The inhibition of the Human Immunodeficiency Virus type 1 activity by crude and purified human pregnancy plug mucus and mucins in an inhibition assay

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Abstract

**Background:** The female reproductive tract is amongst the main routes for Human Immunodeficiency Virus (HIV) transmission. Cervical mucus however is known to protect the female reproductive tract from bacterial invasion and fluid loss and regulates and facilitates sperm transport to the upper reproductive tract. The purpose of this study was to purify and characterize pregnancy plug mucins and determine their anti-HIV-1 activity in an HIV inhibition assay.

**Methods:** Pregnancy plug mucins were purified by caesium chloride density-gradient ultracentrifugation and characterized by Western blotting analysis. The anti-HIV-1 activities of the crude pregnancy plug mucus and purified pregnancy plug mucins was determined by incubating them with HIV-1 prior to infection of the human T lymphoblastoid cell line (CEM SS cells).

**Results:** The pregnancy plug mucus had MUC1, MUC2, MUC5AC and MUC5B. The HIV inhibition assay revealed that while the purified pregnancy plug mucins inhibit HIV-1 activity by approximately 97.5%, the crude pregnancy plug mucus failed to inhibit HIV-1 activity.

**Conclusion:** Although it is not clear why the crude sample did not inhibit HIV-1 activity, it may be that the amount of mucins in the crude pregnancy plug mucus (which contains water, mucins, lipids, nucleic acids, lactoferrin, lysozyme, immunoglobulins and ions), is insufficient to cause viral inhibition or aggregation.
lions of micro-organisms a day are reported to be cleared from the reproductive tract by cervical secretions that are the tract’s most effective first line of defence [7].

Thus far six mucin genes have been reported to be expressed by the female reproductive tract, namely MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6 [6]. The genes for MUC2, MUC5B, MUC5AC and MUC6, are found on chromosome 11p15.5 and express the secreted gel forming mucins, whereas MUC1 and MUC4 are membrane associated mucins expressed by the epithelium of the ecto-cervix and vagina [7]. Of these, MUC4 and MUC5B are reported to be the major mucin genes expressed by the endo-cervix [8]. The variation, under hormonal influence, of the viscoelastic and rheological properties of these mucins during the menstrual cycle is well documented [4].

Human crude saliva is known to inhibit Human Immunodeficiency Virus type 1 (HIV-1) activity in an in vitro assay [9,10]. These authors speculated that it was the mucus component that inhibited the virus. We very recently showed that both crude saliva and its purified mucin components MUC5B and MUC7 inhibited HIV-1 activity [11] and so did the purified MUC1 of breast milk [12]. The MUC1 of breast milk also showed anti-pox viral activity [13]. Our hypothesis is that cervical mucins should have a similarly inhibitory effect on HIV-1 activity, an important question considering that the vagina and cervix are significant routes for HIV transmission. The aim of this study therefore was to extract and purify the mucins in the pregnancy plug mucus and to determine their anti-HIV-1 activity using an HIV inhibition assay.

We therefore extracted and purified mucins from the pregnancy plug mucus which occludes the cervical canal throughout the pregnancy period [2,14]. This large mucus plug which is more like the mucus of the luteal phase than the mucus of the mid-cycle [2] was obtained during labour and just prior to delivery.

Sub-Saharan Africa is reported to be home to about 25 million adults and children who are HIV positive [15]. In Southern Africa 25.7% of the population has HIV/AIDS, making this the most highly prevalent region of infection compared to the Eastern and the Western regions with 11.4% and 4.3% prevalence respectively [16]. In South Africa alone, between 4.68 and 7.03 million people were living with HIV/AIDS in 2004 [17], of whom 55% were female [18]. Thus this preliminary study could make a significant contribution to the efforts being made in controlling this epidemic.

In this study we report the anti-HIV-1 activities of crude and purified human pregnancy plug mucus and mucins in an in vitro inhibition assay. We have demonstrated that
the purified mucins from the pregnancy plug mucus inhibited HIV-1 infection of the CEM SS cells. However, the crude pregnancy plug mucus failed to inhibit HIV-1 infection of these cells.

