Endogenous Mucosal Antiviral Factors of the Oral Cavity

Diane C. Shugars

The oral cavity represents a unique site for mucosal transmission of human immunodeficiency virus type 1 (HIV-1). Unlike other mucosal sites, the oral cavity is rarely a site of HIV transmission despite detectable virus in saliva and oropharyngeal tissues of infected persons. One reason for this apparent paradox is the presence of endogenous mucosal antiviral factors. Innate inhibitory molecules, such as virus-specific antibodies, mucins, thrombospondin, and soluble proteins, have been identified and partially characterized from saliva. A recent addition to the growing list is secretory leukocyte protease inhibitor (SLPI), an 12-kDa non-glycosylated protein found in serous secretions. Physiologic concentrations of SLPI potently protect adherent monocytes and activated peripheral blood mononuclear cells against HIV-1 infection. SLPI levels in saliva and semen but not breast milk approximate levels required for inhibition in vitro. Characterization of SLPI and other endogenous antiviral molecules may enhance our understanding of factors influencing mucosal HIV-1 transmission.

Mucosal transmission accounts for most infections with the human immunodeficiency virus type 1 (HIV-1). Unlike other mucosal sites (e.g., genital tract), the oral cavity is an infrequent route of HIV-1 transmission (reviewed in [1]). Infectious virus is rarely isolated from saliva, confirming the virtual lack of oral transmission reported by numerous epidemiologic studies. This finding is not due to an absence of virus in the oral cavity: HIV-1 RNA, proviral DNA, and infected cells are readily detectable in oropharyngeal tissues and salivary secretions of infected persons. Rather, saliva contains endogenous mucosal antiviral factors that block HIV-1 infection through a variety of mechanisms. Only when oral tissues are challenged with a bolus of virus (e.g., during experimental infection of macaques with the related simian immunodeficiency virus or receptive sex among humans; see Ruprecht et al., this issue) can the protective effects of saliva be overcome. Identification and characterization of innate mucosal antiviral molecules, such as those present in saliva, may strengthen our understanding of mucosal HIV transmission and aid in the development of novel strategies to reduce infection. This review summarizes the oral barriers to viral transmission and discusses endogenous salivary factors that have documented anti–HIV-1 activity, focusing on the most recently described mucosal protein, secretory leukocyte protease inhibitor (SLPI).

Protective Barriers to Oral HIV-1 Transmission

Numerous factors contribute to the relative lack of viral infection through oral fluids. The mucosal membrane itself serves as a physical barrier against invading microorganisms. Oral fluids lubricate and constantly bathe intra-oral tissues, flushing HIV-1 and other pathogenic microorganisms into the gastrointestinal tract. Although HIV-1 is present in the oral cavity, salivary virus titers typically are low compared with those in blood and genital secretions [2], which are well-recognized sources of transmission. In addition, saliva contains several antiviral factors that naturally hinder viral infection through the oral route.

The antiviral activity of saliva was first demonstrated by Patricia Fultz [3] over a decade ago. Fultz reported that pretreatment of HIV-1 with human or chimpanzee whole saliva significantly reduced the infectivity of activated peripheral blood mononuclear cells (PBMC) in vitro. Subsequent studies confirming and extending the initial observation (reviewed in [1]) have led to the recognition of multiple mechanisms through which oral secretions might thwart HIV-1 transmission: virus-specific antibody responses, aggregation of virus particles by high-molecular-mass glycoproteins, and blockade of viral or cellular targets (or both) by inhibitory endogenous proteins.

Virus-Specific Salivary Antibodies

The presence of immunoglobulins in mucosal secretions, including saliva, has been well-documented (reviewed in [4] and Janoff et al., this issue). HIV-1–specific immunoglobulins of IgA, IgG, and IgM isotypes are detected readily in salivary...
secretions of infected individuals [5, 6]. A simple testing system has been developed recently to measure HIV-1-specific IgG responses in oral mucosal transudate, serum-derived fluid that bathes the teeth (reviewed in [7]). This highly sensitive and specific alternative to blood provides a noninvasive, portable method for diagnostic investigations and population surveillance. Adaptation of this method for use in the detection of secretory IgA (S-IgA), the predominant immunoglobulin in mucosal fluids, may prove a useful tool in vertical transmission studies.

