1 activities of the salivary MUC5B and MUC7 mucins from the three groups of HIV patients (i.e. patients with CD count <200, 200-400 and >400) was determined in an *in vitro* inhibition assay. The result demonstrated that irrespective of their CD4 count both MUC5B and MUC7 mucins from HIV patients failed to inhibit HIV-1 activity and 100% viral infection of the CEM SS cells was measured by the p24 antigen assay after a 30min incubation period (Figure 7.1A, B and C). This was unlike MUC5B and MUC7 mucins from HIV negative individuals (Section 6.2.2). When HIV-1 was treated with the media instead of mucins as a control, 100% HIV-1 replication or infection of the CEM SS cells was detected (Figure 7.1A, B and C). However, no HIV-1 infection was seen when heat inactivated HIV-1 was used (Figure 7.1A, B and C).

The effect of the length of the incubation period on the rate of inhibition of the HIV-1 infection of the CEM SS cells was determined by incubating the CEM SS cells with the mixtures for 1h and 2h. There was no difference due to the length of the incubation period (Figure 7.1A, B and C). As in the case of the MUC5B and MUC7 from HIV negative individuals (Section 6.2.2), serial tenfold dilution, (10^{-1} to 10^{-4}) of the MUC5B and MUC7 mucins, starting at 0.9mg mucin, from HIV patients of all the

different CD4 counts (<200, 200-400 and >400) were done in triplicate and this made no difference to infectivity.

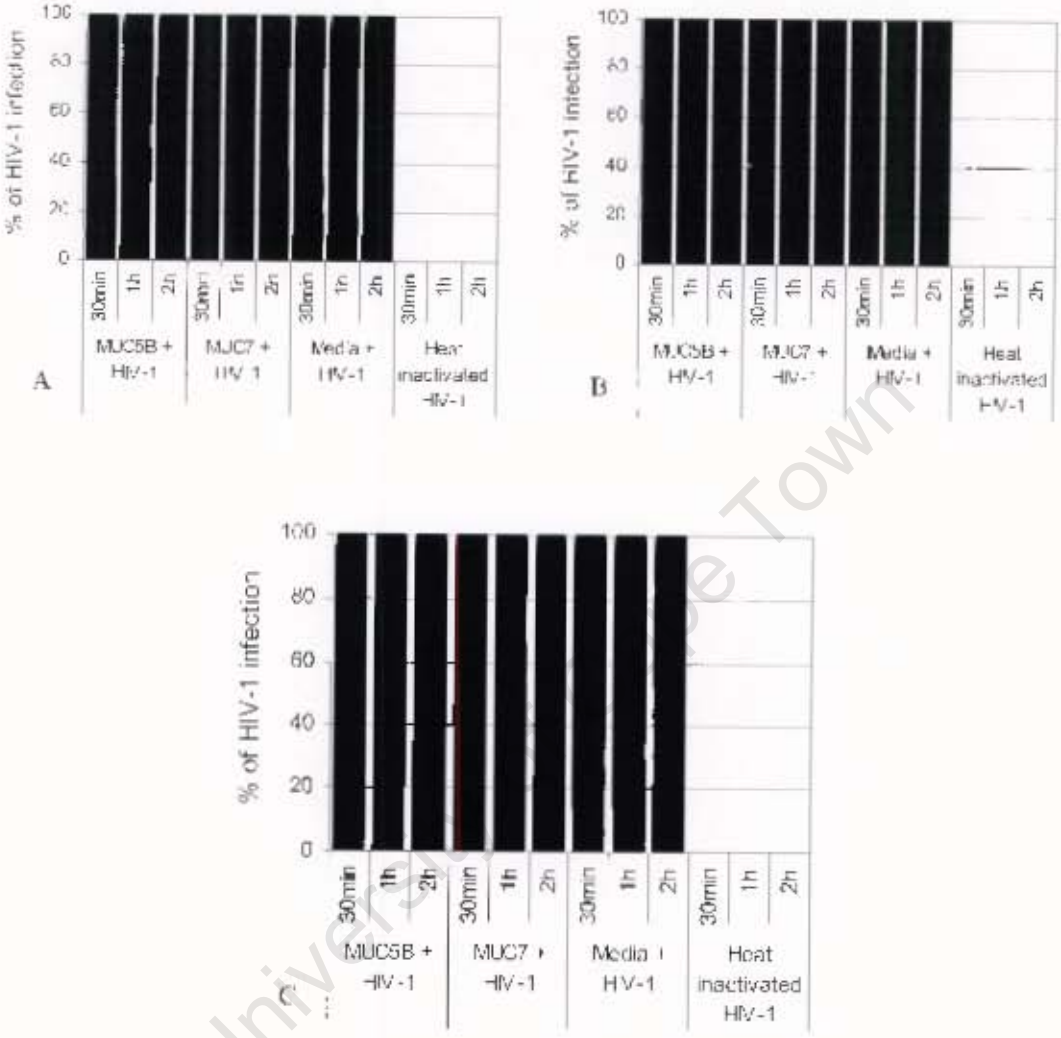


Figure 7.1 Anti-HIV-1 activities of salivary MUC5B and MUC7 mucins from HIV patients with CD4 count (<200, 200-400 and >400). Salivary MUC5B and MUC7 mucins (500µl or 0.9mg each) from patients with CD4 count (<200, 200-400 and >400) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The unfiltered samples were then incubated with CEM SS cells at a concentration of 0.5 × 10^6cells/ml for 30min, 1h and 2h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B and C indicate the anti-HIV-1 activity of salivary MUC5B and MUC7 mucins from HIV patients with CD4 counts <200, 200-400 and >400 respectively.

To determine if the salivary MUC5B and MUC7 mucins from HIV positive patients trap or aggregate the viruses as the MUC5B and MUC7 mucins from HIV negative individuals did (Section 6.2.2), the mixtures were filtered through 0.45µm pore size

cellulose acetate filter at the end of the incubation period (60min), and the filtrates were subsequently incubated with the CEM SS cells for 30 min. Unlike the filtrates from the mixtures of HIV-1 plus MUC5B and MUC7 from HIV negative individuals (Section 6.2.2), these filtrates caused 100% viral infection of the CEM SS cells (Figure 7.2A, B and C). Even if these filtrates were incubated with the CEM SS cells for 1h and 2h, no change from the above results were observed (Figure 7.2A, B and C).

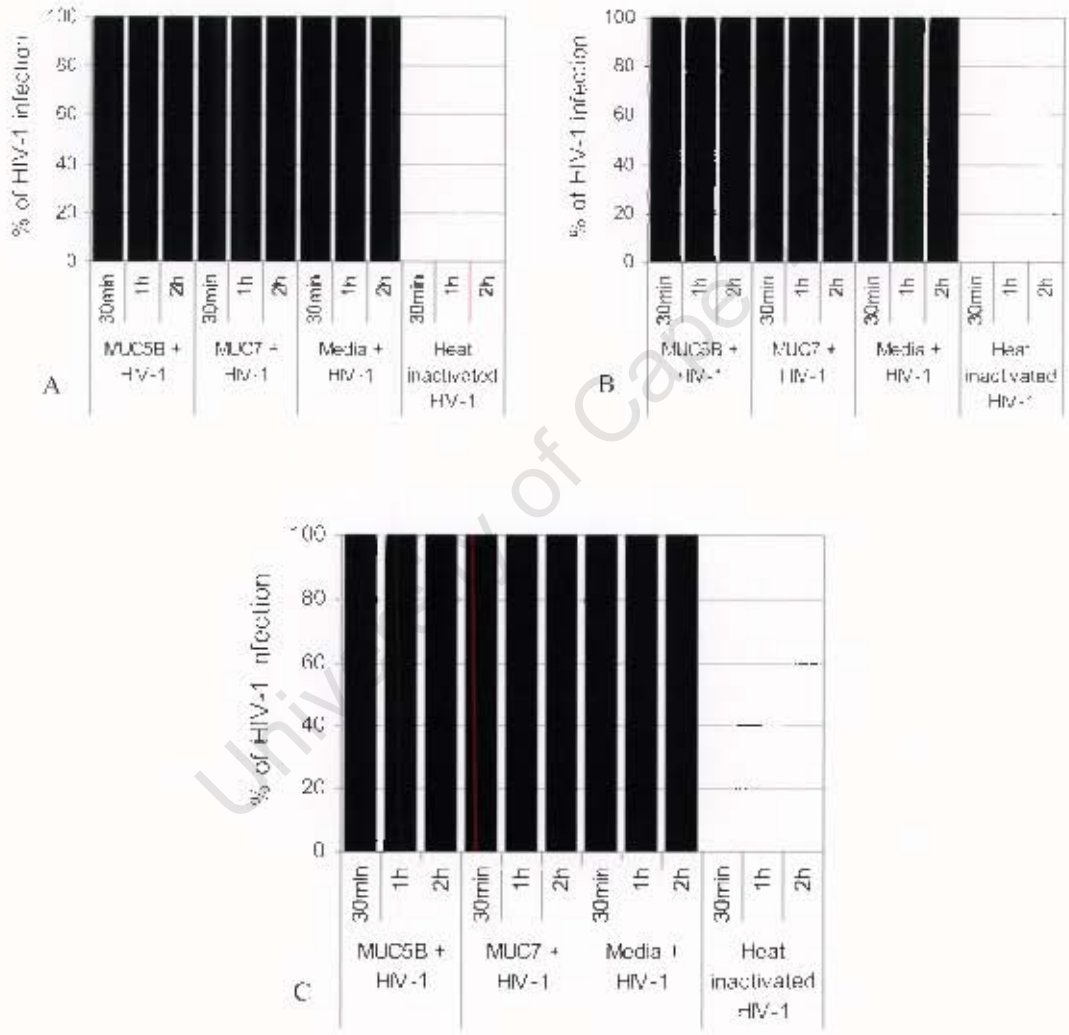


Figure 7.2 Anti-HIV-1 activities of salivary MUC5B and MUC7 mucins from HIV patients with CD4 count (<200, 200-400 and >400). Salivary MUC5B and MUC7 mucins (500µl or 0.9mg each) from patients with CD4 count (<200, 200-400 and >400) were incubated with subtype D HIV-1 for 60min and filtered through 0.45µm pore size cellulose acetate filter. As controls HIV-1 treated with media and heat inactivated HIV-1 were used. The filtrates of the mixtures were then incubated with CEM SS cells at a concentration of 0.5×10^6cells/ml for 30min, 1h and 2h. After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay. Letters A, B and C indicate the anti-HIV-1 activity of salivary MUC5B and MUC7 mucins from HIV patients with CD4 counts <200, 200-400 and >400 respectively.

7.2.3 Gradient gel analyses of salivary MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400)

To assess if the HIV infection induced any structural or size difference on the salivary MUC5B and MUC7 mucins, salivary MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400), were dissolved in a gel loading buffer and were subjected to a 4-20% gradient gel alongside the salivary MUC5B and MUC7 mucins from HIV negative individuals as a control (Figure 7.3).

