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What is This?
The Anti-HIV-1 Activity Associated with Saliva

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Abstract. This review summarizes the data on the anti-human immunodeficiency virus (HIV) activity associated with saliva and the possible routes of oral transmission of HIV. Saliva can be passed from an HIV-infected individual to an uninfected person via sexual or non-sexual activities. The relative risk of HIV transmission through saliva is a subject of continuing concern for dental practitioners. HIV-infected individuals frequently have oral lesions that can cause bleeding and release of the virus into the oral cavity. In addition, viral p24 and HIV-1 RNA were detected in tonsils and adenoids even in asymptomatic seropositive individuals. Nevertheless, the potential HIV-infectivity of saliva is low, although both infectious HIV-1 and HIV DNA have been detected in saliva. This observation has led to the suggestion that saliva may contain factors that inhibit HIV-1 infectivity. At least two anti-HIV activities have been partially characterized: (i) physical entrapment of HIV by high-molecular-weight molecules (e.g., mucins), and (ii) inhibition of viral infection by soluble proteins. Several studies have indicated that, of the salivary proteins evaluated, recombinant secretory leukocyte protease inhibitor (rSLPI) could inhibit HIV-1 infection in macrophages at physiological concentrations. The anti-HIV activity of the serine protease inhibitor rSLPI is most likely due to its interaction with a cell-surface molecule(s) other than the primary HIV-1 receptor, CD4, and may involve (i) inhibition of cell-surface serine protease(s), and/or (ii) interaction with other human-specific co-factors essential for viral entry.

Key words: HIV, saliva, mucins, SLPI.

Introduction

Human immunodeficiency virus-1 (HIV-1) has been identified as the etiological agent of acquired immunodeficiency syndrome (AIDS). Two of the major factors in viral transmission are the amount of infectious virus in a body fluid and the extent of parenteral contact with that fluid. While blood and genital fluids can have high levels of HIV-1 particles and/or HIV-1-infected cells, other body fluids are not likely sources of viral transmission (e.g., see Levy, 1993; Table 4). Although the presence of infectious virus in saliva of HIV-infected persons has been reported (e.g., see Malamud and Friedman, 1993), transmission of the disease through saliva has not been proven (Friedland et al., 1986; Klein et al., 1988). In a study of 1309 dental professionals, in which 94% had reported puncture wounds and 72% had treated high-risk patients, only one, without other risk factors, was HIV-positive (Klein et al., 1988). This has led to the suggestion that saliva may contain factors that inhibit HIV-1 infectivity. Indeed, several in vitro studies have shown the anti-HIV activity of saliva (e.g., Fox, 1993), and the isolation and characterization of the anti-HIV salivary components have been the subject of several investigations (Bergey et al., 1993, 1994; McNeely et al., 1995; Turpin et al., 1996).