**Results**

**Mucin purification**

Pregnancy plug mucins were purified by density gradient centrifugation, twice in caesium chloride/4 M GuHCl with a buoyant density between 1.39 and 1.40 g/ml to remove proteins and nucleic acids. The purification profile in Fig. 1 demonstrates a clear separation of the lower density proteins positive for Lowry from the higher-density glycoproteins positive for PAS. The mucin-rich fractions (fractions number 3, 4 and 5) (Fig. 1b) were pooled, dialysed against three changes of distilled water and freeze-dried.

**SDS-PAGE analysis**

Pregnancy plug mucus (20 μg) was dissolved in gel loading buffer containing 0.2 M 2-mercaptoethanol and loaded onto 10% SDS-PAGE (Fig. 2). Gels were stained either with PAS for carbohydrate or Coomassie Brilliant Blue G-250 for protein. An intense PAS positive band (M, >220 kDa) appeared on the top of the running gel below which there was another band of size <220 kDa (Fig. 2a, lane 3). Coomassie Blue staining also showed material at the top of the running gel and a number of bands of higher electrophoretic mobility and therefore of relatively smaller size within the gel (Fig. 2a, lane 2).

Caesium chloride density gradient ultra-centrifugation removed most of the contaminant protein from crude mucus as shown clearly by subsequent gel electrophoresis (Fig. 2b, lane 4). Bands at the top of the running gel, staining both for protein and carbohydrate confirmed the presence of the mucin and its purity (Fig. 2b, lanes 4 and 5).

**Western blotting**

Western blot analysis was performed to determine the identity of the mucins present in the pregnancy plug mucus. Samples (40 μg each) 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 (Fig. 3 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. The Western blotting result confirmed the presence of MUC1, MUC2, MUC5AC and MUC5B mucins in the pregnancy plug mucus (Fig. 3 lanes 3, 6, 9 and 12 respectively). While MUC5AC was strongly expressed (Fig. 3 lane 9) MUC2 appeared in relatively smaller amounts and as a doublet (Fig. 3 lane 6, arrows) [19]. While the positive controls MUC1 (lane 1), colonic mucus (lane 4), pseudomyxoma peritonei (lanes 7 and 10) [20] reacted with the anti-MUC1, anti-MUC2, anti-MUC5AC and anti-MUC5B antibodies respectively, the negative controls namely the salivary MUC5B (lane 2), tracheal sputum (lane 5), 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.

However, due to the lack of Western blotting antibodies against MUC4 and MUC6 the identification of these mucins was not done in this study.

**Toxicity assay**

Prior to the HIV inhibition assay the toxicity of the crude pregnancy plug mucus and purified pregnancy plug mucins to the CEM SS cells was determined by toxicity assay. As shown in Table 1, no toxicity of these components or no cell death was detected.

**Inhibition assay**

The anti-HIV-1 activities of the crude pregnancy plug mucus and purified pregnancy plug mucins were determined by HIV inhibition assay. When HIV-1 was incubated with crude pregnancy plug mucus for an hour and the mixture subsequently added to or incubated with the...
CEM SS cells for 30 min, a 100% HIV-1 infection of the CEM SS cells was measured by the p24 antigen assay (Fig. 4). However, when the virus was first incubated with purified mucins from the pregnancy plug for an hour and then the mixture subsequently incubated with the CEM SS cells for 30 min, an approximately 97.5% inhibition of the viral activity or an approximately 2.5% infection of the CEM SS cells was detected. This suggests that compared to the crude pregnancy plug mucus the purified pregnancy plug mucins reduce the infection of CEM SS cells by an approximately 39 fold (Fig. 4).

To determine the effect of time (incubation period) on the rate of viral infection or inhibition ability of the samples, the mixtures of (HIV-1 plus crude pregnancy plug mucus) and (HIV-1 plus purified pregnancy plug mucins) were incubated with the CEM SS cells for longer time periods (1 h and 3 h). However, no difference in the rate of viral infection or inhibition ability of the samples due to incubation time difference was observed (Fig. 4). To determine the anti-HIV-1 activity of the purified pregnancy plug mucins at the highest dilution or lowest concentration, serial tenfold fold dilutions (i.e. 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) of the mucins were also done. Again, no difference in the anti-HIV-1 activity of the purified pregnancy plug mucins was detected down to 10⁻⁴ (Fig. 4a,b,c and 4d).