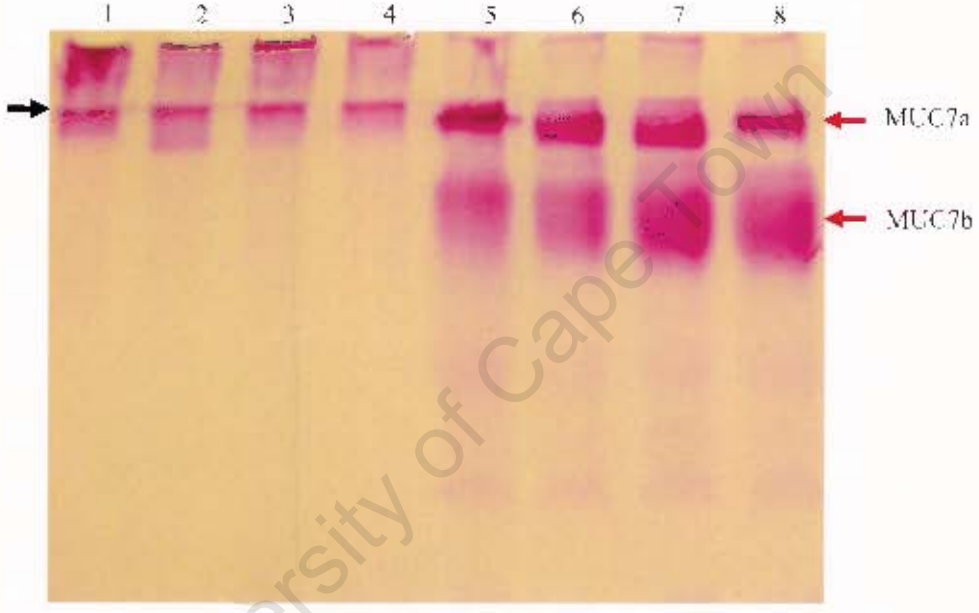


Figure 7.3 Gradient gel analysis of salivary MUC5B and MUC7 mucins from HIV negative and positive individuals. Freeze-dried samples (30µg) of MUC5B from HIV negative individual (lane 1), MUC5B from HIV positive individuals with CD4 counts <200 (lane 2), 200-400 (lane 3), >400 (lane 4), MUC7 from HIV negative individual (lane 5), MUC7 from HIV positive individuals with CD4 counts <200 (lane 6), 200-400 (lane 7) and >400 (lane 8) were prepared in a gel loading buffer and separated in a 4-20% gradient gel. Following electrophoresis the gel was stained with PAS. While arrows in red indicates the two glycoforms of MUC7 on top of the running gel (MUC7a) and slightly entering the running gel (MUC7b), the arrow in black is at the start of running gel.

The PAS stained gel showed that the MUC5B from normals had slightly more material in the stacking gel and less penetration into the running gel (lane 1) than that from the HIV patients with CD4 count <200 (lane 2), 200-400 (lane 3) and >400 (lane 4) which showed less material in the stacking gel and high penetration into the running gel. MUC5B from patients with CD4 count <200 (lane 2) appeared as a broader band on the top of the running gel. The MUC7a from normals had slightly

more material and showed less penetration into the running gel (lane 5) than that from the HIV patients with CD4 count <200 (lane 6), 200-400 (lane 7) and >400 (lane 8) which showed slightly less material of MUC7a and more material of MUC7b than the normal. The MUC7a from patients with CD4 count >400 (lane 8) showed less material than the rest.

7.2.4 Immunoreactivity analyses of salivary MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400)

To determine if there are any immunoreactivity differences between salivary MUC5B and MUC7 mucins from HIV negative and HIV positive individuals towards the same antibodies as the result of the HIV infection, salivary MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400) were coated in an ELISA plates alongside the salivary MUC5B and MUC7 from HIV negative individuals and probed with anti-MUC5B and anti-MUC7 polyclonal antibodies (Figure 7.4).

Although the difference in immunoreactivity between mucins from HIV negative and positive samples is very small, MUC5B (Figure 7.4A) and MUC7 (Figure 7.4B) from HIV negative individuals have shown the highest reactivity towards their respective antibodies. Interestingly, we detected immunoreactivity differences between the mucins from HIV patients of different CD4 counts as well. However none of these differences were significant.

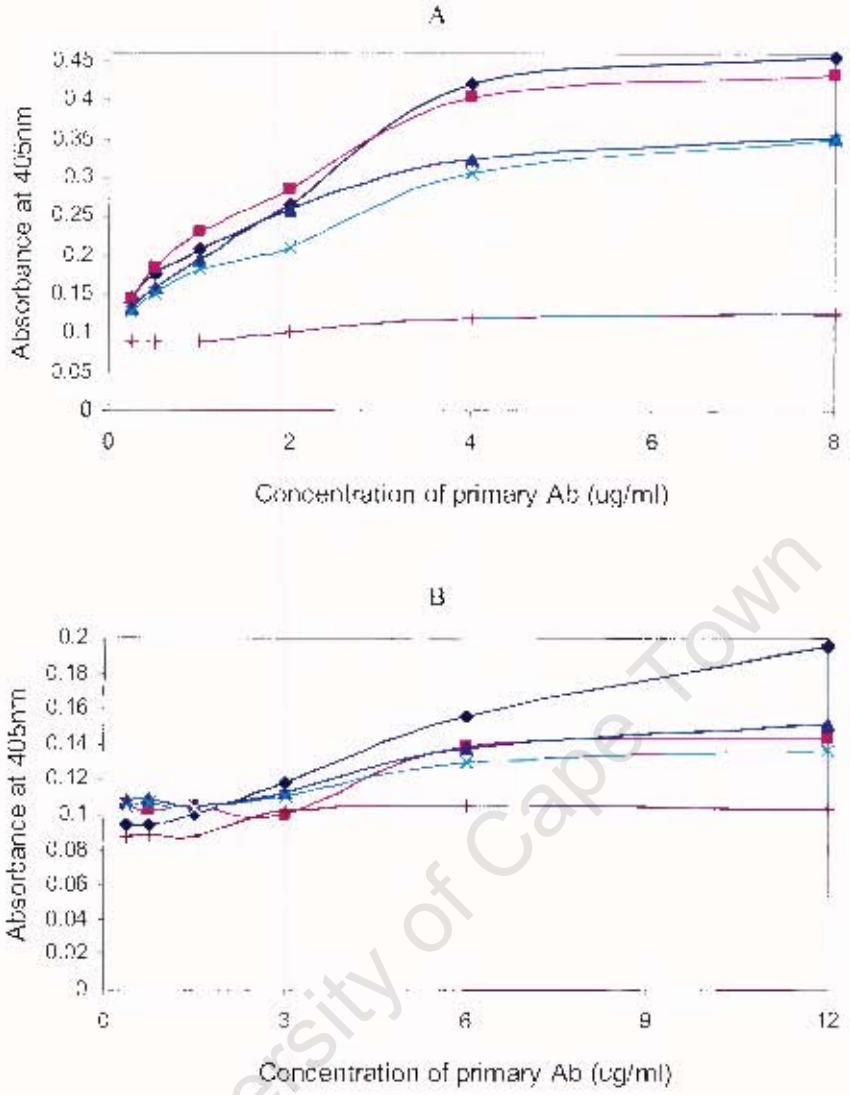


Figure 7.4 ELISA monitoring immunoreactivity of salivary MUC5B and MUC7 mucins from HIV negative and positive individuals. Plate (A) was coated with MUC5B from HIV negative individual (♦). MUC5B from HIV positive individuals with CD4 counts <200 (■), 200-400 (▲) and >400 (×) and plate (B) was coated with MUC7 from HIV negative individual (♦). MUC7 from HIV positive individuals with CD4 counts <200 (■), 200-400 (▲) and >400 (×). Plates were incubated with serial two-fold dilutions of goat anti-MUC5B (A) and goat anti-MUC7 (B) polyclonal antibodies at concentrations between 8µg/ml and 0.25µg/ml (goat anti-MUC5B) and 12µg/ml and 0.375µg/ml (goat anti-MUC7). Antibody binding was detected using rabbit anti-goat HRPO linked secondary antibody and visualized with TMB/H2O2 substrate. Absorbance values were read at 405nm in a Titertek ELISA reader. Each point is the average absorbance of duplicate samples. As a negative control wells were coated with PBS (+).

7.2.5 Western blotting analyses of salivary MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400)

To determine if there are any charge differences between the salivary MUC5B and MUC7 mucins from HIV negative and HIV positive individuals or among the mucins from HIV patients of different CD4 counts, MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400) were run in an agarose gel alongside the MUC5B and MUC7 from HIV negative individuals as a control. Mucins were then transferred to nitrocellulose membranes and probed with rabbit anti-MUC5B and mouse anti-MUC7 antibodies (Figure 7.5).

The MUC5B from HIV negative individual (lane 4) clearly had more material and a wide range of charge after equal loading, than the MUC5B from the HIV positive patients with CD4 count >400 (lane 1), 200-400 (lane 2) and <200 (lane 3) which showed relatively small range of charge. The mobilities between groups hardly differed (lanes 1-5).

Differences in the charge was observed between the MUC7 from HIV negative and HIV positive individuals and within the group of HIV patients of different CD4 counts (lanes, 7, 8, 9 and 10). MUC7 from HIV negative individuals (lane 10) was of lower charge than the MUC7 from HIV patients with CD4 count >400 (lane 7), 200-400 (lane 8) and <200 (lane 9). As shown in the figure, while the positive controls crude saliva (lanes 5 and 11) reacted with the anti-MUC5B and anti-MUC7 antibodies, as expected the negative controls MUC7 (lane 6) and MUC5B (lane 12) did not react with the anti-MUC5B and anti-MUC7 antibodies respectively.

