Cationic Polypeptides Are Required for Anti-HIV-1 Activity of Human Vaginal Fluid

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*J Immunol* 2005; 175:7560-7567; doi: 10.4049/jimmunol.175.11.7560

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Cationic Polypeptides Are Required for Anti-HIV-1 Activity of Human Vaginal Fluid¹

Nitya Venkataraman,²* Amy L. Cole,²* Pavel Svoboda,† Jan Pohl,‡ and Alexander M. Cole³*

Mucosal surfaces of the vagina are the portals for heterosexual transmission of HIV-1 and therefore play a fundamental role in the pathogenesis of primary infection. In the search for direct biological evidence for the role of human vaginal fluid in innate host defense, we characterized the anti-HIV-1 function of cationic polypeptides within minimally manipulated vaginal fluid. In the current study we revealed that vaginal fluid confers intrinsic anti-HIV-1 properties against both X4 and R5 strains of HIV-1 and could protect against HIV-1 infection and reduce proviral genome integration in organotypic cultures of human cervicovaginal tissue. The majority of this activity was contained in the cationic polypeptide fraction, and the depletion of cationic polypeptides using a selective cation exchange resin ablated most of the intrinsic activity against HIV-1. By adding the cationic polypeptide fraction to depleted vaginal fluid, we were able to restore activity against HIV-1. Using a proteomic approach, we identified 18 cationic polypeptides within vaginal fluid, nearly all of which are either known antimicrobials or have other purported roles in host defense. Interestingly, physiologic concentrations of 13 of the cationic polypeptides were not active alone against HIV-1, yet in concert they partially restored the anti-HIV-1 activity of cation-depleted vaginal fluid. These results suggest that synergism between cationic polypeptides is complex, and full anti-HIV-1 activity probably involves the aggregate of the cationic peptides and proteins in vaginal fluid. The Journal of Immunology, 2005, 175: 7560–7567.

A

pproximately 40 million people have been infected with HIV-1 worldwide according to the 2004 World Health Organization estimates (1). There has been a dramatic increase in the global spread of HIV-1, especially via the heterosexual mode of transmission (2, 3). At present, nearly 60% of infected individuals are women (4, 5). The natural sexual transmission of HIV occurs through mucosal surfaces, such as vaginal or rectal mucosa (6). Vaginal and rectal subepithelial stromal tissues are densely populated with dendritic cells, macrophages, and T cells that express both CD4 and the HIV-1 coreceptors, CXCR4 and CCR5 (7, 8). The mechanisms by which HIV-1 journeys across the mucosal epithelia are not completely understood, but may directly involve the epithelial cells (9). Once the virus reaches the lamina propria, it can either directly infect macrophages or T lymphocytes or adhere to (or infect) dendritic cells, whose traffic to the regional lymph nodes converts them into sites of vigorous viral replication (10, 11). Although considerable attention in immunopathogenetic research on HIV-1 has been focused on acquired immu

1 This work was supported by Grants AI052017 and AI065430 (to A.M.C.) from the National Institutes of Health. Emory Microchemical and Proteomics Facility is supported by National Institutes of Health-National Center for Research Resources Grants 02878, 12878, 13948, and 016692.

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4 Abbreviations used in this paper: SLPI, secretory leukocyte protease inhibitor; AU, acid urea; CM, carboxymethyl; HBD, human β-defensin; HNP, human neutrophil peptide; NET, neutrophil extracellular trap; VFB, vaginal fluid buffer.

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infection by HIV-1. We reveal that the cationic proteins in human vaginal fluid inhibit the entry of HIV-1 in human epithelial cell lines and organotypic cervicovaginal tissues. We used a proteomic approach to identify 18 different cationic polypeptides in vaginal fluid, most of which have been previously reported to exhibit antimicrobial properties. Although individual polypeptides at physiological concentration did not exhibit antiviral activity against HIV-1 infection, a combination of the peptides partially restored the antiviral activity. Selective depletion of cationic polypeptides from whole vaginal fluid reduced the intrinsic anti-HIV-1 activity. Most importantly, the anti-HIV-1 activity of depleted fluid was restored upon repletion with the cationic polypeptide extract. Collectively, these studies suggest that the intrinsic anti-HIV-1 activity of vaginal fluid is an aggregate effect of all its active cationic polypeptide components.

