Purified Human Breast Milk MUC1 and MUC4 Inhibit Human Immunodeficiency Virus

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Abstract
Background: The HIV-AIDS pandemic is prevalent in sub-Saharan Africa. Breastfeeding is a risk factor, with transmission from mother to child being as high as 40%. *Objectives:* To determine the antiviral activity of crude breast milk and its purified mucins MUC1 and MUC4 against HIV-1 in patients who were HIV positive compared to those who were not. *Methods:* Twenty-one human milk samples were taken from both groups. Breast milk mucins were purified by density-gradient ultracentrifugation in caesium chloride and analyzed by SDS-PAGE, Western blotting and amino acid content. The inhibition of the virus by crude milk and purified mucin was assayed by an in vitro HIV-1 p24 assay. *Results:* SDS-PAGE for purified mucin showed several high-molecular-weight bands for the HIV-negative group and prominently stained single bands on the stacking gel with faintly periodic acid Schiff-positive glycoprotein bands observed in some cases in the running gel for the HIV-positive mucins. Western blot analysis identified the mucins in both groups to be MUC1 and MUC4. Both mucins showed more intensity on Western blotting for the HIV-positive group. There was no difference in the content of serine, threonine and proline of purified mucins for both groups. HIV-1 was not inhibited by crude breast milk from normal (13/14 samples) and infected individuals (19/19 samples). Fifteen of 20 and 16/18 samples of purified mucin from the uninfected and HIV-positive groups, respectively, inhibited the virus. *Conclusions:* Crude breast milk does not inhibit HIV-1, whilst purified mucins do in an in vitro assay.

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Introduction

Crude mucus is a viscous secretion synthesized by goblet cells of the columnar epithelium that line the major internal tracts of the body. The main component responsible for its viscous and elastic gel-like properties is the mucous glycoprotein (mucin) [1].

Gel-forming mucins are high-molecular-weight glycoproteins with complex O-linked oligosaccharide side chains found both in crude mucus gels and as trans-membrane proteins on the apical cell surface of glandular and ductal epithelia of various organs [2]. There are a whole

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host of trans-membrane mucins, with MUC1, MUC4 and MUC16 being very well characterized [3]. Human breast milk is a natural emulsion in which lipids are present in the form of small droplets called fat globules [4]. Proteins present in milk such as lactoferrin, lysozyme and secretory leukocyte protease inhibitor have been shown to possess anti-HIV properties [5].

In 1996, Rossi et al. [6] reported the presence of MUC1 in human milk, with MUC4 demonstrated later [7]. About 70% of mucins are present in the fat globule membrane (cream fractions) with the rest in skim milk [8]. Although human milk is rich in mucin [9], mother-to-child transmission of HIV-1 through breastfeeding accounts for the majority of HIV-1 infections among children.

Our laboratory showed that human saliva and its purified mucin from uninfected individuals inhibit HIV-1 in an in vitro assay [10], whilst mucins from HIV-positive patients did not [11]. We also showed that crude virus-free breast milk did not inhibit the virus, while purified mucin did [12].

The objectives of this study were to isolate, purify and characterize milk mucins and to compare the anti-HIV-1 activities of crude milk and its purified mucins in HIV-negative and, for the first time, HIV-positive patients, in an in vitro p24 inhibition assay. The study is meant to verify previous findings [12] and to further investigate the behavior of infected milk towards the virus.

Methods

Ethics
This study was approved by the University of Cape Town Health Sciences Faculty Research and Ethics Committee (Rec. Ref.: 096/2009).

Preparation of Milk Fat Globule Membrane and Purification of Mucins
Human breast milk samples were collected from HIV-negative (n = 21) and HIV-positive (n = 21) lactating mothers from the Maternity Unit at Groote Schuur Hospital (Cape Town, South Africa). Samples were collected into 6 M guanidinium chloride, 1 mM phenylmethylsulfonyl fluoride and 10 mM ethylenediaminetetraacetic acid at an approximate 1:5 ratio and immediately frozen at –20°C and stored. Just prior to analysis, the milk was thawed and subsequently dialyzed overnight at 4°C against three changes of distilled water and then freeze dried.