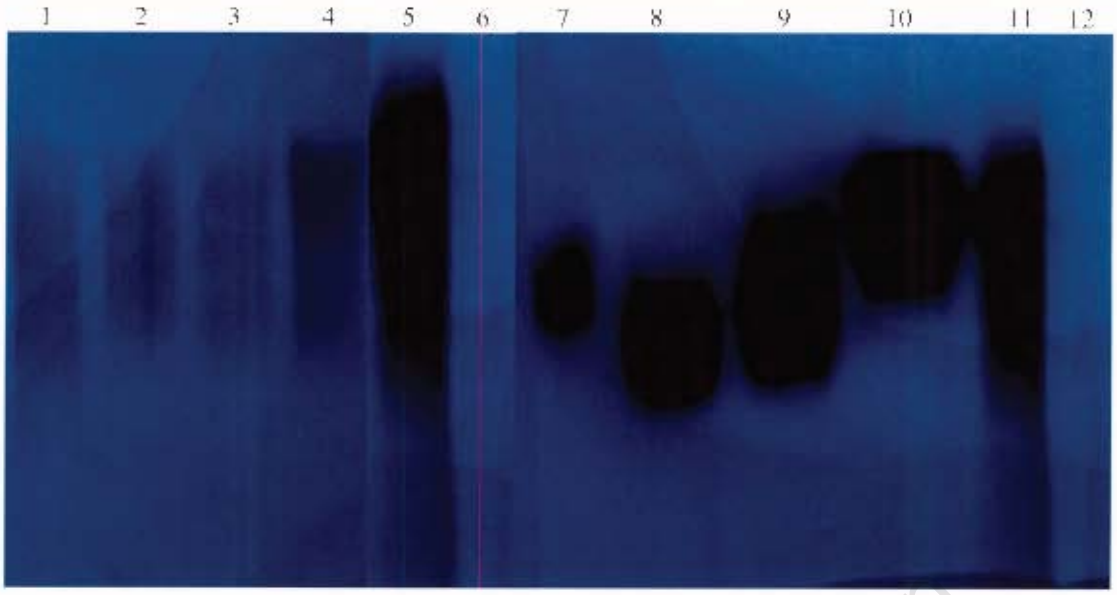


Figure 7.5 Western blotting analyses of salivary MUC5B and MUC7 mucins from HIV positive individuals with different CD4 counts (<200, 200-400 and >400). Lane 1, MUC5B from patients with CD4 >400, lane 2, MUC5B from patients with CD4 200-400, lane 3, MUC5B from patients with CD4 <200, lane 4, MUC5B from HIV negative individual, lane 5, crude saliva (positive control), lane 6, MUC7 (negative control), lane 7, MUC7 from patients with CD4 >400, lane 8, MUC7 from patients with CD4 200-400, lane 9, MUC7 from patients with CD4 <200, lane 10, MUC7 from HIV negative individuals, lane 11, crude saliva (positive control) and lane 12, MUC5B (negative control) were separated by a 1% agarose gel and transferred to nitrocellulose membrane. Following overnight blocking, the membranes were incubated for 2h with rabbit anti-MUC5B polyclonal (lanes 1-6) and mouse anti-MUC7 monoclonal (lanes 7-12) antibodies diluted in 5% (m/v) low fat milk powder in TBST at 1 in 2000 (rabbit anti MUC5B) and 1 in 1000 (mouse anti-MUC7). Membranes were then washed 3 × 5min with TBST and incubated for 1h with HRPO linked goat anti-rabbit (lanes 1-6) and goat anti-mouse (lanes 7-12) secondary antibodies diluted in 5% (m/v) low fat milk powder in TBST at dilutions of 1 in 5000 and 1 in 1500 respectively. After another TBST wash (3 × 5min), bands were detected using an ECL detection kit.

7.2.6 Comparison of the HIV virulence in saliva, breast milk, cervical secretion and plasma of the HIV infected individuals

To check whether HIV in saliva, breast milk, cervical secretion and plasma can infect the CEM SS cells at the same or different rates, CEM SS cells were infected or incubated with crude saliva, breast milk, cervical plug and plasma from HIV positive individuals and HIV-1 as a control. The result demonstrated that while the live virus (positive control) infected the CEM SS cells by almost 100%, the crude saliva, breast milk, cervical plug and plasma failed to cause viral infection of the CEM SS cells. As expected media (negative control) did not cause viral infection of the CEM SS cells (Figure 7.6).

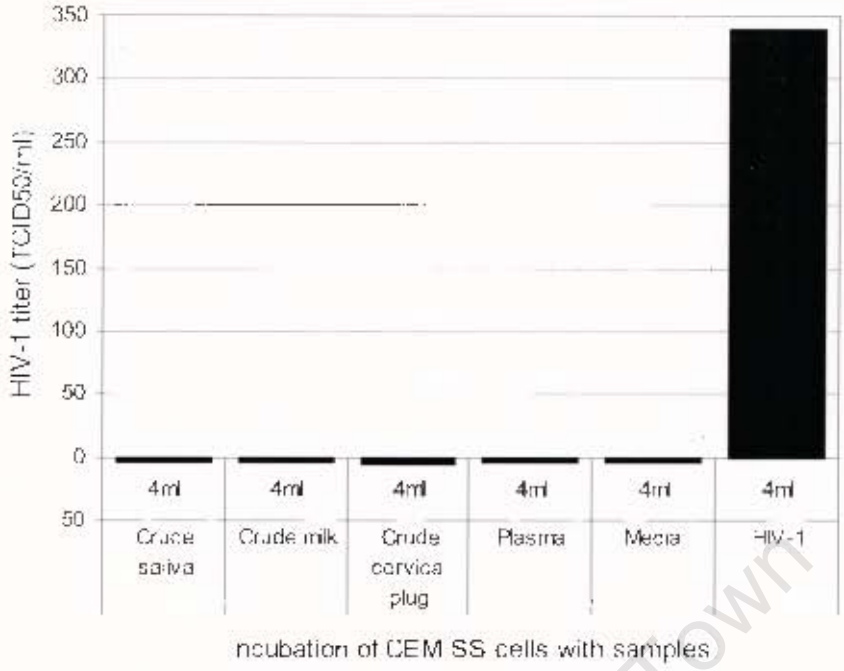


Figure 7.6 Comparison of the HIV virulence in saliva, breast milk, cervical secretion and plasma of the HIV infected individuals. Crude saliva, breast milk, cervical secretion and plasma (4ml each) from HIV positive individuals were incubated with CEM SS cells at a concentration of 0.5 × 10^6cells/ml for 60min. As controls CEM SS cells were incubated with live virus or HIV-1 (positive) or media (negative). After PBS wash cells were cultured and viral replication was measured by a qualitative p24 antigen assay.

7.3 Discussion

It was shown that irrespective of their CD4 count (<200, 200-400 and >400) both MUC5B and MUC7 mucins from HIV patients did not inhibit HIV-1 activity; instead 100% viral replication was detected by the p24 antigen assay. Size, charge and immunoreactivity differences between the mucins from HIV negative and positive individuals and among the mucins from HIV patients of different CD4 count was observed by SDS-PAGE, Western blot and ELISA. The crude saliva, breast milk, cervical plug and plasma from HIV positive individuals did not infect the CEM SS cells.

Mucins of higher charge migrate further into the gel than those of lower charge (Thornton *et al.*, 1999). The MUC7 from disease migrated further than that from normal individuals. Therefore these diseased species of MUC7 were more highly

charged than normal MUC7. If this is due to an altered glycosylation then one can expect shorter side chains and an exposure of more charged groups on the mucin, with a consequent increase in the charge per density ratio of the mucin. It would also be expected that MUC7 from patients with a CD4 count >400 would migrate closer to where normal MUC7 is positioned. Surprisingly this was not to be the case because it seemed like the mobility of MUC7 from patients with CD4 <200 (Figure 7.5, lane 9) migrated less than CD4 200-400 (lane 8) and CD4 >400 (lane 7). Also the gels could be over loaded and thus we have not highlighted the mobility of the different mucins adequately.

In the preceding Chapter (Chapter 6) it was shown that salivary MUC5B and MUC7 mucins from HIV negative individuals inhibited HIV-1 activity by approximately 97.5%. Here we investigated whether salivary MUC5B and MUC7 mucins from HIV positive individuals would have a similar inhibitory effect to that from HIV negative individuals in an *in vitro* inhibitory assay. We have shown that irrespective of their CD4 count (<200, 200-400 and >400) both MUC5B and MUC7 mucins from HIV patients failed to inhibit HIV-1 activity. There was a 100% infection of the CEM SS cells as detected by the p24 antigen assay. Although the reason is not clear, it is possible that HIV infection induces changes of the salivary glands which results in a decline in the amount of saliva and a change in its constituents (Wagner *et al.*, 1996). This in turn may affect the glycosylation pattern or sugar composition of the salivary mucins, and if inhibition of the virus is through aggregation by the carbohydrate side chains, and if changes in these sugar side chains occurs as a result of infection (Lal *et al.*, 1992), it is conceivable that this is the reason for the inability of mucins from HIV positive individuals to inhibit the virus in an in *vitro* assay. Furthermore HIV infection is also reported to suppress the production of saliva inhibitory factors, or elicit blocking molecules (Crombie *et al.*, 1998). Mucins are also reported to lose their carbohydrate side chains as a result of infections which could affect their ability to adhere or recognize antigens or micro-organisms (Taylor *et al.*, 1998). Thus there is a higher possibility that the HIV infection induced change in the glycosylation pattern of the mucins.

To check whether the HIV infection affected the ability of the mucins to trap or aggregate the virus, the filtrates of the mixtures were incubated with the CEM SS

cells. The filtrates caused 100% infection of the CEM SS cells, unlike the filtrates from the mixtures of HIV-1 and normal mucins. This suggests that both MUC5B and MUC7 mucins from HIV positive individuals, irrespective of their CD4 count failed to aggregate the virus. Hence there were free viruses in the filtrates that could enter the CEM SS cells and cause infection. As the carbohydrate moieties of salivary mucins serve as an attachment sites for bacteria and viruses (Bosch *et al.*, 2000; Prakobphol *et al.*, 1999), changes in charge or glycosylation pattern as a result of HIV infection could affect the ability of the mucins to aggregate the virus.

The PAS stained 4-20% gradient gel demonstrated that there was a size difference between the mucins from HIV negative and positive individuals as well as between the mucins from HIV patients of different CD4 counts, with the mucins from HIV positive individuals showing slightly more penetration or higher electrophoretic mobility. Again changes in the glycosylation pattern could be implicated. Furthermore as the degree of glycosylation affects the electrophoretic mobility (Pallesen *et al.*, 2001; Patton *et al.*, 1995), the appearance of the mucins from HIV negative and positive individuals on the gradient gel was different. In summary as the structural differences in mucins are related to physiologically different functions (Bolscher *et al.*, 1995), the size or structural difference of the salivary mucins from HIV patients may affect their ability to trap or aggregate the virus. However, the differences we detected were too small to form any conclusion.

Enzyme linked immunosorbent assay (ELISA) was also performed to determine if the immunoreactivity of the salivary mucins was altered due to the HIV infection. As shown in the result section immunoreactivity differences between mucins from HIV negative and positive individuals was observed, with both MUC5B and MUC7 mucins from HIV negative individuals showing the highest reactivity. If not for the shortage of antibody, better immunoreactivity difference between MUC7 mucins from HIV negative and positive individuals could have been shown by increasing the concentration of the primary antibody. Although the reason is not clear, as variation in glycosylation can affect antibody binding (Patton *et al.*, 1995), the HIV infection may have affected the glycosylation pattern of the mucins which could result in epitope modification. This finding seems to strengthen the outcomes of the Western blot (charge) and gradient gel (size) analysis (the preceding paragraphs).

The role of salivary MUC5B and MUC7 in protecting the oral cavity from bacteria, viruses, yeasts, and toxins is well documented (Bosch *et al.*, 2000; Liu *et al.*, 1999; Prakobphol *et al.*, 2005; Situ and Bobek, 2000). However, Lal *et al.* (1992) reported that compared to HIV negative individuals, saliva from HIV positive individuals possess considerably lower anti-candicidal activity. This was supported by the findings of Gururaja *et al.* (1999) and Situ *et al.* (2003) that fungal infections specifically *Candida albicans* has increasingly colonized the oral cavity of HIV positive patients. This suggested that the HIV infection may have induced functional alteration on the salivary mucins which are very potent in normal circumstances. For instance MUC7 in immunocompromised individuals is reported to lose the expression of sugar receptor (sLe^x) hence making the individual more susceptible to oral diseases (Prakobphol *et al.*, 1998). Furthermore, as covalently modified MUC7 is reported to lose its potency against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (Liu *et al.*, 1999), the idea that HIV infection might induce structural changes to the mucins is the most likely phenomenon can be think of. In summary the SDS-PAGE (gradient gel), ELISA and Western blot analysis strengthens these findings.