Materials and Methods

Reagents
Human neutrophil lysozyme and human milk lactoferrin were purchased from Sigma-Aldrich. Recombinant calgranulin A (S100A8) and calgranulin B (S100A9) were purchased from Abnova. Recombinant cystatin B and SLPI were purchased from R&D Systems. Histone H2A was purchased from Upstate USA. Cathepsin G was purchased from Bachem Bioscience. Recombinant β-defensins, HBD-1 and -2, were gifts from Dr. T. Ganz (David Geffen School of Medicine, University of California, Los Angeles, CA). The α-defensins, HNP-1, -2, and -3, were purified from human leukocytes and were gifts from Drs. Ganz and R. I. Lehrer (David Geffen School of Medicine).

Collection and processing of vaginal fluid
Vaginal fluid was collected from postmenarcheal, but premenopausal, healthy female donors after obtaining informed consent according to the guidelines of the institutional review board of University of Central Florida. Donors with a history of current or recent vaginal infections and those under antherapeutic treatment for any reason were excluded from the study using a questionnaire. To collect undiluted vaginal fluid, an Instead SoftCup (Ultrafem) was inserted into the vagina according to the manufacturer’s instructions and was removed after 30 min. The SoftCup was then centrifuged for 10 min at 1000 x g in a 50-ml sterile conical tube to collect the fluid sample (28). Retrieved samples were homogenized by sonication on ice using a microtip ultrasound probe (10–2 to 3-s pulses). These mini-fluid sample (28). Retrieved samples were homogenized by sonication on ice using a microtip ultrasound probe (10–2 to 3-s pulses). These mini-

The cationic polypeptides bound to the CM resin were extracted in sub-

Two-dimensional gel electrophoresis of vaginal fluid
Acid-extracted vaginal fluid samples were electrophoresed on a 12.5% native acid urea-polyacrylamide gel (AU-PAGE) in the first dimension at 75 V for 16–18 h (29, 31). The gel was then stained with 0.1% Amido Black (0.04% naphthol blue-black, 2.5% isopropanol, and 1% acetic acid) to visualize the protein bands. The entire lane of the first dimension AU gel was excised, washed twice for 5 min each time in dH2O, followed by two 5-min washes with 50 mM Tris (pH 8.8), and soaked for 10 min in equilibration buffer (50 mM Tris, 6 mM urea, 2% SDS, 20% glycerol, and bromophenol blue ad libitum (pH 8.8)) containing 10 mg/ml DTT. The gel strips were electrophoresed in a 16% Tricine-SDS-PAGE as the second dimension for 2 h at 40 mA (32). Protein spots were visualized by SYPRO Ruby gel stain (Bio-Rad), excised, and stored at 4°C in 1% acetic acid until analyzed by mass spectrometry.

The identification of cationic polypeptides from vaginal fluid
The proteins were then subjected to trypsin digestion and mass spectrometric analysis (MALDI-TOF-MS/MS analysis) (33) at the microchemical and proteomics facility at Emory University as described previously (34, 35). GPS Explorer 2.0 software (Applied Biosystems) and a MASCOT (www.matrixscience.com/) search engine were used for identification of peptide fragments. The National Center for Biotechnology Information nonredundant database and the Mannomax database were used for the searches.