Mucins from milk fat globule membrane (MFGM), prepared according to the method of Schroten et al. [9], were purified by density-gradient ultracentrifugation in 3.5 M caesium chloride (CsCl) and 4 M guanidinium hydrochloride at 105,000 g for 48 h, twice [13]. Protein was estimated by the method of Lowry et al. [14], and glycoproteins by the periodic acid Schiff (PAS) proce-

dure of Mantle and Allen [15]. Mucins were further analyzed by 3–5% gradient SDS-PAGE (stained with Coomassie Blue and PAS) after being dissolved in sample application buffer containing 0.2 M 2-mercaptoethanol [16] and their identities characterized by Western blotting after 0.7% agarose gel electrophoresis [17]. Nitrocellulose membranes were incubated overnight either with 1:100 mouse anti-MUC1 NCL monoclonal antibody or with 1:200 diluted human MUC4-specific 1G8 monoclonal antibody. The amino acid analysis further confirmed the identity of the mucins [18].

HIV-1 p24 Antigen Assay
The anti-HIV-1 activities of crude breast milk and its purified mucins (at a concentration of 1.0 mg/ml in 0.25% PBS) from HIV-negative and HIV-positive women were tested in an HIV-1 p24 antigen assay which was a variation of the method of Nagashunmugam et al. [19] and Peacocke et al. [20]. Peripheral blood mononuclear cells (PBMCs) from 3 different HIV-negative donors were isolated using the Ficoll density-gradient method, and were stimulated with PHA and interleukin-2 for 4 and 2 days before the assay, respectively.

A dual-tropic (CCR5 and CXCR4) subtype C HIV-1 virus strain (TV167) was used for these experiments. Viral supernatant fluid in culture media (RPMI 1640 with 10% fetal calf serum, penicillin and streptomycin) and PBMCs served as the positive control, while culture media with and without PBMCs were used as negative controls. A second dual-tropic subtype C HIV-1 strain (TV671) was included in the experiments as an additional positive control and the HIV-1 assay was done according the method of Peacocke et al. [20]. HIV-1 viral infection in humans is characterized by periods of antigenemia in which HIV-1 antigens are detectable in the blood. The HIV p24 antigen assay is an ELISA assay for the quantitative estimation of p24 antigens, which are detectable in the blood after an individual has been exposed to the virus.

Results

Mucin Preparation, Purification, Identification and Analysis
The total protein in the milk samples in the HIV-negative and HIV-positive groups was 106.2 (SEM ± 11.48) and 144.01 mg (SEM ± 11.24), respectively. There was no significant difference in the mean amount of μg mucin/mg protein in the HIV-negative (17.17 SEM ± 3.48) compared to the HIV-positive (18.86 SEM ± 3.05) groups.

After the second purification step, a clear separation of proteins by Lowry assay from PAS-positive glycoprotein (mucin) was observed, denoting purity of mucin (fig. 1). Mucin-rich fractions with a density of 1.32–1.41 g/ml for the HIV-negative samples (fractions 4, 5 and 6; fig. 1a) and 1.39–1.46 g/ml for the HIV-positive samples (fractions 4, 5 and 6; fig. 1b) were pooled, dialyzed against three changes of distilled water and then freeze dried.
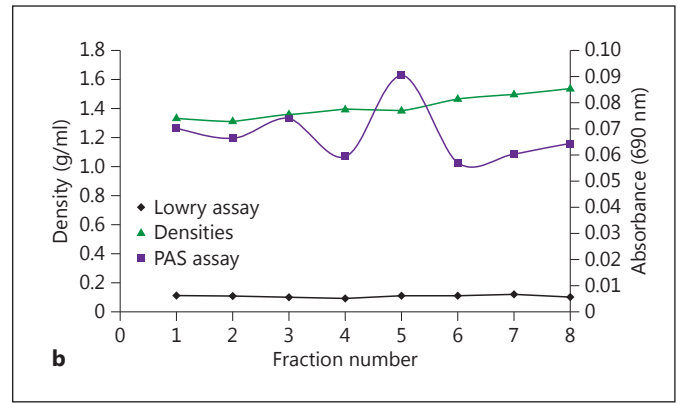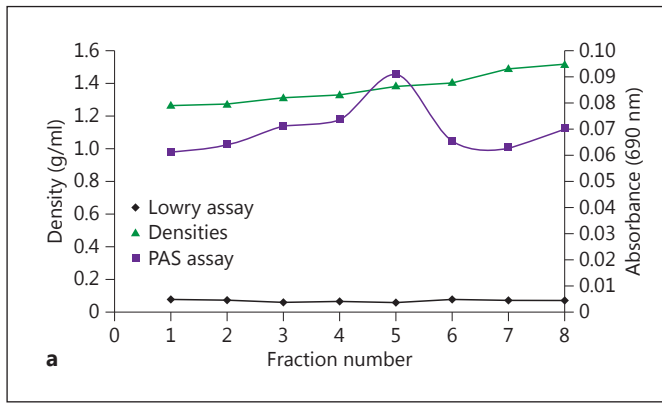
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Fig. 1. Second CsCl density-gradient centrifugation of the MFGM samples prepared from HIV-negative (**a**) and HIV-positive (**b**) samples. The purification of mucins after a second centrifugation step in a CsCl density gradient. Solid CsCl and 4 M guanidinium chloride containing 10 mM ethylenediaminetetraacetic acid, 5 mM NEM and 0.05% CHAPS pH 6.5 were added to the semi-purified mucin-rich fractions obtained from the first density centrifugation spin to give a starting density of 1.39–1.40 g/ml. After centrifugation, the tubes were fractionated into 8 equal fractions, and an aliquot of each fraction was used for density measurements and the estimation of mucin and protein. The figures show the clear removal of all the protein (Lowry assay line) contamination, with the mucin-rich (PAS assay line) fractions 4, 5 and 6 at a density (Densities line) of 1.32–1.41 and 1.39–1.46 g/ml, respectively.