As shown in Fig. 4, when HIV-1 was incubated with the media (positive control) instead of the pregnancy plug mucins prior to addition to the CEM SS cells at all time points (30 min, 1 h and 3 h), HIV-1 infection of the CEM SS cells was not inhibited and 100% HIV-1 replication or infection of the CEM SS cells was measured by the p24 antigen assay. Surprisingly the heat inactivated HIV-1 (negative control) was also shown to cause an approximately 30% infection of the CEM SS cells at all time points (Fig. 4).

To determine or compare the efficiency of HIV-1 aggregation by the crude pregnancy plug mucus and purified pregnancy plug mucins, at the end of the incubation period (1 h), the mixtures of (HIV-1 plus crude pregnancy plug mucus), (HIV-1 plus purified pregnancy plug mucins) and the control (HIV-1 plus media) were filtered through 0.45 μm pore size cellulose acetate filter (25 mm diameter) and the filtrates were added to or incubated with the CEM SS cells at different time-points (30 min, 1 h and 3 h). The result demonstrated that the filtrates from the mixtures of (HIV-1 plus crude pregnancy plug mucus) and (HIV-1 plus media) caused 100% HIV-1 infection of the CEM SS cells (results not shown).

**Discussion**

According to various studies [9,10,21,22], salivary macromolecules (possibly mucins) aggregate HIV-1 prior to host cell entry, thus preventing transmission of HIV-1 through saliva. Wiggins *et al.* [7] reported that mucus is the first line of defence against pathogenic microorganisms. Studies in our laboratory have also confirmed these findings [11]. Crude saliva (from individuals with a self-declared risk free lifestyle and thus presumably uninfected), and its purified mucins MUC5B and MUC7 [11] and purified MUC1 from breast milk [12] show anti-HIV-1 activity in an *in vitro* inhibition assay.

It thus remains to be asked why other areas such as the female reproductive tract and breast milk, so rich in mucus and mucins quite similar in substance and conformation to those in saliva, still remain major routes of transmission of the virus. In the case of breast milk we showed that its MUC1 component inhibited the HIV-1 from infecting CEM SS cells in an *in vitro* assay only after it was dissociated from the milk fat globules and isolated and purified by caesium chloride density gradient ultracentrifugation. Crude breast milk had no such inhibitory effect on HIV-1 [12]. In the light of this we decided to investigate whether cervical mucus and mucin display any anti-HIV-1 properties, considering that the cervix is a significant route of transmission in women.

The quality 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 [8,23]. 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 mucus plugs at the mouth of the cervix rich in mucin [2,14], were obtained from women in labour. However, a comparison of the effect of purified plug mucin versus purified cervical mucin on HIV is being planned.

**Table 1: Toxicity of crude pregnancy plug mucus and purified pregnancy plug mucins to CEM SS cells.**

| Sample                  | Con  | CEM SS cells | % of dead cells | % of live cells |
|-------------------------|------|--------------|-----------------|-----------------|
| Pregnancy plug mucus    | 0.9 mg  | 2.5 × 10⁶/ml | 0               | 100             |
| Pregnancy plug mucins   | 0.9 mg  | 2.5 × 10⁶/ml | 0               | 100             |
In this study we have demonstrated that the purified mucins from the pregnancy plug inhibited HIV-1 infection of the CEM SS cells. However, the crude pregnancy plug mucus and the media failed to inhibit HIV-1 infection of these cells. Though the mechanism of inhibition is not clear, it is likely that when the HIV-1 was incubated with the mucins, the virus was trapped by aggregation through the sugar side-chains of the mucins, a purely physical phenomenon [10,24-26], resulting in preventing the virus from entering the host cells (CEM SS cells). This was supported by our finding that salivary MUC7 inhibited HIV-1 infection of the CEM SS cells when it was incubated with the virus prior to addition to the CEM SS cells. However, the mucin failed to inhibit viral infection of these cells when it was incubated with CEM SS cells prior to addition of the virus (unpublished data). This suggests that the mucin inhibits HIV-1 infection by physically aggregating the virus than by blocking putative viral binding sites or receptors on the cells.