In this study the difference in the degree of infectivity or virulence of the viruses in the saliva, breast milk, cervical secretion and plasma of HIV positive individuals was also determined by incubating these secretions with CEM SS cells. As shown in the results section none of these secretions were able to infect the CEM SS cells. Though the reason is not clear maybe the viral load in these secretions was too low to cause infection. Thus secretions with higher viral load are required to tell whether there is difference in the virulence or ability of the viruses in these secretions to infect host cells.

CHAPTER 8

THE ROLE OF MUC7 IN INHIBITING OR REDUCING VIRAL INFECTION OF THE PERIPHERAL BLOOD MONONUCLEAR CELLS AND MINIMIZING THE SPREAD OF HIV-1 FROM HIV-1 POSITIVE TO HIV-1 NEGATIVE CELLS

8.1 Introduction

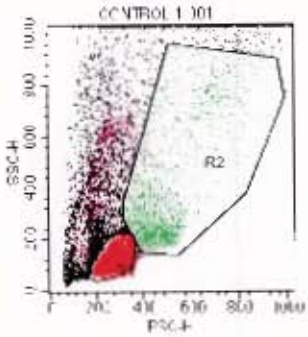
The aims of this part of the study were to investigate whether MUC7 i) could inhibit viral infection of the peripheral blood mononuclear cells (PBMCs) ii) reduce viral infection of the HIV-1 infected PBMCs and iii) can minimize the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs in an *in vitro* inhibition assay.

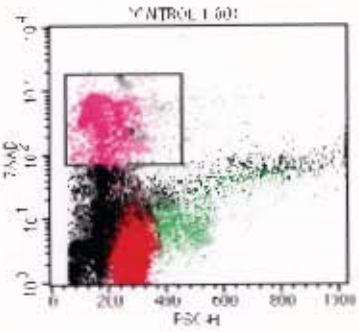
In i) HIV-1 negative PBMCs were incubated with different concentrations of MUC7 prior to the addition of HIV-1 Subtype C. In ii) HIV-1 infected PBMCs were treated with different concentrations of MUC7 prior to cell culture. In iii) the HIV-1 positive PBMCs were incubated with different concentrations of MUC7 prior to the addition of HIV-1 negative PBMCs. The mixtures were then cultured and viral replication was measured by a qualitative p24 antigen assay.

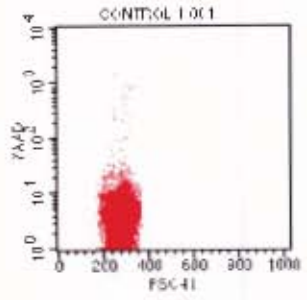
8.2 Results

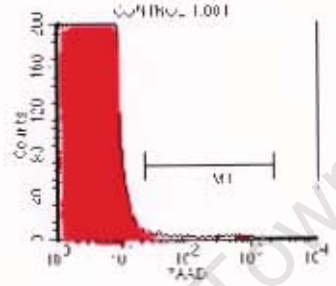
8.2.1 Toxicity of MUC7 to white blood cells

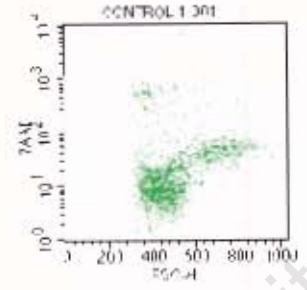
The toxicity of salivary MUC7 to white blood cells namely monocytes, lymphocytes and granulocytes at different mucin concentrations (1mg, 0.5mg and 0.25mg) was determined using the toxicity assay. The result (flow cytometry reading) revealed that even at the highest mucin concentration (1mg), MUC7 was found to be non-toxic to the white blood cells and as a result no cell death was observed (Figure 8.1).

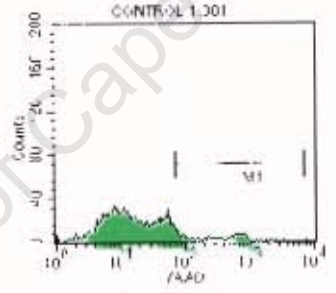












Histogram Statistics

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Gated Events: 142541
X Parameter: FL3-H 7AAD (Log)

Patient ID:
Gate: G1
Total Events: 166535

Marker	Left, Right	Events	% Gated	% Total
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M1	24, 237	222	0.16	0.12

Histogram Statistics

Sample ID: CONTROL 1
Acquisition Date: 17-Aug-05
Gated Events: 5560
X Parameter: FL3-H 7AAD (Log)

Patient ID:
Gate: G2
Total Events: 166535

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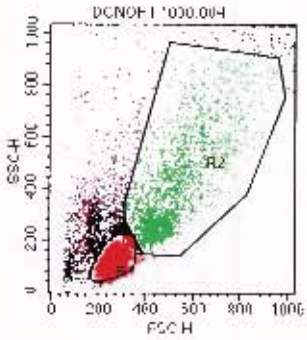

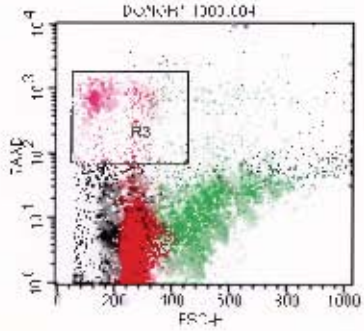

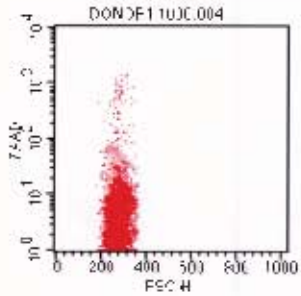

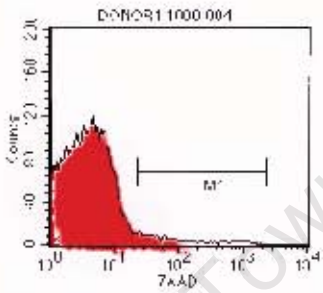

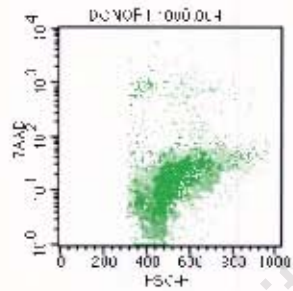

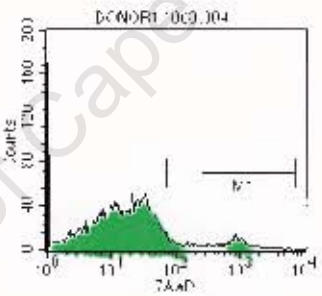

Histogram Statistics

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Gated Events: 26960 — Total Events: 44664
X Parameter: FL3-H 7AAD (Log)

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All	1	9910	26960	100.00	60.36
M1	24	2371	790	2.95	1.78

Histogram Statistics

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Acquisition Date: 17-Aug-06 — Gate: G2
Gated Events: 10443 — Total Events: 44664
X Parameter: FL3-H 7AAD (Log)

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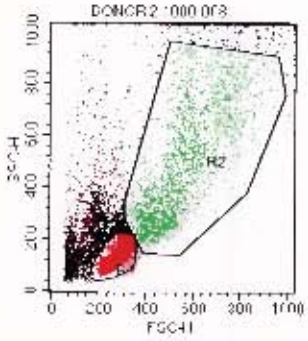
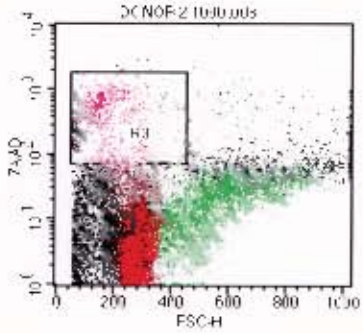
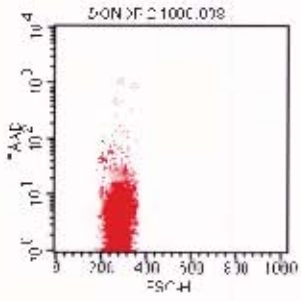
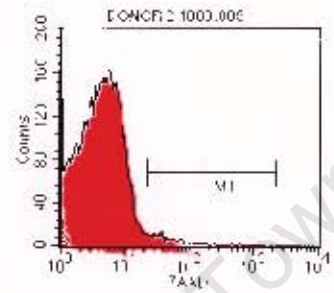
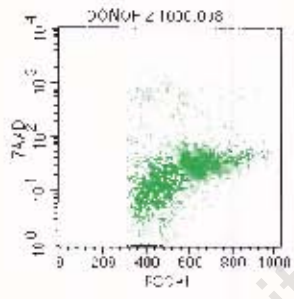
M1	75	7254	1038	9.94	2.32

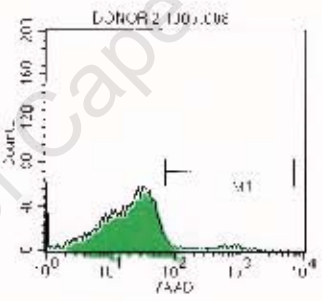












Histogram Statistics

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X Parameter: FL3-H 7AAD (Log)

Patient ID:
Gate: G1
Total Events: 54274

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All	1	9910	32850	100.00	60.53
M1	24.	2371	617	1.88	1.14

Histogram Statistics

Sample ID: DONOR 2 1000
Acquisition Date: 17-Aug-06
Gated Events: 9499
X Parameter: FL3-H 7AAD (Log)

Patient ID:
Gate: G2
Total Events: 54274

Marker	Left	Right:	Events	% Gated	% Total
All	1.	9910	9499	100.00	17.50
M1	73.	7234	473	4.98	0.87

Figure 8.1 Determination of MUC7 toxicity to white blood cells using a toxicity assay. White blood cells (1.5ml or 6.4 - 16.2 × 10^6 cells) in RPMI containing 10% Fetal Calf Serum and 1% Penicillin/Streptomycin antibiotic were mixed with different concentrations of salivary MUC7 (1mg, 0.5mg and 0.25mg) and incubated in a CO_2 incubator for 48h. As a control, cells without MUC7 were incubated in a CO_2 incubator for 48h. At the end of the incubation period cells were washed and resuspended in PBS. Thereafter cells were incubated with 7-Amino-actinomycin D (7AAD) and cell viability was monitored by flow cytometry. Blood samples from two donors were used and a description is presented below each assay. NB as the results for the different mucin concentrations are similar; a representative which is a control and the results at the highest mucin concentration (1mg) of each donor are presented.

8.2.2 Toxicity of MUC7 to red blood cells

The toxicity of salivary MUC7 to red blood cells at different mucin concentrations (1mg, 0.5mg and 0.25mg) was also determined using the toxicity assay. The result revealed that even at the highest mucin concentration (1mg), MUC7 was found to be non-toxic to the red blood cells and no haemolysis of the red blood cells was observed (Figure 8.2).