The purity of the MFGM mucins was confirmed by 3–5% SDS-PAGE gradient stained with Coomassie brilliant blue. The gels show that the purification of mucins by density-gradient ultracentrifugation in CsCl was successful (fig. 2, lanes 4–5) as compared to the unpurified MFGM material that showed the presence of contaminant proteins (fig. 2, lanes 2–3).

Purity was also confirmed by amino acid analysis. Serine, threonine and proline amounted to 11.2, 11.6 and 8.1% (30.9% of the mucin) for the HIV-negative group (table 1) and 11.5, 10.9 and 11.7% (34.1% of the mucin) for the HIV-positive group (table 1), very much in the range of what is expected for mucins [18].

Purified mucins were loaded (30 μg) onto 10% SDS-PAGE gels and stained with PAS. The HIV-negative samples (fig. 3a, lanes 2–8) demonstrated bands that stained prominently in the stacking and on top of the running gel with a molecular weight >170 kDa and of varying intensity. Additionally, 3 samples showed smeared bands with sizes ranging from 72 to 170 kDa that have entered the running gel (fig. 3a, lanes 2, 6 and 7). The HIV-positive mucin samples showed a prominent band, running as an intensely stained smear of molecular weight size >170 kDa in the stacking gel (fig. 3b, lanes 2–8). Faintly staining smears with sizes ranging from 72 to 170 kDa were observed in the running gel for the HIV-positive mucins (fig. 3b, lanes 2, 5, 6 and 8).

Western blotting confirmed the presence of MUC1 (fig. 4a) and MUC4 (fig. 4b) as the mucin in human breast
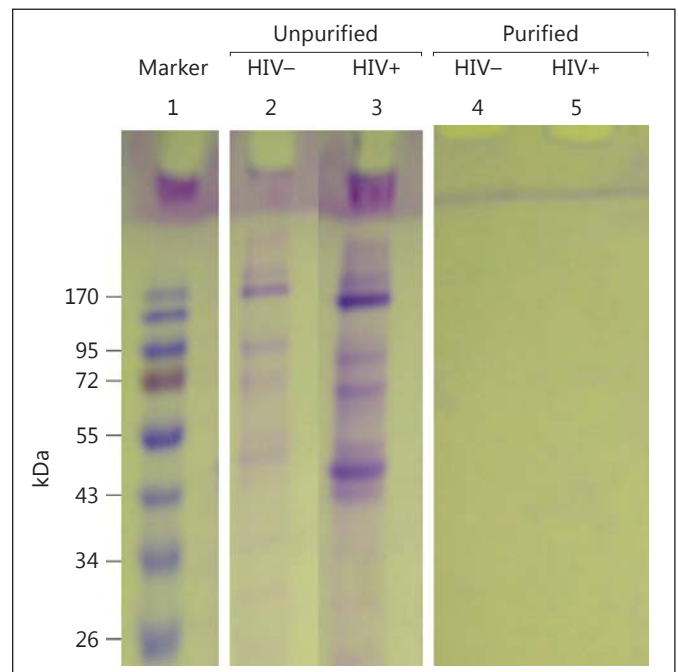