Presence of HIV in the oral cavity

Studies on the ability to isolate HIV from saliva have produced various results, ranging from eight isolates from 20 patients (Groopman et al., 1984) to one in 21 (Moore BE et al., 1993), one in 55 (Levy and Greenspan, 1988), one in 83 (Ho et al., 1985), and none of seven (Schioldt et al., 1989). The potential HIV-infectivity of saliva is far less than that of serum. Samples of serum and whole saliva were collected simultaneously from 75 HIV-infected individuals and tested for viral p24 antigen and infectious virus. Of the 218 sera and saliva samples co-cultured with phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC), 38% and 1%, respectively, were positive for cell-free infectious virus (Barr et al., 1992). The low risk of acquiring AIDS via salivary transmission was confirmed by Yeung et al. (1993). Even in the presence of severe periodontal disease, very low concentrations of infectious virus were present in 21% of saliva samples from HIV-seropositive patients at all stages of HIV infection.
For dental practitioners, in particular, the relative risk of HIV transmission through saliva from HIV-seropositive persons is a subject of continuing concern (Serb and Yeung, 1994). HIV-infected individuals frequently have mucosal and gingival lesions such as gingivitis and candidiasis. These lesions can cause bleeding into the oral cavity, releasing virus into saliva and increasing the potential risk of transmission (Yeung and Serb, 1994). The presence of viral DNA has been detected by polymerase chain reaction (PCR) in centrifuged cell pellets from saliva (Yeung et al., 1993; Liuzzi et al., 1994) and in monocytes, lymphocytes, and mucosal epithelial cells obtained from saliva (Qureshi et al., 1995). Most recently, it has been suggested that HIV can be disseminated through the upper aero-digestive tract mucosa from intra-epithelial dendritic cells, submucosal macrophages, and T-lymphocytes (Wenig et al., 1996). Histomorphologic changes in tonsils or adenoids were shown to correlate with the viral p24 protein in situ, even in asymptomatic HIV-positive individuals (Frankel et al., 1996; Wenig et al., 1996). Significant amounts of HIV-1 RNA were detected in 9 of 10 tonsil biopsies from HIV-seropositive patients (Faust et al., 1996). The interaction of dendritic cells and T-cells, activated continuously by environmental stimuli within the lymphatic tissue of the nasopharynx, may support HIV-1 replication even in a subclinical stage of infection. There are two possible routes of infection of the tonsils and adenoids: (i) hematogenous spread of HIV-infected peripheral blood cells and/or (ii) direct infection of dendritic cells present in oral mucosa of the upper aero-digestive tract. Interestingly, it has been reported recently that the non-traumatic oral exposure of adult macaques to cell-free simian immunodeficiency virus (SIV) resulted in infection and development of AIDS (Baba et al., 1996). These results support the suggestion that dendritic cells located in the tonsils might be directly infected after oral exposure.

Mucosal transmission of HIV
The exact mechanism of HIV transmission after exposure of mucosal surfaces to HIV-infected fluids is unknown. Two important questions with respect to the mechanism of mucosal transmission are: (i) What is the infectious agent, and (ii) which cells are infected? The infectious agent in these fluids may be HIV-infected cells, cell-free virus, and/or immune complexes of virus and antibody (e.g., see Levy, 1993). During exposure of mucosal surfaces, HIV could be transmitted by (i) entering the body through lesions in the epithelium (Padian, 1987), (ii) infecting CD4+ cells, e.g., Langerhans' cells and macrophages, and (iii) infecting intact CD4+ epithelial cells.

Dendritic cells
A well-identified population of dendritic cells, Langerhans' cells, has been proposed as a target especially well-suited for HIV infection in mucosa (Miller et al., 1993; Soto-Ramirez et al., 1996). Originating from the bone marrow, Langerhans' cells migrate into the peripheral epithelia and act as antigen-presenting cells. These CD4-expressing cells are located in the skin and on the surface of the oral and vaginal mucosa. HIV-1 infection of Langerhans' cells has been demonstrated by electron microscopy, immunochemistry, RNA and DNA polymerase chain-reaction (PCR) analysis, and p24 production (Rappstberger et al., 1988; Delorme et al., 1993; Ayeunie et al., 1995; Schmitt and Dezutter-Dambuyant, 1994; Soto-Ramirez et al., 1996). The productive infection of Langerhans' cells has been demonstrated in vitro after either culture with HIV-1-infected cells or exposure to cell-free virus.