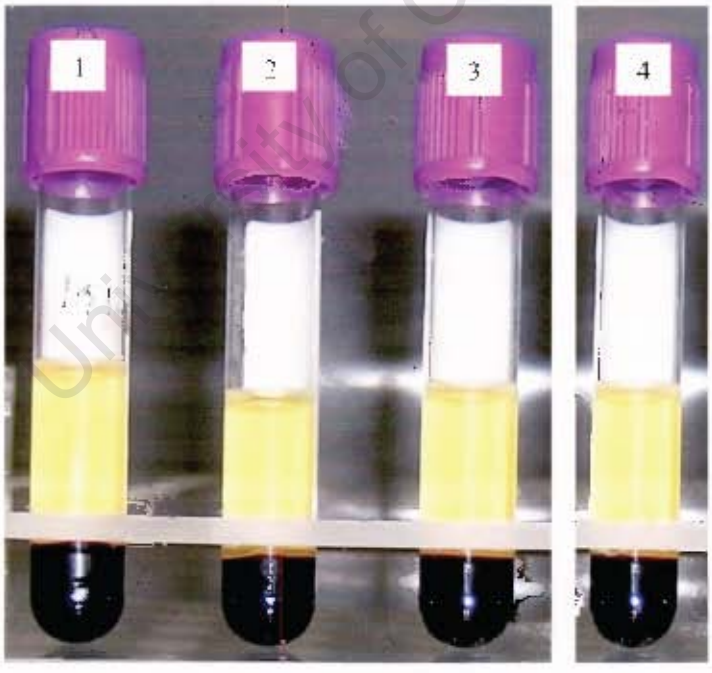


Figure 8.2 Determination of MUC7 toxicity to red blood cells using a toxicity assay. Red blood cells were mixed with different concentrations of MUC7 (1mg, 0.5mg and 0.25mg) and incubated for 60min at room temperature. As a control, cells without MUC7 were incubated for 60min at room temperature. At the end of the incubation period the blood was centrifuged (1000g, 15min, 4°C) and haemolysis was visualized. NB. Tubes number 1, 2, 3 and 4 (2.5ml each or 10.5 - 14.7 × 10^9 cells) were incubated with 1mg, 0.5mg and 0.25mg of MUC7 and control respectively.

8.2.3 Viral load of the blood samples from HIV positive and negative individuals

The viral load of the blood samples from both the HIV positive and negative individuals was determined prior to the HIV inhibition assays. As presented in Table 8.1 most of the blood samples from the HIV patients were found to have high viral loads. As expected no viral particles were detected in the samples from the HIV negative individuals.

Table 8.1 Viral load of blood samples from HIV positive and negative individuals

HIV positive individual	**Viral load (IU/ml)**
1	8800
2	2100
3	400
4	81
5	35
6	2200
7	3800
8	19000
9	120000
HIV negative individual	
1	-
2	-

8.2.4 Inhibition of HIV-1 activity by blocking the putative viral binding sites (receptors) of the peripheral blood mononuclear cells using MUC7

The same experiment as in Section 6.2.5 was done to determine whether MUC7 can inhibit HIV-1 infection of the PBMCs by blocking their putative viral binding sites (receptors). The result showed that, as in the case of CEM SS cells (Section 6.2.5), even at the highest concentration (1mg), MUC7 did not inhibit HIV-1 infection of the PBMCs (Figure 8.3). Instead a high percentage of viral infection was detected. However, no viral infection was detected when the PBMCs from HIV negative individuals were used as a negative control (Figure 8.3).

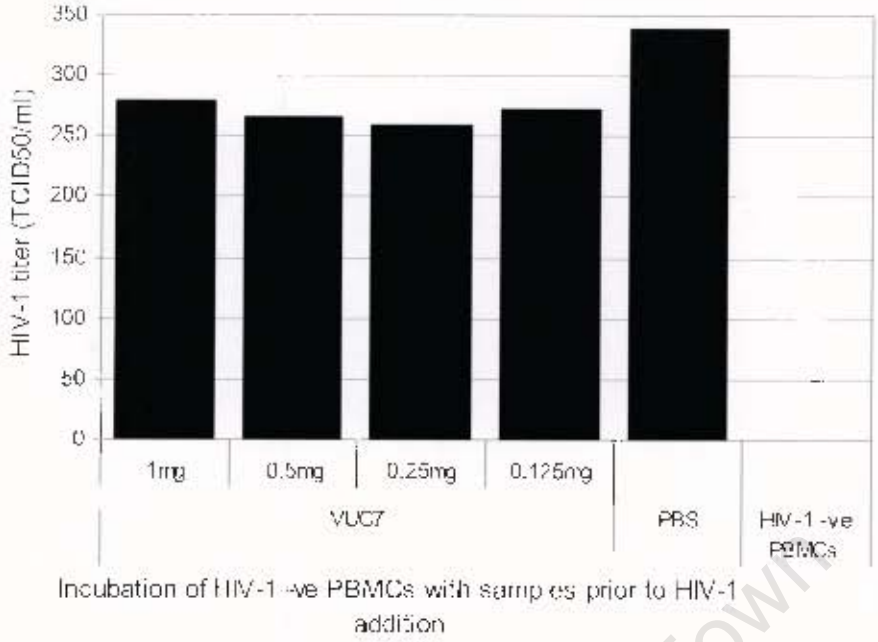


Figure 8.3 Inhibition of HIV-1 activity by blocking the putative viral binding sites (receptors) of the peripheral blood mononuclear cells using MUC7 in an *in vitro* assay. The PBMCs from HIV-1 negative individuals were incubated with different concentrations of salivary MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) for 60min. As controls PBMCs incubated with PBS (positive) and PBMCs from HIV-1 negative individuals (negative) were used. At the end of the incubation period, HIV-1 Subtype C was added to the mixtures except to the negative control. Cells were then cultured and viral replication was measured by a qualitative p24 antigen assay

8.2.5 The role of MUC7 in reducing viral infection of the HIV-1 infected peripheral blood mononuclear cells

To check whether MUC7 could reduce viral infection of the HIV-1 infected PBMCs, PBMCs from HIV-1 infected individuals were treated or incubated with different concentrations of MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg). The result demonstrated that like PBS (positive control) MUC7, even at its highest concentration (1mg) did not reduce viral infection of the HIV-1 infected PBMCs (Figure 8.4).

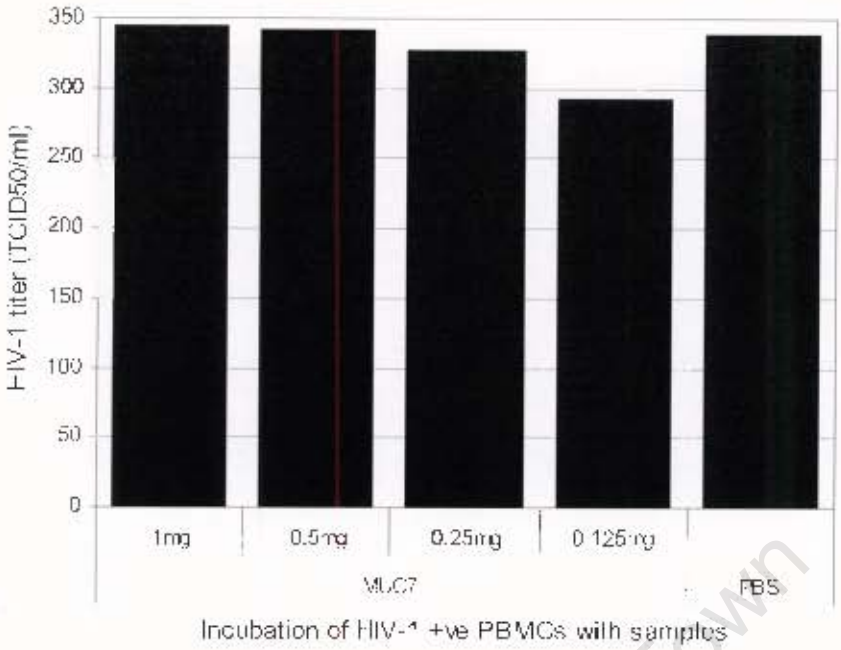


Figure 8.4 The role of MUC7 in reducing viral infection of the HIV-1 infected peripheral blood mononuclear cells in an *in vitro* assay. The PBMCs from HIV-1 positive individuals were incubated with different concentrations of salivary MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) for 60min and cells were then cultured and viral replication was measured by a qualitative p24 antigen assay. As a control PBMCs from HIV-1 positive individuals were incubated with PBS.

8.2.6 The role of MUC7 in minimizing the spread of HIV-1 from HIV-1 positive peripheral blood mononuclear cells to HIV-1 negative peripheral blood mononuclear cells

To determine whether MUC7 could minimize the spread of HIV-1 infection from HIV-1 positive PBMCs to HIV-1 negative PBMCs, PBMCs from patients with high viral load (Table 8.1) were treated or incubated with different concentrations of MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) prior to the addition of HIV-1 negative PBMCs. As shown in the result section no HIV-1 infection or inhibition of HIV-1 activity by MUC7 at all concentrations was detected by the p24 antigen assay after three days of cell culture (Figure 8.5). Surprisingly only minimal viral infection or activity was detected by the p24 antigen assay when the HIV-1 infected PBMCs were directly mixed with the HIV-1 negative PBMCs as a control (Figure 8.5).

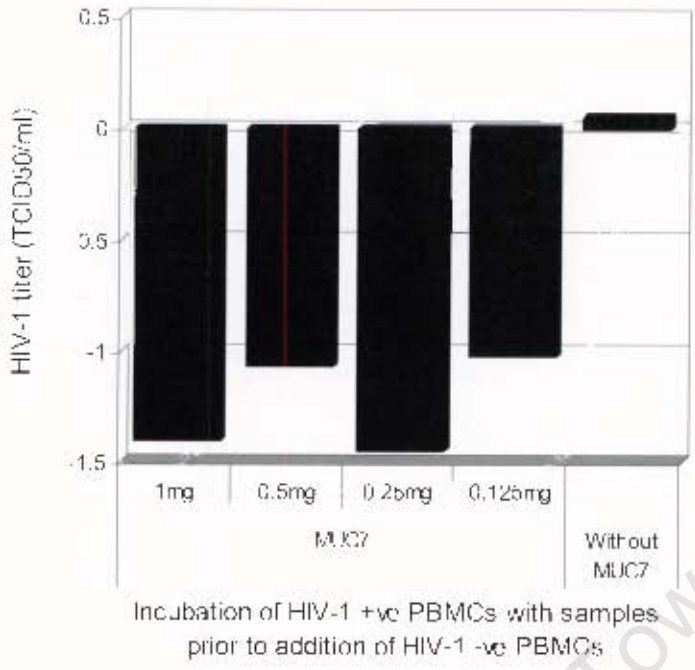


Figure 8.5 The role of MUC7 in minimizing the spread of HIV-1 from HIV-1 infected peripheral blood mononuclear cells to HIV-1 negative peripheral blood mononuclear cells in an *in vitro* assay. The PBMCs from HIV-1 positive individuals were incubated with different concentrations of salivary MUC7 (1mg, 0.5mg, 0.25mg and 0.125mg) for 60min. At the end of the incubation period the mixtures were added to HIV-1 negative PBMCs and cells were then cultured and viral replication was measured by a qualitative p24 antigen assay. As a control PBMCs from HIV-1 positive individuals were directly incubated with HIV-1 negative PBMCs.