Human cervicovaginal tissue model
Organotypic EpiVaginal cultures of normal human vaginal-ectocervical epithelial cells and immunocompetent dendritic cells were propagated as suggested by MatTek. Each 60 mm2 of tissue adhered tightly atop a microporous membrane insert and was maintained at the air-liquid interface using 5 ml of maintenance medium (MatTek). Tissues (three per treatment condition) were pretreated in with 50 μl of PBS or 50 μl of vaginal fluid diluted 1/1 with PBS for 30 min and then rinsed twice with warm PBS. Tissues were topically applied with 100 μl of PBS (control), PBS containing 25 ng p24 of HIV-1 BaL, or PBS containing BaL and vaginal fluid (equivalent to 50% of whole fluid) for 24 h. Treatments were then removed, and tissues were washed with 100 μl of warm PBS, then vaginal fluid (50%) or PBS vehicle was reapplied in 50 μl. A one-time dose of 1 × 106/ml HIV-1 BaL-infected PM1 cells was then added to the cultures after 72 h of treatment and the microsporadic virus to determine the initial HIV-1 infection, and these were removed after 2 days. Basal maintenance medium was changed every other day, and the topical (apical) treatments were removed and
reapplied on days 3 and 6 after infection. On day 9 after infection, DNA was extracted from two tissues per treatment condition using Qiagen’s DNA Micro Kit. Total protein was extracted from the third tissue per treatment condition and assayed by ELISA for HIV-1 p24\textsuperscript{eq}.

**Detection of HIV-1 provirus in human cervicovaginal tissue**

HIV-1 infection of cervicovaginal tissues was assessed by real-time PCR quantitation of the HIV-1 BaL env gene (relative to \( \beta \)-actin controls) in total tissue DNA isolated 9 days after infection. The HIV-1 BaL primers used were 5′-AAACCTCAGTCTACAC-3′ and 5′-TACATTGCTTCTCTACTTC-3′, which amplify a 700-bp region of BaL gp120. The \( \beta \)-actin primers used were 5′-CTCTCCGAGCAGATGTTG-3′ and 5′-GGTAAACGCACTAAG-3′, which amplify a 105-bp region of human \( \beta \)-actin. Two hundred nanograms of DNA was mixed with 2 μL of each primer, and dH\textsubscript{2}O. Triplicate 20-μL reactions were conducted using the MyiQ real-time PCR detection system (Bio-Rad), and HIV-1 BaL levels were normalized to \( \beta \)-actin. Data were analyzed with iCycler IQ Optical System software. Melt-curve analysis and gel electrophoresis revealed that single PCR products were amplified for each gene. Moreover, the env PCR product was verified by subcloning into pCR4-TOPO (Invitrogen Life Technologies), followed by DNA sequence analysis (Biomolecular Sciences Genomics Core Laboratory, University of Central Florida).

**Statistics**

Luciferase assays were performed in triplicate for each treatment condition in each experiment, with relative light units in vehicle-only control wells set at 100% infection. Each treatment condition was analyzed by one-way ANOVA, followed by Tukey’s pairwise comparison. Mass spectrometric analysis for each polypeptide identified was performed in duplicate, and protein spots with a confidence index >85% combined with ion scores of ≥40 for one or more peptides matched to each protein were considered positively identified (33).

**Results**

**Human vaginal fluid is intrinsically active against HIV-1**

The mucosal layer lining the vaginal epithelial cells is rich in antimicrobial polypeptides that provide a crucial barrier against invading microbial and viral pathogens (15). Although some of these polypeptides have been shown to exhibit antiviral properties (19, 23, 25, 36–38), detailed analysis of the intrinsic anti-HIV-1 activity of vaginal fluid has not been reported. In this study we explored the activity of the cationic polypeptide components of vaginal fluid against HIV-1. TZM-bl cells were treated with either PBS (vehicle control) or vaginal fluid diluted in DMEM/high glucose medium with 10% FBS and infected with both R5 (HIV-1 BaL; Fig. 1A) and X4 (HIV-1 IIIB; Fig. 1B) strains of HIV-1. After 24 h, excess virus was removed, and infection was quantitated as a measure of luciferase expression. Compared with vehicle-only controls, vaginal fluid extracts significantly reduced the infection of both viral strains in a dose-dependent manner. As measured by a standard MTT tetrazolium assay, the vaginal fluid extracts were not cytotoxic (data not shown). These results indicate that human vaginal fluid intrinsically inhibits the entry of HIV-1 into host cells.