Epithelial cells
Epithelial cells lining wet cavities (mouth, esophagus, larynx, vagina, anal canal), histologically classified as stratified squamous non-keratinized cells, do not express the CD4 antigen, and it was originally thought that these cells could not be infected by HIV. It was found subsequently that HIV can infect CD4+ cells, including epithelial cells (Adachi et al., 1987; Tan et al., 1993; Philips et al., 1994). The human cervix-derived epithelial cells, ME180, are infected efficiently in culture by direct contact with HIV-infected H9/HIV-1MN cells, although they are difficult to infect with cell-free virus (Tan et al., 1993). Interestingly, similar observations were reported when primary or established oral epithelial cells were co-incubated with HIV-1-infected H9 cells, and entry of HIV was studied by electron microscopy (Qureshi et al., 1995). Within 30 min, infected cells interact with and adhere to the oral epithelial cells, with polarized budding of the virus at the point of contact with the epithelial cells. Electron microscopic analysis has shown that this interaction leads to vacuolization of the epithelial cells followed by viral uptake, which is consistent with receptor-mediated endocytosis and some non-specific uptake in vacuoles. When in situ polymerase chain-reaction was used, HIV-1 proviral DNA was detected in oral mucosal squamous cells and mononuclear cells in the saliva of HIV-infected patients, suggesting active replication of HIV-1 in these cells (Qureshi et al., 1996). These findings suggest that epithelial cells could be infected by HIV-1 in vivo, and a similar mode of cell-associated mucosal transmission may mediate the entry of HIV-1 into intact epithelial cells of different origin. Productively infected epithelial cells would subsequently produce new virus which may infect CD4+ T-lymphocytes and macrophages in the connective tissue below the epithelium.

Anti-HIV activity in saliva
Several studies have indicated that saliva may inhibit HIV infectivity (e.g., see Malamud and Friedman, 1993). Saliva from human submandibular/sublingual (HSMSL) and parotid (HPS) glands, as well as whole saliva, reduces HIV-1 infectivity in vitro (Fultz, 1986; Fox et al., 1988, 1989; Archibald and Cole, 1990; Yeh et al., 1992; Bergey et al., 1993; Malamud et al., 1993; Robinovitch et al., 1993). HSMSL and the whole salivas exert more potent and consistent anti-HIV activity than HPS. The anti-HIV effect of HSMSL saliva (measured in a plaque assay on HeLa/CD4+ cell monolayers infected with HIV-1_MDI) ranged from 44 to 100% (an average of 66.3 ± 17.5%) compared with an average of 34.7 ± 29.3% for HPS collected from the same individuals (Bergey et al., 1993). Filtration of both HSMSL and whole saliva greatly reduces the antiviral activity. For example, fresh HSMSL saliva filtered through a 0.22-µm filter prior to the addition of the virus retains only 14% inhibitory activity as compared...
with non-pre-filtered HSMSL saliva (Bergey et al., 1993). Filtration of whole saliva after incubation with the virus for 1 hr at 37°C significantly reduces the level of HIV-1 in the filtrate (~ 50% by p24 antigen measurement) (Yeh et al., 1992). These data suggest that large-molecular-weight compounds present in saliva that are retained by the filter may inhibit HIV infectivity. Subsequent electron microscopic studies have revealed the aggregation and/or entrapment of the virus particles by the HSMSL salivary components (Bergey et al., 1993).

High-molecular-weight anti-HIV component: mucins
Salivary aggregates can contain a number of proteins and glycoproteins, including mucins, IgA, lysozyme, peroxidase, amylase, statherin, and proline-rich proteins (Bergey et al., 1993). Several lines of evidence indicate that HIV-1 is entrapped mainly by the salivary mucins. First, there is no significant difference in the anti-HIV-1 activity observed for filtered (38%) vs. unfiltered (35%) HPS, and parotid saliva does not cause aggregation of HIV particles (Bergey et al., 1993). This is expected, since HPS is serous and contains no mucins. In addition, examination of partially purified components from HSMSL saliva indicated that the filtration-sensitive inhibitory activity is associated with the mucin-rich fractions (Bergey et al., 1994). However, the anti-HIV-1 activity of saliva is only partially explained by aggregation of the virus by mucins. The fact that anti-HIV-1 activity is retained in both the filtrate from the HSMSL and whole saliva, as well as in the purely serous HPS, indicates that some soluble, filterable component(s) present in saliva may be involved in HIV inhibition.

Soluble anti-HIV components
Several studies have focused on identifying and characterizing the soluble anti-HIV-1 factor(s) present in saliva. The ability of cystatin SN, cystatin S1, basic proline-rich peptides and statherin purified from HSMSL, and amylase purified from parotid saliva to inhibit HIV replication was measured in a plaque assay on HeLa/CD4+ cell monolayers (Bergey et al., 1994). The cystatins demonstrated a 30% inhibitory effect; no significant inhibition was observed for the other salivary components tested.