To determine whether lengthy duration of cell culture could affect the outcome of the above result, the mixtures (HIV-1 positive PBMCs plus different concentrations of MUC7) were incubated with HIV-1 negative PBMCs and cultured for longer time (six days). However, lengthy incubations (cell culture) made no difference to the above results (data not shown).

8.3 Discussion

This study proved the ability of salivary, breast milk and pregnancy plug mucins to inhibit HIV-1 activity. The question is what can be done to exploit the anti-HIV-1 activity of these mucins. Thus the focus of this Chapter was to investigate whether salivary MUC7 can be used as an anti-HIV-1 agent in the blood stream which is one of the main routes of HIV-1 transmission.

The PBMCs which were used in this study are known to express all the three chemokine receptors or co-receptors (CCR5, CXCR4, and CCR3) and several adhesion molecules such as MHC II, ICAM-3, ICAM-1 and LEA-1, in addition to CD4 (Lallos *et al.*, 1999). The HIV-1 Subtype C which is the most prevalent strain in the Southern African countries was the virus strain used in this experiment.

The mucin (MUC7) did not inhibit infection of the PBMCs by the Subtype C virus by blocking the putative viral binding sites as mentioned in Chapter 6. MUC7 failed to inhibit infection of the CEM SS cells by the Subtype D virus. Again the reason could either be that MUC7 did not block the viral binding sites or the viruses were quick to bind or enter the PBMCs before being trapped or aggregated by the mucin (Bergey *et al.*, 1994; Bolscher *et al.*, 2002; Malamud *et al.*, 1993). However, as previously mentioned, electron microscopy is required to determine exactly the interaction of the mucin and PBMCs.

The other point of interest was that if mucins were added to the already infected PBMCs would they reduce the infectivity of these cells. To address these questions HIV-1 positive PBMCs were incubated or treated with different concentrations of MUC7 and cultured. However, no reduction in viral infection as a result of MUC7 treatment was observed. HIV-1 is an intracellular pathogen which integrates with the host cells nucleic acids, and the likelihood of there being any physical contact between the virus in the HIV-1 positive PBMCs and the mucin, is unlikely to occur (Bergey *et al.*, 1994; Bolscher *et al.*, 2002; Malamud *et al.*, 1993).

As shown in the previous Chapter (Chapter 6), salivary (MUC5B and MUC7), breast milk (MUC1) and pregnancy plug (MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) mucins inhibited HIV-1 activity by aggregating the virus. The question in our minds is whether mucins can act as therapeutic or anti-HIV-1 agents in areas such as blood which is not known to be a rich source of mucins but one of the main routes of HIV-1 transmission. As HIV is an intracellular pathogen which integrates with the host cells nucleic acids, the idea of reducing HIV-1 infection of infected cells using mucins (see the previous paragraph) which inhibit HIV-1 by aggregating or trapping the virus (Bergey *et al.*, 1994; Bolscher *et al.*, 2002; Malamud *et al.*, 1993) is out of the question. Furthermore as a cure for HIV is yet to be found, the possible solution

would be to keep the virus at the lowest level possible or to minimize the generation of new virus particles by destroying the infected cells as $CD8^+$ T cells do (McCune, 2001; McMichael and Rowland-Jones, 2001).

Although the information about viral spread among lymphocytes is limited (Sol-Foulon *et al.*, 2002) an attempt was made to check if mucins could minimize the spread of HIV-1 from HIV-1 positive to HIV-1 negative PBMCs by aggregating the free viruses which could be released by the infected cells before entering the uninfected ones. In this study, MUC7, which is the smallest mucin and simplest in structure was used (Bolscher *et al.*, 1999; Lagow *et al.*, 1999; Situ and Bobek, 2000; Thornton *et al.*, 1999). As it is only found in saliva (Thomsson *et al.*, 2002) its lack of toxicity to both white and red blood cells was confirmed prior to the HIV assay. As shown in the result section interestingly enough MUC7, at all mucin concentrations, inhibited HIV-1 activity by 100%. Theoretically the spread of HIV-1 from the HIV-1 positive to the HIV-1 negative PBMCs should be minimized by mucin trapping or aggregating free viruses released by the infected cells prior to their entry to the uninfected cells. It is assumed that viral particles released from the infected PBMCs should be detectable, which did not seem to be the case here. Also surprisingly, hardly any virus was detected when the mucin untreated HIV-1 positive PBMCs were directly added to the HIV-1 negative PBMCs, raising the question of whether any viral exchange occurred between uninfected and infected cells.

Another question that arose from these results was whether three days of cell culture was sufficient time for the viruses to spread from the HIV-1 positive PBMCs to HIV-1 negative PBMCs to cause infection. To address this the experiment was repeated followed by a six day cell culture. However, this did not influence the above result. In this assay the occurrence of any viral spread from HIV-1 positive PBMCs to HIV-1 negative PBMCs remains ambiguous. Thus further studies should include the establishment of an assay with a capacity to confirm viral spread from HIV-1 positive PBMCs to HIV-1 negative PBMCs and an indirect co-culture HIV assay to exactly determine the role of MUC7 in minimizing the spread of HIV-1 from the infected to the non infected blood cells.

In summary MUC7 neither inhibited HIV infection of the PBMCs by blocking the putative viral binding sites nor reduced viral infection of the HIV-1 infected PBMCs in an *in vitro* assay. Although MUC7 seemed to inhibit or minimize the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs as mentioned in the previous paragraph further study is required for the future to answer the questions raised by the study.

CHAPTER 9

ANTI-VACCINIA VIRUS ACTIVITIES OF SALIVARY, BREAST MILK AND PREGNANCY PLUG MUCINS IN AN *IN VITRO* INHIBITION ASSAY

9.1 Introduction

The objective of the part of the study reported in this Chapter was to check if salivary, breast milk and pregnancy plug mucins can inhibit the activity of other enveloped viruses besides HIV-1. In this study the activities of purified MUC5B and MUC7 from saliva, MUC1 from breast milk and pregnancy plug mucins against a laboratory adapted enveloped virus, vaccinia virus (vGK-5 strain), which is a member of the poxvirus family was determined by an inhibition assay. Vaccinia virus (vGK-5) was incubated with MUC5B, MUC7, MUC1 and pregnancy plug mucins separately and subsequently added to the African green monkey kidney cells (BSC-1). Cells were then cultured and the state of infection was monitored by Crystal Violet Dye solution staining.

9.2 Results

9.2.1 Toxicity assay

The toxicity of MUC5B and MUC7 from saliva, MUC1 from breast milk and pregnancy plug mucins to the BSC-1 was determined by a toxicity assay. The result revealed that none of the mucins were toxic and no cell death was observed (Table 9.1).

Table 9.1 Toxicity of MUC5B and MUC7 from saliva, MUC1 from breast milk and pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) to the BSC-1.

Sample	Con	BSC-1	% of dead cells	% of live cells
MUC5B	0.1mg	3×10^6/ml	0	100
MUC7	0.1mg	3×10^6/ml	0	100
MUC1	0.1mg	3×10^6/ml	0	100
Pregnancy plug mucins	0.1mg	3×10^6/ml	0	100

The percentage of live cells was calculated as the number of live cells over total cells (live plus dead) × 100.

9.2.2 Anti-vaccinia virus activities of salivary mucins

The result demonstrated that MUC5B and MUC7 mucins inhibited vGK-5 infectivity by about 60% and 70% respectively. However no inhibition (100% infection) of the BSC-1 was reported when the control, untreated viruses (vGK-5 without mucins) were added to the BSC-1 (Figure 9.1).

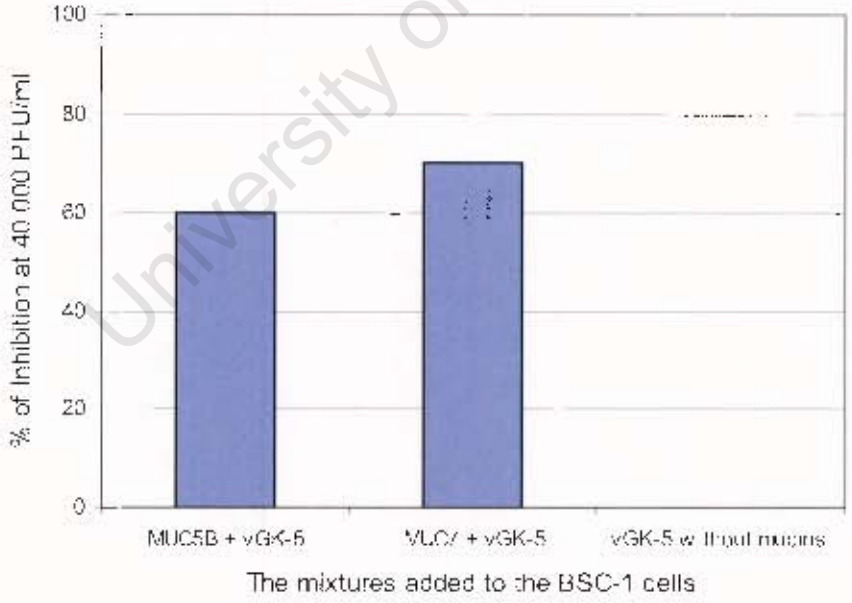


Figure 9.1 Anti-vaccinia virus activity of salivary MUC5B and MUC7 mucins. Salivary MUC5B and MUC7 (0.1mg each) were incubated with vGK-5 for 15min at 37°C. The mixtures were added to the BSC-1 in the Minimum Essential Medium and cultured at 37°C in a CO_2 incubator (5% CO_2) for 48h to allow the formation of viral plaques. As a control untreated viruses (vGK-5 without mucins) were added to the BSC-1. Evidence of inhibition was then demonstrated by staining of the cell monolayers with Crystal Violet Dye.

9.2.3 Anti-vaccinia virus activities of pregnancy plug mucins

The result revealed that at viral concentrations of 3 million Plaque Forming Unit/ml (PFU/ml) and 2.4 million PFU/ml, pregnancy plug mucins (containing MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) inhibited vGK-5 activity by 55% and 100% respectively. However when the same concentrations of untreated viruses (vGK-5 without mucins) were added to the BSC-1 no inhibition (100% infection) of the BSC-1 was observed (Figure 9.2).