The virus and mucins were incubated together with the cells for different incubation periods, i.e. 30 min, 1 h and 3 h to determine the effect of time on infection or lack thereof. Cultures were then washed three times after each incubation period to remove free virus and cultured for another 4 days in IL-2 rich media. This was done to determine if the virus had entered the cells during the initial incubation step and was able to replicate inside the cells for the extended incubation period to produce p24 antigen, or if the mucins were successful in preventing viral...
entry into the cells and therefore prevent the production of p24 antigen.

To further confirm the hypothesis that mucins inhibit HIV-1 activity by physically aggregating the virus, the CEM SS cells were incubated with the filtrates from the mixtures. The lower infection (2.5%) of the CEM-SS cells by the filtrate from the mixture of HIV-1 plus purified

pregnancy plug mucins suggests the presence of insignificant amount of viruses in the filtrate or almost complete aggregation of the virus by the mucins, leaving no free viruses to pass through the filter paper into the filtrate to cause viral infection. On the other hand the 100% infection of the CEM-SS cells caused by the filtrates from the mixtures of HIV-1 plus crude pregnancy plug mucus and HIV-1 plus media suggests the presence of higher amount

Figure 4
Inhibition of HIV-1 activity by crude pregnancy plug mucus and purified pregnancy plug mucins in vitro assay.
Crude pregnancy plug mucus and purified pregnancy plug mucins (0.9 mg each) were incubated with subtype D HIV-1 for 60 min and filtered through 0.45 μm 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 × 10⁶ cells ml⁻¹ for 30 min, 1 h and 3 h. 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.
of viruses in the filtrates or the failure of the crude pregnancy plug mucus and the media to aggregate the viruses. This finding agreed with the report that HIV-1 may bind to the high-molecular weight components which results in macromolecular complex formation which is removable by filtration through 0.45 μm pore filter paper [10,24-26].

The lack of inhibition by crude pregnancy plug mucus compared to the inhibition by purified pregnancy plug mucs is not clear. However it should be considered that mucins constitute only about 0.5–1% of total crude mucus [27] which is known to contain water, glycoproteins, lipids, nucleic acids, lactoferrin, lysozyme, immunoglobulins and ions [7]. It is likely therefore that the potency of mucins would in this case be in their purified form rather than when they are a minor part of a larger secretion in which their concentration would be diluted. This was quite different in the case of crude saliva, the inhibitory effect of which was similar to that of its purified mucins, separable by gel filtration and individually effective against the virus [11]. However, quantification of the amount of mucins in the crude mucus prior to any assay should be considered before drawing this conclusion.

The heat inactivated HIV-1 (negative control) caused an approximately 30% infection of the CEM SS cells suggesting that the viruses, when inactivated but not completely killed are still infective, albeit to a lesser degree. To determine whether there is a dose/effect relationship and the lowest possible effective concentration with anti-HIV-1 activity, ten fold serial dilutions (10⁻¹ to 10⁻⁴) of the mucins were also done from a starting concentration of purified mucin of 0.9 mg. The mucins showed strong anti-HIV-1 activity down to a dilution of 10⁻⁴, but in this study the lowest possible concentration which can cause inhibition of HIV-1 activity was not identified. Thus a lower starting concentration of purified mucin than 0.9 mg would be advisable.

There was also no effect of time (incubation period) on the inhibitory effect of mucins or the infectivity of the virus. This suggested that the mucins aggregated the virus immediately and permanently. However, shorter starting times of incubation of mucins and the virus would be necessary to determine the shortest time mucins take to aggregate the virus.