Fig. 2. Three to 5% SDS-PAGE gradient gel analyses of the milk mucins before and after purification. 30 μg of the freeze-dried purified MFGM material was prepared in reducing gel loading buffer and separated on a 3–5% SDS-PAGE. The gel was stained for protein with Coomassie brilliant blue R-250. Lane 1, molecular weight marker; lanes 2–3 show the bands of proteins from unpurified MFGM samples from the HIV-negative and -positive groups, respectively; lanes 4–5 represent the purified MFGM samples from the HIV-negative and -positive groups, respectively. HIV– = HIV negative; HIV+ = HIV positive.
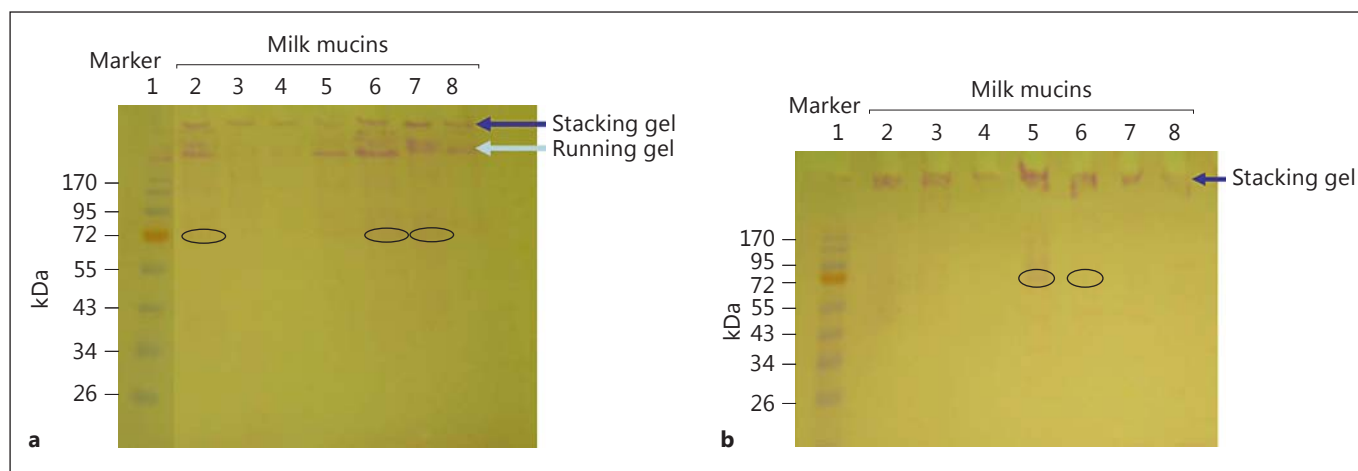
Fig. 3. Ten percent SDS-PAGE analysis of breast milk mucins. Freeze-dried milk mucins from HIV-negative samples (**a**) and HIV-positive samples (**b**) were prepared in reducing gel loading buffer and subjected to 10% SDS-PAGE, and the gels were stained with PAS. Lane 1, protein molecular weight markers; lanes 2–8 indicate the mucins on the gel. Lanes 2, 6 and 7 (**a**) and lanes 5 and 6 (**b**; arrows) show the presence of mucins which have entered the running gel.

Table 1. Analysis of the amino acid composition of purified mucins from HIV-negative and HIV-positive groups

| Amino acids	One-letter symbol	μmol% HIV–	μmol% HIV+
Aspartic acid	D	7.1	7.8
Threonine	T	11.6	10.9
Serine	S	11.2	11.5
Glutamic acid	E	11.2	8.7
Proline	P	8.1	11.7
Glycine	G	5.1	5.4
Alanine	A	8.6	7.3
Valine	V	5.1	5.5
Methionine	M	0.0	0.8
Isoleucine	I	3.0	3.0
Leucine	L	4.7	5.1
Tyrosine	Y	0.4	1.6
Phenylalanine	F	1.0	1.4
Lysine	K	3.7	2.9
Histidine	H	15.1	12.5
Arginine	R	3.4	3.8

milk. Cervical mucins were used as positive controls (fig. 4, lane 1). The results show that the MUC1 and MUC4 mucins were detected in both groups. The staining for MUC1 in the milk mucins for the HIV-negative group (fig. 4a, lanes 3–4) showed less staining intensity than the MUC1 detected in the milk mucins from the HIV-positive group (fig. 4a, lanes 5–6). Also the MUC1 from the HIV-positive group had a wider spectrum of charge and size (fig. 4a, lanes 5–6) than the MUC1 from the HIV-negative group (fig. 4a, lanes 3–4). The detection of MUC4 in the HIV-negative group (fig. 4b, lanes 3–4) also showed less intense staining than in the HIV-positive group (fig. 4b, lanes 5–6), with a particularly intense reaction for the sample in lane 5 (fig. 4b).