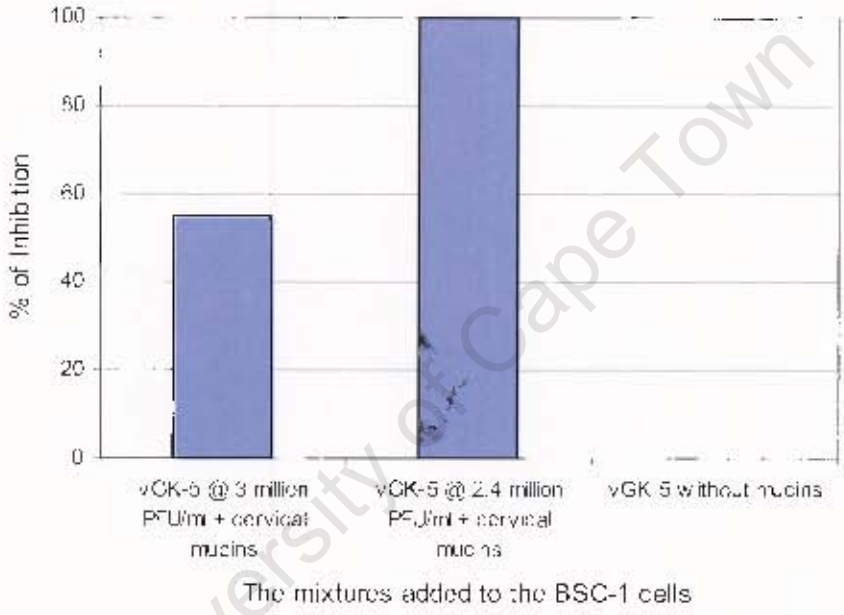


Figure 9.2 **Anti-vaccinia virus activity of pregnancy plug mucins.** Pregnancy plug mucins (0.1mg) were incubated with vGK-5 at viral concentrations of 3 million PFU/ml and 2.4 million PFU/ml for 15min at 37°C. The mixtures were added to the BSC-1 in the Minimum Essential Medium and cultured at 37°C in a CO_2 incubator (5% CO_2) for 48h to allow the formation of viral plaques. As a control untreated viruses (vGK-5 without mucins) were added to the BSC-1. Evidence of inhibition was then demonstrated by staining of the cell monolayers with Crystal Violet Dye.

9.2.4 Anti-vaccinia virus activity of breast milk mucin

The activity of MUC1 (milk mucin) against vGK-5 activity at different viral concentrations was also determined by an *in vitro* inhibition assay. The assay revealed that at viral concentrations of 3 million PFU/ml and 2.4 million PFU/ml MUC1 inhibited vGK-5 activity by 75% and 100% respectively. On the other hand when the

BSC-1 were infected by the same concentrations of untreated viruses (vGK-5 without MUC1), no inhibition (100% infection) of the BSC-1 was detected (Figure 9.3).

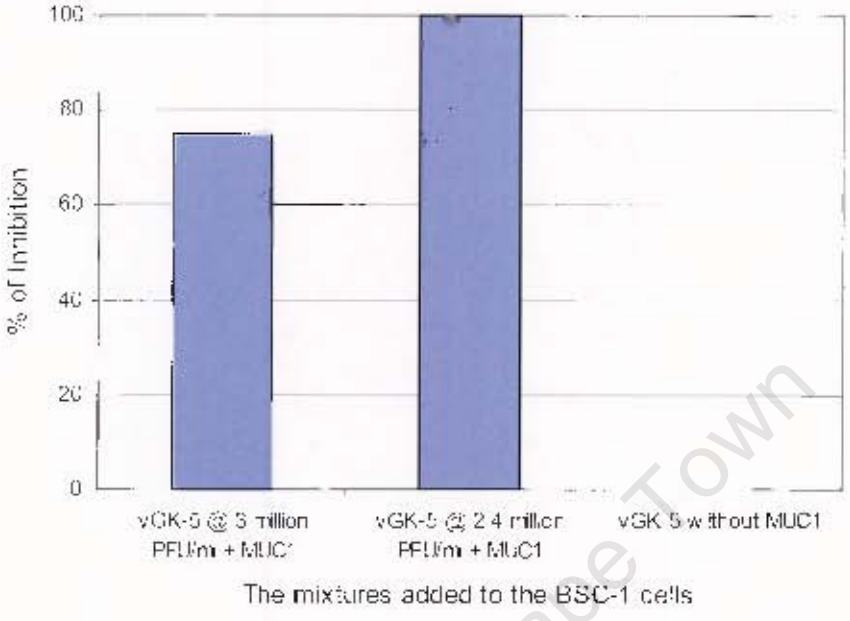


Figure 9.3 Anti-vaccinia virus activity of breast milk mucin. Milk mucin (MUC1) (0.1mg) was incubated with vGK 5 at viral concentrations of 3 million PFU/ml and 2.4 million PFU/ml for 15min at 37°C. The mixtures were added to the BSC-1 in the Minimum Essential Medium and cultured at 37°C in a CO_2 incubator (5% CO_2) for 48h to allow the formation of viral plaques. As a control untreated viruses (vGK-5 without MUC1) were added to the BSC 1. Evidence of inhibition was then demonstrated by staining of the cell monolayers with Crystal Violet Dye.

9.3 Discussion

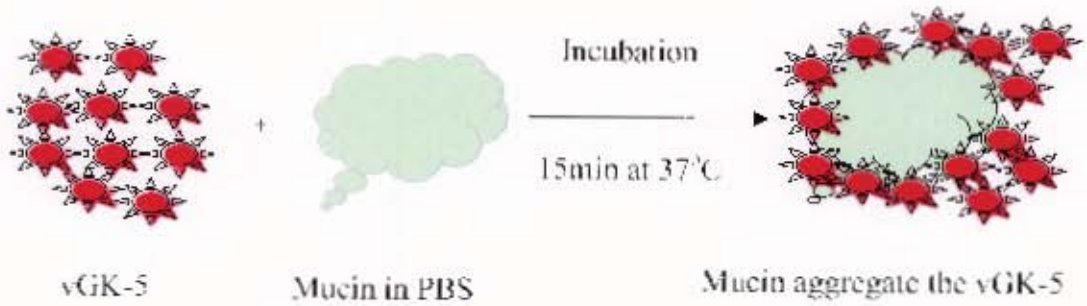
As salivary, breast milk and pregnancy plug mucins inhibit HIV-1 activity, we tested the effectiveness of mucins against the activity of other enveloped viruses. In this Chapter we describe the anti-vaccinia virus activity of these mucins.

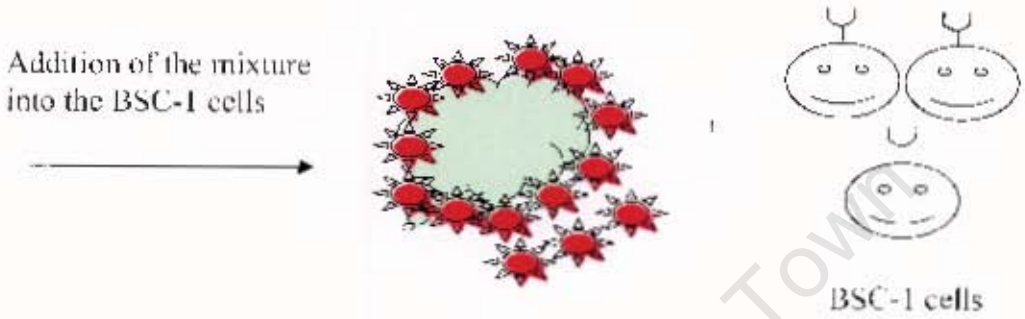
The virus which was used in this study was a highly attenuated recombinant vaccinia virus strain (vGK5) of the enveloped poxvirus family (Billings *et al.*, 2004). According to Billings *et al.* (2004) vGK5 is a double-stranded DNA virus and 185 kbp in size. Like the wild-type Western Reserve strain of vaccinia virus it can grow in cell culture but lacks a neuro-virulence factor to replicate in the brain which makes it relatively safe to handle (Kotwal *et al.*, 2004).

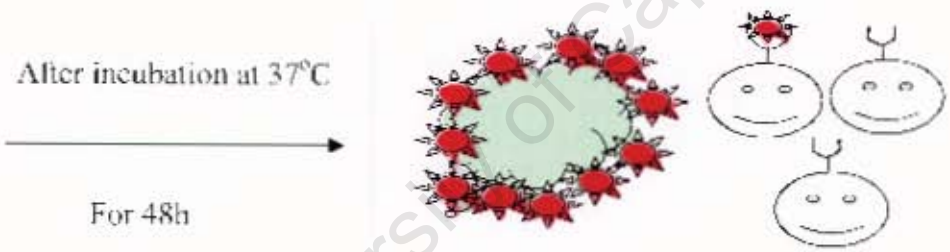
Upon showing that salivary MUC5B and MUC7 mucins inhibited HIV-1 activity (Chapter 6) we wondered whether these mucins showed the same inhibitory activity against other enveloped viruses such as vGK-5. When the vGK-5 was treated with salivary MUC5B and MUC7 mucins prior to addition to the host cells (BSC-1) the infectivity of the vGK-5 was reduced. It is likely that, as in the case of HIV-1 (Chapter 6), the carbohydrate moieties of the salivary mucins could trap or aggregate the vGK-5 (Bosch *et al.*, 2000) and inhibit host cells entry (Klein *et al.*, 1992, Prakobphol *et al.*, 1998, Prakobphol *et al.*, 1999, Prakobphol *et al.*, 2005). However, to confirm this hypothesis deglycosylation of both MUC5B and MUC7 prior to the incubation with vGK-5 is required.

As explained earlier the mucins may have inhibited viral activity by aggregating the virus prior to host cell entry. However the rate of aggregation or inhibition could be influenced either by mucin concentrations or viral load. To prove this hypothesis inhibition assays at different viral or mucin concentrations were required.

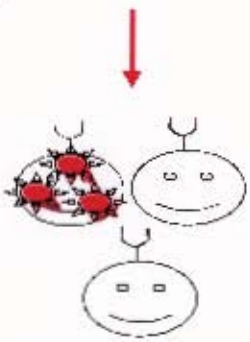
As was shown in the result section, the pregnancy plug mucins and MUC1 (milk mucin) inhibited vGK-5 activity in a dose dependent manner up to 100%. Although the reason is not completely clear, at higher viral concentration (3 million PFU/ml), it is possible that some viruses are left un-aggregated by the mucins, hence free to bind to the surface of the BSC-1 and cause viral infections. This could be the reason why there was only 55% and 75% inhibition of the vGK-5 activity by pregnancy plug mucins and MUC1 respectively. On the contrary when fewer viruses (2.4 million PFU/ml) were used it is possible that enough space on the surface of the mucins should be available for all the viruses to bind and, as a result, no free viruses are left to bind and infect the BSC-1 (Figure 9.4). Therefore 100% inhibition of the vGK-5 activity was achieved by both the pregnancy plug mucins and MUC1.