Altered systemic and secretory immune responses are characteristic features of HIV infection. Serum levels of total IgA, IgA1, IgA2, and IgG are markedly elevated during disease progression, a condition referred to as hypergammaglobulinemia. In contrast, patients with HIV or AIDS, compared with uninfected controls, exhibit significantly reduced levels of total S-IgA and isotypes (IgA1, IgA2) in whole and parotid salivary secretions [8, 9]. Virus-specific salivary S-IgA antibodies to p24 and gp160 are present in patients early in their disease course, but titers decline with progression to AIDS [10]. Available immunoglobulin in the oral cavity is further reduced by diminished salivary flow rates due to advancing HIV-1 disease, use of xerostomic medications, and HIV-associated salivary gland disease [9].

Despite clear differences among these salivary antibody profiles, HIV and AIDS patients can mount substantial local immune responses to oral opportunistic infections, such as Candida albicans [11, 12], a fungal infection that causes oral candidiasis or thrush. Neutralizing salivary responses can be generated in persons vaccinated with either gp120 V3 loop synthetic peptides [13] or cholera toxin-conjugated peptide antigen [14], indicating that local salivary responses may lower virus transmission through the oral route. It is important to note, however, that neutralizing salivary antibodies may reduce viral infectivity in the donor but do not protect the unvaccinated recipient against infection. In addition, saliva contains inhibitory components of nonimmune origin since secretions from uninfected individuals also block infection in vitro (reviewed in [1]).

### Inactivation of Salivary HIV-1 via Aggregation

Another mechanism through which salivary HIV-1 is inactivated is through physical entrapment and sequestration by high-molecular-mass glycoproteins, such as mucins [15] and thrombospondin-1 [16]. Mucins are sulfated polysaccharides of high (MG1) and low (MG2) molecular mass that are present in mucous secretions, such as sublingual or submandibular gland fluids. Thrombospondin-1 is a high-molecular-mass trimeric sulfated glycoprotein that belongs to the extracellular matrix family of glycoproteins. Both types of anionic molecules bind the virus envelope glycoprotein gp120 through charge interactions, thus creating a block to cellular infection by preventing complex formation between gp120 and the high-affinity HIV-1 receptor CD4 [15, 16].

Yet, on the basis of the following observations, viral aggregation only partially accounts for the inhibitory activity in saliva [17]: First, parotid secretions, which are purely serous and contain no mucins, also block HIV-1 infection in vitro. Second, filtration of whole saliva to remove high-molecular-mass molecules reduces but does not eliminate the inhibitory effect.

### Soluble Salivary Anti-HIV-1 Factors

Several soluble molecules that are naturally present in saliva have been proposed, largely on the basis of their previously characterized antimicrobial activities, to have anti-HIV-1 activity (table 1). Many of these molecules have been tested as purified preparations for inhibition of HIV-1 in various cell culture models. Defensins (cyclic antimicrobial peptides) significantly diminished HIV-1-associated cytopathicity in the human T cell line MT-4 [18], presumably via disruption of viral and cellular membrane fusion during virus entry [19]. A role for proline-rich proteins, a heterogeneous group of small (6–9 kDa) proteins that are highly concentrated in parotid fluids, has also been described [20].

Two studies have directly compared the inhibitory activities of various purified salivary proteins within a given cell culture model. Utilizing an HeLa-CD4 cell plaque assay, Bergey et al. [15] assessed cystatins, basic proline-rich peptide, statherin, and amylase purified from salivary secretions for activity against the lymphotropic HIV-1 strain IIIB. Only the cystatins demonstrated modest (30%) antiviral activity; no inhibition was observed with the other salivary components. In a separate investigation, McNeely et al. [17] evaluated a panel of salivary proteins (proline-rich peptides, statherin, histidine-rich peptides, lysozyme, lactoferrin, and cystatin) for protection of human adherent monocytes against infection with the macrophage-tropic HIV-1 strain BaL. The authors reported that pretreatment with lysozyme, cystatins, or lactoferrin lowered subsequent virus production by >90% compared with virus production in the untreated control but required concentrations greatly exceeding physiologic levels. Only recombinant SLPI (rSLPI; Amgen, Boulder, CO) significantly blocked infection at physiologic concentrations (1–10 μg/mL). The authors further showed that SLPI depletion of whole saliva resulted in a cor-

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<th>Table 1</th>
<th>Proposed anti-HIV-1 factors in salivary secretions.</th>
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<tr>
<td>HIV-1-specific antibodies</td>
<td>Mucins</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Amylase</td>
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<tr>
<td>Peroxidases</td>
<td>Statherin</td>
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<tr>
<td>Complement</td>
<td>Proline-rich peptides</td>
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<tr>
<td>Cystatins</td>
<td>Thrombospondin-1</td>
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<tr>
<td>Lactoferrin</td>
<td>Histatins</td>
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<tr>
<td>Defensins</td>
<td>Secretory leukocyte protease inhibitor</td>
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responding loss of inhibitory activity. Thus, SLPI is likely to be a major deterrent of HIV-1 transmission in oral secretions.