HIV-1 p24 Antigen Assay

The anti-HIV-1 activities of crude breast milk and its purified mucins (at a concentration of 1.0 mg/ml in 0.25% PBS) from HIV-negative and HIV-positive women were tested in an HIV-1 p24 antigen assay which was a variation of the method of Nagashunmugam et al. [19] and Peacocke et al. [20]. PBMCs from 3 different HIV-negative donors were isolated using the Ficoll density-gradient method, and were stimulated with PHA and interleukin-2 for 4 and 2 days before the assay, respectively. A dual-tropic (CCR5 and CXCR4) subtype C HIV-1 virus strain (TV167) was used for these experiments. Viral supernatant fluid in culture media (RPMI 1640 with 10% fetal calf serum, penicillin and streptomycin) and PBMCs served as the positive control, while culture media with and without PBMCs were used as negative controls. A second dual-tropic subtype C HIV-1 strain (TV671) was included in the experiments as an additional positive control, and the HIV-1 assay was done according the method of Peacocke et al. [20]. HIV-1 viral infection in humans is characterized by periods of antigenemia in which HIV-1 antigens are detectable in the blood. The HIV p24 antigen

Fig. 4. Identification of MUC1 (**a**) and MUC4 (**b**) in purified human breast milk mucins by Western blotting analyses. Samples (400 μg) were separated on a 0.7% agarose gel electrophoresis and then transferred onto a nitrocellulose membrane before blocking overnight. Primary antibodies: mouse anti-MUC1 and anti-MUC4. Secondary antibodies: goat anti-mouse horseradish peroxidase. **a** MUC1 Western blot analysis: lane 1, cervical mucus positive control; lane 2, negative control; lanes 3–4, MUC1 expressed in HIV-negative purified milk mucins; lanes 5–6, MUC1 expressed in HIV-positive purified milk mucins. **b** MUC4 Western blot analysis: lane 1, cervical mucus positive control; lane 2, negative control; lanes 3–4, MUC4 expressed in HIV-negative purified milk mucins; lanes 5–6, MUC4 expressed in HIV-positive purified milk mucins. Pos con = Positive control; Neg con = negative control.
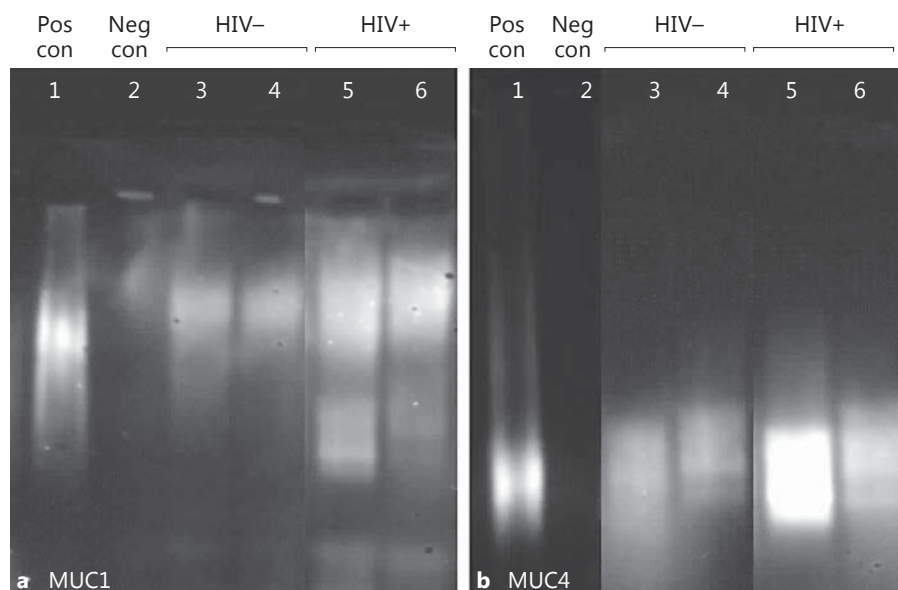
assay is an ELISA assay for the quantitative estimation of p24 antigens, which are detectable in the blood after an individual has been exposed to the virus.