Although HIV-1 Subtype C is currently the most prevalent in South Africa, the Subtype D which was used in this study was found during the early HIV epidemic in the country and is quite prevalent here, albeit to a lesser degree. Even though we wished to use the Subtype C strain, the Subtype D strain is unfortunately the only lab adapted strain we had available to us in the vicinity of Cape Town and it is possible that this is the only laboratory based HIV assay in the country. As described in the Methods section, this 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 it was fully characterised and sequenced subsequently [28]. The human T lymphoblastoid cell line (CEM SS cells), which was used in this study, is reported to express CD4, CXCX4, ICAM-3 and MHC class II molecules [29]. These cells are capable of developing easily quantifiable syncytia formation in four to six days upon the addition of HIV-1 [30]. Although Subtype C predominantly uses CCR5, several instances of co-receptor switch to CXCX4 or even dual tropism have been observed in Subtype C, especially later in infection. Therefore this study could be relevant to in vivo situations, where transmitted viruses are most often CCR5 tropic.

Extraction of mucus was in 6 M GuHCl and proteolytic inhibitors which included 10 mM EDTA, 5 mM NEM, and 1 mM PMSF to reduce endogenous proteolysis of mucins [2]. PMSF and EDTA inhibit serine and metallo-protease activity respectively whilst NEM inhibits thiol proteases and minimizes thiol-disulfide exchange [1].

Caesium chloride density gradient purification removes all contaminants such as non-mucin proteins, lipids, proteoglycans and nucleic acids from mucins [31]. Purification of the mucins was confirmed by SDS-PAGE [32]. The removal of these contaminants from mucins was believed to be by dissociative conditions through the presence of GuHCl [1], known to be a widely used denaturant [33] which in this case could well dismantle the tertiary structure of mucins [14].

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 small amount compared to the other mucins. Immunohistochemistry confirmed previous reports of the expression of MUC4 and MUC6 by the endometrial tissue (data not shown), 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. [6], Wiggins et al. [7], Gipson et al. [23] and Wickstrom et al. [34], studies which reported the expression of MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC6 by the female reproductive tract.

Conclusion

In summary, we have shown the in vitro inhibition of HIV-1 activity by purified mucins from the pregnancy plug. However, the crude pregnancy plug mucus failed to inhibit HIV-1 activity. Although it is not clear why the crude sample did not inhibit HIV-1 activity, it is likely that
the amount of mucins in the crude pregnancy plug mucus is of too low a concentration to cause viral inhibition or aggregation. Future studies will attempt to establish the lowest amount of purified mucin required to cause aggregation of the virus. Also different HIV strains, cell lines and samples from different donors for statistical validity to strengthen this preliminary finding, will be carried out. A comparison between the anti-HIV-1 activity of each cervical mucin from the different stages of the menstrual cycle has also been planned.

Materials and methods

Ethics

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

Materials

Mouse anti-MUC1 monoclonal (NCL-MUC1, 201607) and goat anti-mouse horse radish peroxidase (HRPO) linked secondary antibodies were kindly provided by Sara Kirkham (Manchester, UK). The CEM SS cells were from AIDS Research and Reference Reagent Programme (Germantown, USA). The p24 antigen kit was from Vironostika HIV-1 Antigen kit Biomérieux (France). Sepharose CL-4B and reagent solvents such as guanidinium chloride (GuHCl) and caesium chloride (CsCl) were from Sigma (UK). Trypan Blue Dye solution was from Merck (Germany).

Pregnancy plug mucus collection

Pregnancy plug mucus was obtained from the Groote Schuur Hospital Maternity Division at the University of Cape Town. The pregnancy plug mucus was retrieved prior to delivery and collected into cold 6 M GuHCl containing proteolytic inhibitors, namely 10 mM EDTA, 5 mM NEM and 1 mM PMSF pH 6.5 and stored at -20°C.

Mucus preparation

Crude pregnancy plug mucus was prepared according to the method of Carlstedt et al. [2]. The pregnancy plug mucus was collected into 0.1 M Tris-HCl, 2% (w/v) EDTA and 5 mM PMSF pH 7.5 and prepared for the HIV inhibition assay. After gentle stirring for 15 h at 4°C, insoluble materials were removed by high-speed centrifugation at 9 000 g for 2 h at 4°C. The supernatant was dialysed against three changes of distilled water at 4°C and freeze-dried.