Due to the large number no enough space in the mucin was available for all the viruses to bind thus some viruses were left free to bind and infect the BSC-1 cells



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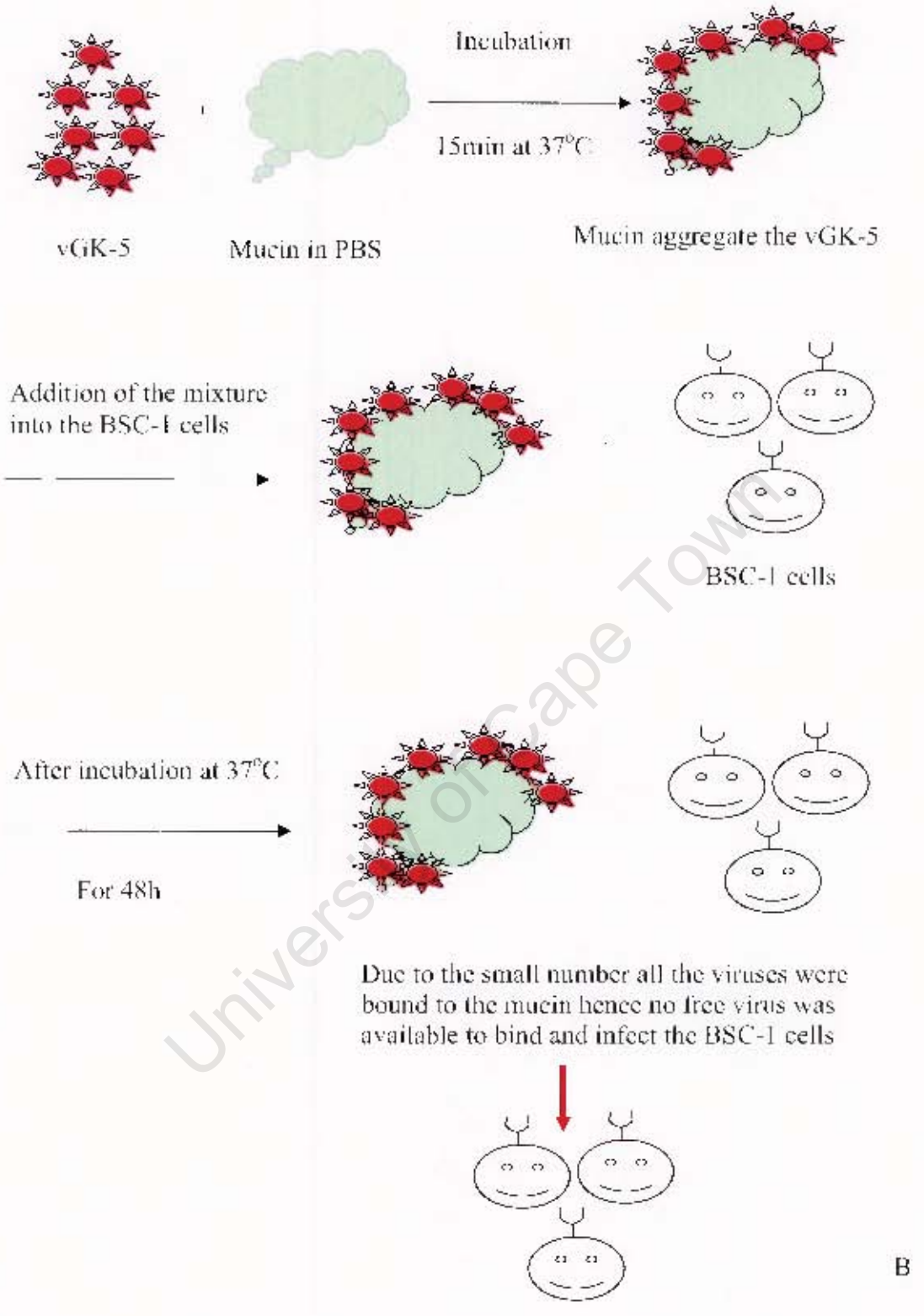

Figure 9.4 A sketch to explain the effect of viral dose in vaccinia virus inhibition assay. Vaccinia virus (vGK-5) at viral concentrations of 3 million PFU/ml (A) and 2.4 million PFU/ml (B) were incubated with mucins for 15min at 37°C. The mixtures were added to the BSC-1 in the Minimum Essential Medium and cultured at 37°C in a CO_2 incubator (5% CO_2) for 48h. Evidence of inhibition was then demonstrated by staining of the cell monolayers with Crystal Violet Dye.

In conclusion the lack of effective and long lasting vaccines against enveloped viruses makes humans very susceptible for the outbreak of any new virulent strains (Kotwal *et al.*, 2005). Therefore more attention should also be given to the natural anti-viral components such as mucins in the search for safe, affordable and effective vaccines.

CHAPTER 10

CONCLUSIONS, DISCUSSION AND THE FUTURE

Since its outbreak in 1981 in sub-Saharan Africa, about 25 million adults and children have been reported to live with HIV/AIDS with approximately 2.2 million deaths in 2003 alone (Losina *et al.*, 2006). According to Shaikh *et al.* (2006) Southern African countries have the highest prevalence of HIV/AIDS with 25.7% compared to East and West Africa, which respectively have 11.4% and 4.3%. In South Africa alone, between 4.68 and 7.03 million people were living with HIV/AIDS in 2004 (Strode *et al.*, 2005), of whom 55% were females (Kagee *et al.*, 2005) and which puts the country among the worst affected countries in the world (Shaikh *et al.*, 2006). Jaspan *et al.* (2005) reported that of the 1 million annual births in South Africa, more than a quarter of the infants are born to HIV-infected mothers. By the year of 2010 this epidemic is expected to cost South Africa approximately 17% of its GDP growth (Stevens *et al.*, 2006). Furthermore the incidence of opportunistic diseases such as TB is also reported to increase with HIV prevalence (Lawn and Wood, 2006; Soeters *et al.*, 2005). Therefore the need for the development of a safe, natural and affordable vaccine is very urgent.

This study raises questions of the possibility of novel compounds, in this case with mucus as a possible component, to prevent transmission of the virus or to reduce the load of free virus in bodily substances such as the blood. Obviously more work is required to develop these ideas further.

The HIV-1 Subtype C (see Chapter 8) is currently the most prevalent Subtype in Southern African countries and has only recently become available to us. Thus our intention is to use it for future inhibition assays in conjunction with a range of different cell lines with appropriate dose response curves. The very high costs of the *in vitro* inhibition assays have been a huge limiting factor in this study.

This study focused on the role of human salivary, breast milk and pregnancy plug mucus and mucins from HIV negative individuals in HIV-1 inhibition. This was

compared to the anti-HIV-1 activity of the salivary mucins from HIV positive individuals in an *in vitro* inhibition assay. Furthermore we investigated the role of mucins in inhibiting HIV-1 infection, reducing viral infection of the HIV-1 positive PBMCs and minimizing the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs. The role of mucus and mucins in the inhibition of other enveloped viruses such as poxvirus was also a part of this study. The possibility of mucus or mucins being eventually used as an anti-viral agent is a long term goal of our research.

We have shown that normal saliva and its mucin component together with purified mucin from breast milk and the pregnancy plug inhibit the virus. Crude breast milk and pregnancy plug mucus from normals and salivary mucins from patients who are HIV positive have no effect on viral infectivity. Mucins failed to reduce viral infection of the HIV positive cells or prevent the infectivity of cells with which they were incubated. There is also an urgent need to test the effect of heated breast milk, which we think could release the MUC1 and consequently have an effect on viral load and transmission.

The lack of inhibition by infected salivary mucin component is of great interest. Very early indications, by agarose gel electrophoresis, are that these mucins have different charge properties, implying altered glycosylation of the mucins which could well result in altered viral binding properties of the mucins. More agarose gel electrophoresis data followed by HPLC sugar analysis, comparing the length of carbohydrate side-chains and monosaccharide composition of mucins in normal and infected states will contribute further to the understanding of our findings. The failure of HIV-1 positive secretions to infect cells in culture would be due to a low viral load, something to consider for the future.

This study has been largely a qualitative one, the idea of which arose from an earlier finding that crude saliva and possibly its mucus component inhibited the HIV-1 virus in an *in vitro* assay. We were able to confirm this finding, together with isolating the purified mucins from saliva, breast milk and pregnancy plug mucus identifying them and further showing their anti-HIV-1 activity. Inhibition of the virus seemed immediate and the effect of the mucins was not lessened by considerable dilution of

their amount. However we were unable to establish a suitable dose-response curve down to the smallest amount of mucin that would show inhibitory activity. Also, our findings need to be verified statistically and we are designing a study to do this. We have found that salivary, breast milk and pregnancy plug samples had to be pooled to have enough of a mucin yield; also the cost of the inhibition assays was daunting to say the least and we are currently seeking funding to address the many questions this study has raised. Kirkham *et al.* (2002) has described a method of quantifying mucin in crude mucus and it will be worth our while to have this data in future studies.

Another future consideration would be to use an indirect co-culture for HIV inhibition assay to accurately determine the role of mucins in minimizing the spread of HIV-1 from HIV-1 positive PBMCs to HIV-1 negative PBMCs. In this method the HIV-1 positive PBMCs and HIV-1 negative PBMCs can be cultured in a tissue culture plate with compartments separated by a membrane with the HIV-1 positive PBMCs in the lower and the HIV-1 negative PBMCs in the upper compartment. Viruses and growth factors can pass through the membrane but not the PBMCs and mucins; therefore there should not be any physical contact between the PBMCs in the lower and the PBMCs in the upper compartments of the tissue culture plate. Thus the principle of the experiment is the HIV-1 positive PBMCs in the lower compartment will be treated with different concentrations of mucins and their role in minimizing HIV spread from the HIV-1 positive PBMCs in the lower compartment to the HIV-1 negative PBMCs in the upper compartment will be determined by p24 antigen assay. That is, if the mucins trap or aggregate the free viruses which are released from the HIV-1 infected PBMCs, no viral infection of the HIV-1 negative PBMCs in the upper compartment should be recorded but if the mucins failed to trap or aggregate the free viruses, as the viruses are free to pass through the membrane to the upper compartment viral infection of the HIV-1 negative PBMCs in the upper compartment is expected. However, confirmation of viral spread from HIV-1 positive PBMCs to HIV-1 negative PBMCs is required prior to this assay.

Furthermore we wish to examine whether there is any association between gastro-intestinal mucin gene polymorphisms and susceptibility to infection with HIV. Mucin genes are highly polymorphic due to the presence of long stretches of variable number of tandem repeats (VNTRs) that are heavily glycosylated. Several studies reported an

association between disease and the length of the VNTR region, for example in asthma (Kirkbride *et al.,* 2001; Vinall *et al.,* 2000) or polymorphisms at the level of a single nucleotide, as in ulcerative colitis (Kyo *et al.,* 2001), and an association between short alleles of MUC1 and gastric cancer (Carvalho *et al.,* 1997). The variation of alleles in subjects of different ancestry has also been reported (Kirkbride *et al.,* 2001).

MUC7 is of interest to us because of the reported association between a haplotype carrying a shorter MUC7 allele of five tandem repeats (MUC7*5) with higher Forced Expiratory Volume (FEV_1) at 53 years, reduced age-related decline of FEV_1 and reduced incidence of wheeze has recently been reported. A 20mer MUC7 peptide has been shown to possess a broad-spectrum anti-microbial activity (Bobek and Situ 2003). Situ *et al.* (2003) further showed that a 12-mer peptide of the original 20-mer retains potent anti-fungal activity against *Candida albicans* and *Cryptococcus neoformans.*

Therefore one of our future projects is on the association of MUC7 and HIV susceptibility.

Purified salivary MUC5B and MUC7, breast milk mucin (MUC1) and pregnancy plug mucins (MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6) also inhibited the activity of vaccinia virus (vGK-5 strain) which is a family of the enveloped poxvirus (Billings *et al.,* 2004) in a dose dependent manner up to 100%. This suggests that mucins may also possess a strong activity against other enveloped viruses.

Finally this study has raised important questions that justify further investigation and requests for appropriate funding.

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