**Anti-HIV-1 activity resides in the cationic fraction of vaginal fluid**

Experiments were designed to selectively remove the cationic polypeptides from whole vaginal fluid to determine whether this depletion reduced the anti-HIV-1 activity of the fluid. Whole, undiluted vaginal fluid was collected from healthy donors using an Instead SoftCup. A weak cation exchange resin, CM-Prep (Bio-Rad), was used to deplete the cationic peptides and proteins from vaginal fluid while sparing the concentrations of remaining proteins and electrolytes. We pioneered the CM-resin-mediated depletion technique and have characterized the selective depletion of cationic polypeptides from nasal fluid (29). The activities of whole vaginal fluid extract, CM-depleted vaginal fluid, and the polypeptides extracted from the CM resin were tested individually against HIV-1 BaL (Fig. 2A) and HIV-1 IIIB (Fig. 2B) in TZM-bl cells for 24 h. Cells treated with whole vaginal fluid showed a significant reduction in infection compared with the PBS-treated control (\( p < 0.0002; n = 13 \)), whereas the CM-depleted fluid did not inhibit infection. Similar to whole vaginal fluid, polypeptides extracted from the CM resin exhibited significant anti-HIV-1 activity compared with both the PBS control and CM-depleted vaginal fluid (\( p < 0.0002; n = 14 \)). Taken together, these data indicate that the anti-HIV-1 activity of vaginal fluid is contained in the cationic fraction. Whole vaginal fluid, CM-depleted vaginal fluid, and the extracted cationic polypeptides were used in subsequent proteomic and reconstitution assays.

**Identification of cationic polypeptides of vaginal fluid**

We next used a novel proteomic technique to identify cationic polypeptide components in vaginal fluid. The cationic polypeptide fraction from whole, undiluted vaginal fluid was subjected to AU-PAGE (the first dimension of a two-dimensional gel), which separates polypeptides based on cationic charge density (31, 39). A slice from the AU-PAGE was inserted into a Tricine-SDS-PAGE...
Table I. Cationic polypeptides identified in vaginal fluid by two-dimensional analysis and MALDI-TOF MS/MS analysis

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Spot ID</th>
<th>Accession</th>
<th>Molecular Mass (Da)</th>
<th>Score</th>
<th>C.I.%</th>
<th>Role in Host Defense</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Albumin</td>
<td>gi</td>
<td>23241675</td>
<td>45,130.4</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Neutrophil Gelatinase Associated Lipocalin</td>
<td>gi</td>
<td>4261868</td>
<td>20,534.5</td>
<td>51</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Cathepsin-G (Chain A)</td>
<td>gi</td>
<td>20664220</td>
<td>26,740.9</td>
<td>227</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Neutrophil Gelatinase Associated Lipocalin</td>
<td>gi</td>
<td>7767000</td>
<td>19,976.3</td>
<td>41</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Unnamed protein product</td>
<td>gi</td>
<td>14041892</td>
<td>23,795.5</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Histone H2B</td>
<td>gi</td>
<td>184086</td>
<td>11,324.1</td>
<td>55</td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td>H2A histone family</td>
<td>gi</td>
<td>4504251</td>
<td>14,086.9</td>
<td>113</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Fatty acid binding protein 5 E-FABP</td>
<td>gi</td>
<td>4557581</td>
<td>15,154.5</td>
<td>196</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Galectin-7</td>
<td>gi</td>
<td>3891470</td>
<td>14,934.8</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>Lysozyme</td>
<td>gi</td>
<td>3660304</td>
<td>14,599.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>Lysozyme (dimer)</td>
<td>gi</td>
<td>14278473</td>
<td>14,693.1</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>Cystatin A</td>
<td>gi</td>
<td>68783</td>
<td>11,166.6</td>
<td>54</td>
<td>98</td>
</tr>
<tr>
<td>13</td>
<td>Calgranulin B</td>
<td>gi</td>
<td>7417329</td>
<td>13,102.5</td>
<td>88</td>
<td>89</td>
</tr>
<tr>
<td>14</td>
<td>Histone H2A family</td>
<td>gi</td>
<td>10800144</td>
<td>13,927.8</td>
<td>126</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>Histone H4 family</td>
<td>gi</td>
<td>223582</td>
<td>11,230.3</td>
<td>127</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>H2A histone family</td>
<td>gi</td>
<td>18105045</td>
<td>13,897.8</td>
<td>118</td>
<td>81</td>
</tr>
<tr>
<td>17</td>
<td>Cystatin A</td>
<td>gi</td>
<td>4885165</td>
<td>10,999.7</td>
<td>120</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>Calgranulin A</td>
<td>gi</td>
<td>29888</td>
<td>10,930.8</td>
<td>197</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>Unnamed protein product</td>
<td>gi</td>
<td>14041892</td>
<td>23,795.5</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>HNP 1–3</td>
<td>gi</td>
<td>229858</td>
<td>3,489.6</td>
<td>63</td>
<td>99</td>
</tr>
</tbody>
</table>