Mucin preparation

Pregnancy plug mucus was thawed and stirred gently for 15 h at 4°C in 6 M GuHCl and a cocktail of proteolytic inhibitors as described above. Insoluble materials were removed by high-speed centrifugation at 9 000 g for 2 h at 4°C. The soluble material was then pooled and subjected to density gradient ultra-centrifugation, twice for 48 h at a 105 000 g at 4°C in a Beckman L45 ultra-centrifuge [31]. Briefly, samples in 4 M GuHCl containing 10 mM EDTA, 5 mM NEM and 0.05% CHAPS pH 6.5 were adjusted to a density of 1.39 to 1.40 g/ml with caesium chloride prior to centrifugation. Mucin rich fractions were pooled, dialysed against three changes of distilled water at 4°C and freeze-dried.

SDS-PAGE analysis

Pregnancy plug mucins (20 μg) 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 2 min prior to loading. Electrophoresis was performed by the method of Laemmli [35] in a 10% (w/v) running gel and a 4% (w/v) stacking gel using the Hoeffer Mighty Small mini-electrophoresis system. After electrophoresis gels were stained for carboxyhydate with Periodic Acid Schiff (PAS) and for protein with Coomassie Brilliant Blue G-250.

Agarose gel electrophoresis

Purified pregnancy plug mucins (40 μg) were prepared in a sample loading buffer containing 40% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and boiled for 2 min prior to loading. Electrophoresis was carried out according to the method of Thornton et al. [36], in a 1% (w/v) agarose gel (15 × 15 cm) prepared in running buffer containing 40 mM TAE, 1 mM EDTA, and 0.1% SDS pH 8.0. Briefly, agarose (1.6 g in 160 ml 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 subcell gel apparatus. Upon polymerization the apparatus was filled with running buffer and electrophoresis was performed at 100 V for 2.5 h at room temperature.

Western blotting

After agarose gel electrophoresis the purified pregnancy plug mucins were transferred to nitrocellulose membrane (Nitrocellulose, 0.22 μ) by vacuum blotting for 1 h at a suction pressure of 40 mbar, according to the method of Thornton et al. [36]. The transfer buffer contained 4 × SSC (0.6 M NaCl, 60 mM Tri-sodium citrate, pH 7.0). After electro-blotting non-specific binding was 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 × 5 min and incubated for 2 h with mouse anti-MUC1 monoclonal and rabbit anti-MUC2, anti-MUC5AC and anti-MUC5B polyclonal antibodies diluted in 5% (m/v) low fat milk powder in TBST at a dilution of 1 in 100 (mouse anti-

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MUC1), 1 in 5000 (rabbit anti-MUC2 and anti-MUC5AC) and 1 in 2000 (rabbit anti-MUC5B). The membranes were washed 3 × 5 min with TBST and incubated for 1 h with HRPO linked goat anti-mouse and goat anti-rabbit secondary antibodies diluted in 5% (m/v) low fat milk powder in TBST at 1 in 1500 and 1 in 2000 respectively. After another TBST wash (3 × 5 min) bands that interacted with the antibody were detected by exposing the membranes to ECL detection kit.

Toxicity assay
The toxicity of crude pregnancy plug mucus and purified pregnancy plug mucins to the phytohaemagglutinin (PHA) stimulated CEM SS cells was tested. Briefly 500 μl of the CEM SS cells in RPMI complete containing 10% Fetal Calf Serum, 1% Penicillin/Streptomycin antibiotic, 10 μmol L-fungin and 50 μmol 2-mercaptoethanol (final concentration 2.5 × 10^6 cells/ml) were incubated with 250 μl of IL-2 and 250 μl (0.9 mg) of crude pregnancy plug mucus and purified pregnancy plug mucins in CO2 incubator for 24 h. As controls CEM SS cells with IL-2 only and IL-2 without CEM SS cells (blank) were used. After spinning at 100 g for 5 min cells were re-suspended in 500 μl 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.