In a recent study, whole saliva, fractions of submandibular saliva obtained by gel filtration, and purified and recombinant salivary proteins were investigated for their ability to inhibit infection of primary macrophages with HIV-1_{mac} or primary T-cells with HIV-1_{imm} (McNeely et al., 1995). The protein-rich fractions of submandibular saliva which retained HIV-1 inhibitory activity are in the low-molecular-weight range of 10 to 14 kDa. Nine saliva proteins—including two proline-rich proteins, two histatins, statherin, cystatin, lysozyme, lactoferrin, and recombinant secretory leukocyte protease inhibitor (rSLPI)—were screened for antiviral activity. While several of these proteins (cystatin, lysozyme, and lactoferrin) have anti-HIV-1 activity at levels exceeding physiological concentrations, only rSLPI significantly inhibits HIV-1 infection in macrophages (> 90% inhibition of reverse transcriptase [RT] activity, compared with controls) at physiological concentrations. SLPI also inhibits HIV-1 infectivity of primary T-cells, although to a lesser extent. Whole saliva depleted of SLPI by elution from an affinity column containing anti-SLPI antibody exhibits a corresponding decrease in anti-HIV-1 activity. These data suggest that SLPI may be the main soluble factor responsible for the HIV-inhibitory effect of saliva.

Anti-HIV activity of recombinant secretory leukocyte protease inhibitor (rSLPI)
Given the observation that rSLPI inhibits HIV-1 infection, further studies were conducted to determine the mechanism(s) of this inhibition (McNeely et al., 1995). The anti-HIV-1 effect was also observed when macrophages were pre-incubated with rSLPI and washed prior to the addition of the virus. Immobilized rSLPI does not bind recombinant soluble CD4 (rsCD4) or the recombinant viral proteins rgp160 and rgp120. Treatment with rSLPI does not change CD4 expression on macrophages. If it is assumed that rSLPI is not altered by immobilization, the anti-HIV-1 activity of rSLPI is most likely due to its interaction with a host cell molecule(s) other than the HIV-1 receptor, CD4. The inhibition of HIV-1 infection of macrophages by rSLPI has also been observed by Shugars et al. (1996). These investigators have concluded, based on assays utilizing PCR to detect internalized virus, that SLPI inhibits the virus-cell fusion and/or uncoating step of the HIV infection cycle.

The possibility that SLPI might be the salivary component responsible for the anti-HIV activity had been proposed earlier (Rice et al., 1991). However, in the studies conducted at that time, no inhibition of HIV replication or cytopathicity was observed with rSLPI. In a recent publication (Turpin et al., 1996), Rice and co-workers have re-evaluated the anti-HIV activity of rSLPI. Under one set of conditions, HIV-1 infection of macrophages was inhibited by rSLPI; however, since the p24 levels return to those of the untreated controls after 24 and 30 days, the authors suggest that the inhibition observed at 14 days can be explained by the loss of cells during the initial washings. Indeed, in an experiment identical except for the absence of the washing step, less inhibition of infection was observed. It was found that rSLPI does not inhibit replication of HIV-1 in the human T-cell line, CEM-SS, or in stimulated human peripheral blood lymphocytes (PBLs). Rice and co-workers also found that rSLPI does not inhibit binding of the virus to the host cell, cell-cell fusion, or the enzymatic activities of HIV-1 reverse transcriptase or protease.