Footnotes:

*a* The numbers correspond to the labeled spots in Fig. 3.

*b* Ion score of one or more peptide fragments that match a protein in the database.

*c* Confidence index percentage.

*d* Note that this spot has a C.I.% index of <85% but was considered positive because it was identified in multiple samples.

FIGURE 3. Identification of cationic polypeptides in vaginal fluid. Whole vaginal fluid (10 µl) was subjected to one-dimensional electrophoresis in an AU-PAGE, followed by Tricine-SDS-PAGE as the second dimension. The SYPRO Ruby-stained two-dimensional gel of whole vaginal fluid extract shows the protein spots identified by MALDI-TOF mass spectrometric analysis. Each polypeptide spot and their reported roles in host defense are listed in Table I. Unlabeled arrows are fragments of human albumin.

Comparison of proteomic profiles of whole and CM-depleted vaginal fluids reveals cationic polypeptides that contribute to anti-HIV-1 activity

Two-dimensional gel electrophoresis was next used to characterize the cationic polypeptides that remained in the vaginal fluid after CM depletion as well as those that were extracted with the CM resin. Fig. 4 compares two-dimensional gel electrophoretograms of whole vaginal fluid, CM-depleted vaginal fluid, and the polypeptides extracted from the resin. Spots without arrows were not identified. Among the polypeptides that were absent in CM-depleted fluid yet recovered from the resin include lysozyme, cystatin B, calgranulin B, histone H2A, HNP1–3, lipocalin-2, and cathepsin G (indicated by arrows in Fig. 4, A and C). Some components are reportedly active against HIV-1 (lysozyme and HNP1–3), whereas the anti-HIV-1 activities of the others have not been reported. We next explored which of the cationic polypeptide components of vaginal fluid were the principal effectors active against HIV-1.
A polypeptide was tested for anti-HIV-1 activity at its measured physiological concentration. TZM-bl cells were treated with individual polypeptides at the final concentrations given in Table II, then infected with HIV-1 BaL or HIV-1 IIIB; at 24 h, anti-HIV-1 activity was measured by quantifying luciferase expression. At physiological concentrations, none of the polypeptides alone inhibited viral entry, as shown by the absence of reduction in luciferase expression compared with the control (data not shown). These data suggest that the ability of vaginal fluid to prevent HIV-1 entry may be due to two or more cationic antimicrobial polypeptides acting in synergy.

Cationic polypeptides of vaginal fluid synergize to inhibit HIV-1 infection

The abundance of antimicrobial peptides in vaginal fluid with often overlapping roles in host defense suggests that the anti-HIV-1 activity is not a result of actions of individual peptides. Moreover, our studies indicate that the individual polypeptides at physiological concentrations do not prevent entry of HIV-1 into host cells. We therefore hypothesized that these polypeptides must act in concert to prevent HIV-1 infection. To test our hypothesis, we prepared a mixture of 13 available recombinant or natural peptides at physiological concentrations, as shown in Table II. TZM-bl cells were treated with the polypeptide mix, either alone or added to CM-depleted vaginal fluid, and were subsequently infected with HIV-1 (Fig. 5). Although the polypeptide mix alone reduced infectivity ~40%, this was not significant compared with the vehicle-only control. Moreover, the addition of the polypeptide mix to CM-depleted fluid was not completely restorative. Due to availability, not every polypeptide identified was represented in the mixture, which may have contributed to the incomplete restoration of CM-depleted fluid. This hypothesis was supported in our next experiment.