HIV inhibition assay
The anti-HIV-1 activities of the crude pregnancy plug mucus and purified pregnancy plug mucins were tested by the inhibition of HIV-1 by the crude pregnancy plug mucins and purified pregnancy plug mucins isolated from human cervical mucus on the CEM SS cell line. The antiviral activity of the crude pregnancy plug mucins and purified pregnancy plug mucins on the CEM SS cell line was calculated as live cells/total cells × 100.

Analytical determinations
Glycoprotein was estimated by the PAS procedure of Manosh and Allen [37] and protein according to the method of Lowry et al. [38].

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
HHH carried out the biochemical studies and drafted the manuscript. CdB established and carried out the HIV inhibition assay. ZEL and MGT participated in the biochemical studies. LS participated in pregnancy plug mucin collection and analysis. DK contributed ideas to the design and coordination of the study. ASM conceived of the study, participated in its design and coordination and finalised the manuscript. All authors read and approved the final manuscript.

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References
1. Carlstedt I, Sheehan J, Ulmsten U, Wingerup L: Isolation and purification of the mucin component of human cervical mucus. Adv Exp Med Biol 1982, 144:273-275.
2. Carlstedt I, Lindgren H, Sheehan JK, Ulmsten U, Wingerup L: Isolation and characterization of human cervical-mucus glycoproteins. Biochem J 1983, 211:13-22.
3. Gipson IK, Spurr-Michaud SJ, Tisdale AS, Kublin C, Cintron C, Keutmann H: Stratified squamous epithelium produce mucin-like glycoproteins. Tissue Cell 1995, 4:397-404.
4. Idris N, Carraway KL: Sialomucin complex (MUC4) expression in the rat female reproductive tract. Biol Reprod 1999, 61:1431-1438.
5. Venegas MF, Navas EL, Gaffney RA, Duncan JL, Anderson BE, Schaeffer AJ: Binding of type I-derivatised Escherichia coli to vaginal mucus. Infect Immun 1995, 63:416-422.
6. Gipson IK, Ho SB, Spurr-Michaud SJ, Tisdale AS, Zhan Q, Torklakovic E, Pudney J, Anderson DJ, Toribara NW, Hill JA: Mucin genes expressed by human female reproductive tract epithelia. Biol Reprod 1997, 56:999-1011.
7. Wiggins R, Hicks SJ, Soothill PW, Millar MR, Corfield AP: Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. Sex Transm Infect 2001, 77:402-408.
8. Arguero P, Spurr-Michaud S, Tisdale A, Gipson IK: Variation in the amount of T antigen and N-acetylactosamine oligosaccharides in human cervical mucus secretions with the menstrual cycle. J Clin Endocrinol Metab 2002, 87:5641-5648.
9. Barry EJ, Cho ML, Blumberg BM, Hammerskold ML, Rekosh D, Epstein LG, Levine MJ: Interaction of HIV-1 and human salivary mucins. J Acquir Immune Defic Syndr 1994, 7:995-1002.
10. Nagashunmugam T, Friedman HM, Davis C, Kennedy S, Goldstein LT, Malanud D: Human submandibular saliva specifically inhibits HIV type 1. AIDS Res Hum Retroviruses 1997, 13:371-376.

11. Habte HH, Mall AS, de Beer C, Lotz ZE, Kahn D: The role of crude human saliva and purified mucins MUC5B and MUC7 in the inhibition of Human Immunodeficiency Virus type 1 in an inhibition assay. Virol J 2006, 3:99.

12. Habte HH, de Beer C, Lotz ZE, Tyler MG, Kahn D, Mall AS: Inhibition of Human Immunodeficiency Virus type 1 activity by purified human breast milk mucin (MUC1) in an inhibition assay. Neonatology 2008, 93(3):162-170.

13. Habte HH, Lotz ZE, Tyler MG, Abrahams M, Rodrigues J, Kahn D, Kotwal GJ, Mall AS: Anti viral activity of purified human breast milk mucin. Neonatology 2007, 92:96-104.

14. Eriksen GV, Carlstedt I, Uldbjerg N, Ernst E: Cervical mucins affect the mobility of human spermatozoa in vitro. Feril Sterl 1978, 70:350-354.