**SLPI Blocks HIV-1 Infection of Primary CD4+ Cells**

SLPI is a small (11.7 kDa) non-glycosylated, highly basic, acid-stable protein. The protein, which is distributed widely in mucosal secretions, is secreted by acinar cells of submucosal glands and epithelial cells lining mucous membranes [21]. SLPI contributes to local defenses by protecting mucosal surfaces against attack by serine proteases (e.g., granulocyte elastase and cathepsin G) from leukocytes during inflammation. The single-chained protein is organized into two structurally homologous domains containing four disulfide bonds each [22]. The C-terminal domain includes the protease-inhibitory site [23], while the N-terminal domain stabilizes the protease-antiprotease complex and mediates binding to heparin [24].

Most [17, 25, 26] but not all [27] reports have demonstrated that HIV-1 BaL infection of adherent monocytes is significantly inhibited by rSLPI. In typical experiments, a single pretreatment of rSLPI during initial virus exposure reduced infectivity by 90% as measured by reverse transcriptase activity or p24 antigen content. Inhibition is dose dependent and occurs maximally at 1–10 μg/mL, which is consistent with physiologic concentrations in saliva. Similar results have been observed utilizing PBMC cultures and the HIV-1 lymphotropic viruses IIIB [17, 25] and NL4-3 [25]. rSLPI also protects monocytes and PBMC against infection with HIV-2 but requires higher concentrations (10–20 μg/mL) for a more modest effect (Shugars, unpublished data). On-going experiments are assessing rSLPI for activity against clinical HIV-1 isolates. In the experiments described above, rSLPI preparations were clinical grade, bacterially expressed and purified, non-cytotoxic at the concentrations tested, and unmodified compared with endogenous SLPI [17].

The SLPI effect appears to be more pronounced in primary (nontransformed) cells. rSLPI did not block either virus-mediated cell-cell fusion or infection with cell-free virus in selected CD4-expressing cell lines [1, 17, 25, 26]. It is not yet known whether rSLPI blocks cell-cell fusion of primary cell cultures. The inhibitor also exhibits inhibitory activity against Sendai and influenza A viruses [28] but not cytomegalovirus [26], herpes simplex virus type 1 [26], or murine leukemia virus [25].

**Mechanism of SLPI-Mediated Anti-HIV Activity**

Several lines of evidence indicate that SLPI targets a host cell molecule rather than the virus itself. SLPI does not bind directly to the virus envelope proteins gp120 or gp160 [17, 27]. As described by McNeely et al. [17], antiviral activity was retained when rSLPI-pretreated cells were extensively washed prior to virus exposure. In the converse experiment, no inhibition was observed following rSLPI pretreatment of virus. In a follow-up report, the authors revealed that radiolabeled rSLPI bound specifically and with high affinity to a single class of receptors on monocytes (~7000 receptor sites/cell, KD = 3.6 nM) in a dose-, pH-, and time-dependent manner [29]. That report also described co-precipitation of a 55-kDa cell surface protein from monocytes using anti-SLPI antibodies [29]; the identity of the putative SLPI receptor has not yet been described.

Additional experiments have implicated SLPI activity at an early stage in cellular infection. Analysis by nested polymerase chain reaction revealed that the inhibitor disrupts a step that occurs prior to viral reverse transcription [29]. Experiments in which rSLPI failed to bind recombinant soluble CD4, disrupt binding of virus to CD4+ target cells, interfere with CD4-gp120 interactions, or modify monocytic CD4 expression [17] suggest that this step follows initial interactions between the virus and its high-affinity cellular receptor, CD4. As supporting evidence, SLPI does not interfere with later stages of cellular infection, such as reverse transcription, protease activity, or virus budding from infected cells [17, 25, 26]. Thus, SLPI blocks HIV-1 internalization of primary CD4-expressing cells through a mechanism most likely involving virus entry (perhaps via interactions with the newly described chemokine co-receptors) or capsid uncoating.