Interestingly, the cationic polypeptide extract (cleaved from the CM resin) was highly restorative to CM-depleted fluid (p = 0.00012; n = 14), and the combined anti-HIV-1 activity was equivalent to the activity of whole vaginal fluid (Fig. 5). These data suggest that the anti-HIV-1 activity of vaginal fluid is primarily contained in the cationic fraction, and that the activity is complex and requires the collective polypeptides.

Vaginal fluid protects against HIV-1 infection of human cervicovaginal tissue

We next tested whether vaginal fluid could protect organotypic human cervicovaginal tissues against HIV-1 infection. This ex vivo model closely resembles the native mucosae of the ectocervix

### Table II. Physiological concentration of cationic proteins that contribute to anti-HIV-1 activity of vaginal fluid

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (µg/ml)</th>
<th>Method of Detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calprotectin</td>
<td>34 ± 7</td>
<td>Semiquantitative Western blot and densitometry</td>
<td>(15, 72, 73)</td>
</tr>
<tr>
<td>Cystatin B</td>
<td>32.16</td>
<td>Densitometry</td>
<td>This study</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>13 ± 2</td>
<td>Semiquantitative Western blot and densitometry</td>
<td>(15, 68)</td>
</tr>
<tr>
<td>Histone H2A</td>
<td>11.04</td>
<td>Densitometry</td>
<td>This study</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>10.88</td>
<td>Densitometry</td>
<td>This study</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.9 ± 0.2</td>
<td>Semiquantitative Western blot and densitometry</td>
<td>(15, 16, 19)</td>
</tr>
<tr>
<td>SLP1</td>
<td>0.7 ± 0.1</td>
<td>Semiquantitative Western blot and densitometry</td>
<td>(15, 22, 23, 78)</td>
</tr>
<tr>
<td>HBD-2</td>
<td>0.57 ± 0.13</td>
<td>Semiquantitative Western blot and densitometry</td>
<td>(15, 20)</td>
</tr>
<tr>
<td>HNP-1–3</td>
<td>0.35 ± 0.07</td>
<td>Semiquantitative Western blot and densitometry</td>
<td>(15, 27, 38, 79)</td>
</tr>
<tr>
<td>HBD-1</td>
<td>0.04 ± 0.02</td>
<td>ELISA</td>
<td>(15, 20)</td>
</tr>
</tbody>
</table>

* Represents the arrows indicated in Fig. 4, A and C.
* Calprotectin, heterodimer of calgranulin A and calgranulin B, was tested as individual peptides.
* HNP-1, HNP-2, and HNP-3 were tested as individual polypeptides.
and vagina, containing a full-thickness epithelia composed of vaginal-ectocervical cells that are interspersed with immunocompetent dendritic (Langerhans) cells in the basal and suprabasal layers. To study the role of vaginal fluid in reducing HIV-1 infection and integration of the proviral DNA into the host genome, cervicovaginal tissues infected with HIV-1 BaL in the absence or the presence of an apical film of vaginal fluid were compared. The tissues were treated with PBS (vehicle control) or vaginal fluid diluted 1/1 in PBS for 30 min before infection with HIV-1 BaL (p24, 25 ng/tissue) diluted in PBS (control) or in 50% vaginal fluid. Twenty-four hours after infection, excess virus was removed, and PBS control or 50% vaginal fluid was reapplied to the apical tissue surface. Total tissue DNA was extracted 9 days after infection, and the proviral DNA levels in BaL- vs BaL- plus V- infected tissues. Human vaginal organotypic cultures were treated with PBS control or 50% vaginal fluid and infected with HIV-1 BaL or IIIB (not shown). Luciferase was then measured as described in the text, and the percent infection was calculated. * p < 0.00015. Experiments were performed in triplicate, and error bars represent the SEM.

FIGURE 5. Cationic polypeptides of vaginal fluid synergize to inhibit HIV-1 infection. TZM-bl cells were treated as indicated in the figure and infected with BaL (p24, 2 ng/ml) for 24 h or with IIIB (not shown). Luciferase was then measured as described in the text, and the percent infection was calculated. * p < 0.00015. Experiments were performed in triplicate, and error bars represent the SEM.