We have examined the ability of rSLPI obtained from R&D Systems (Minneapolis, MN) to inhibit cell-cell fusion in an HIV-induced syncytium formation assay (unpublished data). Uninfected CD4+ Sup-T1 or H9 cells were co-incubated with TF228.1.16 cells expressing the HIV envelope glycoproteins, gp 120 and gp41 (Jonak et al., 1993), in the absence and presence of rSLPI (0.1, 1, and 10 μg/mL). rSLPI did not inhibit syncytium formation at any of the concentrations tested. This is consistent with prior observations (Bu et al., 1995; T. McNeely, personal communication; Turpin et al., 1996). We did not observe any
inhibition of syncytium formation in the presence of 10 to 50 µL of whole saliva. These results indicate that although SLPI inhibits infection by cell-free virus (McNeely et al., 1995; Shugars et al., 1996), it does not inhibit cell-cell fusion. Although the mechanisms of virus-cell and cell-cell fusion are presumed to be similar, several studies have indicated that there are significant differences between these processes (e.g., see Konopka et al., 1995). Monoclonal antibodies (5D4, 7C2) to the D3/D4 domains of CD4 inhibit HIV-1 infection but have no effect on CD4-gp120 binding or HIV-induced syncytium formation (Hasunuma et al., 1992). The lectin jacalin inhibits HIV infectivity, but it does not interact with gp120, nor does it block virus-cell binding or HIV-induced syncytium formation (LaFont et al., 1994).

Possible mechanisms of anti-HIV activity of rSLPI

The precise physiological function of SLPI is not known. Its major role appears to be the protection of the mucosal epithelium against excessive proteolytic activity (Rice and Weiss, 1990). SLPI has been widely studied and well-characterized as a potential therapeutic agent for the treatment of inflammatory pulmonary disorders such as emphysema (Lucey et al., 1990; Rudolphus et al., 1991) and cystic fibrosis (Birrer, 1995). Also known as antileukoprotease or mucus proteinase inhibitor (MPI), SLPI is a small, 11.7-kDa, non-glycosylated, basic, serine protease inhibitor. The 2.5-Å x-ray crystal structure of rSLPI complexed to α-chymotrypsin has been determined (Gruetter et al., 1988). The 107-amino-acid SLPI is organized into two homologous domains which have similar folds and four disulfide bonds each. SLPI is found in a variety of fluids, including saliva (Ohlsson et al., 1983), bronchial mucus (Ohlsson et al., 1977), tears (Kuppers, 1971), cervical mucus (Wallner and Fritz, 1974), and seminal plasma (Schiessler et al., 1976), and it is widely distributed in submucosal glands (Franken et al., 1989). SLPI is present as the free active inhibitor in parotid secretions at concentrations four-fold higher than that found in whole saliva, where approximately 15% is found as the esterase-SLPI complex (Ohlsson et al., 1983). SLPI inhibits a range of proteolytic enzymes, including the neutrophil proteases, elastase and cathepsin G, and the pancreatic proteases, chymotrypsin and trypsin (Thompson and Ohlsson, 1986).

Inhibition via cell-surface serine proteases

Interestingly, certain cell-surface serine proteases may play a role in HIV infection. Several studies have suggested that proteolytic cleavage of the third variable domain (V3) of the HIV-1 gp120 is a prerequisite to the entry of HIV-1 virions into CD4+ cells (Hattori et al., 1989; Kidu et al., 1990, 1991; Clements et al., 1991; Callebaut et al., 1993; Schulz et al., 1993; Avril et al., 1995; Oravec et al., 1995; Handlej et al., 1996; Niwa et al., 1996). It has been shown that the serine proteases thrombin and trypsin specifically cleave HIV-1 gp120 at the tryptic site in the V3 loop. Neither SLPI nor its half-length derivative, Arg59-Ala107, shows any inhibitory activity against thrombin (Masuda et al., 1994); however, SLPI may inhibit trypsin or some other as-yet-unidentified serine protease involved in V3 loop cleavage. The membrane-bound serine esterase, trypsinase TL2, purified from human T-lymphocytes, binds specifically to gp120 and cleaves the V3 loop (Kido et al., 1990, 1991; Niwa et al., 1996). It has been suggested that the T-cell activation antigen CD26, an atypical serine protease, dipeptidyl-peptidase IV, may serve as a cofactor for HIV-1 entry (Callebaut et al., 1993; Oravec et al., 1995). Another cell-surface-associated serine protease, cathepsin G, has also been identified as a possible complementary factor for HIV-1 infection of U937 cells (Avril et al., 1995). A urokinase-type plasminogen activator, a protease produced by macrophages, has been found to bind and cleave gp120 at the V3 loop and significantly increase the HIV-1 infection of macrophages (Handlej et al., 1996). Thus, the anti-HIV-1 activity of SLPI may involve inhibition of cell-surface serine protease(s). Alternatively, this inhibitory activity may be due to the interaction of SLPI with other cell-surface factor(s).