SLPI’s antiviral activity appears to be distinct from its known antiprotease activity. SLPI mutants containing disruptive point mutations within the protease-binding site exhibited wild-type antiviral activity [29]; however, an intact SLPI molecule is required for the effect because mutants containing either the N-terminal or C-terminal domain exhibited no inhibition [29].

**Table 2.** Endogenous concentrations of secretory leukocyte protease inhibitor in unpaired biologic fluids, as measured by an ELISA.

<table>
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<tr>
<th>Biologic fluid</th>
<th>Mean ± SE SLPI concentration (μg/mL)</th>
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<tr>
<td></td>
<td>HIV-uninfected donor</td>
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<tr>
<td>Filtered whole unstimulated saliva</td>
<td>1.14 ± 0.28</td>
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<tr>
<td>Seminal plasma</td>
<td>16.0 ± 2.5</td>
</tr>
<tr>
<td>Breast milk</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Cerebral spinal fluid</td>
<td>0.002 ± 0.0001</td>
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NOTE: SLPI = secretory leukocyte protease inhibitor.
Further studies are needed to identify the site within SLPI responsible for its antiviral activity and to determine its exact mechanism of action.

A Role for SLPI in Mucosal HIV-1 Transmission?

It is not known whether SLPI plays a role in mucosal transmission of the virus in vivo. To begin to address this question, I used an ELISA to quantitate endogenous SLPI concentrations in unpaired biologic fluids. As summarized in table 2, endogenous SLPI levels in filtered saliva and seminal plasma fall within the range required for antiviral activity in vitro. No significant difference between SLPI levels was observed on the basis of HIV-1 status. Higher SLPI levels in semen (15- to 20-fold) compared with levels in saliva suggest a complex role of SLPI in genital secretions. The obvious lack of correlation between seminal SLPI levels and risk of transmission may be explained by physiologic differences between saliva and semen.

For example, Ohlsson et al. [30] found SLPI to be partially degraded in semen due to proteolytic cleavage by prostate-specific antigen, thus potentially reducing free inhibitor to levels suboptimal for antiviral activity. Alternatively, the failure of rSLPI to bind monocyte surfaces under basic conditions [29] suggests that seminal SLPI may not interact with its cognate target in the alkaline environment of semen. In contrast, SLPI levels in breast milk are low (table 2) and, on the basis of infectivity assays, are predicted to be below the level required for inhibition in vivo. Experiments conducted by Wahl et al. [26] indicate that this is indeed the case, and this is consistent with the recognized risk of transmission through breast milk. As expected, cerebral spinal fluids, which carry little known risk of HIV-1 transmission, contain negligible amounts of SLPI (table 2).

Further evidence for SLPI being a natural salivary inhibitor of HIV-1 was recently described by Wahl et al. [31]. In situ hybridization detected HIV-1 in 30% of salivary glands obtained from infected persons; however, SLPI mRNA was abundantly expressed in these tissues. The apparent discrepancy was resolved by demonstrating that the virus and inhibitor are physically dissociated from one another. That is, SLPI accumulated in salivary acinar cells or ductal epithelium, while the virus localized to interstitial mononuclear cells and did not co-localize with SLPI. Only within the oral cavity do virus and inhibitor meet, further supporting a role for SLPI in thwarting oral HIV-1 transmission.

Summary

Saliva contains endogenous inhibitory molecules that contribute to the relative lack of HIV-1 transmission orally. Virus-specific immunoglobulins neutralize and inactivate the virus. High-molecular-mass glycoproteins, such as mucins and thrombospondin-1, sequester HIV-1 into aggregates for clearance by the host. Soluble proteins, such as SLPI, protect target cells against infection. These factors work in concert to reduce virus entry at mucosal sites, making the oral cavity an attractive model for developing new strategies to reduce mucosal transmission of this virus.

Acknowledgments

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References


Addendum. Other mechanisms for HIV-1 inhibition by saliva have been described recently. Nagashunnugam et al. [32] have demonstrated that submandibular saliva reduces viral infectivity by stripping gp120 from the surface of virions, presumably through a mechanism involving salivary agglutinin and/or MG2 mucin. Baron et al. [33] have reported saliva-mediated disruption and lysis of infected mononuclear cells and the CD4+ CEM lymphocytic cell line and have proposed that the activity is due to the hypotonicity of oral secretions. Although intriguing, these proposed mechanisms await confirmation.