15. N'Dri-Yoman T, Walensky RP, Dakoury-Dogbo N, Goldie SJ, Messou C, Weinstein MC, Deuffic-Burban S, Salamon R, Freedberg KA: Impact of opportunistic diseases on chronic mortality in HIV-infected adults in Côte d’Ivoire. S Afr Med J 2006, 96(26-27):306-310.

16. Shaikh N, Abdullah F, Lombard CJ, Smit L, Bradshaw D, Makubalo L: Masking through averages-intraprovincial heterogeneity in HIV prevalence within the Western Cape. S Afr Med J 2006, 96:538-543.

17. Strode A, Stack C, Mushariwa M: HIV vaccine research-south Africa’s ethical-legal framework and its ability to promote the welfare of trial participants. S Afr Med J 2005, 95:598-601.

18. Kagee A, Toefy Y, Simbayi L, Kalichman S: HIV prevalence in three predominantly Muslim residential areas in the Cape Town Metropole. S Afr Med J 2006, 95:512-516.

19. Govender U: The biochemical and molecular characterization of respiratory mucins in TB. In MSc thesis University of Cape Town, Surgery Department; 2006.

20. Chirwa N, Tyler M, Govender D, Kavin B, Goldberg P, Krige J, Lotz Z, Alistair H, Kahn D, Malanud D: The biochemical and immunohistochemical characterisation of mucins in colonic disease. A pilot study. S Afr J Surg 2007, 45:18-23.

21. Malanud D, Davis C, Berthold P, Roeth E, Friedman H: Human submandibular saliva aggregates HIV. AIDS Res Hum Retroviruses 1993, 9:663-667.

22. Malanud D, Nagashunmugam T, Davis C, Kennedy S, Abrams WR, Kream R, Friedman HM: Inhibition of HIV infectious by human saliva. Oral Dis 1997, 3:58-63.

23. Ginpson IK, Maccia R, Spurr-Michaeld SJ, Argueso P, Garriguid AR, Hill JA, Offner GD, Keutmann HT: The amount of MUC5B mucin in cervical mucus peaks at midcycle. J Clin Endocrinol Metab 2001, 86:594-600.

24. Archibald DW, Cole GA: In vitro inhibition of HIV-1 infectivity by human salivas. AIDS Res Hum Retroviruses 1990, 6:1425-1432.

25. Shugas DC, Alexander AL, Fu K, Freal SA: Endogenous salivary inhibitors of human immunodeficiency virus. Arch Oral Biol 1999, 44:445-453.

26. Shugas DC, Wahi SM: The role of the oral environment in HIV-1 transmission. J Am Dent Assoc 1998, 129:851-858.

27. Creeth JM: Constituents of mucus and their separation. Br Med Bull 1978, 34:17-24.

28. Treurnicht FK, Smith TL, Engelbrecht S, Claassen M, Robson BA, Zeeier M, van Rensburg EJ: Genotypic and phenotypic analysis of the env gene from South African HIV-1 subtype B and C isolates. J Med Virol 2002, 68:141-146.

29. Lallos LB, Laal S, Hoxie JA, Zolla-Pazner S, Bandres JC: Exclusion of HIV Coreceptors CXCR4, CCR5 and CCR3 from the HIV envelope. AIDS Res Hum Retroviruses 1999, 15:895-897.

30. Nara PL, Hatch WC, Dunlop NM, Robey WG, Arthur LO, Gonda MA, Fischinger PJ: Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. AIDS Res Hum Retroviruses 1997, 3:283-302.

31. Creeth JM, Denborough MA: The use of equilibrium-density-gradient methods for the preparation and characterization of blood-group-specific glycoproteins. Biochem J 1970, 117:879-91.

32. Mall AS: Gastro-duodenal mucin isolation and structure. In PhD thesis University of Newcastle upon Tyne, Department of Physiological Sciences; 1988.

33. Francis RD, Bradford HB: Some biological and physical properties of molluscan contagiosum virus propagated in cell culture. J Virol 1976, 19:382-388.

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

35. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227:680-685.

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

37. Mantle M, Allen A: A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain [proceedings]. Biochem Soc Trans 1978, 6:607-609.

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

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