FIGURE 6. Vaginal fluid inhibits HIV-1 infection of human vaginal tissues. Human vaginal organotypic cultures were treated with PBS control or 50% vaginal fluid and infected with HIV-1 BaL or IIIB (not shown). Nine days after infection, tissues were harvested for DNA or protein analysis. A, Real-time quantitative PCR of HIV-1 BaL proviral DNA corresponding to a 700-bp region of the env gene was performed in BaL- vs BaL- plus vaginal fluid (VF)-infected tissues (n = 4). B, HIV-1 p24 protein levels in BaL- vs BaL- plus V- infected tissues. * p = 0.0091. Error bars represent the SEM.

Discussion

The mechanisms by which vaginal mucosa protects against sexually transmitted and other infections are not completely understood. Although several studies have focused on the adaptive immune system of mucosal surfaces of the female reproductive tract, scant attention has been focused on the innate immune factors in vaginal secretions (41–44). Evidence is accumulating that vaginal epithelia are more than simple physical barriers to protect against invading pathogens (15, 45, 46). On the contrary, this surface and its overlying fluid are replete with antimicrobial polypeptides that act as effectors of innate host defense.

The current study provides evidence that cationic polypeptides contribute significantly to the intrinsic biological activity of vaginal fluid against HIV-1 infection. Proteomic analysis of the cationic polypeptide fraction of vaginal fluid revealed numerous cationic antimicrobial and host defense polypeptides. Polypeptides with known microbicidal effects that have been identified in our study and previously identified in mucosal secretions include lysozyme, lactoferrin, cathelicidin (47, 48), β-defensins, α-defensins, and SLPI (15, 45). Although each of these polypeptides reportedly prevented HIV-1 infection, their activities were realized only when assayed at supraphysiologic concentrations, and they were not active against HIV-1 when tested individually at physiologic concentrations. However, a mixture of the peptides added back to CM-depleted vaginal fluid partially restored the activity. Partial (rather than complete) restoration of activity may be reflective of the following. 1) Some of the recombinant proteins may not exhibit the same anti-HIV-1 activity as that of the purified or natural proteins in the secretions. 2) Although we created the mixture with individual polypeptides, the full activity of certain proteins (e.g., calgranulins A and B) may be best realized in their heterodimeric form. 3) Due to availability, several polypeptides that we identified could not be included in the polypeptide mixture. Any or all of these conditions support the premise that the collective cationic polypeptide fraction is responsible for anti-HIV-1 activity of vaginal fluid. Indeed, when the extracted cationic polypeptide fraction (bound to the CM-resin) was used to reconstitute the CM-depleted fluid, anti-HIV-1 activity was restored completely.

Whole vaginal fluid was collected from healthy donors using a diaphragm-like device (Instead SoftCup), which enabled the collection of whole undiluted cervicovaginal fluid (28). In contrast, other commonly used methods of cervicovaginal fluid collection, such as extraction from preweighed tampons or vaginal lavage (15, 49, 50), can suffer from protein adhesion to the tampon or a dilute lavage of unknown protein concentration. Unlike lavage, the Instead SoftCup is convenient and can be self-inserted; thus, women are more receptive to donating cervicovaginal fluid. Although no one method of collection is perfect, approaches that enable the retrieval of whole, undiluted fluid may afford the best representation of the condition in vivo.

Lactic and other organic acids that result in the low pH of human vaginal fluid (normally pH 3.8–4.5) as well as volatile compounds, such as H2O2, are thought to contribute to microbial host defense (13, 14). Our studies were designed to minimize or eliminate the effects of these factors, because the acidity of the vaginal fluid was neutralized (pH 7.4) before subjecting the fluid to anti-HIV-1 assays. Moreover, although all the anti-HIV-1 activity was

tified by p24 PM ELISA was significantly lower in cervicovaginal tissues treated with vaginal fluid compared with control tissues (p = 0.0091; n = 2; Fig. 6B). These studies imply that vaginal fluid plays an important role in preventing HIV-1 transmission in cervicovaginal mucosa.

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Disclosures

The authors have no financial conflict of interest.

References