Discussion

Mother-to-child transmission of the HIV through breastfeeding accounts for a significantly high proportion of the burden of HIV infection in sub-Saharan Africa. The idea of investigating the role of breast milk and its mucin constituents in the inhibition of HIV-1 arose after our laboratory showed that saliva and its mucins inhibited the virus in an in vitro assay [10].

Our aim, in this expanded study which included an HIV-positive group, was to verify the original qualitative study by Habte et al. [12] showing that crude breast milk did not inhibit HIV-1 whilst its purified mucin component did. Whilst Habte et al. [12] identified MUC1 in HIV-negative breast milk, we were further able to identify both MUC1 and MUC4 in breast milk of HIV-negative and -positive patients. We were unable to separate MUC1 from MUC4 to test them individually. Our results, like those of Habte et al. [12] and unlike those of another study [21], showed that crude breast milk does not inhibit HIV-1.

On SDS-PAGE, there was an interindividual variation in the electrophoretic behavior of the mucins from the HIV-negative group. In some cases, there were several bands present in the stacking and at the beginning of the running gel (fig. 3a, lanes 2, 5–8). One prominent band of slightly varying intensity was present at the beginning of the stacking gels for all the samples (fig. 3b, lanes 2–8) of the HIV-positive group. It is not certain why the PAS-positive glycoprotein bands in the running gel on SDS-PAGE for the HIV-positive group appeared faint. MUC1 in disease has been reported to have an altered (deficient) glycosylation [22], which is possible in HIV-infected milk.

Both MUC1 and MUC4 were detected by Western blotting in both groups (fig. 4) after purification of mucins by CsCl density-gradient ultracentrifugation in which proteins, which fractionate at lower densities, are separated from mucins, which fractionate as a distinct PAS-positive peak at an approximate density of 1.4 g/ml. MUC1 from the HIV-positive group showed an electrophoretic behavior quite different from that of the HIV-negative group, indicating that there was a range of charge and size far wider in the former than in the latter group. This suggests that there was altered glycosylation of MUC1 in infected milk, perhaps explaining the absence of detection of MUC1 in the HIV-positive group on SDS-PAGE. The difference between MUC4 in the two groups was not as stark as that of MUC1 on Western blotting. However, for both MUC1 and MUC4, Western blotting showed a far higher intensity for mucins from the HIV-

positive group, for equal loadings, suggesting a possible defense mechanism coming into effect in the HIV-positive state. This would complicate the explanation for the absence of MUC1 in the HIV-positive group on SDS-PAGE. Although the identity of the mucins was further confirmed by amino acid analysis, there was hardly a difference between the groups [23].

HIV-1, when incubated with crude breast milk samples, gave a positive expression of the p24 antigen on day 4, suggesting that no inhibition of the virus had occurred. The reason for this is unclear, but it could be that the mucins are of low concentration in milk, enclosed in fat globules [9, 12] and not exposed to the virus, and thus unable to prevent mother-to-child transmission during breastfeeding. It could also be that human breast milk has far more virus (240–8,100 copies/ml) [21], compared with less than 1 copy/ml in saliva, making oral transmission so rare [24].

When the HIV-1 virus was incubated with purified mucins, no expression of p24 occurred, suggesting that the mucins inhibited the virus in the in vitro assay. Mucins of both the HIV-negative (15/20) and HIV-positive (16/18) groups inhibited the virus. We are unable to explain the absence of inhibition by 5 samples in the infection-free group and 2 in the HIV-positive group. It is possible that the pattern of glycosylation plays a role in this phenomenon, considering that mucins display microheterogeneity in their sugar moieties, but that has to be further investigated. The moieties of the mucin carbohydrate act as receptors which aggregate pathogenic microorganisms and thus prevent them from entering and causing infection of the host cells. Although this study did not look at the mechanism of how mucins aggregate the HIV-1, we suggest that the aggregation of the virus by the mucins is purely a physical entanglement through a charge interaction with carbohydrate side chains considering that mucins have a high density of negatively charged sialic acid and sulphate residue [12].

Our results, with respect to the behavior of breast milk and its components confirm the findings of Habte et al. [12] for normal and for a new group of infected patients. The assay used in this study was limited in that it was not a dose-response curve which would have enabled us to calculate an IC_{50} value for inhibition. It would be of future interest to compare the inhibition of the individual mucins MUC1 and MUC4 in the inhibition of the virus using a pseudo-viral assay which would establish a dose-response curve.

Conclusion

This study has shown that crude breast milk does not inhibit HIV-1, but its purified mucins from HIV-positive and -negative groups do.

Acknowledgements

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