Inhibition via interaction with non-CD4 cell-surface cofactors

Reports from several laboratories have indicated that, although the cell-surface molecule CD4 serves as the primary receptor for HIV-1, other human-specific cofactor(s) are essential for virus-cell and/or cell-cell membrane fusion required for viral entry and syncytium formation (e.g., see Levy, 1993). Accordingly, other cell-surface proteins such as LFA-1, HLA class I, CD7, and CD44S have been proposed as cofactors in HIV infection (Moore et al., 1993; Sato et al., 1994; Dukes et al., 1995). Recently, chemokines (chemotactic cytokines) and their receptors which are members of the seven-transmembrane, GTP-binding protein (G protein)-coupled receptors, have been shown to play essential roles in early events in HIV-1 infection. The chemokine “orphan” receptor LESTR (fusin) was identified as an accessory cofactor for T-cell-tropic, but not macrophage-tropic, HIV-1 strains (Berson et al., 1996; Feng et al., 1996). Fusin is widely distributed in human tissues and is expressed at high levels in human PBMC and in a number of hematopoietic cell lines (e.g., see Paxton et al., 1996). On the other hand, the β-chemokines RANTES, MIP-1α, and MIP-1β were found to inhibit infection by primary or macrophage-tropic HIV-1, but not by T-cell line-tropic strains (Cocchi et al., 1995). Subsequently, it has been shown that macrophage-tropic viruses use a β-chemokine receptor (CC-CR-5) for fusion and entry into target cells (Deng et al., 1996; Dragic et al., 1996). In light of these observations, it is of interest that rSLPI inhibits HIV-1 infection by the macrophage-tropic strain, HIV-1{	extsubscript{BaL}}, more effectively than by the T-cell tropic strain, HIV-1{	extsubscript{IIIB}} (McNeely et al., 1995). It is possible that the inhibition of HIV infection by rSLPI involves an interaction with chemokine receptors.

Inhibition via interaction with salivary mucins

The cationic SLPI carries 20 positively charged groups, 15 lysines and 5 arginines, and has been shown to form tight complexes with several sulfated glycosaminoglycans. SLPI forms a tight complex with negatively charged heparin
Heparin accelerates the inhibition of elastase by SLPI. In human bronchial secretions, SLPI is tightly bound electrostatically to the highly glycosylated mucins (Van-Seuningen et al., 1992). Both the anti-chymotrypsin and anti-elastase activities of SLPI are attenuated in the presence of mucin (Nadzieko and Finkelstein, 1994). Heparin (Ito et al., 1987) and mucins (Berger et al., 1994) are both effective anti-HIV-1 agents. Heparin is not expected to be present in saliva, but it is possible that the salivary mucins (e.g., see Tabak, 1995) bind SLPI and that an SLPI-mucin interaction plays a role in the anti-HIV-1 activity of saliva. In fact, SLPI-mucin complexes could be involved in the aggregation/entrapment mechanism of HIV inhibition by saliva.

Conclusions

It is clear from the above discussion that further studies are needed for both (i) the possible routes of oral transmission of HIV infection and (ii) the precise role of oral secretions in inhibiting HIV infection to be fully understood. The most important question for dental practitioners is the relative risk of HIV transmission through oral secretions from HIV-infected persons. While there is ample evidence for the presence of anti-HIV factors in salivary secretions, the extent to which these factors are active in vivo is not known and needs to be evaluated carefully. It is important to establish the correlations among the amount of HIV, its infectivity in salivary secretions, and the level of anti-HIV factors present in these secretions. Such studies will help to clarify the role of oral secretions in HIV pathogenesis.

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